TARGETING THE SODIUM IODIDE SYMPORTER (NIS) AND APOPTOTIC GENES IN EXTRATHYROIDAL TUMORS

By

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(M.Sc. Biotechnology)

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INDEX

List of Figures .......................................................................................................................... vii
List of Tables ............................................................................................................................ ix
ABBREVIATIONS .................................................................................................................. xi
ACKNOWLEDGEMENTS ................................................................................................... xiii
ABSTRACT ............................................................................................................................... 1
1. Review of Literature ........................................................................................................ 11
   1.1. Sodium Iodide Symporter (NIS) ...................................................................................... 11
       1.1.1. Cloning of NIS ........................................................................................................ 12
       1.1.2. Functions of NIS .................................................................................................... 14
       1.1.3. Post translational modifications of NIS ................................................................. 15
       1.1.4. NIS in thyroid cancer ............................................................................................. 16
       1.1.5. NIS in extra-thyroidal tissues ................................................................................. 17
       1.1.6. NIS as a reporter gene .......................................................................................... 18
       1.1.7. NIS as a therapeutic gene ..................................................................................... 19
   1.2. Retinoblastoma ............................................................................................................. 19
       1.2.1. Heritable versus non-heritable RB ......................................................................... 21
       1.2.2. Genetics of retinoblastoma ..................................................................................... 22
       1.2.3. Diagnosis and classification .................................................................................. 23
       1.2.4. Treatment and management ................................................................................... 24
       1.2.5. Surgical treatments ................................................................................................. 26
       1.2.6. Chemotherapy ....................................................................................................... 27
       1.2.7. Radiotherapy ......................................................................................................... 29
       1.2.8. Targeted therapy ..................................................................................................... 29
   1.3. Lactoferrin ....................................................................................................................... 30
       1.3.1. Structure of Lf ......................................................................................................... 30
       1.3.2. Forms of Lf ............................................................................................................. 31
       1.3.3. Potential benefits of bLf in cancer medicine .......................................................... 32
   1.4. Breast cancer .................................................................................................................. 33
       1.4.1. Risk factors ............................................................................................................... 34
       1.4.2. Breast cancer therapy ............................................................................................. 34
       1.4.3. Chemotherapy ....................................................................................................... 35
       1.4.4. Targeted therapy ..................................................................................................... 35
1.13. Research questions addressed........................................................................................................ 68

2. Materials and Methods............................................................................................................................. 71

2.1. RB Tumor processing............................................................................................................................. 71

2.2. RB Tumor specimens and ethics approval............................................................................................ 71

2.3. Immunohistochemistry for NIS in RB tumors....................................................................................... 80

2.4. Flow cytometric (FC) analysis of NIS protein in RB tumors............................................................... 82

2.5. Immunoblotting for NIS protein in RB tumors..................................................................................... 82

2.6. Determination of NIS protein in RB cell line (Y79 cells)................................................................. 83

2.6.1. Maintenance of Y79 cell lines.......................................................................................................... 83

2.6.2. Flow cytometric analysis of NIS protein in Y79 cells ..................................................................... 83

2.6.3. Immunofluorescence of NIS protein in Y79 cells ......................................................................... 83

2.6.4. Immunoblotting of NIS protein in Y79 cells .................................................................................. 84

2.6.5. RNA isolation of Y79 cells .............................................................................................................. 84

2.6.6. cDNA conversion or Reverse Transcriptase PCR (RT-PCR) ......................................................... 85

2.6.7. Quantitative Real Time PCR (qRT-PCR) for lactoferrin receptors (Lf)........................................ 86

2.7. Preparation of bovine lactoferrin (bLf).............................................................................................. 87

2.7.1. Treatment of glasswares ................................................................................................................. 87

2.7.2. Preparation of Native bLf .............................................................................................................. 87

2.7.3. Preparation of Apo bLf ............................................................................................................... 87

2.7.4. Preparation of Fe bLf .................................................................................................................. 88

2.7.5. Iron estimation in Fe-bLf .............................................................................................................. 89

2.7.6. Cellular internalization of bLf in Y79 cells by confocal microscopy ............................................. 90

2.7.7. Cellular internalization of bLf in Y79 cells by flow cytometry ...................................................... 91

2.8. Molecular cloning ............................................................................................................................... 91

2.8.1. Competent cell preparation by CaCl₂ method ................................................................................. 92

2.8.2. Transformation by heat-shock method ......................................................................................... 92

2.8.3. Minipreparation of plasmid DNA ............................................................................................... 93

2.8.4. Maxi-preparation of plasmid DNA ............................................................................................. 94

2.8.5. Restriction digestion .................................................................................................................... 95

2.8.6. Gel elution .................................................................................................................................... 95

2.8.7. Ligation ......................................................................................................................................... 96

2.9. Establishment of CMV-NIS and EpCAM-NIS stable transfectants of MCF-7 cells 96

2.9.1. Maintenance of cell lines.............................................................................................................. 96
2.9.2. Generation of stable MCF-7 transfectants ..................................................... 97
2.9.3. Overexpression of NIS in stable transfectants determined at mRNA level by qRT-PCR  97
2.9.4. Overexpression of NIS in stable transfectants determined at protein level by Western blotting ................................................................. 98
2.9.5. Migration assay ............................................................................................. 98
2.9.6. Radioiodide Uptake Assay in the stable transfectants ................................... 98
2.9.7. Non-radioactive iodide uptake assay in the stable transfectants .................... 99
2.9.8. Inhibition of uptake by Sodium perchlorate ............................................... 100
2.9.9. Efflux of iodide ........................................................................................... 100
2.10. Preparation and characterisation of nanocomplex ........................................... 101
2.10.1. PEI nanocomplex preparation ................................................................ 101
2.10.2. Dynamic Light Scattering ..................................................................... 101
2.10.3. Transmission Electron Microscope ....................................................... 102
2.10.4. Determination of NIS protein expression in the nanocomplex delivered cells 102
2.10.5. Nonradioactive iodide and radioactive iodide uptake assay ..................... 102
2.11. RB Tumor processing ..................................................................................... 102
2.11.1. RNA isolation of the tumors ................................................................ 106
2.11.2. cDNA conversion of the tumor RNA ..................................................... 106
2.11.3. qRT-PCR for survivin splice variants, Bax and Bcl-2 ........................... 107
2.1. Silencing of Survivin-WT and Survivin-ΔEx3 in RB cell line (Y79 cells) ...... 108
2.1.1. Maintenance of Y79 cell lines ................................................................. 108
2.1.2. qRT-PCR for survivin splice variants, Bax and Bcl-2 in Y79 cells .......... 108
2.1.3. Transfection of Sur-WT siRNA and Sur-ΔEx3 siRNA in Y79 cells ...... 109
2.1.4. qRT-PCR for Sur-WT and Sur-ΔEx3 silenced Y79 cells .......... 109
2.1.5. Immunoblotting for Sur-WT and Sur-ΔEx3 silenced Y79 cells ............ 109
2.1.6. Annexin V staining of the Sur-WT and Sur-ΔEx3 silenced Y79 cells ....... 110
2.1.7. Caspase 3/7 activity of the Sur-WT silenced Y79 cells ......................... 111
2.2. Statistical analysis .......................................................................................... 111
3. Chapter 3 .............................................................................................................. 114
3.1. INTRODUCTION ............................................................................................. 114
3.2. Hypothesis ....................................................................................................... 118
3.3. Aims .................................................................................................................... 118
3.4. MATERIALS AND METHODS .................................................................................. 119
3.5. RESULTS ............................................................................................................... 122
  3.5.1. NIS protein is localised in membrane and cytoplasm of RB tumors .......... 122
  3.5.2. Quantitative analysis of NIS by flow cytometry shows elevated expression in invasive tumors ................................................................. 125
  3.5.3. Western Blotting reveals glycosylation status of NIS in RB ....................... 130
  3.5.4. Quantitative analysis of NIS by flow cytometry in Y79 cells ................. 132
  3.5.5. Immunofluorescence shows NIS positivity in Y79 cells ....................... 133
  3.5.6. Western Blotting shows NIS expression at 75 kDa in Y79 cells .......... 135
  3.5.7. Endogenous level detection of Lf receptors in Y79 cells .......................... 136
  3.5.8. Uptake of three forms of bLf in Y79 cells by flow cytometry ............... 137
  3.5.9. Cellular uptake of three forms of bLf in Y79 cells by immunofluorescence 139
  3.6. Determination of NIS protein expression after bLf treatment .................. 141
3.7. DISCUSSION ..................................................................................................... 143
4. CHAPTER 4 ............................................................................................................. 152
  4.1. INTRODUCTION ................................................................................................. 152
  4.2. Hypothesis ........................................................................................................ 156
  4.3. Aims .................................................................................................................... 156
4.4. MATERIALS AND METHODS ............................................................................ 157
4.5. RESULTS ............................................................................................................. 162
  4.5.1. PART A: NIS gene expression via EoCAm specific promoter ................... 162
  4.5.2. Part B: NIS gene delivery via EpCAM targeting nanocomplex ............. 175
4.6. DISCUSSION ..................................................................................................... 187
5. Chapter 5 ............................................................................................................... 196
  5.1. INTRODUCTION ................................................................................................. 196
  5.2. Hypothesis ........................................................................................................ 201
  5.3. Aims .................................................................................................................... 201
5.4. MATERIALS AND METHODS ............................................................................ 202
5.5. RESULTS ............................................................................................................. 205
  5.5.1. Expression of survivin variants, Bax and Bcl-2 in RB tissues .............. 205
  5.5.2. Association with the clinicopathological characteristics of tumor ........ 207
  5.5.3. Correlation among the survivin variants, Bcl-2/Bax in RB .................. 215
  5.5.4. Survivin and its variants mRNA expression in Y79 cells .................... 217
5.5.5.  
*In vitro* silencing of Survivin-WT and Survivin-ΔEx3 in Y79 cells ..........219

5.5.6.  Inhibition of Survivin-WT and Survivin-ΔEx3 leads to significant induction of cell apoptosis .................................................................222

5.5.7.  Effects of Survivin-WT silencing on the splice variants ...............224

5.6.  DISCUSSION .................................................................................................224

CONCLUSION AND FUTURE PERSPECTIVES ..............................................235

APPENDIX .........................................................................................................241

REFERENCES ......................................................................................................249
List of Figures

Figure 1.1 Schematic representation of function of NIS .......................................................... 12
Figure 1.2 Schematic representation of NIS structure ............................................................... 13
Figure 1.3 Schematic representation of NIS expression detected in various normal cells and
cancer cells .................................................................................................................................. 18
Figure 1.4 Anatomical picture of an eye with retinoblastoma ................................................ 20
Figure 1.5 Genetics of heritable and non-heritable retinoblastoma ........................................ 22
Figure 1.6 Structure of Lactoferrin .......................................................................................... 31
Figure 1.7 Protein structure of EpCAM ................................................................................... 39
Figure 1.8 Schematic representation of survivin protein .......................................................... 46
Figure 1.9 Schematic illustration of 6 survivin splice variants containing common exon 1 and
exon 2 ...................................................................................................................................... 47
Figure 1.10 Schematic representation of molecular pathways of apoptosis ........................... 52
Figure 1.11 Chemical structures of PEI .................................................................................. 57
Figure 1.12 Schematic representation of delivery of DNA via polymer ................................... 58
Figure 1.13 Schematic illustration of SELEX methodology ...................................................... 64
Figure 2.1 Schematic representation of deparaffinisation of RB tumor sections .................... 81
Figure 2.2 SDS-PAGE showing the purified form of bLf ....................................................... 89
Figure 2.3 Iron content in bLf ................................................................................................ 90
Figure 3.1 Schematic representation of the NIS work performed in this study ..................... 119
Figure 3.2 Schematic representation of the bLf work performed in this study ..................... 120
Figure 3.3 Immunostaining of NIS protein in RB tumor tissues .......................................... 123
Figure 3.4 Immunostaining of NIS protein in different sets of RB tumor tissues ............ 124
Figure 3.5 Flow cytometric analysis of NIS in invasive RB tumors ..................................... 127
Figure 3.6 Flow cytometric analysis of NIS in non-invasive RB tumors ............................. 128
Figure 3.7 Computation of NIS positivity determined in flow cytometry ........................ 129
Figure 3.8 Western blot of NIS protein expressed in RB tumor tissues ............................. 131
Figure 3.9 Flow cytometric analysis of NIS in Y79 cells ..................................................... 132
Figure 3.10 Immunofluorescence of Y79 cells ..................................................................... 134
Figure 3.11 NIS protein is expressed in Y79 cells ............................................................... 135
Figure 3.12 Y79 cells have endogenous Lf receptors mRNA expression .......................... 136
Figure 3.13 Cellular uptake of Apo-bLf, Fe-bLf and Native-bLf in Y79 cells ........................ 138
Figure 3.14 Apo-bLf, Fe-bLf and Native-bLf bind to cell membrane and get efficiently
internalized by 30 min .............................................................................................................. 140
Figure 3.15 Western blot of NIS protein detected after three forms of bLf treatments in Y79
cell line ................................................................................................................................... 142
Figure 4.1 Schematic representation of the first part of the work performed in this chapter 158
Figure 4.2 Vector maps of plasmids .................................................................................... 159
Figure 4.3 Schematic representation of the cloning procedure employed in the current study
to generate EpCAM-NIS clone .............................................................................................. 160
Figure 4.4 Schematic representation of the second work performed in this chapter ............ 161
Figure 4.5 Schematic representation of synthesis of nanocomplex ..................................... 162
List of Tables

Table 1.1 Classification of intraocular and extraocular Retinoblastoma ........................................... 24
Table 1.2 Table showing surgical treatments for retinoblastoma with its advantages and disadvantages .......................................................................................................................................................... 25
Table 1.3 Table showing important non-surgical treatments for retinoblastoma with its advantages and disadvantages ........................................................................................................................................................................... 26
Table 1.4 Advantages and disadvantages of aptamers and antibodies ............................................. 63
Table 2.1 Characteristics of tumor cohort used in NIS expression study ........................................ 73
Table 2.2 Components of a mastermix for RT-PCR reaction .......................................................... 85
Table 2.3 List of primers used in the bLf study ................................................................................. 86
Table 2.4 List of primers used in the NIS study ................................................................................. 97
Table 2.5 Characteristics of tumor cohort used in survivin splice variants, Bax and Bcl-2 expression study ........................................................................................................................................................................... 103
Table 2.6 Cycling conditions for RT-PCR ....................................................................................... 106
Table 2.7 List of primers used in the survivin splice variants, Bax and Bcl-2 study ....................... 108
Table 3.1 Clinicopathological information of all RB tumors analysed for the study .................. 121
Table 3.2 Association of NIS immunoscore by IHC with clinicopathological features ............. 125
Table 3.3 Association of NIS positivity with clinicopathological features on the basis of flow cytometry ........................................................................................................................................................................... 126
Table 3.4 Association of NIS protein expression analysed in flow cytometry with clinicopathological features ........................................................................................................................................................................... 129
Table 4.1 Zeta potential of the PEI, PEI/NISpDNA complex and PEI/NIS/EpDT3 nanocomplex prepared in water determined by Zetasizer Nano ZS ......................................................... 180
Table 4.2 Biostability of the nanocomplexes .................................................................................... 180
Table 5.1 Histopathological features of RB tumor tissues analysed ............................................. 204
Table 5.2 Association of mRNA expression of survivin splice variants, Bcl-2 and Bax with gender and invasion status in RB tumors ........................................................................................................................................................................... 209
Table 5.3 Correlation among the expression levels of survivin variants, Bcl-2 and Bax in RB tumors ........................................................................................................................................................................... 216
Table 5.4 Spearman correlation coefficients of variables like survivin variants, Bcl-2 and Bax in RB tumors ........................................................................................................................................................................... 217
List of conference presentations


4) **J Samuel**, R K Kanwar, J R Kanwar, S Krishnakumar. “Sodium Iodide Symporter is differentially expressed in retinoblastoma and correlates with the aggressiveness of tumors”. Oral presentation at the International Society for Genetic Eye Diseases and Retinoblastoma (ISGEDR), Ghent, Belgium on 22/08/2013-24/08/2013. **Recipient of a travel grant of 1200$ and waiver of 400$ registration fee.**

Manuscripts under preparation


2) **J Samuel**, R K Kanwar, J R Kanwar, S Krishnakumar. “Study of survivin splice variants, Bcl-2 and Bax expression profiles in Retinoblastoma by Real Time PCR.”

ABBREVIATIONS

1. ATCC- American type culture collection
2. Bax- Bcl-2 associated X protein
3. Bcl-2- B-cell lymphoma 2
4. BSA- Bovine serum albumin
5. CSC- Cancer stem cell
6. CTCs- Circulating tumor cells
7. DAPI- 4,6-diamidino-2-phenylindole
8. DNA – Deoxyribonucleic Acid
9. DTT- Dithiothreitol
10. ECM- Extracellular matrix
11. EDTA- Ethylenediaminetetraacetic acid
12. EGFR- Epidermal growth factor receptor
13. ELISA- Enzyme linked immune sorbent assay
14. EpCAM- Epithelial cell adhesion molecule
15. FBS- Foetal bovine serum
16. FDA- Food and Drug Administration
17. FITC – Fluorescein isothiocyanate
18. HPV- Human papillomavirus
19. HSP- Heat shock protein
20. Ig- Immunoglobulin
21. IHC - Immunohistochemistry
22. kDa- Kilo Dalton
23. LNA- Locked nucleic acid
24. MB- Methylene blue
25. MMPs- Matrix metalloproteinases
26. mRNA- microRNA
27. MS- Mass spectrometry
28. NSCLC- Non-small-cell lung cancer
29. N-terminal- amino terminal
30. NIS-Sodium Iodide Symporter
31. OSCC- Oral squamous cell carcinoma
32. PBS- Phosphate buffered saline
33. PCR- Polymerase chain reaction
34. PF- Paraformaldehyde
35. PI- Propidium iodide
36. PLGA- Poly(D,L-lactic-co-glycolic acid)
37. PMSA- Prostate specific membrane antigen
38. RNA- Ribonucleic Acid
39. RT-PCR- Reverse transcriptase-polymerase chain reaction
40. SDS-PAGE- Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
41. SELEX- Systemic evolution of ligands by exponential enrichment
42. SEM- Standard error of the mean
43. shRNA- Short hairpin RNA
44. siRNA- Small interfering RNA
45. SNPs- Single nucleotide polymorphisms
46. Tm- Melting temperature
47. TNM- Tumor, nodal, metastasis
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Sodium Iodide Symporter (NIS), an intrinsic membrane glycoprotein is a key regulator of iodide access to thyroid gland. NIS co-transports active I$^-$ and Na$^+$ with the help of Na$^+/K^+$-ATPase for the normal thyroid function. NIS protein expression occurs not only in thyroid cells but also in other extra thyroidal tissues like salivary gland, gastric mucosa, lactating mammary gland, placenta and kidney. Its expression has been reported in thyroid cancer which is exploited for diagnostic imaging and molecular radionuclide-based therapy of differentiated NIS expressing thyroid cancer. Retinoblastoma (RB), a malignant tumor of the eye arising from the developing retina is the most frequent primary intraocular malignancy of childhood. Delay in diagnosis and lack of awareness about the disease in resource-poor countries ultimately leads to high mortality rate and poor rate of eye salvage. The primary treatment for RB is chemotherapy with a regimen of etoposide, vincristine and carboplatin. Apart from chemotherapy, radiotherapy is another effective mode in treating RB. RB is a radiosensitive tumor and brachytherapy, a mild form of radiotherapy is used to treat small RB tumors with the help of a plaque containing radioactive material. Radiotherapy can be mediated via NIS, the possibility of NIS presence in RB tumors was therefore explored in this study. Since many of the RB patients in India are diagnosed late which end up in enucleation, the study was aimed to explore the expression of NIS protein in Grade D and Grade E RB tumors. Thus, RB, a non-thyroidal malignancy was investigated for the expression of NIS in human RB tumor samples as well as in an established standard RB cell line (Y79). The presence of therapeutic protein like NIS in a non-thyroidal tumor like RB could open a lot of therapeutic opportunities.

NIS expression was analyzed in a cohort of 41 advanced stages RB tumor samples, classified as Grade D and E as per International Intraocular Retinoblastoma Classification (IIRC).
There were 23 Grade D and 18 Grade E tumors; 25 invasive and 16 non-invasive tumors within the cohort of 41 tumors. Invasive tumors analysed were either choroidal, optic nerve, scleral or with extra ocular invasion. Choroidal invasion was further divided into <3mm and >3mm invasion and optic nerve invasion as pre laminar, post laminar and surgical end of the optic nerve. In 21 tumor samples, NIS expression was analysed by immunohistochemistry, and in another 20 samples NIS protein expression was analysed by Flow cytometry. NIS protein expression was also studied by Western blotting in 6/41 tumor samples. NIS protein expression was observed in all 41 RB tumor samples. Membrane and cytoplasmic expression of NIS protein was prevalent in RB tumors when analysed immunohistochemically. Flow cytometry revealed that NIS protein expression was significantly higher in the RB cases of choroid and/or optic nerve invasion when compared to non-invasive tumors (P<0.05). Western blotting of NIS protein in RB showed bands at 75 kDa, 50 kDa and 25 kDa which revealed the different glycosylation status of the protein. Immunofluorescence, Flow cytometry and Western blotting studies in Y79 cell line also showed similar results to primary RB tumor samples. Immunofluorescence revealed cell surface as well as membrane staining in Y79 cells similar to IHC findings in tumor samples. Furthermore, a single protein band of 75 kDa was observed in Y79 on Western blotting revealing a differentially glycosylated status of NIS protein. Thus NIS is expressed in RB in various glycosylation forms. In conclusion, using RB tumor samples the presence of NIS protein in a non-thyroidal tumor has been demonstrated. This study being the first report for RB thus opens a lot of opportunity for further research in modulating NIS expression for therapeutic opportunities in RB tumors. From the translational perspective of these findings, it will be critical to determine in vivo, if the NIS expression in RB tumours is functional for a radioiodide based therapy.
In the past two decades, studies on NIS expression in many cancers (thyroidal and extrathyroidal) reveal that there are multiple regulatory levels of NIS functional expression. These include transcriptional (gene), translational, posttranslational, distribution to intracellular organelles and targeting to the plasma membrane. Use of several compounds with known tumor-inhibitory effects has been shown to partially succeed in inducing endogenous NIS expression in thyroid and breast cancers. Lactoferrin (Lf) is a glycoprotein with iron-binding ability. Being a component of innate immunity, it is known to present at higher concentrations in mammalian milk colostrum and exocrine secretions. Bovine lactoferrin (bLf) is a multifunctional protein with its emerging anti-cancer biodrug potential. It can act also as a cell growth/cell differentiating agent as well as a transcriptional agent. Studies from our laboratory have recently shown that internalization of bLf through receptor mediated endocytosis, into the cell and nucleus can regulate gene transcription. bLf’s effect on NIS expression is not known. It was hypothesized that bLf could upregulate the NIS protein expression. Since the status of lactoferrin receptors in Y79 cell line is not known, it was therefore aimed to determine first if Y79 cells express lactoferrin receptors. The mRNA expression of Lf receptor was detected in Y79 cells by qRT-PCR. Initial studies on Lf receptors in Y79 cells revealed prominently higher extent of endogenous expression of transferrin receptors and low-density lipoprotein receptor-related proteins-1 and -2 (LRP1 and LRP2). It was aimed further to test whether bLf in partially iron saturated (Native-bLf), iron saturated (Fe-bLf) (>97 % saturation) and iron free (Apo-bLf) forms internalized in Y79 cells. Interestingly, rapid internalization of all the three forms of bLf, Native-bLf, Fe-bLf and Apo-bLf at concentration of 12.5 nmol, in above 90 % of cells was observed, at 30 min incubation. Flow cytometric studies in Y79 cells also confirmed the uptake of three forms of bLfs at various concentrations. NIS protein expression after the three forms of bLf treatment was determined by Western blotting and found that the NIS protein expression was doubled.
in 50 nmol concentration of Fe-bLf and 25 nmol concentration of Apo-bLf. Although it was important to ascertain the functional activity of the bLf induced NIS, functional assay like non-radioactive iodide uptake assay could not be performed. Y79, being a suspension cell line and non-radioactive iodide uptake assay being a sensitive colorimetric assay performed usually in monolayer cultures, the technical difficulty of adding and removing successive reagents for the time dependant assay in suspension cell lines restricted the determination of functional activity of expressed NIS.

Since, the initial attempts to conduct iodide uptake assays for investigating functional status of NIS in RB cell line were unsuccessful, the focus of the study was shifted towards another strategy of overexpressing NIS through exogenous NIS gene delivery via receptor targeting. Therefore gene cloning experiments were performed to overexpress NIS gene in another extrathyroidal cancer cell line, MCF-7 (derived from a human breast cancer adenocarcinoma), as well as in Y79 cells for functional iodide uptake assay. Vectors containing CMV, and EpCAM specific promoter driven NIS gene were used to establish stable transfectants. However, the attempts of establishing stable Y79 transfectant overexpressing NIS were again unsuccessful in preliminary experiments because of the difficulty in establishing a stable transfectant in suspension cells.

An expression vector system with NIS gene and a tissue specific promoter, epithelial cell adhesion molecule (EpCAM) directed towards the EpCAM expressing tumor cells was cloned. EpCAM, a membrane glycoprotein is expressed at low level on the basolateral membrane in normal epithelial cells whereas expressed at high level on the apical membrane in cancer cells. This overexpression has been utilized in many cancers like breast cancer for receptor targeted therapeutic approaches. MCF-7 cells overexpress EpCAM therefore further studies were undertaken with this cell line. Although NIS is expressed endogenously in breast cancer, its expression is at insufficient level to exploit for radionuclide-based therapy. It was
hypothesized that if NIS was specifically overexpressed in breast cancer cells (MCF-7) via a tissue specific promoter (EpCAM) through receptor targeted gene delivery, it would induce functional activity of NIS leading to higher cellular iodide uptake. Stable transfectants of MCF-7, expressing NIS protein with CMV-promoter (used as a control) and EpCAM promoter were established. Real time PCR and Western blot analysis of the stable transfectants of MCF-7 confirmed significant overexpression of CMV-NIS over EpCAM-NIS at the mRNA (P<0.005) and protein levels due to constitutional activity of the CMV promoter. These stable transfectants, MCF-CMV-NIS and MCF-EpCAM-NIS were employed for non-radioactive iodide uptake study which is a sensitive colorimetric assay to evaluate the functional expression of NIS protein in these transfectants. Iodide uptake was specifically observed in both the transfectants and no iodide uptake was observed in the untransfected MCF-7 cells. The functionality of NIS in these transfectants was also proved by conventional radioactive iodide uptake studies using 4µCi/mL Na\textsuperscript{125}I. The uptake of non-radioactive iodide and radioactive iodide was significantly higher (P<0.05) in MCF-EpCAM-NIS cells than the MCF-CMV-NIS cells. The uptake of radioiodide by NIS was significantly (P<0.05) inhibited by Sodium perchlorate, an inhibitor of NIS. This shows that iodide uptake has been specifically via NIS. Efflux studies were performed and it was observed that 75% of the iodide has been released in first few min. This shows that the iodide taken up is associated with high efflux rate with minimal retention time. This proves the functionality of the NIS protein expressed by the stable transfectants. NIS overexpressing stable transfectants showed higher migratory ability than the control cells confirming the functional activity of overexpressed NIS and its role in cell migration. Altogether, the obtained results show that delivering NIS gene via a tissue specific promoter like EpCAM promoter seems to be the promising strategy for radionuclide based therapy in breast tumors.
Another attempt was made in delivering NIS plasmid DNA in EpCAM positive MCF-7 cell line via a nanoformulation using a polymer. Polyethylenimine (PEI) is a widely used polymer and a substitute for viral vectors for gene delivery. This study was further aimed to deliver NIS gene specifically to breast cancer cells (MCF-7) via PEI coated with EpCAM aptamer which served as a targeting ligand. It was hypothesised that the nanocomplex would specifically target breast cancer cells overexpressing EpCAM, a surface biomarker. Flow cytometry studies showed EpCAM aptamer (EpDT3)’s higher affinity towards EpCAM positive cells (MCF-7) and lower affinity towards EpCAM-low cells (MIOM-1) thus confirming EpDT3’s specificity. The nanocomplex was prepared by incorporating NIS pDNA inside PEI and by coating EpDT3 outside. The size and zeta potential of the nanocomplex was analysed by Zeta sizer and its shape and size by Transmission Electron Microscopy (TEM). NIS protein expression after gene delivery via nanocomplex was studied by Western blotting. Functional activity of the NIS gene delivered by the nanocomplex was also determined by both non-radioactive iodide uptake assays as well as by radioactive iodide uptake assay. Dynamic light scattering (DLS) revealed that the nanocomplexes were ~ 100 nm and with a zeta potential of -14.2 mV. The nanocomplexes remained stable in serum for 72 h at a pH of 7.0. Western blotting after transfection of nanocomplexed NIS gene showed overexpression of NIS protein. Non-radioactive and radioactive iodide uptake assays demonstrated significant increase (P<0.05) in the functional activity of NIS indicating the efficacy of nanocomplex to deliver NIS gene. Higher uptake of iodide by the cells following delivery of nanocomplex confirms the capacity of the delivery system for selective and efficient targeting. Thus, in this study, a PEI based nanocomplex was formulated which carries a therapeutic gene inside and delivers it specifically to EpCAM positive breast cancer cells. Based on these findings, it is concluded that the nanocomplex retains the integrity of NIS pDNA, leads to its functional activity and is efficient at targeting EpCAM positive
cancer cells. This can be a potential mode of NIS gene therapy via non-viral vectors in EpCAM positive non-thyroidal cancers.

In the last phase of the study, the gene expression status of important apoptotic regulatory molecules in RB tumors was analysed. Apoptosis is a natural process of programmed cell death which is regulated by both apoptotic and anti-apoptotic molecules. The important families involved in the apoptosis are Inhibitor of Apoptosis (IAP) and B-cell lymphoma 2 (Bcl-2) family. Since the endogenous expression of apoptotic regulatory genes have not been explored before in RB, this study hypothesized that the expression of Bax and Bcl-2 from Bcl2 family, and survivin and four of its variants from the IAP family would be associated with clinical parameters like histopathological high risk factors, and the gene showing dominant status could be targeted by siRNA technology. This study was aimed to investigate the mRNA expression of Bcl-2, Bax, survivin and its splice variants in a cohort of 20 RB tumors. The information about these molecules is still lacking as far as RB tumors and their progression is concerned. The differential expression of these apoptosis regulatory genes in relation to clinicopathological features of RB was analysed at mRNA level by Real Time PCR. Bcl-2 was expressed in all the RB tumors whereas Bax was expressed in 80% of the tumors analysed. The Bcl-2 expression was significantly higher (P<0.001) in RB and the Bcl-2/Bax ratio was remarkably higher in poorly differentiated tumors than well differentiated tumors, although no statistical significance was obtained. No positive or negative correlation between Bcl-2 and Bax with respect to their expression levels was found in RB tumors.

Among survivin and its variants, Survivin-wild type (WT) was found to be the predominant form and all the anti-apoptotic variants such as Survivin-ΔEx3 and Survivin-3β were highly expressed in the tumor samples studied compared to pro-apoptotic variants such as Survivin-
2β and Survivin-2α. A statistically significant higher expression was seen in RB tumor samples for Survivin-WT compared to Survivin-2β (P<0.05). A positive correlation with respect to the expression levels in tumor samples was observed among all the five variants of survivin. A statistical significant difference was found in Survivin-2α, Survivin-2β and Survivin-ΔEx3 for the differentiation of RB tumors (P<0.05). No significant correlation between Bcl-2/Bax and survivin was observed indicating that their expression levels are independent of each other. Considering the positive correlation of survivin members with RB tumor progression, it was further aimed to study the apoptotic effects on silencing the Survivin-WT and one of the anti-apoptotic splice variant, Survivin-ΔEx3 in Y79 cells. Silencing via siRNA technology of Survivin-WT in Y79 cell line showed higher pro-apoptotic effects and increased caspase 3/7 activity in Y79 cells because silencing of Survivin-WT induced silencing of all variants. No pro-apoptotic effects were observed on silencing of Survivin-ΔEx3 alone. The findings thus suggest that overexpression of both survivin along with its variants and higher Bcl-2/Bax ratio cause the inhibition of apoptosis and thereby lead to progression of RB cancer. This study also suggests targeting survivin (a validated cancer target) in RB could be a viable option for targeted therapy.

Taken together, it can be concluded from the entire study that for the first time expression of NIS in RB has been analysed and correlated with clinicopathological invasiveness of the tumor. This finding of a validated therapeutic gene such as NIS in RB can possibly pave the way for newer and specific treatment regimens in RB. The specificity of EpCAM based NIS gene therapy was demonstrated by employing breast cancer cell line as a proof of concept model via two modes 1) tissue specific promoter and 2) nanoformulation, both of which showed encouraging outcomes. The high prevalence of RB NIS expression in 100% of human samples studied indicates that it is upregulated with high frequency during malignant transformation of RB tumors, and therefore holds a potential diagnostic and therapeutic value
in RB. Moreover, delivering NIS gene through tissue specific promoter targeted delivery (to extrathyroidal cancers), it can serve both as a reporter and as a therapeutic gene, so that it is possible to image, monitor, and treat the tumors with radioiodide, just as in differentiated thyroid cancer. Importantly, further studies are needed to understand the functional NIS expression in RB cells and its regulatory factors. The radioiodide based therapeutic efficacy in RB will thus likely depend on manipulating NIS regulation. The NIS induction potential of emerging anti-cancer natural biodrug bLf needs to be investigated in detail for RB and other cancers.

In addition, this study also showed for the first time the upregulated expression of splice variants of survivin, Bax and Bcl-2 in RB tumors indicating their possible role in tumor progression and differentiation through apoptosis dysregulation. This study not only showed positive correlation of survivin and its variants with histopathological features of RB tumor but also presented survivin gene silencing as a viable option for targeted therapy in RB that should be investigated further in in vivo models at preclinical levels.
Chapter 1

Review of Literature
1. **Review of Literature**

1.1. **Sodium Iodide Symporter (NIS)**

Sodium Iodide Symporter (NIS) is an integral plasma membrane glycoprotein which plays a pivotal role in thyroid hormonogenesis by mediating iodide (I⁻) transport into thyroid cells. Since I⁻ is scarce in the environment, body has adapted to provide itself a sufficient I⁻ for normal thyroid function. This process of active I⁻ uptake in the thyroid is ensured by the NIS (Dai G, Levy O & N 1996; Smanik PA et al. 1996). This ability to concentrate iodide is utilized clinically for imaging and ablation of thyroid carcinomas by administration of radioactive iodide after thyroidectomy. Radioactive iodide therapy uses very small amounts of radioactive material to diagnose and treat abnormalities. Radioactive isotopes when administered in small dose are absorbed into the bloodstream in the gastrointestinal tract. This isotope is being concentrated to the specific target for its delivery and destroys the cells in the location. Radiodine imaging is an important diagnostic tool for the evaluation of well differentiated thyroid carcinoma. Apart from diagnosis, iodide uptake also permits treatment for thyroid cancers, using I-131(Kogai & Brent 2012). Under normal physiology, NIS also mediates active I⁻ transport into several extrathyroidal tissues, including lactating mammary gland, salivary glands and gastric mucosa. NIS cotransports two sodium ions (Na⁺) along with one I⁻. The driving force for the uptake process is transmembrane sodium gradient while electrochemical sodium gradient that allows NIS to be functional is maintained by Na⁺/K⁺-ATPase pump (Figure 1.1).
Figure 1.1 Schematic representation of function of NIS

*NIS transports 2 Na\(^+\) and 1 I\(^-\) into the cytoplasm simultaneously by utilizing the energy generated Na\(^+\)/K\(^+\) ATPase pump.*

1.1.1. **Cloning of NIS**

Cloning of human NIS (hNIS) gene was done by screening human thyroid cDNA library based on homologous sequence of rat NIS (rNIS) (Smanik PA et al. 1996) which in turn was cloned from the FRTL-5 Fisher rat thyroid cell line derived cDNA library in *Xenopus laevis* oocytes (Dai G, Levy O & N 1996). hNIS and rNIS exhibit 84% identity and 92% similarity between them according to aminoacid sequence analysis. Apart from rNIS and hNIS, mouse NIS (mNIS) and porcine NIS (pNIS) have also been isolated (Perron B et al. 2001; Pinke LA et al. 2001; Selmi-Ruby S et al. 2003). hNIS gene which is located at chromosome 19p12-13.2 consists of 15 exons intervened by 14 introns (Smanik PA et al. 1997). hNIS has 643 aminoacids which encodes a protein of predicted molecular mass of 69 kDa and a transcript of 3.7 kb while rNIS transcript is 2.8 kb and encodes a protein of 618 amino acids with a
predicted molecular mass of 65 kDa. The main dissimilarity between hNIS and rNIS is that there is an additional 5 amino acids in the extracellular loop between transmembrane domains XII and XIII and extra 20 amino acids in the cytoplasmic COOH terminus of hNIS. NIS protein contains 13 transmembrane domains with an intracellular carboxyl-terminus and an extracellular amino-terminus (Figure 1.2). In addition, loops between transmembrane domains I and II, III and IV, V and VI, VII and VIII, IX and X, and XI and XII are located intracellularly while loops between transmembrane domains II and III, IV and V, VI and VII, VIII and IX, X and XI, and XII and XIII are located extracellularly (Chung 2002).

Figure 1.2 Schematic representation of NIS structure

Schematic representation of NIS containing 13 transmembrane domains depicted in red colour. The three sites of N-linked glycosylation are depicted in green colour. The N-terminal is located extracellularly and C-terminal is located intracellularly (Micali et al. 2014)
1.1.2. Functions of NIS

NIS belongs to the solute carrier family 5 (SLC5A) or the sodium/solute symporter family (SSSF) (De la Vieja et al. 2000). Majority of the members in this family are symporters and transport different molecules such as sugars, amino acids, vitamins, urea and anions (Jung 2002; Wright 2013; Wright & Turk 2004). NIS like other members in the family uses a secondary active transport mechanism in order to translocate its substrate relies on the electrochemical gradient of Na\(^+\) as the driving force to transport iodide across the cell membrane. This electrochemical gradient of Na\(^+\) is maintained by the activity of Na\(^+\)/K\(^+\)-ATPase (Bizhanova et al. 2009). Electrophysiological analysis of NIS revealed the translocation of two Na\(^+\) down their electrochemical gradient with the simultaneous translocation of one I\(^-\) against its electrochemical gradient into cells (Figure 1.1). In addition, it has been reported that binding of Na\(^+\) to NIS occurs before iodide binding (Eskandari S et al. 1997). The affinity constants of Na\(^+\) and I\(^-\) were determined to be 30mM and 30µM respectively for rat NIS (Eskandari S et al. 1997). Apart from iodide, NIS can also transport other monovalent anions like perchlorate (ClO\(_4^-\)), astatine (At\(^-\)), rhenium (ReO\(_4^-\)), and technetium (TcO\(_4^-\)) (Dadachova E & N 2004; Eskandari S et al. 1997; Van Sande J et al. 2003; Zuckier LS et al. 2004). One iodide ion uptake by NIS is escorted by 162 molecules of water and one thiocyanate is escorted by 253 water molecules (Zeuthen, Belhage & Zeuthen 2006). Perchlorate can act as a competitive inhibitor for NIS mediated iodide transport. Furthermore, radioactive forms of astatine, rhenium, and technetium have important implications for NIS-mediated radionuclide imaging and therapy. It has been reported that high energy alpha particle-emitter At\(^{211}\) efficiently kills tumor than I\(^{131}\) for NIS-mediated radioiodide therapy (Petrich T et al. 2006). In normal thyroid, thyroid stimulating hormone stimulates NIS expression (Bizhanova et al. 2009; Schlumberger et al. 2007) by activating the thyroid stimulating hormone (TSH) receptor which in turn initiates a signaling cascade.
mediated by G-protein. This signaling cascade eventually increases NIS expression in the thyroid follicular cells and also modulates the plasma membrane targeting of NIS (Harun-Or-Rashid M et al. 2010; Hou, Bojdani & Xing 2010; Huang et al. 2011a).

1.1.3. **Post translational modifications of NIS**

Glycosylation sites on NIS were found to be conserved among species and three N-linked glycosylation sites on asparagines residues at position 225, 297 and 485 were reported (Levy O et al. 1998). Inside the extracellular loop between VI and VII lies position 225 whereas positions 485 and 297 are located between XII and XIII within the last extracellular loop. Mutant NIS with abolished glycosylated sites were functionally similar to the wild type NIS and did not affect surface trafficking of the NIS protein (Levy O et al. 1998).

Phosphatidylinositol 3-kinase (PI3K) pathway activation in MCF-7 cells lead to increased expression of underglycosylated NIS protein with no trafficking to the plasma membrane (Knostman et al. 2007). In another study, it was revealed that in thyroid cells, NIS regulation is affected by MEK (mitogen-activated protein kinase or extracellular signal-regulated kinase kinase) signaling (Vadysirisack et al. 2007) whereas a recent study in breast cancer showed MEK inhibition resulted in NIS lysosomal degradation (Zhang, Beyer & Jhiang 2013). Other than glycosylation, NIS is also a phosphoprotein and gets phosphorylated at positions S43, T49, S227, T577, and S581. Out of these sites, the potential sites which are responsible for NIS iodide transport velocity are S43 and S581 (Vadysirisack DD et al. 2007). Apart from glycosylation and phosphorylation, NIS also contains leucine motif which is responsible for oligomerization of NIS at the cell membrane. Four leucine residues at positions 199, 206, 213 and 220 present in transmembrane domain VI make up leucine zipper motif. One of the properties of NIS is its existence in different oligomeric forms which regulates targeting, stability and function (Sitte, Farhan & Javitch 2004; Veenhoff, Heuberger & Poolman 2002). An elaborate study by light scattering coupled with size exclusion chromatography on the
biochemical characteristics of NIS showed that NIS majorly exists as dimers or multimers and that at least one disulphide bond is involved in the dimeric formation (Huc-Brandt et al. 2011).

1.1.4. **NIS in thyroid cancer**

Of all human malignancies, thyroid cancer accounts for approximately one percent and is considered to be the most frequent endocrine malignancy (SI 2003). Out of various therapies for cancer, radioiodide therapy brings down the rate of recurrence and improves clinical outcome (Mazzaferri EL & SM 1994; Wartofsky L et al. 1998). For treatment of residual thyroid cancer, I\textsuperscript{131} is used for differentiated thyroid cancer which brings down the chances of recurrent cancer (Sawka et al. 2004). *The ability of the NIS to uptake iodide and retain it in thyroid by organification is the basis for radioiodide therapy in clinical use.* Thus NIS is used in the detection and targeted reduction of residual thyroid tumors in patients (EL 1999; Singer PA et al. 1996). Majority of the differentiated thyroid cancers has the ability to take up I\textsuperscript{131} because they express NIS. But, de-differentiated thyroid cancer poses a problem of no I\textsuperscript{131} uptake as it has cold nodules (Kogai, Taki & Brent 2006). Apart from radioiodide uptake, the radioiodide also has to be accumulated, and this accumulation is the outcome of iodide influx and efflux. *Due to dedifferentiation in some thyroid tumors, NIS expression/function is decreased and thus radioiodide therapy is rendered inactive* (Jhiang SM et al. 1998; Ringel MD et al. 2001; Ryu KY et al. 1999). In such cases the functional NIS expression has to be increased for effective radioiodide therapy. Functional NIS expression can be increased by increased levels of TSH (30U/L). A recent study reported recombinant human thyroid stimulating hormone (rhTSH) was used for enhancing the expression of functional NIS protein (Liu et al. 2011). This not only ensures radioiodide uptake but also stores iodide by increasing the thyroglobulin expression. However, many redifferentiating reagents like
Histone Deacetylase (HDAC) inhibitors, demethylating agents and retinoic acids are used to increase NIS expression and function in thyroid cancer cells (Kitazono et al. 2001).

1.1.5. **NIS in extra-thyroidal tissues**

NIS protein expression and NIS mediated radioiodide accumulation occurs not only in thyroid cells but also in few extra thyroidal cells (Figure 1.3) like salivary gland, gastric mucosa, lactating mammary gland, placenta and lacrimal glands (Cho JY et al. 2000; Jhiang SM et al. 1998; Josefsson M et al. 2002; Spitzweg C et al. 1999; Tazebay UH et al. 2000; Vayre L et al. 1999; Wapnir RA & S 2002). Though NIS is expressed functionally, these extra thyroidal cells can neither organify iodide for retaining the iodide nor the expressed NIS is responsive to TSH stimulation (N 1993). NIS is functionally expressed in the mammary gland during late pregnancy and lactation. The physiological importance of NIS expression in lactating mammary gland is to concentrate iodide in lactating milk for nursing newborns to synthesize their own thyroid hormone biosynthesis (N 1993). Like in mammary gland, hNIS is also expressed in salivary gland (Jhiang SM et al. 1998) and in gastric mucosa (Jhiang SM et al. 1998; Vayre L et al. 1999). The physiological significance of NIS expression in both salivary glands and stomach was suggested to be important for entero-thyroid circulation of iodide (Josefsson M et al. 2002). As functional NIS is expressed in these extra thyroidal tissues, administration of radioactive iodide for thyroid cancer treatment can result in damaging side effects like gastritis, sialadenitis and xerostomia (Van Nostrand D, Neutze J & F 1986). A natural antimicrobial system called lactoperoxidase system producing H₂O₂, hypoiodite (IO-) with bactericidal activities, is also expressed in NIS-expressing extra thyroidal tissues (Bosch, Van Doorne & De Vries 2000).
1.1.6. **NIS as a reporter gene**

The first study used thyroid cancer cells at *in vivo* level to experiment the NIS transgene as a non-invasive molecular imaging tool (Shimura et al. 1997). Following this study, various studies were then performed with non-replicating adenovirus in cervical cancer (Boland et al. 2000) and engineered oncolytic RNA virus in myeloma using NIS as an imaging gene (Dingli et al. 2004). Recent use of NIS as an imaging gene has been the focus of gene therapy research related to cardiovascular diseases. One of these studies showed that in NIS transfected hearts, imaging was possible using $^{99m}$TeO$_4$ and I$^{123}$ scintigraphy (Miyagawa et al. 2005). Another group was able to quantify cardiac gene expression via micro-SPECT/CT imaging after NIS transfer in cardiac transplants (Ricci et al. 2008). NIS was also used as a reporter gene to track migration of immune cell like macrophage towards inflammation site (Seo et al. 2010).
1.1.7. NIS as a therapeutic gene

After the successful cloning of hNIS and the application of radioiodide in the treatment of thyroid cancer, many studies have stretched the use of NIS-mediated radioiodide therapy to non-thyroid cancers as well. *Various studies have shown transfer of NIS gene to different cancer cells like colon cancer, breast cancer, glioma, melanoma, pancreatic cancer, myeloma, prostate cancer, ovarian cancer and follicular thyroid carcinoma* (Boland A et al. 2000; Dingli D, Russell SJ & 3rd 2003; Dwyer RM et al. 2006; Dwyer RM et al. 2005; Groot-Wassink T et al. 2002; Mandell RB, Mandell LZ & Jr 1999; Petrich T, Knapp WH & E 2003; Shen DH et al. 2004; Sieger S et al. 2003; Spitzweg C et al. 2000). Transfecting NIS gene to cancer cells has to be specific in order to decrease the effect of radioiodide on the surrounding normal cells. For this reason, selective targeting of NIS to cancer cells was accomplished by using tissue specific promoters. Such selective targeting study was done in prostate adenocarcinoma LNCap cells, which were transfected with NIS gene driven by prostate-specific antigen (PSA) promoter. This resulted in prostate specific NIS expression, radioiodide accumulation and eventually an increased cancer cells specific cytotoxicity (Spitzweg C et al. 2000). In order to enhance iodide retention time in cells that have no NIS expression, thyroperoxidase (TPO) was co-expressed with NIS (Huang M et al. 2001). NIS as a therapeutic gene offers effective radioiodide therapy in thyroid as well as in non-thyroid cancers. Thus NIS with its diagnostic ability as well as with its therapeutic ability holds great possibilities in the clinical front.

1.2. Retinoblastoma

Retinoblastoma (RB), a malignant tumor of the eye arising from the developing retina is the most common primary intraocular malignancy of childhood (Figure 1.4). The estimated worldwide occurrence of RB is between 1 in 15,000 to 20,000 live birth children independent of ethnic group and sex (Broaddus E, Topham A & AD 2009; Suckling RD et al. 1982). In
most cases, diagnosis is made under the age of five years. In adults, RB is very rare but has been reported in certain cases (Biswas J et al. 2000). It is suggested that retinoblastoma diagnosed after childhood age develop from preexisting progenitor lesions. RB is the first disease in clinical history for which a genetic etiology of cancer has been described (Saxena P & J 2011). Furthermore, RB1 gene is also the first tumor suppressor gene to be identified (Jr 1971).

Figure 1.4 Anatomical picture of an eye with retinoblastoma.

*RB picture (H&E stained) taken at the Ocular Pathology Laboratory, Sankara Nethralaya, India (Vandhana S et al. 2011)*
1.2.1. Heritable versus non-heritable RB

Majority of the patients (60%) develop RB in one eye only (unilateral RB) with occasional occurrence of multiple tumor foci (unilateral multifocal RB). In the rest 40% of the cases, both eyes are affected (bilateral RB) generally with more than one focus per eye (bilateral multifocal RB). In children, usually bilateral disease is diagnosed earlier than unilateral disease (Bishop JO & EC 1975). Biallelic RB1 gene inactivation has to occur for both heritable and non-heritable RB to develop. The first mutation (M1) of the RB1 gene is constitutional whereas the second mutation (M2) arise in somatic retinal cells, thus giving arise to heritable RB (Dimaras H et al. 2012). When both RB1 mutational events (M1 and M2) occur in one somatic retinal cell causing biallelic RB1 loss, it leads to the non-heritable RB (Figure 1.5). In addition to developing new tumors until retinal differentiation stops, such children are also at the risk of developing extraocular tumors throughout their lives (Eng C et al. 1993; Mohney BG et al. 1998; Wong FL et al. 1997). A person is prone to secondary cancers such as brain, skin, lungs if he/she carries a constitutional mutation of RB1 gene (Eng C et al. 1993). Few cases of bilateral RB also develop as trilateral RB (pinealoblastoma). Trilateral RB arises at the pineal region of the brain and is associated with intracranial neuroblastic tumor (Kivelä 1999). Most of the unilateral RB cases are sporadic while majority of the bilateral RB cases are also sporadic and the remainder bilateral RB will have familial RB i.e., positive family history. Familial disease in a relative of children with RB is indicated by the presence of retinal scars or quiescent tumors (retinomas) which is identified by the examination of the fundus of the eye in all first degree relatives of children with RB (Gallie BL et al. 1982).
Heritable RB occurs when both M1= constitutional RB1 mutation & M2=somatic RB1 mutation occurs, whereas Non-heritable RB occurs when both the mutation occurs in the somatic cell.

1.2.2. Genetics of retinoblastoma

Occurrence of RB is explained by Knudson’s “two hit hypothesis” which points out that both copies of the RB1 gene has to be mutationally inactivated in the same developing retinoblast which would result in RB (Jr 1971; Knudson AG Jr, Hethcote HW & BW 1975). In the hereditary form one copy of the RB1 gene is already mutated in the germ line and the next mutation i.e. “second hit” which occurs frequently during retinal development will inactivate the second copy of the RB1 gene. Another problem of the inherited mutation is that it is more likely to give rise to cancer in other tissues. When compared to hereditary form, somatic
mutations must occur in both alleles of the \textit{RB1} gene in the same retinoblast for nonhereditary RB. The \textit{RB1} gene which encodes for an important tumor suppressor protein is present on chromosome 13q14 and in normal conditions functions to inhibit cancer not only in the eye but also throughout the body (Bartek J, Bartkova J & J 1997; Friend SH et al. 1986; Fung YK et al. 1987). It was found that though loss of \textit{RB1} gene can commence retinoma, it was inadequate for the growth of RB (Dimaras H et al. 2008). For the development of retinoma to RB, multiple genetic changes or mutational events like M3-Mn apart from mutation of both RB1 alleles (M1 and M2 mutation) is required (Corson TW & BL 2007; Dimaras H et al. 2008). The major genetic changes or mutational events that occurs are periodic gains and losses of chromosomes 1q,2p,6p and 13q,16p respectively which ultimately lead to RB (Corson TW & BL 2007). Recent studies have shown the correlation of \textit{RB1} gene mutational status with clinicopathological features of RB (Mohammad Javed Ali et al. 2010) and also its use in genetic counselling (Abidi et al. 2011). The other functions of \textit{RB1} gene apart from its involvement in RB development are management of important processes like miRNA regulation, DNA methylation and histone modification (Benetti R et al. 2008; Chi P, Allis CD & GG 2010; Lu J et al. 2007 ; Wen H et al. 2008 ).

\textbf{1.2.3. Diagnosis and classification}

Based on the location of the tumor, the signs of RB are leukocoria, strabismus, red eye, nystagmus (Abramson DH et al. 2003). Retinoblastoma has varying growth patterns with exophytic tumors growing into the subretinal space under the retina and endophytic tumors growing into the vitreous (Abramson DH et al. 2003). In 1960s, the Intraocular RB tumors were classified using Reese-Ellsworth (R-E) classification based on size and location of tumors (REESE AB & RM 1963). Later, international classification of retinoblastoma developed for intraocular tumors because of chemotherapy, which gave prominence on intraocular seeding (A 2005). For extraocular tumors, International RB staging system was
proposed for uniform staging of patients with RB to cover disease’s entire (Chantada G et al. 2006) (Table 1.1).

### Table 1.1 Classification of intraocular and extraocular Retinoblastoma

<table>
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<tr>
<th>Classification</th>
<th>Stages</th>
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<tbody>
<tr>
<td><strong>Intraocular</strong></td>
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<tr>
<td>Reese-Ellsworth</td>
<td>Group 1 Group 2 Group 3 Group 4 Group 5</td>
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<tr>
<td></td>
<td>Small tumor less than 4 disc diameters</td>
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<tr>
<td><strong>Intraocular</strong></td>
<td></td>
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<tr>
<td>International Classification of Intraocular Retinoblastoma</td>
<td>Group A Group B Group C Group D Group E</td>
</tr>
<tr>
<td></td>
<td>Small tumors less than 3 mm</td>
</tr>
<tr>
<td><strong>Extraocular</strong></td>
<td></td>
</tr>
<tr>
<td>Stage 0</td>
<td>Stage I Stage II Stage III Stage IV</td>
</tr>
<tr>
<td>International Retinoblastoma Staging System</td>
<td>Includes patients treated conservatively</td>
</tr>
</tbody>
</table>

### 1.2.4. Treatment and management

Untreated RB tumors metastasizes via the optic nerve to central nervous system, through lymphatics to lymph nodes and through blood to bone marrow (Meel R, Radhakrishnan V &
S 2012). There are various treatments available for RB once the initial diagnosis has been
done. These treatments mainly depend on various factors such as age of the child, tumor size,
stage, localization, presence of vitreous seeds etc. The primary goal of treatment in RB
management is child survival followed by saving vision and globe diagnosis at an early
period helps in decreasing the mortality of the disease (Leander C et al. 2007). Currently,
various treatments are available for RB which includes enucleation, laser photocoagulation,
external beam radiotherapy, cryotherapy, brachytherapy and chemotherapy (Table 1.2 &
1.3). Frequent follow up examinations are required even after successful treatment of RB.
Retinoblastoma treatments are widely divided into three types. 1) Surgery 2) Chemotherapy
and 3) Radiotherapy.

Table 1.2 Table showing surgical treatments for retinoblastoma with its advantages and
disadvantages

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Used for</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enucleation</strong></td>
<td>Advanced RB (Group E eyes)</td>
<td>Prevents systemic metastasis</td>
<td>Loss of vision</td>
<td>(Wattiaux et al. 2000)</td>
</tr>
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<tr>
<td><strong>Thermotherapy</strong></td>
<td>Small tumors with no vitreous or subretinal seeds</td>
<td>Provides complete regression of tumors</td>
<td>Major complications like peripheral focal lens opacity and focal iris atrophy</td>
<td>(Shields CL et al. 1999)</td>
</tr>
<tr>
<td><strong>Cryotherapy</strong></td>
<td>Small anterior RB tumors</td>
<td>Rapid necrosis of tumor tissue</td>
<td>Increased risk of developing myopia, retinal detachment and shrinkage of sclera</td>
<td>(Shields et al. 1989)</td>
</tr>
<tr>
<td><strong>Laser photocoagulation</strong></td>
<td>Small posterior RB tumors</td>
<td>Acceptable option for selected intraocular tumors</td>
<td>Retinal detachment and retinal ischaemia</td>
<td>(Behr &amp; Jean-Paul 1997)</td>
</tr>
</tbody>
</table>
Table 1.3 Table showing important non-surgical treatments for retinoblastoma with its advantages and disadvantages

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Used for</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systemic</strong></td>
<td>Systemic chemotherapy</td>
<td>Reduces the risk of metastasis</td>
<td>Not suitable for advanced tumors as it is non-responsive</td>
<td>(Mintzer &amp; Simanek 2008; von Gersdorff et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>Group A/B retinoblastomas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Intra-arterial</strong></td>
<td>Less advanced cases of retinoblastoma</td>
<td>Regression of subretinal seeds and vitreous seeds</td>
<td>Mild complications like eyelash loss and forehead hyperemia</td>
<td>(Abramson et al. 2008)</td>
</tr>
<tr>
<td><strong>Chemotherapy</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td><strong>Brachytherapy</strong></td>
<td>Usually for intraocular melanoma, selective for retinoblastoma</td>
<td>Effective in treating tumors with no vitreous or subretinal seeding</td>
<td>Cataract and radiation retinopathy</td>
<td>(Shields et al. 2001)</td>
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<td></td>
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<tr>
<td><strong>External beam</strong></td>
<td>Large or multiple tumors unsuitable for other therapies</td>
<td>Effective in preserving vision in eyes</td>
<td>Facial deformity, retinopathy and neovascular glaucoma</td>
<td>(Chan et al. 2009)</td>
</tr>
<tr>
<td><strong>Radiotherapy</strong></td>
<td></td>
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</tr>
</tbody>
</table>

* Carboplatin, Vincristine, Etoposide and Melphalan are mainly used

1.2.5. **Surgical treatments**

a) **Enucleation**: Enucleation is carried out for unilateral group E intraocular retinoblastoma and also in cases where chemotherapy has failed.

b) **Thermotherapy**: Thermotherapy is a type of treatment where the tumor is relatively small and they are heated upto 42-60 degrees. Multiple techniques are followed for tumor ablation, whether it is ≤3 mm or greater than 3 mm resulting in regression in
86% of tumors (Shields CL et al. 1999). When the tumors are large, they require rigorous treatment resulting in ocular complications like lens opacity.

c) **Cryotherapy:** In the cryotherapy treatment, tumors are subjected to -90°C and are allowed to freeze which causes crystal formation, cell rupture, and damage to vascular endothelium. Usually tumors that are less than 3 mm are used for this therapy and are cured completely (Abramson, Ellsworth & Rozakis 1982). No serious aftereffects apart from shrinkage of sclera and development of myopia are observed for this treatment (Shields et al. 1989).

d) **Laser photocoagulation:** This technique is used for tumor up to 2 mm in height. Argon (532 nm) laser is used to coagulate blood supply to the tumor. At monthly intervals, multiple sessions of few seconds of 350 mW are given to coagulate the blood supply (Chawla, Jain & Azad 2013). The aftereffect of laser therapy is that it may lead to retinal ischemia (Murphree et al. 1996).

**1.2.6. Chemotherapy**

In advanced tumor cases, intravenous chemotherapy is used. To decrease the probability of trilateral RB in the cases of hereditary retinoblastoma, multiple infusions of chemotherapeutic drugs like carboplatin, vincristine and etoposide are administered (Villegas VM et al. 2013). Apart from the three main drugs, other chemotherapeutic drugs that are in use for RB treatment are cyclophosphamide, melphalan and thiotepa (Meel R, Radhakrishnan V & S 2012).

a) **Neoadjuvant chemotherapy:** Neoadjuvant chemotherapy is used to reduce the tumor bulk before going for radiotherapy or enucleation. Standard doses of vincristine etoposide and carboplatin are used for neoadjuvant chemotherapy and adjuvant chemotherapy (Meel R, Radhakrishnan V & S 2012).
b) **Chemoreduction:** Sufficient reduction in tumor demands at least six cycles of chemotherapy. Anything less than six cycles results in recurrence of subretinal seeds and recurrence of tumor. A combinational treatment of chemotherapy and focal consolidation therapy has been reported to be more potent in tumor management than chemotherapy alone (Gombos DS et al. 2002; Rodriguez-Galindo C et al. 2003; Schefler AC et al. 2007; Shields CL et al. 2006; Shields CL et al. 2004).

c) **Periocular chemotherapy:** Studies on local chemotherapy for treatment are necessary to overcome the systemic side-effects of systemic chemotherapy. Systemically administered drugs have limited entry to avascular structures like sub-retinal and vitreous spaces leading to recurrence of subretinal and vitreous seeds (Shields et al. 2002). Studies have shown that locally delivering carboplatin to periocular structures in an aqueous solution had therapeutic effect in group C and D RB along with adverse effects (Kiratli, Kocabeyoğlu & Bilgiç 2007; Mulvihill et al. 2003; Schmack et al. 2006). Currently clinical trials are on to deliver carboplatin to periocular space where there is a controlled release of the drug (Lin & O’Brien 2009; Van Quill et al. 2005).

d) **Intraarterial chemotherapy:** Earlier studies showed an encouraging outcome after injecting melphalan intraarterially in patients with vision stabilizing (Abramson et al. 2008). But later studies cautioned about carcinogenic effects resulting from several sessions of intraarterial chemotherapy (Vijayakrishnan et al. 2010). In another study, intra-arterial chemotherapy, a local chemotherapy was performed with melphalan drug (Vajzovic LM et al. 2011). Though, there was tumor reduction in 75% eyes, it presented with adverse effects like myostitis, vitreous hemorrhages and high-risk characteristics (Vajzovic LM et al. 2010). Another study with intra-vitreal administration of melphalan (Ghassemi F & CL 2012), showed better tumor control but the higher concentration (50µg) of melphalan caused hypotonia and phthisis bulbi.
1.2.7. Radiotherapy

Radiotherapy is effective in treating RB as it is a radiosensitive tumor.

a) Brachytherapy: Plaque or brachytherapy is known as internal radiation therapy usually performed for small tumors. Radioactive material is placed in a plaque made of gold or lead and the plaque is sewn near the tumor. Brachytherapy is not associated to the risk of secondary cancers (Shields et al. 2001).

b) External Beam Radiotherapy (EBRT): EBRT is the most effective therapy in treating multiple tumors. Over a period of five weeks, a dose of 45 Gray is given to the patient. This treatment is associated with ocular damage and rise of secondary cancers (Roarty, McLean & Zimmerman 1988). However, in a recent study, EBRT has been termed as a highly potent therapy in controlling tumors and preserving vision (Chan et al. 2009).

1.2.8. Targeted therapy - Potential pre-clinical application

Although different treatments are available for RB, newer treatment modalities are always in demand which can overcome the side effects of the current treatments. Newer treatments include newer molecules exclusively expressed in RB which can be targeted for therapy. As a first report from our lab, in a cohort of 43 RB tumor samples, epithelial cell adhesion molecule (EpCAM) was found to be expressed in all the tumors (Krishnakumar S et al. 2004), and this overexpression was then exploited for delivering an EpCAM siRNA via a gold nanoparticle coated with EpCAM antibody in RB cells (Mitra et al. 2013). More recently, the EpCAM molecule was targeted in another study in RB to deliver doxorubicin specifically to EpCAM positive cells thus inhibiting cellular proliferation (Subramanian et al. 2012). In another study in RB, it was shown that overexpression of T-cell lymphoma invasion and metastasis 1 (Tiam1) correlated with the invasion of the tumors (Adithi et al. 2006) and this was proved in a subsequent study where the silencing of Tiam1 in RB results in
modulation of cell invasion. This study thus provided evidence of using Tiam1 as one of the potential target for therapy (Subramanian et al. 2013). Another potential target explored in RB was High Mobility Group AT-hook 2 (HMGA2) (Venkatesan et al. 2009) and its silencing resulted in regulatory changes in RB cells thus showing a potential for targeted therapy (Venkatesan et al. 2012). Role of miRNA such as, miR 17-92 cluster in invasion was shown in RB and therefore it was suggested that targeting this particular cluster would benefit in the therapy of RB (Kandalam MM et al. 2012). A miRNA profiling of RB patients serum sample revealed important miRNAs involved in RB progression and it was thus inferred that their corresponding target genes could provide information about prospective biomarkers in RB (Beta et al. 2013).

1.3. Lactoferrin

Lactoferrin (Lf) is a glycoprotein present in bodily secretions and plays a key role in natural defence of the body. It is a ~78-80 kDa single-chain iron binding multifunctional protein which regulates iron absorption in the intestine (Kanwar RK & JR 2013). Apart from regulating iron absorption, it also regulates immune responses, renders protection to the body against microbial infection and enhances intestinal cell growth (Kanwar et al. 2008). Bovine lactoferrin (bLf) from the milk is also reported to have anti-carcinogenic, anti-inflammatory, anti-microbial and immunomodulatory properties (Kanwar RK & JR 2013). Recent research on bLf has shown its potential to become one of the safest natural food for cancer therapy (Berlutti et al. 2011; Gibbons, Kanwar & Kanwar 2010; Kanwar et al. 2014; Tsuda et al. 2010).

1.3.1. Structure of Lf

Lf protein consists of 690 amino acids and is folded into a homologous N and C terminal lobes each containing two domains namely N1 & N2 and C1 & C2 respectively. The domains contain an iron-binding site and a glycosylation site (Baker & Baker 2005). The N lobe and C
lobe are joined by a unique 10-15 residue, alpha helical peptide (Figure 1.6). Lf also has strong binding affinity towards ferric ion (Fe$^{3+}$) and carbonate ion (CO$_3^{2-}$) which renders antioxidant property to the molecule (Mulder et al. 2008).

Figure 1.6 Structure of Lactoferrin

A polypeptide chain showing two symmetrical lobes: N-lobe and C-lobe. The two lobes are connected via a hinge region containing α-helix which renders flexibility to the molecule. Within each lobe there are two domains depicted as N1, N2, C1 and C2 (García-Montoya et al. 2012).

1.3.2. **Forms of Lf**

Lf in its natural state has less iron content (around 15-20%) and this allows the molecule to strive for iron from outside its environment (Tsuda et al. 2004). There are three states of iron saturation for Lf 1) Apo-Lf which is iron free, 2) monoferric form of Lf containing only one
Fe$^{3+}$ ion and 3) hololactoferrin form which is has two Fe$^{3+}$ ions (Baker & Baker 2005; Kanwar RK & JR 2013). Lf has high affinity for iron mainly because of the presence of 4 protein ligands which impart negative charges to bind Fe$^{3+}$. To balance the binding of CO$_3^{2-}$ ion binding, an Arg side chain and a helix N-terminus is present. Apart from Fe$^{3+}$ binding, Lf has affinity for various other metals like selenium (Se), Gallium (Ga$^{3+}$) and Aluminium (Al$^{3+}$) (Baker & Baker 2005). Lf is internalized into the cells via the lactoferrin receptors (LfR), transferrin receptors, and also the low-density lipoprotein receptor-related proteins −1 and −2 (LRP1 and LRP2) (Naot et al. 2005). Fe-bLf nanocomplexes is shown to internalize via Lf, transferrin and LRP receptors in a recent study from our lab (Kanwar et al. 2014).

1.3.3. Potential benefits of bLf in cancer medicine

1.3.3.A. Anti-cancer effects

bLf’s potential as an anti-cancer agent has been studied in various types of cancers both at in vitro as well as at in vivo level. In lung cancer, tumor suppression was observed in transgenic mice expressing hVEGF-A165 after oral administration of bLf at 300mg/kg of body weight for 6 weeks. Apart from the tumor suppression, level of tumor necrosis factor-α and other anti-inflammatory cytokines like IL-4 and IL-10 was significantly decreased after treatment with bLf (Tung et al. 2013). Similarly, in a murine model of squamous cell carcinoma and fibrosarcoma, bLf showed efficient reduction in tumor growth after direct administration to the tumors (Wolf et al. 2007). In colon carcinoma, combination of azoxymethane and bLf showed better treatment effect than azoxymethane alone (Tsuda et al. 1998). Apart from bLf, human Lf (hLf) treatment in breast cancer cells also showed effective growth arrest of cancer cells at G1 to S transition phase of the cell cycle (Damiens et al. 1999). Studies from our lab have established bLf as a natural product with anti-cancer effect in various cancer models (Gibbons, Kanwar & Kanwar 2010; Kanwar, Mahidhara & Kanwar 2012; Kanwar et al. 2014). One report from our lab has shown that when different stages of iron saturated bLf
was administered to a C57BL/6 mice carrying tumor, the high iron content form of bLf showed higher activity in augmenting cancer chemotherapy (Kanwar et al. 2008). In another study, anti-oxidative effect of bLf was established in H₂O₂ exposed colon cancer cell line, HT29 when Apo-bLf and 100% Fe-bLf treatment showed an increase in antioxidant enzymes (Burrow, Kanwar & Kanwar 2011). Thus the above study proved the anti-oxidative effect of bLf in cancer.

**1.3.3.B. bLf as transcriptional activator**

Lf on binding to the cell surface enters inside the cell through Lf binding sites (LBS) and binds to the nucleus (Garré et al. 1992). There are specific LBS like GGCACCTTGC which is known to activate the transcriptional activity of a reporter gene containing the sequence (He & Furmanski 1995). Out of the two domains of Lf, highly charged N-terminal domain is responsible for binding to DNA (Kanyshkova et al. 1999). There are other recent studies which report that the Lf after binding to nucleus induces changes at the genetic level such as modulating the activity of immune and epithelial cells (Gifford, Ishida & Vogel 2012). Lf is also considered to be a stimulator for cellular growth (Cornish et al. 2004). In a recent study from our lab, Fe-bLf was shown to possess therapeutic effect on mice for osteoarthritis by dissolving calcium pyrophosphate crystals in joints thus proving anti-arthritic effect of Fe-bLf (Samarasinghe, Kanwar & Kanwar 2014).

**1.4. Breast cancer**

Breast cancer is a global disease affecting 1.38 million women per year worldwide (Ferlay et al. 2010). In India, the survival rate of breast cancer patients at 10 years is 77% (Dinshaw et al. 2005). In Australia, the chance of women developing breast cancer in their lifetime is 1 in 8 (Australian Institute of Health and Welfare & Cancer Australia 2012. Breast cancer in Australia: an overview 2012).
1.4.1. **Risk factors**

Age primarily decides the incidence of occurrence of breast cancer among women. The rate of increase in breast cancer slows down after menopause which shows that it is hormone dependent. African Americans suffer from lower survival rate owing to the excessive rate of poorly differentiated tumors than the white population, thus making race or ethnicity as one of the determinants for higher risk for breast cancer (Libson & Lippman 2014). External factors also increase the risk of breast cancer. One of them is the hormone replacement therapy which doubled the risk of breast cancer when it was given along with progestin and oestrogen (Chlebowski et al. 2009). Exposure to radiation and alcohol consumption also increase the likelihood of breast cancer. Younger women are more prone to radiation related risk (Hancock, Tucker & Hoppe 1993) whereas increase in 10g/day of alcohol presents a 9% chance of breast cancer (Smith-Warner et al. 1998).

1.4.2. **Breast cancer therapy**

1.4.2.A. **Surgery**

Ablation of breast and lymph nodes known as “Radical mastectomy” was practiced early in the 19th century which increased the survival rate but later in the 20th century, much benign method was practised where only a part of the breast was removed. Mastectomy leads to the complete removal of the breast which disfigured the body, and this problem was later on addressed via the breast conservation methods. In the breast conservation methods, only tumors were curtailed using neoadjuvant therapy and for tumors that do not respond to chemotherapy, oncoplastic surgery was practised (Fisher et al. 2002).

1.4.2.B. **Radiation**

Radiation therapy not only reduces the mortality rate but also decreases recurrence (Clarke et al. 2005). The overall survival in high risk patients after partial mastectomy increased with a
follow up of radiation therapy (Group 2011). Treatment with radiation is well planned and given in tangential angles to avoid short-term and long-term effects.

1.4.3. **Chemotherapy**

Breast cancer is a systemic disease and adjuvant therapy is given for patients where there is a chance of relapse. The drugs that are administered for chemotherapy are anthracycline based drugs, cyclophosphamide, methotrexate and fluorouracil (CMF) which decreased the mortality among breast cancer patients (Group 2012). Neo-adjuvant chemotherapy prior to surgery gives the advantage to assess the tumor *in vivo*, and also to curtail the size of the tumor thus enabling breast conservation. However, neoadjuvant therapy was found to have no effect on overall survival of patients (Wolmark et al. 2001).

1.4.4. **Targeted therapy**

The principle molecule in breast cancer which is targeted is human epidermal growth factor receptor 2 (HER2) because of its overexpression. In addition to chemotherapy, a monoclonal antibody, Trastuzumab targeting HER2 protein showed beneficial effects such as reducing the recurrence of the disease. Inspite of Trastuzumab reducing the recurrence by half, many patients still had progression in their disease. This unresponsiveness was because of the heterodimerisation of the HER2 members (Gajria & Chandarlapaty 2011). This dimerization was surpassed by introduction of another antibody called Pertuzumab (Libson & Lippman 2014). Various tyrosine kinase inhibitors like lapatinib (Blackwell et al. 2010), neratinib and afatinib are also in use for the treatment of breast cancer (Libson & Lippman 2014). Recently, clinical trials of an antibody-drug conjugate of Trastuzumab and Mertansine showed promising results of progression free survival of patients (Verma et al. 2012).
1.4.5. After effects of breast cancer treatment

Both radiotherapy and chemotherapy attributes to after effects in breast cancer. Radiotherapy is considered to be a better treatment in controlling locally progressed and early stage breast tumors but induces coronary artery disease. Earlier, high radiation treatment was given wide open to the entire chest area, but current radiation techniques are confined to a smaller portion of breast with little or no effects to the heart (Paszat et al. 1998). Congestive heart failure is the cardiovascular toxicity associated with the chemotherapy treatment. Treatment with the drug, Trastuzumab leads to cardiac malfunction but it becomes reversible once the administration is discontinued (Ewer et al. 2005). Another part of the body that chemotherapy affects is bone. In a trial premenopausal women who underwent chemotherapy for breast cancer was shown to have decreased bone mineral densitometry which increases the risk of fractures (Headley et al. 1998). Chemotherapy also affects the reproductive ability in women by exhausting the follicle pool in a dose dependent manner (Sonmezer & Oktay 2004).

1.4.6. Triple negative breast cancer

Triple negative breast cancer’s (TNBC) characteristics are under-expression of HER2 and absence of progesterone receptor (PR) and estrogen receptor (ER) (Schmadeka, Harmon & Singh 2014). In comparison to non-TNBC, the disease tend to metastasize quickly to central nervous system in TNBC patients (Rakha & Chan 2011). Around 75% of tumors from women with triple negative phenotype carries mutation in the BRCA1 gene (Foulkes, Smith & Reis-Filho 2010). The treatment options for TNBCs are limited as they are non-responsive even for the highly potent treatments given for non-TNBCs. Proliferative index of the TNBC tumors play a role in prognosis of disease as higher proliferation rate shows higher pathologic complete response (pCR) whereas lower pCR is associated with lower proliferation rate (Keam et al. 2011). Extensive studies on TNBCs have revealed subtypes such as Basal-like (BL), Immunomodulatory (IM), Mesenchymal (M) and Luminal Androgen Receptor (LAR)
with specific genes expression which may be targeted for therapy (Schmadeka, Harmon & Singh 2014).

1.4.7. NIS based gene therapy

As mentioned earlier in section 1.1.5, NIS is expressed in lactating mammary gland which eventually led to the discovery of NIS expression in more than 80% of breast cancer (Tazebay et al. 2000). Unlike membrane expression of NIS in lactating breast, NIS expression was more in the cytoplasm in the cancer tissues. Cysteinyl-tRNA synthetase gene was found to be associated with the membrane targeting of NIS in estrogen receptor positive breast cancer subtype. Thus, different genes could be responsible for membrane targeting of NIS in different subtypes of breast cancer (Beyer et al. 2011b). A study on TNBC’s revealed 65% of them showing a positive NIS expression (Renier et al. 2010). As radioiodine uptake of NIS is concerned, a recent study showed not only positive NIS gene expression but also radioiodine uptake by 50% of breast cancer tissues (Damle, Narkar & Badwe 2011). Out of twenty three metastatic breast cancer patients, eight showed NIS expression and two out of eight showed positive iodide uptake. This shows that only a fraction of breast cancer metastatic cases can accumulate iodine (Wapnir et al. 2004). NIS based gene therapy could be achieved only by introducing exogenous NIS gene for a significant iodide accumulation. Use of non-replicative adenovirus for NIS gene expression in BC cell line, MDA-MB-231 showed NIS gene expression as well as reduced survival post radioiodine administration (Riesco-Eizaguirre et al. 2011).

1.5. Epithelial Cell Adhesion Molecule (EpCAM)

The EpCAM, a transmembrane glycoprotein first discovered in colon carcinoma (Herlyn M et al. 1979), is expressed in normal epithelium and over expressed in various cancers (Went PT et al. 2004). In normal epithelial cells, EpCAM is expressed on the basolateral side of the
membrane but in cancer cells, over expression across the membrane is observed (Yanamoto S et al. 2007). When compared to EpCAM in healthy epithelia, EpCAM expressed in cancer cells are in hyperglycosylated state (Munz M et al. 2008). It has a multitude of functions owing to its different localization. EpCAM was found to be strongly expressed in carcinomas of various origins, including colon and rectum (Herlyn M et al. 1979), prostate (Poczatek RB et al. 1999; Zhang S et al. 1998), liver (de Boer CJ et al. 1999; Ruck P et al. 2000; Sansonno D & F 1993), esophagus (Kumble S et al. 1996; Martin IG et al. 1999), lung (Kubuschok B et al. 1999; Piyathilake CJ et al. 2000), head and neck (Takes RP et al. 1997), pancreas (Szala S et al. 1990) and breast (Edwards DP et al. 1986).

### 1.5.1. EpCAM-discovery and function

EpCAM had been discovered multiple times by different groups independently and with each discovery it received the name of the respective monoclonal antibody or cDNA clone used. Various synonyms of EpCAM are Epithelial GlycoProtein-2 (EGP-2), Epithelial Specific Antigen (ESA), GA733-2 and Tumor-Associated Calcium Signal Transducer 1 (TACSTD1) (Okegawa T et al. 2004). EpCAM mainly has three domains which are extracellular domain (EpEX), a single transmembrane domain and an intracellular domain (EpICD) (Okegawa T et al. 2004). The extracellular domain inturn contains three domains which are epidermal growth factor like (EGF) domain, a thyroglobulin repeat domain and a cysteine-poor domain (Figure 1.7).
Chapter 1

Figure 1.7 Protein structure of EpCAM

*EpCAM* protein structure is shown as a tetramer. The N-terminus is located extracellularly and C-terminus is located intracellularly. The polypeptide chain is depicted as a bent loop within *TY* domain positioning the *EGF*-like domain towards the plasma membrane. The red arrows depict the cleavage sites for two proteases which releases the intracellular region.

*Figure adapted from (Baeuerle PA & O 2007).*

One of the basic functions in multicellular organisms is cell to cell interaction and cell adhesion molecules (CAMs) are necessary for this process. Apart from cell adhesion, CAMs are involved in various other processes like cell migration, signaling, cell cycle and morphogenesis (Litvinov SV et al. 1997). Cell adhesion is crucial in regulating various cellular functions including contact-inhibition (Craig & Brady-Kalnay 2011) which plays a critical role in organogenesis (Fagotto & Gumbiner 1996). There are basically four CAM
families: the cadherins, the selectins, the integrins and the immunoglobulin CAM superfamily. EpCAM which was reported by different groups independently does not fall under any of the four families. When compared to other CAMs which are expressed in almost all normal tissues, EpCAM expression is limited to only epithelial cells. Since in the early days of EpCAM discovery its function was associated only with cell adhesion, it was proposed that EpCAM is a CAM (Basak S et al. 1998). However recent work on EpCAM has revealed its role in many other processes such as cell migration, signaling, differentiation and proliferation apart from cell adhesion. In addition to this being a biomarker, EpCAM has a regulatory function at morphological level in stem cells/progenitor cells (Schnell, Cirulli & Giepmans 2013). Differential glycosylation is considered to be one of the elements for altered functions of EpCAM in malignant cells compared to normal cells. In human, EpCAM has three N-glycosylation sites, Asn198, Asn111 and Asn74 (Schön et al. 1993; Thampoe, Ng & Lloyd 1988). Among the three, Asn198 is important of all as its glycosylation is linked with protein stability and expression at the cell surface (Munz et al. 2007). Moreover, the half-life of the protein decreased to 7 h from 21 h on removal of all the three glycosylation sites establishing the importance of the post-translational modification (Schnell, Cirulli & Giepmans 2013). Such differential glycosylation in cancer compared to normal epithelia has been reported in various molecules such as E-cadherin and integrins (Hakomori 1996; Pinho et al. 2011). While EpCAM was considered as an adhesion molecule, in comparison to E-cadherin, it had a reduced adhesion property (Litvinov et al. 1994). Interestingly, a study showed that when both EpCAM and E-cadherin were co-expressed, EpCAM interrupted E-cadherin association with the cytoskeleton thus weakening the adhesion property of E-cadherin (Litvinov et al. 1997).
1.5.2. EpCAM gene

The EpCAM gene is present on the chromosome 2p21 with a total of nine exons and is approximately of the size 14 kb (Calabrese G et al. 2001; Linnenbach AJ et al. 1993). EpCAM is conserved across many species not only at gene level but also at protein level among man, mouse and gorilla (Schnell, Cirulli & Giepmans 2013). The mRNA is around 1.5 kb and all the reported open reading frames encode a protein of 314 aminoacids (Perez MS & LE 1989). Mutations in EpCAM gene have been reported in Lynch syndrome and congenital tufting enteropathy with different germLine deletions are the cause in disrupting the 3’ end of the EpCAM gene in Lynch syndrome (Ligtenberg MJ et al. 2009), then in congenital tufting enteropathy it’s the four different mutations that cause decreased expression of EpCAM protein (Al-Mayouf SM et al. 2009; Sivagnanam M et al. 2008; Sivagnanam M et al. 2010). Another study showed that the primary cause of uncontrollable diarrhea in infants to be associated with mutations in the EpCAM gene (Sivagnanam M et al. 2008). Cloning and characterization of EpCAM promoter has been done (Gires O et al. 2003; Gires O et al. 2004; McLaughlin PM et al. 2004) and the promoter activity has been restricted to EpCAM expressing cells (Baeuerle PA & O 2007; McLaughlin PM et al. 2004). The specific expression of EpCAM is contributed by the region 687 to 341bp which is upstream of the transcription start site (TSS) (McLaughlin PM et al. 2004). The amount of EpCAM expression is directly related to the transcriptional activity of a 1.1 kb EpCAM fragment upstream of TSS (Gires O et al. 2004). Moreover it was reported that only 177 bp of the 5’ flanking sequence is required to drive reporter gene expression (McLaughlin PM et al. 2004).

1.5.3. EpCAM signaling

Regulated intracellular membrane proteolysis (RIP) is a conserved mechanism in transmembrane proteins where ectodomain is shed after the ligand induction followed by the shedding of the intracellular domain (Brown et al. 2000). RIP is shown to be involved in the
EpCAM induced proliferation and the proteases associated with it are metalloprotease ADAM17 also known as TACE (Tumor necrosis factor-α-converting enzyme) and γ-secretase containing PS-2 (Maetzel et al. 2009). EpCAM is an oncogenic signaling molecule involving the Wnt/β-catenin pathway, thus promoting cell invasion (Yamashita T et al. 2009). β-catenin is accumulated in the cell in the presence of Wnt signaling, interacts and activates various genes like c-myc, vascular endothelial growth factor which are involved in carcinogenesis (Huelsken & Behrens 2002). Several other possible proteolytic pathways have been suggested which regulates EpCAM signaling thus controlling EpCAM’s numerous functions (Schnell, Kuipers & Giepmans 2013).

1.5.4. EpCAM in cancer

There are various studies that contradicts EpCAM’s role in cancers. Earlier it was shown that when EpCAM cDNA was transfected into murine colorectal carcinoma cells, there was a decrease in tumor incidence and metastasis (Edwards DP et al. 1986). In contrast, later on other studies showed that either silencing of EpCAM using siRNA or inducing expression of EpCAM in HEK293 cells causes either decreased invasion of cancer (Osta WA et al. 2004; Yamashita T et al. 2009; Yamamoto S et al. 2007) or enhanced colony formation and metabolism (Münz M et al. 2004) respectively. EpCAM is also reported as a marker for various cancer initiating stem cells. For instance both EpCAM positive hepatocellular carcinoma stem cells and EpCAM positive pancreatic cancer stem cells showed potential to initiate tumors (Yamashita T et al. 2009). EpCAM expression in tumors has been correlated with improved clinical outcomes only in few cases. Improved patient survival in renal clear cell carcinoma was associated with high EpCAM expression in primary tumor (Klatte T et al. 2009; Seligson DB et al. 2004). Likewise in thyroid carcinoma, high EpCAM expression in tumors was associated with improved survival of the patient (Ensinger C et al. 2006; Ralhan R et al. 2010). Various studies indicating the contribution of EpCAM towards promoting
carcinogenesis have also been reported. High EpCAM expression was associated with decreased survival and large tumors in breast cancers (Gastl G 2000; Spizzo G et al. 2004). EpCAM expression increased proportionally from low grade to high grade intraepithelial neoplasia in cervical squamous epithelia (Litvinov SV et al. 1996). Similarly in prostate cancer, EpCAM expression increased from normal via prostatic intraepithelial neoplasia to adenocarcinoma (Poczatek RB et al. 1999).

In the case of head and neck squamous cell carcinoma, inconsistent connection of EpCAM is mainly attributed to the heterogeneity of tumors (Laimer et al. 2008). In certain group of esophageal cancer patients, high survival rate was related to high EpCAM expression (Rossen K & HK 2001) whereas one research group found EpCAM to be involved in decreased survival. There are certain groups who found no such correlations with grade of the tumors (Stoecklein NH et al. 2006). Similarly no differences in EpCAM expression were found among different grades of epithelial ovarian cancer. In another study on stomach, lung and prostate cancer, EpCAM expression had no significant correlation with any of the tumor properties or survival (Went P 2006). But another study in epithelial ovarian cancer, reveals differences among different histological subtypes and that overexpression is directly related to decreased overall survival of the patient (Spizzo G et al. 2006). The basic problem for such contradictions in the role of EpCAM lies in the use of different monoclonal antibodies and different scoring methods while analyzing tumors. Nonetheless the part of EpCAM in carcinogenesis cannot be overlooked.

1.5.5. Therapy based on EpCAM

Using EpCAM, vaccination strategies have been developed: 1) fragment of EpCAM-specific antibody combined with TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand) (Bremer E et al. 2004; Bremer et al. 2004; Mosolits et al. 2004) and 2) toxin fused with
EpCAM antibody fragments (Di Paolo et al. 2003; Flatmark et al. 2013). One of the monoclonal antibodies, adecatumumab in combination with taxotere is currently under clinical trials for prostate cancer (Marschner et al. 2010) is a human IgG of intermediate affinity and is showing encouraging results (Schmidt et al. 2012). A bispecific antibody called Catumaxomab (Removab®); anti-EpCAM mouse-rat hybrid was developed and was used in EpCAM positive patients to treat malignant ascites. But, the treated patients produced antibodies against the bispecific antibody in a Phase II study (Ruf et al. 2010; Sebastian et al. 2007; Zeidler et al. 1999). EpCAM aptamer based therapy showed some promise when it was experimented on multiple tumor cell lines and resulted in rapid internalization and renal clearance (Shigdar et al. 2011). However, more studies have to be performed before it can be considered as a viable option.

Another way of EpCAM based therapy was to generate immunoliposomes or nanoparticle which were loaded with anti-cancer drugs and coated with EpCAM specific ligand to show high therapeutic index. A study on immunoliposomes loaded with doxorubicin coated with anti-EpCAM fragment, scFv 4D5MOCB showed good tumor localization. But such high localization was also observed in non-targeted liposomes owing to enhanced permeability and retention effect (Hussain et al. 2007; Maeda et al. 2000). In the case of EpCAM targeted nanoparticle loaded with paclitaxel for treating retinoblastoma cells, a study from our laboratory showed higher specificity and moderate uptake (Mitra et al. 2011). In another study from our laboratory targeting EpCAM, showed conjugate of EpCAM aptamer and doxorubicin to deliver the drug selectively to RB cells (Subramanian et al. 2012). In a recent study, anti-EpCAM fusion toxin was developed using bioorthogonal click chemistry for tumor targeting which reported a better therapeutic execution (Simon et al. 2013).
1.6. Survivin

Survivin has been extensively studied and considered to be a quintessential target for cancer therapy. One of the many reasons for it to be considered to be an ideal target is its over expression in cancer cells and its role in blocking the apoptosis (Altieri 2003a; Kanwar et al. 2001; Samarasinghe et al. 2012). Apart from its role in apoptosis inhibition it also plays a major role in regulating cell division in normal cells (Johnson & Howerth 2004). Reports from our laboratory and others have shown that survivin is involved in a number of downstream signaling cascade mechanisms which enhance cancer proliferation (Kanwar, Kamalapuram & Kanwar 2011).

1.6.1. Structure and function

Survivin belongs to the inhibitor of apoptosis protein (IAP) family and is the smallest member of the IAP family with survivin gene encoding a 16.5 kDa protein. The protein contains only one Baculovirus IAP repeat (BIR) domain which is at the N-terminal and has an amphipathic C-terminal-helix (Chantalat et al. 2000). The main function of survivin in normal cells is in mitosis and the chromosomal passenger complex (CPC) which regulates mitosis contains survivin apart from Aurora B kinase, INCENP and Borealin (Altieri 2010; Baratchi, Kanwar & Kanwar 2010; Coumar et al. 2013) (Figure 1.8). In cancers, survivin functions by inhibiting the programmed cell death (Rudin et al. 2012) via inhibition of caspase-3 and caspase-7. The inhibition could be either direct mechanism where the survivin binds to caspases or indirect inhibition where survivin binds to XIAP, another member of IAP to form a complex which prevents activation of caspases (Dohi et al. 2004). Recent study revealed that survivin expression regulates autophagy in a way that up-regulation of survivin completely inhibits autophagy process (Wang et al. 2011).
Figure 1.8 Schematic representation of survivin protein.

The Baculovirus IAP repeat (BIR) domain and coiled coil domain are depicted along with nuclear export signal (NES). The functional domains are the phosphorylation sites at Thr 34, Thr 117 and Ser 20. The protein partners for survivin are Aurora B, INCENP, Borealin, Smac/Diablo and Hsp90 are also depicted (Pennati, Folini & Zaffaroni 2008).

1.6.2. Splice variants of survivin

Alternative splicing is a process which generates distinct protein isoforms from a common mRNA precursor (Schwerk & Schulze-Osthoff 2005). Splice variants of survivin are with definite functions of their own and are also conserved among species from which it can be comprehended that they possess a role in the apoptotic machinery of survivin (Wenzel et al. 2000). In humans, six splice variants have been reported and they are Survivin-WT, Survivin-2α, Survivin-2β, Survivin-3α, Survivin-3β and Survivin-ΔEx3 (Huang et al. 2011b) (Figure 1.9). The protein arising from the variants have similar N-terminal sequence, Baculovirus IAP repeat (BIR) domain but a different C-terminal region. Specific residues that are conserved within the BIR domain among the splice variants are S20 which is key in maintaining the survivin and XIAP binding and its phosphorylation disrupts the antiapoptotic properties (Dohi, Xia & Altieri 2007). Second residue that is shared by the variants is T34, dephosphorylation of this residue initiates mitochondrial apoptotic pathway (O'Connor et al. 2000). Other common residues among the variants that have been reported are T48 (Barrett, Colnaghi & Wheatley 2011), L64 (Sun et al. 2005) and D71 (Song, Yao & Wu 2003), which
are all involved in the anti-apoptotic functions. Out of all variants, only two splice variants 2β and 3β share sequence identity in the carboxyl domain with the wild type survivin (Necochea-Campion et al. 2013). Survivin homodimerises and the splice variants modulates its activity by forming heterodimers (Caldas, Honsey & Altura 2005).

![Diagram of Survivin splice variants](image_url)

Figure 1.9 Schematic illustration of 6 survivin splice variants containing common exon 1 and exon 2.

### 1.6.2.A. Survivin-WT

The wild type survivin contains 4 exons, a 426 bp transcript translated into a 142 aminoacids (Caldas, Honsey & Altura 2005). The localization of Survivin-WT is in the mitochondria of cancer cells which shows a role in pathogenesis of tumor (Jha et al. 2012).
1.6.2.B. **Survivin-2α**

Survivin 2α is formed by the addition of 197 bp from intron 2 but only two nucleotides are coding as it has an early stop codon giving rise to shortest survivin variant. When compared to normal cells, Survivin 2α expression is as high as 95 times in lung cancer, colorectal cancer and 150 times in medulloblastoma (Caldas, Honsey & Altura 2005). This elevated expression in cancers is correlated with poor survival outcome and drug resistance (Span et al. 2006).

1.6.2.C. **Survivin-2β**

This splice variant is derived by the introduction of 69 bp nucleotide fragment from intron 2 (Mahotka et al. 1999). Survivin 2β expression and its link to clinicopathological features have been controversial due to contradictory reports. While few studies in colorectal carcinoma and astrocytoma have reported that upregulation in survivin 2β expression is correlated to worse survival outcomes (Antonacopoulou et al. 2010; Huang et al. 2011b) , whereas other studies on the same cancers have reported expression of survivin 2β to be associated with positive outcomes (Yamada et al. 2003). In various other cancers, positive and negative correlation of survivin 2β expression has been drawn out with clinical stages and survival outcome, thus making survivin 2β as one of the most contradicted splice variants (Necochea-Campion et al. 2013).

1.6.2.D. **Survivin-3α**

Introduction of 209 bp from intron 2 generates survivin 3α (Huang et al. 2011b). The status of survivin 3α in cancer is not much studied. In one of the studies in neoplasms of brain, survivin 3α was not detected (Huang et al. 2011b). A recent study on breast cancer suggested a possible role of survivin 3α in tumor development because survivin 3α was absent in normal breast tissues whereas it was detected in breast tumors (Moniri Javadhesari et al. 2013).
1.6.2.E. **Survivin-3β**

Survivin 3β is generated on the introduction of 165 bp nucleotide fragment from intron 3 (Badran et al. 2004). The expression of survivin 3β in cancers is not as high as the other variants’s expression in cancers. A study in oral squamous cell carcinoma showed a slight increase in survivin 3β expression compared to normal mucosa but it did not mount up to statistically significant (De Maria et al. 2009). In colorectal cancers, studies did show statistically significant higher levels of survivin 3β expression, but it did not correlate with the clinicopathological features of the tumors (Antonacopoulou et al. 2010). However in breast cancer though, higher expression of survivin 3β was associated with decreased survival (Span et al. 2006).

1.6.2.F. **Survivin-ΔEx3**

Survivin ΔEx3 is created by deletion of 102 bp of exon 3 and a frameshift (Mahotka et al. 1999). It is one of the splice variants that have been discovered early. Its expression and its significance in the prognosis of the disease have been studied in various cancers. In majority of the cancers studied like breast (Span et al. 2006), cervical (Futakuchi et al. 2007) and thyroid (Vandghanoooni et al. 2011), expression of ΔEx3 have been elevated in cancer tissues when compared to normal tissues. The higher expression has been associated with advanced tumor grade and stages in prostate cancer (Lopergolo et al. 2012), breast cancer (Span et al. 2006) and glioblastoma (Lopergolo et al. 2012). Survivin ΔEx3 localises in the nucleus because of the nuclear localization signal (NLS) found in the sequence which also helps in removing cytoplasmic survivin ΔEx3 (Mahotka et al. 2002b). The subcellular localization of survivin ΔEx3 was found to be in the mitochondria and this mitochondrial accumulation was responsible for inhibition of mitochondrial mediated apoptosis in cancers. Inhibition of apoptosis is via the binding of survivin ΔEx3 with apoptotic molecule, Bcl-2 with the help of
a BH2 domain (Caldas et al. 2005). A recent study showed that survivin ΔEx3 is involved in angiogenesis and regulation in endothelial cell homeostasis (Caldas et al. 2007).

**1.6.3. Survivin as a target for therapy**

Survivin is considered as a prime target for cancer therapy mainly because of its high turnover rate and selective expression in cancers (Altieri 2013; Kanwar et al. 2001; Morrison et al. 2011). Antisense nucleotides were the first rival molecules used against survivin. LY2181308 and locked nuclei acid oligonucleotides were synthesized and these molecules showed encouraging anti-cancer activity and safety (Hansen et al. 2008; Molckovsky & Siu 2008). Blocking survivin expression in lymphoma generated the activity of cytotoxic T-lymphocyte and made the tumors susceptible to B7-1-mediated immunotherapy (Mao et al. 2006). RNA interference mechanism was introduced in targeting survivin to decrease survivin at mRNA and protein level. In in vivo studies, survivin gene silencing resulted in promising results (Davis et al. 2010). In a recent study on breast cancer, targeting both survivin WT and survivin ΔEx3 proved to be comprehensive strategy as promotion in proliferation and apoptosis was observed (Zheng et al. 2011). Although, survivin is stabilized by molecular chaperone Hsp90, inhibition of either of the molecules individually does not mount upto therapeutic value but its dual inhibition has the potential of targeted therapy (Kunath et al. 2003a). Multiple survivin dominant mutants have been used to target survivin in cancer. T34A dominant mutant of survivin was shown to enhance the sensitivity of cisplatin and doxorubicin in breast cancer cells (Xu et al. 2012). Another survivin mutant, C84A mutant fused to cell-penetrating poly-arginine peptide has shown to induce apoptosis in cancer cells (Cheung et al. 2010; Kanwar et al. 2001). A combination of both T34A and C84A mutants have shown to have powerful killing of cancer cells than when these were used alone (Zhang et al. 2008). From our lab, a mutant form of survivin, SurR9-C84A have been shown in a study where it enhances the expression of neuronal and cell cycle markers in
differentiated neurons along with increasing the cell viability (Baratchi et al. 2010). In another recent study from our lab, Fe-bLf in nanoformulation was shown to target survivin in colon cancer stem cells thus inducing apoptosis by activating both extrinsic and intrinsic pathways (Kanwar et al. 2014).

1.7. Bcl-2 family

Bcl-2 gene (B-cell lymphoma 2) was discovered in follicular B-cell lymphoma gene. This gene is present on the chromosome 18q21.3 and its translated product was involved in inhibition of cell death (Tsujimoto & Croce 1986). Apoptotic pathways are divided into two 1) intrinsic pathway and 2) extrinsic pathway (Figure 1.10). The intrinsic pathway is regulated by the Bcl-2 family of proteins, and is stimulated by various activities within the cell like cytokine deprivation, cytotoxic drugs and radiation (Cory & Adams 2002; Pickering et al. 2006). From the permeabilized mitochondria, cytochrome c releases first which binds to APAF-1 and activates pro-caspase to caspase 9, which then stimulate a multitude of caspases activation leading to apoptosis. The extrinsic pathway is induced by the activation of CD95, TNF-related apoptosis inducing ligand (TRAIL), Tumor necrosis factor receptor 1 family proteins which in turn triggers the caspase cascade by enrolling caspase-8 (Walczak & Krammer 2000). In human, approximately 17 Bcl-2 proteins have been identified and each characterized with 4 regions of sequence homology known as Bcl-2 homology (BH) domains. All the Bcl-2 members share at least one of the BH domains. The members of the Bcl-2 family are classified into three groups (Dewson & Kluck 2010).
Figure 1.10 Schematic representation of molecular pathways of apoptosis.

Extrinsic pathway depicts the DISC complex formation and caspase 8 activation. Intrinsic pathway depicts the release of cytochrome c from mitochondria which activates caspase 9. Caspase 3 is then activated by both caspase 8 and 9 which leads ultimately to death of the cell (Favaloro et al. 2012).
1.7.1. Anti-apoptotic members of Bcl-2 family

The anti-apoptotic members of this sub-family are Bcl-2, Bcl-xl, Bcl-w and induced myeloid leukemia cell differentiation (Mcl-1) proteins. The characteristic of these members are they have all the four BH domains including a hydrophobic C terminal part. The difference between anti-apoptotic and pro-apoptotic members are the absence of BH4 domain in pro-apoptotic members which may be responsible for the anti-apoptotic activity (Khemtémourian et al. 2006). Bcl-2 is present on the mitochondrial membrane and confers a protective effect on mitochondria by preserving the membrane integrity, thus inhibiting apoptosis to occur. Bcl-2 exhibits anti-apoptotic activity mainly in three ways. First method is by inhibiting the oligomerization of Bax/Bak, thus blocking the release of apoptosis inducing molecules. Second is by inactivating Bax by binding to it, thereby inhibiting apoptosis. Third method is by preventing the activation of caspase-9 by directly blocking the release of cytochrome c. Likewise, Bcl-xl through BH3 domains of Bax and Bak interacts with them and prevents apoptosis (Khemtémourian et al. 2006; Simonen, Keller & Heim 1997). Mcl-1 on the other hand is a protein with quick turnover and regulates cell survival by involving in the development of T and B lymphocytes (Yang-Yen & Hsin-Fang 2006).

1.7.2. Pro-apoptotic members of Bcl-2 family

Bax, Bak and Bok are the main pro-apoptotic members and they have similar sequences in all domains except BH4 domain which is absent (Reed 2006). Since Bax does not possess a clear BH3 domain, it is prone to activation by P53. During apoptosis, Bax protein oligomerizes after integration with the mitochondria leading to the release of cytochrome c and stimulation of caspases (Petros, Olejniczak & Fesik 2004). Similarly, during apoptosis Bak also oligomerizes after integration with the mitochondria (Chipuk et al. 2004).
1.7.3. **BH3-only members**

The members of this family are BH3 interacting domain (Bid), Bcl-2-associated death promoter (Bad), Bcl-2-interacting killer (Bik), BCL-2-interacting mediator of cell death (Bim), Bcl-2-modifying factor (Bmf), Harakiri (Hrk), (death inducer binding to vBcl-2 and apoptosis-activating factor, APAF-1) Diva, phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1; also known as NOXA) and p53 upregulated modulator of apoptosis (Puma). The entire above mentioned are pro-apoptotic molecules with only BH3 domains as common. These members are activated by various cellular processes like phosphorylation, transcriptional upregulation and truncation (Zha et al. 1996). BH3-only proteins have a characteristic dual function, where it regulates Bcl-2 family members both positively and negatively. Bid is the most studied molecule and it is involved in the intrinsic pathway by linking death receptors to mitochondrial amplification loop. Bid with its strong pro-apoptotic activity targets mitochondria and it activates Bax and Bak. The rationale behind Bid's pro-apoptotic activity is its subcellular localization (Zinkel, Gross & Yang 2006). Phosphorylation activates Bim and Bmf whereas dephosphorylation releases Bad molecule. Caspases cleaves Bad molecule when transforming growth factor β1 induces apoptosis (Zhu et al. 2002). Bim along with Bid are the only BH3 members which releases cytochrome c from mitochondria (Kuwana et al. 2005).

1.7.4. **Bcl-2 in cancer and therapy**

Trademark of cancers is the inhibition of apoptosis and overexpression of Bcl-2 has been shown in number of cancers. Few cellular processes that induce the Bcl-2 overexpression are DNA hypermethylation, chromosomal translocation and deregulation of miRNAs specific for Bcl-2 (Strasser et al. 1990). Various strategies to counteract Bcl-2 overexpression in cancer have been practiced. The first drug to target Bcl-2 mRNA was Oblimersen sodium, an antisense oligonucleotide. Though initial preclinical and clinical studies were successful, it
failed subsequently because of increased expression of other Bcl-2 members (Marcucci et al. 2003). Another therapeutic molecule was ABT-737, which was a mimic of Bad bound with great affinity to Bcl2, Bcl-xl and Bcl-w (Lee et al. 2007a). The mimic, ABT-737 showed appreciable positive outcome in the preclinical studies (Tse et al. 2008). However, clinical trials in small cell lung cancer patients, ABT-263, an oral derivative of ABT-737 showed a disappointing overall response rate (Rudin et al. 2012).

1.7.5. **Bax in cancer and as a biomarker**

Bcl-2-associated X (Bax) protein was the first pro-apoptotic member to be discovered among the Bcl2 family. As a result of alternative splicing, Bax gene generates four variants, namely Bax α, β, Δ and Σ. Bax is considered as one of the futuristic biomarker related to apoptosis (Zhou et al. 1998). Regulation of apoptosis relies on the amount of homodimerisation or heterodimerisation of Bax and Bcl-2 in the cell. Increased sensitivity to drugs is attained when there is an increased amount of Bax/Bax homodimers in the cell (Toshiyuki & Reed 1995). Several cancers like breast, lung and ovary have analyzed the possibility of Bax being a predictive biomarker (Krajewski et al. 1995; Kupryjańczyk et al. 2003). It was found in a study that loss of Bax function in a gastrointestinal cancer tissue led to the progression of cancer (Duval & Hamelin 2002). Another study with colorectal carcinoma showed that patients who lacked Bax expression had 5.33 times higher mortality rate than the patients with high Bax expression (Katkoori et al. 2010). A recent research conducted in oral squamous cell carcinoma, concluded that high expression of Bax was significantly associated with increased proliferation leading to an increased response to radiotherapy (Bose et al. 2012).

1.8. **Polyethyleneimine: A potent non-viral vehicle for gene delivery**

Polyethyleneimine (PEI), a cationic polymer, has been used as an efficient nonviral vector for gene delivery both *in vitro* as well as *in vivo* (Clamme, Azoulay & Mély 2003; Fischer et al.
1999; Kichler et al. 2001). Every third atom of this organic macromolecule is an amino nitrogen group which can be protonated. Irrespective of PEI form i.e., linear or branched, it forms complexes with DNA. The ability of the PEI to trap DNA is attributed to many linker amino groups which also render PEI buffering capacity (Klotz, Royer & Sloniewsky 1969). Molecular weight of the PEI determines the toxicity and transfection efficiency of PEI. Higher the molecular weight, higher the cytotoxicity and transfection efficiency (Kichler et al. 2001). Although when compared to viral transfection systems, PEI has low transfection efficiency, but is still considered as a potent transfection agent.

### 1.8.1. Forms of polyethyleneimine

PEIs are basically in two forms. One is linear polyethyleneimine (lPEI) and the other one is branched polyethyleneimine (bPEI) (Figure 1.11). Both are widely used as gene delivery vehicles. Since PEI has high positive charge on the surface it readily interacts with DNA and RNA which are negatively charged molecules (Neu, Fischer & Kissel 2005). The synthesis of bPEI can be carried out in two methods. One is by acid catalyzed polymerization of aziridine either in alcohols or aqueous solutions (Kunath et al. 2003b; von Harpe et al. 2000) in which the reaction is controlled by standardizing concentration of the initiator and temperature. Second method of bPEI synthesis is bulk polymerization of anhydrous aziridine at a lower temperature (Dick & Ham 1970). The lPEI is synthesized through polymerization of 2-ethyl-2-oxazoline followed by hydrolysis (Brissault et al. 2003). For in vivo applications, lPEI was considered to be more applicable compared to bPEI because lPEI has less cytotoxicity and high efficient transfection property (Wiseman et al. 2003). For example, commercially available transfection agents for lPEI are jetPEI and ExGen500 (Mintzer & Simanek 2008). Both jetPEI and ExGen500 is linear PEI of 22 kDa. They are superior transfection reagents used both at in vitro and in vivo levels.
1.8.2. Endosmolyis

PEI with its high positive charge effectively condenses DNA forming polyplexes. These PEI/DNA polyplexes are internalized into the cells via endocytosis (von Gersdorff et al. 2006) (Figure 1.12). In 1997, “proton sponge” hypothesis was proposed which hypothesized that PEI with its unprotonated amines absorb protons which are pushed into the lysosome resulting in a high intake of Cl⁻ ions and water. This prompts the osmotic swelling of the vesicle, leading to rupture of lysosomes and release of polyplexes into the cytoplasm (Behr & Jean-Paul 1997). However, the endosmolytic activity depends on the size of the polyplexes. The smaller particles does not efficiently deliver gene without the help of endosmolytic compounds unlike the larger polyplexes (Wattiaux et al. 2000).
Positively charged polymer binds with negatively charged DNA to form an overall positive complex which enters the cell on interaction with negatively charged cell membrane. Nanocomplex enters inside the cell via endocytosis and the dissociated DNA from the complex enters the nucleus after the endosomal escape of the complex (Jin et al. 2014).

1.8.3. Transfection of the PEI complexes

Molecular weight of the PEI determines its transfection efficiency. A study analyzed the transfection efficiency of a range of PEI from 0.6 to 70 kDa and concluded that higher molecular weight PEIs result in higher transfection efficiency but it is also associated with higher cytotoxicity (Fischer et al. 2003; Mintzer & Simanek 2008). The reason for the higher toxicity is necrosis of the cells which is caused as a result of aggregation and accumulation of the complexes on the cell surface (Fischer et al. 1999). For an optimal transfection with PEI, a low molecular weight of 5 to 25 kDa was suggested to be effective (Neu, Fischer & Kisel
Higher transfection efficiency of low molecular weight PEI can be achieved by increasing the PEI amine/DNA phosphate (N/P) ratio (Kunath et al. 2003b).

### 1.8.4. Modifications of the PEI molecule

PEI structure is modified to enhance its transfection efficiency and various strategies have been employed for the improvement. One of the extensive modifications done on PEI is PEGylation which imparts a hydrophilic nature to PEI thus preventing the polymer’s interactions with erythrocytes and plasma proteins (Mintzer & Simanek 2008). Polyethylene glycol (PEG) alone or N-hydroxy succinamide (NHS) activated PEG are conjugated with PEI for transfection studies (Ogris et al. 1999). Two characteristics of PEG attached to PEI 1) length and 2) density, determine the transfection efficiency in vitro. A shorter PEG with high density conjugated to PEI efficiently transfected oligonucleotides (Kunath et al. 2003a). But another study with siRNA showed that PEI conjugated to longer PEG with less density was more effective (Mao et al. 2006). Though at in vivo level, the PEI-g-PEG copolymers showed increased circulation with less toxicity, no gene expression was observed (Merdan et al. 2005). This loss in gene expression was attributed to two factors. The first factor was the PEI-g-PEG copolymer had a reduced binding with the cell membrane due to loss of surface charge which hindered its interaction (Godbey, Wu & Mikos 1999). The second factor is a hampered transfer of gene after cellular uptake (Walker et al. 2005). It was shown by another study that by modifying the primary and secondary amine group of PEI with cholesteryl chloroformate, improved transfection efficiency could be achieved (Wang et al. 2002).

Another study reported a lesser toxicity for both forms of cyclodextrin (CD)-modified PEI. With increasing concentration of cyclodextrin, a reduced cytotoxicity was observed in prostatic carcinoma cells for both forms of CD-modified PEI. Though a higher cellular uptake was observed, less transfection rate was observed for CD-bPEI due to hindrance by the cyclodextrin conjugation. This problem was addressed by conjugating adamantane-
terminated PEG with the CD-PEI and the \textit{in vivo} studies with the new modification showed excellent biodistribution and no toxicity (Pun et al. 2004).

\textbf{1.8.5. Targeting receptors via PEI}

In hepatocytes, specific gene transfer was directed to ASGPr-receptors using bPEI linked to galactose through four-carbon spacer (Zanta et al. 1997). In dendritic cells, mannose was used for gene delivery because of high expression of mannose receptors. DNA was delivered in a mannose receptor mediated by using Mannose-bPEI25 and efficiency via this conjugate was higher than the non-conjugated PEI (Avraméas et al. 1996). Another receptor mediated endocytosis was observed with transferrin conjugated with PEI/DNA polyplexes which showed improved cell internalization. In many cancer cells, epithelial growth factor (EGF) receptors are overexpressed and this was exploited for receptor mediated delivery. An enhanced uptake of EGF polypeptide tagged to bPEI25/DNA was observed in tumor cell lines (Blessing et al. 2001). Peptides have been used to conjugate with PEI for specific and receptor mediated delivery. One such peptide is RGD peptide which was conjugated with bPEI25 and it showed ligand receptor delivery and enhanced gene expression (Kunath et al. 2003a). Antibodies or its fragments have been conjugated with PEI for specific delivery. In one study, anti-CD3 antibody targeting T cell receptor-CD3 molecule was tagged with bPEI800 first followed by the DNA. This antibody-PEI conjugate transfected human T cell leukemia cells with a higher transfection efficiency (Kircheis et al. 1997). However, the drawbacks of the study were the huge size of the antibody and potential rise of immunogenicity.

\textbf{1.8.6. Cytotoxicity of PEI}

The free PEI causes cytotoxicity by membrane damage before the internalization and inducing stress inside the cell after internalization (Godbey, Wu & Mikos 2001). This cytotoxicity effect of PEI has led researchers to synthesize biodegradable PEI by introducing
reducible disulfide linkages or ester groups. PEI treated with dimethyl-3,3’-dithiobispropionimidate showed reduced toxicity (Gosselin, Guo & Lee 2001), whereas a synthesized reducible linear PEI behaved similar to PEI but with increased cell viability (Lee et al. 2007b). Likewise, a synthesized oligo (L-lactic acid-co-succinic acid) was conjugated to 1200 Da PEI for reduced cytotoxicity. Though this modification achieved the goal along with increased transfection efficiency, it underwent rapid degradation (Petersen et al. 2002).

1.9. Aptamers

Aptamers, the in vitro selected single-stranded, short DNA or RNA oligonucleotides selectively bind to target molecules because they form secondary structures with aligned conformations (Kanwar et al. 2010). The coining of the term aptamer (Ellington & Szostak 1990) and its synthesis called “SELEX” (Systemic Evolution of Ligands by Exponential enrichment) (Tuerk & Gold 1990) was published by two different groups at the same time.

1.9.1. Advantages of an aptamer

Aptamers are considered to be more advanced than the antibodies for several reasons. Antibodies can be raised only for those molecules, for which an immune response is elicited and thus the restrictive generation of antibodies. In case of aptamers, it can be generated for any molecules including toxins, proteins, phospholipids, sugars and other nucleic acids (Lauridsen & Veedu 2012). Aptamers are capable of distinguishing targets even with slight difference in their structures such that they can bind to even hidden epitopes which are not accessible for antibodies (Kanwar, Roy & Kanwar 2011). The aptamers retain functionality even at an ambient temperature due to the formation of 3-dimensional structure which makes them resistant to pH and temperature changes. During chemical synthesis, the aptamers can be custom-synthesized for increased stability, for immobilization and also add signaling properties. The synthesis of aptamers is highly reproducible and pure, thus avoiding the batch-to-batch variation like antibody production. In addition, aptamers owing to its small
size does not elicit immune response \textit{in vivo} which is the biggest advantage in terms of therapeutic application (Szeitner et al. 2014).

The few drawbacks that have limited aptamer’s use as therapeutic agents are its rapid clearance from the body due to its small size and its degradation in the presence of nucleases (\textbf{Table 1.4}). The degradation rate can be however slowed down by chemical modification (Hu & Zhang 2013). One of the main modifications to overcome nuclease degradation is addition of unnatural bases like locked nucleic acid (LNA) which imparts high resistance. A study proved this by modifying a R06 aptamer by adding LNA which showed a higher binding affinity along with higher nuclear resistance against trans-activating responsive (TAR) RNA element of HIV (Lebars et al. 2007). Aptamers are generated via a process called as systematic evolution of ligands by exponential enrichment (SELEX) which opened opportunity for various target molecules.
Table 1.4 Advantages and disadvantages of aptamers and antibodies

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<td><strong>Aptamer</strong></td>
<td>1. Chemically synthesized and easily scalable</td>
<td>1. Shorter half-life because of small size</td>
</tr>
<tr>
<td></td>
<td>2. Chemical production, so free of bacterial or viral contamination</td>
<td>2. Highly dependent on chemical modifications for stability in the presence of serum</td>
</tr>
<tr>
<td></td>
<td>3. Smaller size</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. Non-immunogenic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5. High stability</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6. Conjugation with dyes or functional groups easily introduced</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7. High specificity against any targets</td>
<td></td>
</tr>
<tr>
<td><strong>Antibody</strong></td>
<td>2 High circulating half-lives inside the body</td>
<td>• Prone to bacterial or viral contamination because of biological synthesis</td>
</tr>
<tr>
<td></td>
<td>3 Resistant to nuclease degradation</td>
<td>• Large size compared to aptamer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Conjugation partners leads to reduced activity</td>
</tr>
</tbody>
</table>

1.9.2. **SELEX**

The procedure of SELEX requires a solid surface on which a target molecule is attached. This targeted molecule is then subjected to a pool of random RNA or DNA oligonucleotides which binds to the target molecule based on their specificity. This is followed by a washing step which removes unbound oligonucleotides leaving a good amount of bound oligonucleotides. These bound oligonucleotides are then amplified by PCR which results in double-stranded DNA converted to single stranded DNA before subjecting it to another round of selection. Repetitive selection cycles screens the high affinity oligonucleotides and these candidates are
then separated and sequenced (Figure 1.13). The most important step in the SELEX methodology is the initial cycle where the experimental conditions are lenient with prolonged incubation period. However, in the final steps of the selection, conditions turn rigorous (Szeitner et al. 2014). Various modifications of the SELEX were introduced for a better affinity and specificity over a period of twenty years. Negative SELEX (Bassett et al. 2004), Subtractive SELEX (Wang et al. 2003), Cell-SELEX (Shangguan et al. 2006), Chip SELEX (Collett, Cho & Ellington 2005) and Atomic force microscopy-SELEX (AFM-SELEX) (Miyachi et al. 2009) are the few developed forms of SELEX over the native one.

Figure 1.13 Schematic illustration of SELEX methodology.

*Target is first immobilized on the bead which is then subjected to a random nucleotide library, after multiple washes; the bound oligonucleotides are amplified by PCR and cloned.*
1.9.3. **Aptamer as diagnostic agents**

Aptamer, due to their many advantages, are widely explored as diagnostic agents in different fields. An optimal diagnostic agent should possess characteristics such as specificity, stability under physiological conditions, reliability, quick detection and most importantly sensitivity. Since aptamers fulfill the above conditions, they are used not only to detect different molecules but also used as an imaging probe (Radom et al. 2013). Aptamers have been used as a biosensor by combining it with a molecular beacon. A study on human neutrophil elastase (HNE) showed a unique biosensor which had a DNA aptamer to HNE, a fluorescent molecular beacon and a siDNA which was complementary to both the beacon and the aptamer. The siDNA competitively bound to either the aptamer or the beacon thus enhancing the sensitivity of the biosensor (He et al. 2010). A new biosensor with three different aptamers specific for cocaine, adenosine and potassium was generated and it was reported to be highly sensitive even at micromolar ranges (Zhang et al. 2010). Like biosensors, aptamers are also used as electrochemical sensors where the aptamer is coated on an electrode and when the aptamer binds to its target, a measurable change in the current flow occurs. Using the concept of aptamer based electrochemical sensors, studies using cocaine-DNA aptamer (Swensen et al. 2009) and interferon-γ aptamer (Zhang et al. 2012) have shown the concept to be feasible. The conjugation of aptamers with radiolabelled compounds for imaging has been used extensively in clinical front with technologies like Single-Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET). One of the studies explored the possibility of tagging radioactive compound, $^{99m}$Tc with a DNA aptamer targeting matrix metalloprotease-9 for studies in brain tumors (Stamenkovic 2003) and gave satisfactory results.
1.9.4. Aptamers as therapeutic agents: Aptamer-drug conjugates

For therapy, aptamers are mainly used either to deliver a therapeutic compound to the target molecule or to inhibit a particular target molecule. Although, few therapeutic aptamers have advanced to clinical trials, so far FDA have approved only one aptamer named Pegaptanib, directed against vascular endothelial growth factor (VEGF)-165 for vascular ocular disease (Ng et al. 2006). Chemotherapy in cancer lacks selectivity and this is the reason for associated side-effects. This problem was addressed in a study where a commonly used cancer drug, doxorubicin was conjugated with an aptamer targeting tyrosine kinase 7. This conjugate had various advantages like high affinity, preservation of aptamer specificity and drug cytotoxic effects along with reduced toxicity to non-targeted cells due to the conjugate internalization via the receptors (Shangguan et al. 2006). There were studies which made these conjugates into bifunctional by introducing iron oxide nanoparticles. In prostate cancer, an aptamer targeting prostate specific membrane antigen was conjugated to superparamagnetic iron oxide nanoparticles (SPION) which allowed imaging via specifically targeting cancer cells (Wang et al. 2008). Similarly, liposomes are also coated with aptamer to target cells. A recent study showed the preparation of a liposome coated with sgc8 aptamer having affinity towards leukemia CEM-CCRF cells was labeled with fluorophores for both the carrier and the load. The targeted liposome exhibited a specific targeting and release of drugs within 30 mins of incubation thus establishing the advantages of aptamer mediated delivery of drugs (áO’Donoghue & B 2010). Apart from delivering drugs, aptamers are also conjugated with siRNAs to silence specific genes. In another study, a CD4 aptamer-siRNA chimera was shown in humanized mice to have protective effect against HIV vaginal transmission by specifically knocking down HIV genes (Wheeler et al. 2011).
1.10. Conclusion

For the progression of RB, various molecules which are endogenously expressed play major role. The main genes involved are apoptotic regulatory genes which are either over expressed or under expressed in the cancer cells compared to the normal cells. The inter-play between these molecules determines the rate of progression of RB tumors. Apart from apoptotic regulatory genes, various other genes such as NIS, whose role in normal cell is not associated to apoptosis, in cancer condition comes into the forefront in enhancing the disease. The main goal of the study was to deduce the role of NIS in RB and stimulation of basal NIS using transactivating agent such as bLf. Apart from deducing the role of NIS in RB, NIS was also delivered in breast cancer in a targeted and specific approach using modified nanocomplexes. Furthermore, the role of dysregulated apoptotic molecules was also studied in RB. Thus, unraveling the mechanism of various molecules associated with the RB and breast cancer would help in understanding and targeting NIS, Bcl-2, Bax and survivin better.

1.11. Hypotheses

1) NIS expression has been studied in various extra-thyroidal cancers. This study hypothesized that if NIS was expressed in RB tumors, it would be associated with clinical parameters like histopathological high risk factors and tumor differentiation. Further it was hypothesized that bLf could upregulate the endogenous NIS protein expression in Y79, an RB established cell line from ATCC (American Type Culture Collection).

2) Basal level NIS expression in breast cancer has been studied extensively. Although NIS is expressed, the endogenous NIS is insufficient for radioiodide therapy. In this study, it was hypothesized that if NIS was specifically overexpressed in breast cancer cells (MCF-7) via a tissue specific promoter (EpCAM) through receptor targeted gene delivery, it would induce functional activity of NIS leading to higher cellular iodide
uptake. It was also hypothesized that NIS gene could be effectively delivered in a nanoformulation (nanocomplex) decorated with EpCAM aptamer for its specific delivery in EpCAM positive breast cancer cells (MCF-7), the successful delivery would then enhance the iodide uptake.

3) The endogenous gene expression of apoptotic regulatory molecules has never been studied in primary RB tumors before. This study explored the expression of Bax and Bcl-2 from Bcl2 family, and survivin and four of its variants from the IAP family. It was hypothesized that the expression of these apoptotic regulatory molecules would be associated with clinical parameters like histopathological high risk factors.

1.12. Novelty of the project

The novelty of this project is for the first time, NIS expression and its correlation with histopathological features of RB tumors has been studied. Other than RB, the prospect of EpCAM specific overexpression of NIS gene in breast cancer cells and its delivery in nanoformulation has been explored. Also the endogenous status of the apoptotic regulatory molecules in RB has never been studied. Therefore, this study intended to bridge the knowledge gap in the correlation of apoptotic regulatory genes (Survivin-WT, Survivin-2α, Survivin-2β, Survivin-3β, Survivin-ΔEx3, Bax and Bcl-2) with the histopathological high risk factors of the RB tumors. In addition, the prospect of survivin targeting using RNAi technology for silencing the dominant player among the urvivin variants and its apoptotic effects has been explored.

1.13. Research questions addressed

1) Is NIS expressed in RB? If expressed, is it correlated with the histopathological features of the tumor? If NIS is expressed in RB cell line, Y79, can the NIS gene expression be transcriptionally activated?
2) Can Y79 cells efficiently internalize bLf? Can iron free and iron saturated forms upregulate the endogenous NIS expression of Y79 cells?

3) Can NIS gene be specifically over expressed in breast cancer cells? Can NIS plasmid be specifically delivered to breast cancer cells via a nanoformulation?

4) Are survivin & its variants, Bax and Bcl-2 expressed in primary RB tumors? Are survivin, Bax and Bcl-2 correlated with each other and show any correlation with histopathological features of the primary RB tumors? Can the expressed survivin and its variants in RB be effectively targeted via siRNA technology?
Chapter 2

Materials and Methods
2. Materials and Methods

Methodology for Chapter 3 experiments

The project work was undertaken as per the MOU signed between Vision Research Foundation, Sankara Nethralaya and Deakin University.

2.1. RB Tumor processing

RB tumor tissues were harvested immediately after enucleation for molecular biology studies before the fixation of the globe in formalin.

2.2. RB Tumor specimens and ethics approval

The tumor samples were obtained from enucleated eyes as part of therapy at Larsen & Toubro Ocular Pathology laboratory, Sankara Nethralaya, Chennai, India. Normal retinal tissues were collected from cadaveric eyes which were donated to C.U.Shah eye bank, Sankara Nethralaya, Chennai, India. The study with primary RB tumor samples was reviewed by the ethics committee of Vision Research Foundation, Sankara Nethralaya Institute (Ethics clearance number: 186-2009-P). Tumors were diagnosed as RB and graded by ocular oncologist based on MRI and ocular fundus photography. The eye ball was grossed as per The International Retinoblastoma Staging Working Group (IRSWG) provided guidelines (Sengupta et al. 2013). For every eye ball that was grossed, sixteen sections were taken. Four breadloaf sections, four pupil optic sections, four lateral globe and four surgical end of optic nerve were taken. From the pupil optic nerve sections or lateral globe sections, whichever had the best tumor sections, were taken for the immunohistochemistry study. All 41 tumors were classified as group D and group E according to International Intraocular Retinoblastoma Classification (IIRC) (Murphree 2005). Histopathological classification of the tumors was made according to the differentiation and invasion of the ocular coats (Finger PT, Harbour
JW & ZA 2002; Sastre X et al. 2009). RB tumors were considered well differentiated when rosettes were present in more than 80% of their area. In the absence of rosettes, the tumors were considered poorly differentiated. The rest of the RB tumors were considered to be moderately differentiated (Gómez-Martínez et al. 2012). In the study of NIS expression in RB, 41 tumors were collected from 41 eyes from the patients whose mean age was 3 years. Out of the 41 tumors, 30 were poorly differentiated, 3 were moderately differentiated and 8 were well differentiated tumors. 33 tumors were unilateral and 8 were bilateral (Table 2.1). Apart from this, all the tumors were also analyzed for choroidal, optic nerve, scleral and extra ocular invasion. Choroidal invasion was further divided into <3mm and >3mm invasion, whereas for optic nerve invasion, pre laminar, post laminar and surgical end of the optic nerve were analysed (Sastre X et al. 2009). The tumors with invasion of choroid > 3mm and optic nerve invasion such as post laminar and surgical end of OP nerve will be taken further for post enucleation chemotherapy for 6 cycles and brachytherapy when the invasion spill over into orbit or invade to sclera. RPE invasion alone was not considered as invasive case. Only cases with choroidal and/or optic nerve invasion were considered to be invasive cases. The entire study was conducted according to the principle tenets of the Helsinki Declaration. The interpretation of the histology was done by two ophthalmic pathologists, Dr.Jyotirmay Biswas and Dr.S.Krishnakumar from Sankara Nethralaya, Chennai, India.
Table 2.1 Characteristics of tumor cohort used in NIS expression study

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Clinico-pathological information of RB tumors studied for NIS</th>
<th>Histopathological report</th>
<th>IIRC grade</th>
<th>Invasion status</th>
<th>Age /Sex</th>
<th>Laterality</th>
<th>Differentiation</th>
<th>Treatment modality</th>
<th>Follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anterior chamber invasion, focal RPE and focal choroidal invasion&lt;3mm</td>
<td>D</td>
<td>I</td>
<td>2yr/</td>
<td>OD</td>
<td>PD</td>
<td>NC</td>
<td>No specific ocular complaints</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Multiple foci of RPE invasion, choroid invasion measuring &gt;3mm, prelaminar invasion of optic nerve</td>
<td>D</td>
<td>I</td>
<td>2yr/</td>
<td>OS</td>
<td>PD</td>
<td>NC</td>
<td>8 cycles of post chemotherapy</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Multiple foci of choroid invasion measuring &gt;3mm, No optic nerve invasion</td>
<td>D</td>
<td>I</td>
<td>3yr/</td>
<td>OD/OU</td>
<td>PD</td>
<td>C</td>
<td>No specific ocular complaints</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Multiple tumor lobules in contact with RPE and focal choroid invasion &lt;3 mm. No Optic</td>
<td>E</td>
<td>I</td>
<td>2yr/</td>
<td>OS</td>
<td>PD</td>
<td>NC</td>
<td>No specific ocular complaints</td>
<td></td>
</tr>
<tr>
<td>Case</td>
<td>Diagnosis</td>
<td>Age</td>
<td>Gender</td>
<td>Eye(s)</td>
<td>Prior Treatment</td>
<td>Additional Details</td>
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<tr>
<td>5</td>
<td>Nerve invasion</td>
<td>3yr</td>
<td>F</td>
<td>OD MD</td>
<td>NC 5 cycles of post chemotherapy</td>
<td>measuring &gt;3mm prelaminar, laminar and early post laminar invasion of optic nerve.</td>
<td></td>
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</tr>
<tr>
<td>6</td>
<td>Choroidal invasion</td>
<td>2yr</td>
<td>F</td>
<td>OD/OU PD</td>
<td>NC No specific ocular complaints</td>
<td>RPE invasion, focal choroidal invasion approximately &lt;3mm, pre laminar and laminar invasion of optic nerve.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>7</td>
<td>Focal tumors</td>
<td>9yr</td>
<td>F</td>
<td>OD PD NC</td>
<td>6 cycles of post chemotherapy</td>
<td>Focal tumor clusters are seen in post laminar portion of optic nerve. No choroidal invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Choroidal invasion</td>
<td>4yr</td>
<td>F</td>
<td>OD PD C</td>
<td>Post chemotherapy 17 cycles continued in Malaysia</td>
<td>Choroidal invasion measuring &gt;3mm, prelaminar and laminar invasion of optic nerve</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case</td>
<td>Intraocular Disease</td>
<td>Duration</td>
<td>Initial</td>
<td>OS/OD/OS</td>
<td>PD/MD</td>
<td>NC</td>
<td>Treatment Details</td>
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<tr>
<td>9</td>
<td>Diffuse choroidal invasion measuring &gt;3mm, no invasion of optic nerve</td>
<td>3yr</td>
<td>D</td>
<td>10m</td>
<td>OS</td>
<td>NC</td>
<td>6 cycles of post chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>RPE invasion, no choroidal invasion, prelaminar, laminar and post laminar (moderate) invasion of optic nerve</td>
<td>4yr</td>
<td>D</td>
<td>4yr</td>
<td>OD</td>
<td>NC</td>
<td>6 cycles of post chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Diffuse choroidal invasion &gt;3mm</td>
<td>3yr</td>
<td>D</td>
<td>4yr</td>
<td>OD</td>
<td>NC</td>
<td>5 cycles of post chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Focal invasion of choroid measuring &lt;3mm</td>
<td>2yr</td>
<td>D</td>
<td>2yr</td>
<td>OD</td>
<td>NC</td>
<td>No specific ocular complaints</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Non-Invasive</td>
<td>4yr</td>
<td>E</td>
<td>OD</td>
<td>PD</td>
<td>NC</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>14</td>
<td>Non-Invasive</td>
<td>2yr</td>
<td>D</td>
<td>OD</td>
<td>PD</td>
<td>NC</td>
<td></td>
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</tr>
<tr>
<td>15</td>
<td>Non-Invasive</td>
<td>2.5y</td>
<td>E</td>
<td>OS</td>
<td>WD</td>
<td>NC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Non-Invasive</td>
<td>1.5y</td>
<td>D</td>
<td>OS/OU</td>
<td>WD</td>
<td>C</td>
<td>No specific ocular complaints</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Non-Invasive</td>
<td>5yr</td>
<td>D</td>
<td>OD</td>
<td>PD</td>
<td>NC</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>No.</td>
<td>Condition</td>
<td>Gender</td>
<td>Age/Duration</td>
<td>Primary Site</td>
<td>Follow-up</td>
<td>Treatment</td>
<td></td>
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<tr>
<td>18</td>
<td>Non-Invasive DNI</td>
<td>D</td>
<td>7yr/OS</td>
<td>PD NC F</td>
<td></td>
<td></td>
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<tr>
<td>19</td>
<td>Non-Invasive DNI</td>
<td>D</td>
<td>1yr/OD</td>
<td>PD NC M</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>20</td>
<td>Non-Invasive DNI</td>
<td>D</td>
<td>5yr/OS</td>
<td>PD NC F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Prelaminar, laminar invasion of optic nerve. No choroidal invasion.</td>
<td>E</td>
<td>1mo n/M</td>
<td>WD NC</td>
<td>6 cycles of post chemotherapy</td>
<td>E NI 4yr/ OD PD NC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>No choroidal invasion and no invasion of optic nerve.</td>
<td>E</td>
<td>4yr/OD</td>
<td>PD NC F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Choroidal invasion &lt;3 mm. Prelaminar, laminar and minimal post laminar invasion of optic nerve.</td>
<td>E</td>
<td>5yr/OD/OU</td>
<td>PD NC F</td>
<td>No specific ocular complaints</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>RPE invasion. No choroidal invasion and no optic nerve invasion.</td>
<td>E</td>
<td>3yr/OS</td>
<td>PD NC F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>RPE invasion. No choroidal invasion and no optic nerve</td>
<td>E</td>
<td>2yr/OS</td>
<td>PD NC M</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
invasion.

26 | Diffuse choroidal invasion measuring >3 mm invasion into anterior, middle fibres of sclera, full thickness optic nerve invasion. E I 4yr/ OS PD NC Chemotherapy advised. No follow up

27 | Invasion of choroid, measuring <3 mm. Prelaminar, laminar and minimal post laminar invasion of optic nerve. E I 3yr/ OD PD NC 3 cycles of post chemotherapy

28 | No invasion of optic nerve and no invasion of choroid. D NI 3yr/ OS/OU PD C No specific ocular complaints 6 cycles of chemotherapy

29 | RPE invasion. There is no invasion of choroid. Prelaminar invasion of optic nerve is seen. D I 2yr/ OD/OU WD C No specific ocular complaints 2 cycles of chemotherapy
<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Gender</th>
<th>Age</th>
<th>Side</th>
<th>Field of Vision</th>
<th>Treatment</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>30</td>
<td>No invasion of choroid and no invasion of optic nerve.</td>
<td>Male</td>
<td>3yr</td>
<td>OS</td>
<td>WD</td>
<td>C</td>
<td>2 cycles of chemotherap</td>
</tr>
<tr>
<td>31</td>
<td>No choroidal invasion. Prelaminar invasion of optic nerve.</td>
<td>Female</td>
<td>8mo</td>
<td>OS/OU</td>
<td>WD</td>
<td>C</td>
<td>No specific ocular complaints</td>
</tr>
<tr>
<td>32</td>
<td>No invasion of choroid. Minimal prelaminar invasion of optic nerve.</td>
<td>Male</td>
<td>5yr</td>
<td>OS</td>
<td>PD</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Choroidal invasion with &lt; 3mm. No invasion of optic nerve.</td>
<td>Female</td>
<td>2yr</td>
<td>OD</td>
<td>PD</td>
<td>C</td>
<td>No specific ocular complaints</td>
</tr>
<tr>
<td>34</td>
<td>Focal RPE invasion. No choroidal invasion. Prelaminar and laminar invasion of optic nerve.</td>
<td>Male</td>
<td>1mo</td>
<td>OD</td>
<td>WD</td>
<td>NC</td>
<td>No specific ocular complaints</td>
</tr>
<tr>
<td>35</td>
<td>No invasion of choroid and optic nerve</td>
<td>Female</td>
<td>3yr</td>
<td>OS</td>
<td>PD</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>No invasion of choroid, no invasion of optic nerve</td>
<td>Female</td>
<td>3yr</td>
<td>OS</td>
<td>PD</td>
<td>NC</td>
<td></td>
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nerve

<table>
<thead>
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<th></th>
<th>No invasion of</th>
<th></th>
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<tbody>
<tr>
<td>37</td>
<td>choroid,</td>
<td>D</td>
<td>I</td>
<td>1yr/</td>
<td>OD/OU</td>
<td>PD</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>prelaminar</td>
<td></td>
<td></td>
<td>M</td>
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<td>invasion of optic nerve.</td>
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<th>Choroidal invasion</th>
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<tr>
<td>38</td>
<td>&gt;3mm, prelaminar,</td>
<td>E</td>
<td>I</td>
<td>4yr/</td>
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<td></td>
<td>laminar and minimal post</td>
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<td></td>
<td>laminar invasion of optic nerve.</td>
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<tbody>
<tr>
<td>39</td>
<td>&lt;3 mm. No invasion of optic nerve.</td>
<td>D</td>
<td>I</td>
<td>5yr/</td>
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<td>PD</td>
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<th>No invasion of choroid. No invasion of optic nerve.</th>
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<td>E</td>
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<th>Focal RPE invasion. Prelaminar, laminar invasion of optic nerve.</th>
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<td>41</td>
<td></td>
<td>E</td>
<td>I</td>
<td>3yr/</td>
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<td>PD</td>
<td>NC</td>
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<td></td>
<td></td>
<td>M</td>
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*Tumor cohort characteristics: Abbreviations: I – Invasive, NI – Non-invasive, M– male, F – female, yr – year, mon – Month, OD – right eye (unilateral), OS – left eye (unilateral), OU –
both eyes (bilateral), PD – Poorly differentiated, MD – Moderately differentiated, WD – Well differentiated, C-Chemotherapy, NC – Non-Chemotherapy, ON -Optic nerve, RPE – retinal pigment epithelium.

2.3. Immunohistochemistry for NIS in RB tumors

Immunohistochemistry was performed for NIS in 21 RB tumors. In brief, 5-μm-thick paraffin-embedded tumor sections were de-paraffinized through three changes of xylene and re-hydrated through graded alcohols to distilled water (Figure 2.1). Antigen retrieval was performed with 10 mM citrate buffer (pH, of 6.0), using the pressure cooker method. Endogenous peroxidase activity was neutralized in the investigated sections with 3% H₂O₂ in H₂O for 10 min, and the slides were incubated with mouse monoclonal anti-human NIS antibody (clone FP5A; Thermo Scientific, USA) at 1:100 dilution over night at 4°C. Immunostaining was performed as recommended by the supplier, (Novolink Polymer kit, Leica Microsystems Inc., Bannockburn, IL). Novolink polymer provided in the kit was used as the secondary antibody and the tumor sections were incubated in the polymer for 1 h. The immunoreactivity was revealed by 3, 3′-diaminobenzidine (DAB) and counterstained with hematoxylin. Intermittent washing between the steps was performed using TBS (pH of 7.6). For negative controls, the same procedure was performed in the absence of the primary antibody. Any cells that appear brown were considered positive for the expression of NIS antigen. Immunostaining was evaluated based on the percentage of positive cells and its staining intensity. The average percentage positivity was calculated for each case after scanning ten tumor fields. Numerical score was assigned for each staining intensity with 3 as strong, 2 as intermediate, 1 for weak and 0 for negative. Calculation of histochemical score (H-Score) was done as reported (Detre S 1995).
Figure 2.1 Schematic representation of deparaffinisation of RB tumor sections
2.4. Flow cytometric (FC) analysis of NIS protein in RB tumors

RB tumor tissues (20 in number) and were collected, homogenised and washed. The cells were resuspended in FC buffer containing ice-cold phosphate buffered saline, 10% fetal bovine serum, and 1% sodium azide. Primary antibody, mouse monoclonal antibody against hNIS (17795; Abcam) with a final dilution of 1:50 was incubated with the cells for 60 min at 37°C. After the incubation period, cells were washed thrice (at 400× g for 5 min each) with ice-cold PBS. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (Sigma Aldrich, USA) was used with a final dilution of 1:750 and incubated for 30 min in dark at 37°C. After the secondary antibody incubation, cells were washed thrice (centrifugation at 400× g for 5 min each) with ice-cold PBS. Cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA); with the CellQuest software program (BD Biosciences, USA). In flow cytometry, change in the relative protein expression was defined for cells that had a shift of fluorescence intensity when compared to the unstained and secondary antibody (FITC) alone stained cells.

2.5. Immunoblotting for NIS protein in RB tumors

Approximately 100 mg from 6 RB tissues was homogenized in radioimmunoprecipitation assay lysis buffer containing 50 mM of Tris HCl (pH 7.6), 1% NP40, 0.28% of Deoxycholate, 0.1% SDS, 1 mM of EDTA, 150 mM of sodium chloride, 1% PMSF (Sigma Aldrich, USA), and 250 μl of 1 mg/mL Protease Inhibitor Cocktail (Sigma Aldrich, USA). Tissues were then sonicated at 80 cycles/min. Protein estimation was carried out by the method of Bradford (Bradford 1976). 50 μg of each sample was run on a 12% SDS-PAGE and then electrophoretically transferred onto nitrocellulose membrane. Nonspecific sites were blocked with 5% nonfat dry milk. The blots were incubated with mouse monoclonal antibody against hNIS (17795; Abcam) (1:500) and rabbit polyclonal antibody against hNIS (104920; Abcam) (1:500) overnight at 4°C. The blots were then incubated with their respective
horseradish peroxidase-conjugated secondary antibodies Anti-mouse IgG-HRP and Anti-
rabbit IgG-HRP (1:2000) (Sigma Aldrich, USA) for 2 h. After intermittent washes with TBS-
T (Tris buffered saline-Tween 20), the immunoreactive bands were detected by ECL using
the *Supersignal West Femto*-maximum sensitivity substrate (Thermo Scientific, USA).
Antibodies against β-actin (1:2000; Sigma Aldrich, USA) were used as the loading control.
The NIS band intensity was measured using a densitometer (BIORAD GS 800, USA) and
Quantity One software. The NIS band optical density thus obtained was then normalized with
respect to the loading control β-actin using Image J software (NIH).

**2.6. Determination of NIS protein in RB cell line (Y79 cells)**

**2.6.1. Maintenance of Y79 cell lines**

Human Retinoblastoma cell line, Y79 was purchased from American Type Culture Collection
(ATCC, U.S.A) and was maintained in Roswell Park Memorial Institute 1640 medium
(RPMI 1640, Sigma Aldrich, USA) supplemented with 10 % fetal bovine serum. The Y79
cell line was grown in a 5 % CO₂ incubator at 37°C.

**2.6.2. Flow cytometric analysis of NIS protein in Y79 cells**

5x10⁵ Y79 cells were collected and the cells were processed for flow cytometry as mentioned
in section 2.4 with the same primary antibody and secondary antibody dilution.

**2.6.3. Immunofluorescence of NIS protein in Y79 cells**

Y79 cells (4x10⁵ cells/mL) were collected and washed. The cells were fixed with 4 %
paraformaldehyde (PFA) for 20 min. After the fixation, cells were washed and blocked with
blocking buffer containing 0.2% Triton X 100 and 5 % FBS for 1 h at 37°C. Primary
antibody, mouse monoclonal antibody against hNIS (17795; Abcam) with a final dilution of
1:50 was incubated with the cells overnight at 4°C. After the incubation period, cells were
washed thrice (at 400× g for 5 min each) with ice-cold PBS. Fluorescein isothiocyanate
(FITC)-conjugated anti-mouse IgG secondary antibody (Sigma Aldrich, USA) was used with a final dilution of 1:60 and incubated for 2 h in dark at 37°C. After the secondary antibody incubation, cells were washed thrice (centrifugation at 400× g for 5 min each) with ice-cold PBS. The cells were fixed onto a slide and mounted using a mounting medium containing DAPI. The cells were visualized using a Leica SP5 confocal microscopy and the images were processed using LAS version software.

2.6.4. Immunoblotting of NIS protein in Y79 cells

5×10⁵ Y79 cells were collected and the cells were processed for immunoblotting as mentioned in section 2.5 with anti-human NIS mouse monoclonal antibody (17795; Abcam) (1:500) used as primary antibody. Secondary antibody was anti-mouse IgG conjugated to HRP (Sigma Aldrich, USA) (1:1000) used. The protein lysate was tested for endogenous control, GAPDH. Anti-human gapdh rabbit monoclonal antibody (1:1000) was used as primary antibody and anti-rabbit IgG conjugated to HRP (1:80,000) was used as secondary antibody. The immunoreactive bands were detected by the ECL method.

2.6.5. RNA isolation of Y79 cells

Total RNA was isolated from 5×10⁵ Y79 cells using the TRIzol reagent (Life technologies, USA). The cells were collected and washed with PBS. TRIzol® Reagent was added, vortexed and incubated at 37°C for 5 min. After the incubation period, chloroform was added to denature the protein present in the samples. The samples were centrifuged at 14,000 rpm for 15 min to separate the aqueous layer. To the aqueous phase, isopropanol was added to remove all soluble salts and incubated at 37°C for 20 min. After the incubation period, the samples were centrifuged at 14,000 rpm for 10 min. The pellet was washed with 70% ethanol and air-dried. The air-dried pellet was resuspended in RNase free water and was quantified in BioSpec-Nano spectrophotometer (Shimadzu Biotech, Japan).
2.6.6. cDNA conversion or Reverse Transcriptase PCR (RT-PCR)

SuperScript™ III first strand synthesis system (Life Technologies, Australia) was used to convert the isolated Y79 RNA into cDNA. A concentration of 1 μg of total RNA was used for the conversion which was carried out as per manufacturer’s instructions. To 1 μg of RNA, 1 μL of 50 μM oligo (dT) and 1 μL of 10 mM dNTP mix was added. This mixture was made up to 10 μl by adding nuclease-free water (Applied biosystems, Australia). The mixture was then incubated at 65°C for 5 min in a Takara PCR thermal cycler (Scientifix, Australia) and then immediately incubated in ice for 1 min. The cDNA synthesis mixture was prepared as described in Table 2.2 and 10 μl of it was added to each tube. Samples were incubated in a PCR thermal cycler for 50 min at 50°C, 85°C for 5 min and finally at 4°C. The cDNA was stored at -20°C until further use.

### Table 2.2 Components of a mastermix for RT-PCR reaction

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>VOLUME(μL) per reaction</th>
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<tbody>
<tr>
<td>10X RT Buffer</td>
<td>2</td>
</tr>
<tr>
<td>25mM MgCl$_2$</td>
<td>4</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>2</td>
</tr>
<tr>
<td>Rnase Out</td>
<td>1</td>
</tr>
<tr>
<td>Superscript</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total volume per reaction</strong></td>
<td><strong>10.0</strong></td>
</tr>
</tbody>
</table>
2.6.7. Quantitative Real Time PCR (qRT-PCR) for lactoferrin receptors (Lf)

In Y79 cells, mRNA level of three Lf receptors, low-density lipoprotein receptor-related proteins-1 and -2 (LRP1 and LRP2) and Transferrin receptor (Tfr) was investigated by qRT-PCR using SybrGreen RT-PCR kit (BioRad, Australia). The PCR reaction mixture containing 100 ng of cDNA was prepared by adding 7.5 µL of SYBR *Premix Ex Taq*™, 0.4 µL of sense (0.2 µM) and antisense primer (0.2 µM) of LRP1, LRP2, Tfr and GAPDH. GAPDH was used as the endogenous control. The reaction mix was made upto 15 µL by adding nuclease free water. The samples were placed in a PCR thermal cycler (iQ5, Biorad). The cycling conditions were initial denaturation step at 94°C for 5 min followed by 50 cycles of denaturation step at 94°C for 30 s then an annealing step for 30 seconds for respective primers and an extension step at 72°C for 1 min. Lf receptors expression was normalized with *GAPDH* expression. The data was then analysed by Livak method (Relative Quantification) (Livak KJ & TD 2001). The expression of both the genes in each sample was analyzed in triplicate for statistical comparisons. The primers and its respective annealing temperature are described in the Table 2.3.

Table 2.3 List of primers used in the bLf study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRP1</td>
<td>F: 5’-AGAAGTAGCAGGACCAGAGGG-3’</td>
<td>59°C</td>
</tr>
<tr>
<td></td>
<td>R: 5’ ACAGTACCAGGAGTTATGA 3’</td>
<td></td>
</tr>
<tr>
<td>LRP2</td>
<td>F: 5’-CGGAGCAGTGTCATATTTTC-3’</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CAGGTGTATTTGGGTGTAAGGC-3’</td>
<td></td>
</tr>
<tr>
<td>TFR</td>
<td>F: 5’-TGTAAGGCAGCCATTGTAAC-3’</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CAGATTCCCACACATCCACT-3’</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5’-CCATCACCATCTCCAGGAG-3’</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CCTGCCTCCACACCTTTTG-3’</td>
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Chapter 2

Abbreviations: TFR - Transferrin receptors, LRP1- low-density lipoprotein receptor-related proteins-1, LRP2 - low-density lipoprotein receptor-related proteins-2 and GAPDH- Glyceraldehyde 3-phosphate dehydrogenase.

2.7. Preparation of bovine lactoferrin (bLf)

The experimental work involving bLf was performed in N-LIMBR laboratory, Deakin University, Australia.

2.7.1. Treatment of glasswares

All the glasswares used for bLf preparation was treated with 30% nitric acid (HNO₃) for 24 h for removal of metal ions. After the treatment, the glasswares were dried and autoclaved for further use.

2.7.2. Preparation of Native bLf

5 g of Native bLf powder was dissolved in 50 mL of sterile milliQ water with pH adjusted to 5.8. A portion of the prepared Native bLf was aliquoted and stored at -20°C.

2.7.3. Preparation of Apo bLf

Both iron free (Apo) and iron saturated (Fe) forms of bLf were prepared according to the standardized methods mentioned in (Kanwar, Mahidhara & Kanwar 2012). Apo bLf was prepared from the prepared native bLf by adjusting the pH to 2.06 with 6 N HCl. On pH reduction, the reddish brown colour of the Lf changes to ivory white colour indicating the removal of bound iron in bLf molecules. The preparation was then extensively dialysed against 0.1 M citric acid using a 50 kDa molecular weight cut off spectra/pro dialysis tube (Pacific laboratory, Blackburn, Australia). The dialysis was carried out for 48 h at 4°C with continuous stirring and a change in citric acid every 12 h. The pH of Apo-bLf was then
changed to 8.0 with 10 M sodium hydroxide (NaOH) at the end of dialysis. A portion of the Apo bLf was aliquoted and stored at -20°C.

2.7.4. **Preparation of Fe bLf**

An aliquot of Apo bLf was taken as a starting material for the preparation of iron saturated bLf. Initially, 0.1 M NaHCO₃ was added to the Apo bLf solution and the pH was adjusted to 8.2 using 6 M NaOH. Freshly prepared Fe-NTA solution containing 0.3M ferric nonanhydrate and 0.6M nitrilotriacetate acid disodium salt was added drop-wise to the solution. The solution was stirred continuously with pH maintained at 8.0. The colour of the solution changes from ivory to deep marrow red indicating the iron complex formation. The solution was allowed to stir for 1 h. Dialysis of the sample was done against milli Q for 48 h at 4°C with change of milliQ every 12 h. The samples were stored at -20°C for further use. SDS-PAGE of all the three forms of bLfs were performed (**Figure 2.2**).
Figure 2.2 SDS-PAGE showing the purified form of bLf

Purified and dialysed form of Apo-bLf, Fe-bLf and Native-bLf were electrophoresed in a 10% SDS-PAGE gel. 50μg of protein was loaded for all forms of bLf. The protein bands migrated at a molecular weight of 78 kDa.

2.7.5. Iron estimation in Fe-bLf

The procedure for measuring iron saturation in all the three preparations of bLf (Native-bLf, Apo-bLf and Fe-bLf) was modified from the previously described method by King (1982). To 1 mL of each test solution [Native-bLf, Apo-bLf, Fe-bLf, blank (water) and iron standards (Ferric ammonium sulphate)] 50 µL of ascorbic acid was added and mixed well. The solutions were incubated for 5 min and then 100 µL of 65% tricholoroacetic acid was added to each tube. The solutions were allowed to incubate for 10 min after shaking vigorously for 30 s. after the incubation period, the solutions were centrifuged at 1,000 rpm for 20 min. 100 µL of alkaline acetate was added to 500 µL of the supernatant from each bLf sample, iron standards and blank, which was immediately followed by the addition of 75 µL of tripyridyl solution and incubation for 10 min. After the incubation period, 200 µL of each sample was transferred to an optically clear 96-well plate and absorbance was read in Asys Expert Plus microplate reader (Asys Hitech) at 550 nm. Iron concentration was estimated by using the below shown formula in all the samples. Estimated iron is shown in the Figure 2.3.

\[
\text{Iron (μg/mL)} = \frac{\text{Test sample-Blank}}{\text{Iron standards-Blank}} \times \text{Concentration of Iron standards}
\]
Iron content in bLf

Iron was estimated in all three forms of bLf. Native-bLf had 30% of iron content, Apo-bLf had 3% iron content and Fe-bLf had 97.5% bLf content.

2.7.6. Cellular internalization of bLf in Y79 cells by confocal microscopy

Internalization of all three forms of bLf (Native-bLf, Apo-bLf and Fe-bLf) was studied in Y79 cells. 1x10⁵ Y79 cells were seeded in a 6 well plate. The cells were treated with 12.5 nmol of Native-bLf, Apo-bLf and Fe-bLf for 30 min by incubating the plates at 37°C. After the incubation period, the cells were washed with PBS and fixed with 4% paraformaldehyde (4% PFA) for 20 min. Following the incubation period, the cells were washed and were permeabilised using 0.1% Triton X 100 for 1 min on ice. The cells were then washed and blocked with 3% porcine gelatin for 1 h at 37°C. Blocking was followed by washing the cells twice with PBS and incubation with primary antibody, goat anti-bLf (Bethyl Scientific, Australia) at a dilution of 1: 250 for 1 h at 37°C. Post primary antibody incubation, the cells were washed twice with PBS and incubated with secondary antibody, anti-goat FITC at a dilution of 1:100 (Sigma Aldrich, USA) for 1 h at 37°C in dark. Post-secondary antibody incubation, the cells were washed with PBS twice. The cells were coated onto a PLL coated
slide, allowed to dry and mounted using a Fluoro shield 4’, 6-diamidino-2-phenylindole (DAPI). The slides were then viewed using a confocal microscope. A minimum of five images from 5 different fields were captured for quantitative analysis.

2.7.7. Cellular internalization of bLf in Y79 cells by flow cytometry

Cellular uptake/internalization of all three forms of bLf in Y79 cells was studied by flow cytometric analysis. 1x10^5 Y79 cells were seeded in a 6 well plate. The time allowed for uptake was increased to 48 h compared to 30 min used to study internalization by immunofluorescence. The different concentrations used were 12.5 nmol, 25 nmol, 37.5 nmol, 50nmol, 62.5nmol and 75 nmol for all three bLfs. After 48 h of incubation at 37°C, the cells were collected and washed with PBS. Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton-X-100 for 1 min. Cells were then blocked with 3% porcine gelatin for 1 h at 37°C. Following blocking, cells were incubated with anti-bovine bLf specific polyclonal antibody raised in goat at 1:250 dilution (Bethyl Scientific, Australia) for 1 h at 37°C. Cells were washed and then incubated with secondary anti-goat IgG- FITC antibody (Sigma Aldrich, USA) at 1:100 dilution for 1 h in dark at 37°C. After washing with PBS, the cells were suspended in 500 µL of PBS analysed by flow cytometry (Canto II, BD Biosciences, USA).

Methodology for Chapter 4 experiments

2.8. Molecular cloning

A full length human NIS cDNA driven by CMV promoter in the pCMV6-AC vector was purchased from Origene (U.S.A). Another plasmid, EGP2 expressing NIS gene under the influence of EpCAM promoter was cloned. Cloning procedure is described below
2.8.1. **Competent cell preparation by CaCl₂ method**

Competent cells are bacterial cells which have fragile cell walls through which foreign DNA can enter. These bacterial cell walls can be made fragile and competent by exposing them to either chemical environment (CaCl₂) or electrical field (electroporation). Exposing them to such treatments leaves the bacterial cell wall porous thus enabling easy uptake of DNA. For this study, competent cells were prepared by the CaCl₂ method and the competent cells used were E.coli DH5-α. From the glycerol stock of E.coli DH5-α, a small amount was inoculated in a 5 mL Luria Bertani (LB) broth and incubated overnight in a shaking incubator at 37°C. The following day, from the activated culture, 50 µL was added to a 50 mL of LB broth and incubated for 2.5 h in a shaking incubator at 37°C. After the incubation period, an aliquot of 15 mL from the 50 mL culture was centrifuged at 5,000 rpm for 5 min at 4°C. To the pellet, 5 mL of cold 0.1M CaCl₂ was added, mixed and centrifuged at 5,000 rpm for 5 min at 4°C. Supernatant was discarded and addition of 0.1M CaCl₂ and the centrifugation step was repeated thrice. To the pellet, cold 0.1M CaCl₂ was added again and incubated in ice for 1.5 h. After the incubation period, cells were centrifuged and the pellet was resuspended in 0.1M CaCl₂. The competent cells were used immediately for transformation.

2.8.2. **Transformation by heat-shock method**

Transformation is a basic technique where a foreign DNA is inserted into a bacterium for replication of the DNA (Alexandrine Froger & Hall 2007). In order to utilize this property of transformation, most of the plasmids are designed to possess bacterial origin of replication and an antibiotic resistance gene for selection of transformed colonies in bacteria. Heat-shock method of transformation was performed for this study. To the 100 µL of freshly prepared competent cell, 250 ng of CMV-NIS plasmid was added, mixed and incubated in ice for 10 min. After 10 min of incubation, the vial was immediately transferred to a 42°C water bath for 90 s. Immediately after 90 s, the vial containing competent cells and plasmid was
incubated in ice for further 10 min. Following the heat shock treatment, the contents of the vial was transferred to a 1 mL of LB broth free of antibiotics and incubated in a shaking incubator at 37°C for 1 h. From the 1mL of cultured LB broth, 100 µL was plated onto a LB plate containing 25 µg/mL kanamycin antibiotic because of the presence of kanamycin resistance gene on CMV-NIS plasmid. The plate was incubated at 37°C overnight. The following day, kanamycin resistant colonies were picked, inoculated in a 5 mL LB broth containing kanamycin antibiotic and incubated at 37°C in a shaking incubator plasmid isolation procedures.

2.8.3. Minipreparation of plasmid DNA

Isolation of plasmid DNA in a small scale from bacteria is known as mini-prep. The usual method for recovering plasmids is the alkaline lysis method. In this study, a QIAprep Spin Miniprep kit (Qiagen) was used to isolate CMV-NIS plasmid. The overnight grown bacterial culture containing CMV-NIS plasmid was centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended with 250 µL of resuspension buffer, Buffer P1 (RNAse and lyse blue added) (Buffer composition described in Appendix). The suspension was always kept in ice. To the suspension, 250 µL of lysis buffer, Buffer P2 was added and mixed by inverting gently. The suspension turned blue. To the mix, 350 µL of neutralization buffer, Buffer N3 was added and inverted immediately for mixing. The solution turned white and cloudy. The solution was centrifuged at 13,000 rpm for 10 min at 4°C. After the centrifugation, the supernatant was applied onto a spin column and centrifuged for 1 min. The flow-through was discarded and 500 µL of Buffer PB was added to the column and centrifuged for 1 min. The flow-through was discarded and 750 µL of Buffer PE was added to the column and centrifuged for 1 min. The flow-through was discarded and the column was centrifuged for 3 min to remove residual ethanol. The spin column was placed in a fresh 1.5 mL vial and 50 µL of Buffer EB was added to the centre of the column. The
column was allowed to stand for 1 min and centrifuged for 2 min. The eluted CMV-NIS plasmid was run on an agarose gel to confirm the correct size. CMV-NIS plasmid was stored at -20°C until further use.

2.8.4. Maxi-preparation of plasmid DNA
Isolation of plasmid DNA in a large scale from bacteria is known as maxi-prep. In this study, a Plasmid Maxi kit (Qiagen) was used to propagate CMV-NIS plasmid. The overnight grown bacterial culture containing CMV-NIS plasmid (100 mL) was centrifuged at 6,000 rpm for 15 min at 4°C. The bacterial pellet was resuspended in 10 mL of ice-cold resuspension buffer, Buffer P1 (RNase A added) (Buffer composition described in Appendix). The pellet was dissolved until no clumps remained in the solution. To the suspension, 10 mL of lysis buffer, Buffer P2 was added and was mixed gently by inverting. The suspension was allowed to incubate for 5 min at room temperature. After incubation, 10 mL of ice-cold neutralization buffer, Buffer P3 was added to the suspension. The tube was inverted immediately for proper mixing. The solution turned less viscous. The suspension was incubated for 20 min in ice. After incubation period, the sample was centrifuged at 12,000 rpm at 4°C for 30 min. The supernatant was added to a fresh tube and centrifuged at 12,000 rpm at 4°C for 15 min. The Qiagentip-500 was equilibrated by 10 mL of equilibration buffer, Buffer QBT. The column was allowed to empty by gravity flow. To the equilibrated Qiagentip, the supernatant of step 5 was added and allowed to enter the resin. After the supernatant has passed the Qiagentip, it was washed with 2x30 mL of wash buffer, Buffer QC. After the washing step, the Qiagentip was placed in a fresh tube and the DNA was eluted by adding 15 mL of elution buffer, Buffer QF. To the eluted DNA, 10.5 mL of isopropanol was added to precipitate the DNA. The sample was centrifuged at 11,000 rpm for 30 min at 4°C. After the centrifugation, the supernatant was discarded. The pellet was washed with 5 mL of 70% ethanol by centrifuging at 11,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was air-dried.
till the remnant ethanol evaporated. To the air-dried pellet, required volume of 1XTE buffer (pH 8.0) was added and resuspended. The correct size and quality of the CMV-NIS plasmid was analyzed on an agarose gel.

2.8.5. **Restriction digestion**

Restriction digestion enables DNA to be cut with the help of specific restriction enzymes at specific regions which results in compatible ends which are in turn capable of a desired ligation. CMV-NIS plasmid and EGP2-TK plasmid were digested using specific restriction enzymes like *Bam*HI and *Xho*I. Using these restriction enzymes, NIS gene was extracted from the CMV-NIS plasmid and EpCAM vector backbone was extracted from the EGP2-TK plasmid. The reaction mix for restriction digestion consists of 250 ng of the plasmids, 2 µL of 10X Buffer 4, 30 units of *Bam*HI and *Xho*I and nuclease free water to make it up to 20 µL. The components in the reaction mix were mixed gently by pipetting up and down. The restriction digestion reaction was set for 3 h at 37°C. The digested product along with undigested sample was analyzed in an agarose gel.

2.8.6. **Gel elution**

The restricted digested product from the agarose gel was eluted using MinElute Gel Extraction Kit (Qiagen). The NIS gene from the digested CMV-NIS plasmid and EpCAM vector backbone from the digested EGP2-TK plasmid was excised from the agarose gel with a clean sharp scalpel. The gel slice was weighed and 3 volumes of solubilisation buffer, Buffer QG were added to 1 volume of gel. Incubation was done at 50°C for 10 min. One gel volume of isopropanol was added to the sample and was mixed by inverting the tube several times. The sample was applied to the MinElute column and centrifuged for 1 min at 11,000 rpm. The flow-through was discarded and 500 µL of Buffer QG was added to the column and centrifuged for 1 min at 11,000 rpm. The flow-through was discarded and 750 µL of Buffer PE was added to the column and centrifuged for 1 min at 11,000 rpm. The flow-through was
discarded and the column was centrifuged for an additional 1 min at 11,000 rpm. The MinElute column was placed in a 1.5 mL vial and 10-15 µL of Buffer EB was added at the center of the membrane. The column was allowed to stand for a min and then centrifuged for 1 min at 11,000 rpm. The eluted DNA was analyzed on an agarose gel for the correct band size.

2.8.7. **Ligation**

Ligation is one of the key steps in the process of recombinant DNA technology. With the help of ligase enzymes, desired genes can be added or pasted onto a carrying vector. The eluted NIS gene and EpCAM vector backbone containing sticky ends was ligated using T4 DNA ligase enzyme (Promega). The ligation reaction was set in a volume ratio of 1:3 of vector: gene insert. The total ligation reaction contained 1 volume of EpCAM vector backbone, 3 volume of NIS gene, 2 µL of 10X ligase buffer, 1 µL of T4 DNA ligase enzyme with nuclease free water made upto 20 µL. The reaction mixture was incubated at 16°C overnight. The ligated sample (EpCAM-NIS) was run in an agarose gel to check for the correct plasmid size.

2.9. **Establishment of CMV-NIS and EpCAM-NIS stable transfectants of MCF-7 cells**

2.9.1. **Maintenance of cell lines**

Human breast cancer cell lines, MCF-7, purchased from ATCC (U.S.A) was maintained in Dulbecco’s Modified Eagle Medium (DMEM, Sigma Aldrich, USA) supplemented with 10% foetal bovine serum. MIO-M1 (Human Retinal Müller glial cell line) was a gift from G.A. Limb, UCL Institute of Ophthalmology, London, England (Limb GA et al. 2002) and was maintained in DMEM medium supplemented with 10% foetal bovine serum. All the cell lines were grown in a 5 % CO₂ incubator at 37°C.
2.9.2. Generation of stable MCF-7 transfectants

MCF-7 cells were seeded on a 6-well plate at 1x10^5 cells. Transfection was performed after the cells attained 80% confluency. The transfection complexes were prepared with Lipofectamine TM 2000 transfection reagent (Invitrogen, USA) for both CMV-NIS plasmid and EpCAM-NIS plasmid. After 20 min of incubation, the complexes were added to cells in separate wells for each plasmid complex. After 4 h of incubation at 37°C in a 5% CO2 incubator, the old DMEM medium was replaced with fresh DMEM medium containing serum for a further 48 h of incubation at 37°C in a 5% CO2 incubator before adding Geneticin (G418) (1mg/mL), an antibiotic for selection of positive clones. Geneticin containing media was added to the transfected cells for a period of 14 days. After 14 days, the survived cells were transferred to a separate dish till the cells become confluent in the dish. The survived cells was then analysed for NIS expression at mRNA and protein level.

2.9.3. Overexpression of NIS in stable transfectants determined at mRNA level by qRT-PCR

5x10^5 cells of stable MCF-7 transfectants (MCF-CMV-NIS and MCF-EpCAM-NIS) were collected and the cells were processed for RNA isolation, cDNA conversion as mentioned in section 2.6.5 and 2.11.2. qRT-PCR was performed as mentioned in section 2.11.3 with the NIS and GAPDH primers given in Table 2.4.

Table 2.4 List of primers used in the NIS study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Amplion size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIS</td>
<td>FP:5'-TGCGGGACCTTTGCAGTACATT-3'; RP:5'-TGCAGATAATTCCGGTGACAA-3'</td>
<td>132</td>
</tr>
<tr>
<td>GAPDH</td>
<td>FP:5'-ACCCACTCCTCCACCTTTGA-3'; RP:5' CTGTTGCTGTAGCCAAATTCGT-3'</td>
<td>100</td>
</tr>
</tbody>
</table>
2.9.4. Overexpression of NIS in stable transfectants determined at protein level by Western blotting

5x10^5 cells of stable MCF-7 transfectants (MCF-CMV-NIS and MCF-EpCAM-NIS) were collected and the cells were processed for immunoblotting as mentioned in section 2.5 with anti-human anti-NIS mouse monoclonal antibody (Abcam) (1:500) used as primary antibody. Secondary antibody was anti-mouse IgG-HRP conjugate (Sigma Aldrich, USA) (1:1000) used. The loading control was β-actin. Mouse monoclonal anti-human β-actin (Sigma Aldrich, USA) (1:4000) was used as primary antibody and anti-mouse-IgG HRP conjugate (Sigma Aldrich, USA) (1:7,000) was used as secondary antibody. The immunoreactive bands were detected with the Super Signal West Femto Chemiluminescent Substrate (Thermo Scientific, USA). The NIS band intensity was measured using a densitometer (BIORAD GS 800, USA). The NIS band density thus obtained was then normalized with respect to the loading control β-actin using Image J software (NIH).

2.9.5. Migration assay

For the normal functioning of organisms and embryonic development, cell migration and invasion processes play a key role. One of the assays to study cell migration is the wound healing assay. The stable transfectants, MCF-CMV-NIS and MCF-EpCAM-NIS were seeded in a 6-well plate and allowed to grow till it attained full confluence. A wound/scratch was created to the cell monolayer using a 200 µL pipette tip. Fresh DMEM media with serum was added to the cells after washing with PBS to remove floating cells. Photographs of the wound in both controls as well as in the MCF-CMV-NIS and MCF-EpCAM-NIS were captured at 0 and 24 h using confocal microscope.

2.9.6. Radioiodide Uptake Assay in the stable transfectants

The standard assay for sodium iodide symporter (NIS) function is based on the measurement of radioiodide uptake (NaI^{125}) in NIS-expressing cells. Radioactive iodide uptake studies in
the stable transfectants, MCF-CMV-NIS and MCF-EpCAM-NIS were performed as described (Carlin S et al. 2000). 1x10^6 cells of MCF-CMV-NIS and MCF-EpCAM-NIS were collected, washed with ice-cold incubation buffer (composition mentioned in appendix) and then incubated with 1mL of incubation medium containing 4 μCi/mL Na^{125}I and 10µM of NaI for 2h. After the incubation period, the supernatant was discarded and the cells were washed with ice-cold incubation buffer. Cells were lysed using lysis buffer and collected as pellet. Activity was counted in the pellet using 2470 WIZARD^2 Automatic Gamma Counter (Perkin Elmer, USA).

2.9.7. Non-radioactive iodide uptake assay in the stable transfectants

Iodide uptake assay is the basis of determining the function of NIS in NIS expressing cells. A spectrophotometric assay (reaction shown below) measuring the uptake of iodide in the stable transfectants, MCF-CMV-NIS and MCF-EpCAM-NIS was performed as described (Waltz F, Pillette L & Y 2010). Initially NaI standards were assayed for plotting the standard graph. NaI standards at 100, 200, 300, 400, 500, 600 and 700 nM was prepared in water and distributed in triplicates in a 96-well plate. To the NaI, 100 µL of ammonium cerium (IV) sulfate and 100 µL of sodium arsenite solutions were added and incubated for 30 min in dark. The absorbance at 420 nm was recorded immediately on a SpectraMax Plus 384 Absorbance Microplate Reader (Molecular Devices, USA). The stable transfectants, MCF-CMV-NIS and MCF-EpCAM-NIS was seeded at a density of 1x10^4 cells in a 96-well plate. The next day, the cells were washed with cold uptake buffer (composition mentioned in appendix) and the residual supernatant was discarded. Cells were then incubated with NaI (10µM) for 15, 30, 45 and 60 min in dark. After the incubation period, NaI was decanted and washed with cold uptake buffer. To the cells, 100 µL of water, 100 µL of ammonium cerium (IV) sulfate (10.5 mM) and 100µL of sodium arsenite solutions (24 mM) were added and incubated for 30 min in dark. After the incubation period, the plate was immediately read on a SpectraMax Plus
384 Absorbance Microplate Reader (Molecular Devices, USA) at 420 nm. The logarithmic conversion of the means of A420 vs. iodide standard concentrations was taken for plotting the calibration curve. The iodide concentrations in the stable transfectants, MCF-CMV-NIS and MCF-EpCAM-NIS were determined after linear regression of the calibration curve.

\[ \text{Reaction between As(III) and Ce(IV) catalyzed by iodide (Sandell-kolthoff reaction) (Waltz, Pillette & Ambroise 2010).} \]

2.9.8. **Inhibition of uptake by Sodium perchlorate**

Inhibition of iodide uptake was performed to show whether the iodide uptake is via NIS. 1x10^6 cells of MCF-CMV-NIS and MCF-EpCAM-NIS were collected, washed with ice-cold incubation buffer and then incubated with 1mL of incubation medium containing 1mM sodium perchlorate, 4 µCi/mL Na^{125}I and 10 µM of NaI for 2 h. After the incubation period, the supernatant was discarded and the cells were washed with ice-cold incubation buffer. Cells were lysed using lysis buffer and collected as pellet. Activity was counted in the pellet using 2470 WIZARD^2 Automatic Gamma Counter (Perkin Elmer, USA).

2.9.9. **Efflux of iodide**

To determine the rate at which iodide is effluxed out, efflux assay was performed. 1x10^6 cells of MCF-CMV-NIS and MCF-EpCAM-NIS were collected, washed with ice-cold incubation buffer and then incubated with 1mL of incubation medium containing 4 µCi/mL Na^{125}I and 10 µM of sodium iodide for 2 h. After the incubation period, the supernatant was discarded and the cells were washed with incubation buffer. Cells were lysed using lysis buffer and...
collected as pellet. This was noted as 0 mins. To the pellet, incubation buffer was added again and incubated further. Supernatant was collected in various time points (15, 30 and 60 min). Activity was counted at each time point in the supernatant as well as in the pellet using 2470 WIZARD² Automatic Gamma Counter (Perkin Elmer, USA).

2.10. Preparation and characterisation of nanocomplex

Polyethyleneimine (PEI), a cationic polymer, has been used as an efficient nonviral vector for gene delivery in vitro as well as in vivo (Antoine Kichler et al. 2001). In this study, a nanocomplex of PEI/NIS pDNA coated by an EpCAM aptamer (EpDT3) was prepared and characterised.

2.10.1. PEI nanocomplex preparation

For the preparation of the complexes, NIS pDNA and PEI were mixed in water by pipetting at various weight ratios such as 1:1.25, 1:2.5, 1:3.75, 1:5, 1:6.25 and 1:7.5 by keeping NIS pDNA concentration constant. PEI/NIS pDNA complexes were formed on the basis of electrostatic attraction. The complex formation was observed in agarose gel when the complexes were electrophoresed. For the nanocomplex formation, EpDT3 aptamer at increasing concentration starting from 50 nM till 200 nM was added to the PEI/NIS pDNA complex and incubated at 37° for 10 min. The formed nanocomplex was run in an agarose gel after 10 min of incubation.

2.10.2. Dynamic Light Scattering

The ζ-potential and particle size of the nanocomplexes were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom). Biostability of the prepared nanocomplexes in the presence of serum was also measured for 3 days by analyzing the ζ-potential and particle size at various period of time.
2.10.3. Transmission Electron Microscope

Transmission electron microscope is a higher resolution microscope which uses electrons to form an image. The size and shape of the prepared nanocomplexes were imaged by transmission electron microscopy (TEM). A 20 µL of the cell suspension was placed on a carbon coated grid for 10 to 15 min allowing the particles to settle down. The grid was removed and 20 µL of 3% Uranyl acetate was placed on the grid for 5 min. Then the grid was removed, air dried and screened in the JEOL JEM1400 Transmission Electron Microscope at an accelerating voltage of 80 kV. Electron Micrographs were taken using the Olympus Keenview CCD camera attached to the microscope.

2.10.4. Determination of NIS protein expression in the nanocomplex delivered cells

5x10^5 cells of MCF-7 were seeded and transfection of NIS plasmid was done using different delivery vehicle such as lipofectamine, PEI alone and nanocomplex. The NIS expression after the transfection was analysed using immunoblotting. Immunoblotting was performed as mentioned in section 2.5.

2.10.5. Nonradioactive iodide and radioactive iodide uptake assay

The iodide uptake assay by both radioactive assay as well as non-radioactive assay was performed for the nanocomplex as described earlier in section 2.9.6 and 2.9.7.

Methodology for Chapter 5 experiments

2.11. RB Tumor processing

General information about primary RB tumor sample details and RB tumor processing have been mentioned in section 2.1 and 2.2 of this chapter. For the study of Bcl-2, Bax and survivin splice variants expression in RB, twenty tumors were collected from 20 eyes for the study from the patients whose mean age 1.9 years. Out of the 20 tumors, 10 were poorly
differentiated, 5 were moderately differentiated and 5 were well differentiated tumors. Eight
tumors were non-invasive and 12 were invasive and all 20 tumors were unilateral (Table 2.5). Apart from this, all the tumors were also analyzed for choroidal, optic nerve, scleral and
extra ocular invasion. Choroidal invasion was further divided into focal invasion, <3mm and
>3mm, whereas for optic nerve invasion, pre laminar, post laminar and surgical end of the
optic nerve were analysed (Sastre X et al. 2009). The entire study was conducted according to
the principle tenets of the Helsinki Declaration.

Table 2.5 Characteristics of tumor cohort used in survivin splice variants, Bax and Bel-2 expression study

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinico-pathological information of RB tumors studied for NIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Histopathological features</td>
</tr>
<tr>
<td>1</td>
<td>Focal RpE invasion. No Invasion of Choroid.</td>
</tr>
<tr>
<td>2</td>
<td>Choroidal invasion &gt;3mm. Laminar and Post-Laminar invasion of the Optic nerve.</td>
</tr>
<tr>
<td>3</td>
<td>No Invasion of Choroid, No Invasion of Optic Nerve.</td>
</tr>
<tr>
<td>4</td>
<td>Choroidal Invasion</td>
</tr>
<tr>
<td>#</td>
<td>Description</td>
</tr>
<tr>
<td>----</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>5</td>
<td>Focal Invasion of Choroid $&lt;$3mm. Pre-Laminar invasion of Optic Nerve</td>
</tr>
<tr>
<td>6</td>
<td>No Choroidal Invasion. Pre-Laminar invasion of Optic Nerve.</td>
</tr>
<tr>
<td>7</td>
<td>Invasion of Choroid, $&lt;$3mm, Laminar Pre-Laminar and minimal Post-Laminar invasion of Optic Nerve</td>
</tr>
<tr>
<td>8</td>
<td>No Invasion of Choroid, No Invasion of Optic Nerve</td>
</tr>
<tr>
<td>9</td>
<td>Focal Invasion of Choroid $&gt;$3mm. Invasion of Pre-Laminar portion of Optic Nerve</td>
</tr>
<tr>
<td>10</td>
<td>Choroidal Invasion $&lt;$3mm. No Invasion of Optic Nerve</td>
</tr>
<tr>
<td>11</td>
<td>Focal choroidal Invasion measuring $&lt;$3mm. Pre-Laminar invasion of Optic Nerve</td>
</tr>
<tr>
<td>12</td>
<td>Focal choroidal invasion measuring $&lt;$3mm. No</td>
</tr>
<tr>
<td></td>
<td>laminar and post laminar invasion of optic nerve.</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>13</td>
<td>No choroidal invasion, prelaminar invasion of optic nerve.</td>
</tr>
<tr>
<td>14</td>
<td>No Invasion of Choroid, No Invasion of Optic Nerve</td>
</tr>
<tr>
<td>15</td>
<td>Invasion of the choroid measuring &gt;3mm. Prelaminar invasion of the optic Nerve.</td>
</tr>
<tr>
<td>16</td>
<td>Choroidal invasion measuring &gt; 3mm. Prelaminar invasion of optic nerve.</td>
</tr>
<tr>
<td>17</td>
<td>No choroidal Invasion. No Optic Nerve Invasion</td>
</tr>
<tr>
<td>18</td>
<td>Choroidal invasion measuring &gt;3mm, Pre-Laminar Laminar and Minimal Post-Laminar Invasion of Optic Nerve.</td>
</tr>
<tr>
<td>19</td>
<td>Choroidal invasion measuring &gt;3mm. Pre-Laminar and Laminar Invasion of Optic Nerve.</td>
</tr>
<tr>
<td>20</td>
<td>No Invasion of Choroid.</td>
</tr>
</tbody>
</table>
2.11.1. RNA isolation of the tumors

Frozen RB tissues were homogenized and total RNA was isolated using the TRIzol reagent (Life technologies, USA) as mentioned in the section 2.6.5.

2.11.2. cDNA conversion of the tumor RNA

After the total RNA was assessed for its concentration and purity, 1 µg of it was converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Invitrogen, USA). The total volume of the RNA mixture for cDNA conversion is 10 µL consisting of 1 µg of RNA, 1 µL of 10X RT buffer, 0.4 µL of 25X dNTP mix (100mM), 1 µL of 10X RT random primers, 0.5 µL of multiscrbe reverse transcriptase enzyme, 0.5 µL of RNase inhibitor and nuclease free water to make up the volume to 10 µL. The samples were mixed and loaded in a thermocycler (Eppendorf, India) with the thermal cycler conditions as mentioned in Table 2.6. The cDNA obtained was stored at -20°C until further use.

<table>
<thead>
<tr>
<th>TEMPERATURE (°C)</th>
<th>TIME (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.6 Cycling conditions for RT-PCR

2.11.3. qRT-PCR for survivin splice variants, Bax and Bcl-2

In 20 RB tumors, mRNA level of five survivin splice variants, Bax and Bcl-2 was investigated by using RT² SYBR® Green qPCR Mastermix (SABiosciences, USA). The PCR reaction mixture containing 100 ng of cDNA was prepared by adding 5 µL of 2 X SYBR green reagents, 0.2 µL of sense and antisense primer (10µM) of respective primers. The reaction mix was made upto 10 µL by adding nuclease free water. GAPDH was used as the endogenous control. The samples were placed in a PCR thermal cycler, ABI Prism 7500 Sequence Detector (Applied Biosystems, Lab India, Chennai, India). The cycling conditions were initial denaturation step at 50°C for 2 min, 95°C for 5 min followed by 40 cycles of denaturation step at 95°C for 15 s then an annealing temperature at 60°C for 1 min and melt curve stage at 95°C for 15 s, 60°C for 60 s, 95°C for 30 s and 60°C for 15 s. The target gene expression was normalized with GAPDH expression. The data was then analysed by Livak method (Relative Quantification) (Livak KJ & TD 2001). The expression of all the genes in each sample was analyzed in triplicate for statistical comparisons. The primers and its respective amplicon size are described in the Table 2.7.
### Table 2.7 List of primers used in the survivin splice variants, Bax and Bcl-2 study

<table>
<thead>
<tr>
<th>MOLECULE</th>
<th>PRIMER</th>
<th>SEQUENCE</th>
<th>AMPLICON SIZE (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH Forward</td>
<td>5’ACCCACTCCTCCACCTTTTG3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>5’CTGTTGCTGTAGCCAAATTCG3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax Forward</td>
<td>5’CCTGTGCAACCGACTCTTCACTCA3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax Reverse</td>
<td>5’CCACCGTGCCGATCCAGGCC3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2 Forward</td>
<td>5’ATGTGTTGGAGACGCTCAA3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2 Reverse</td>
<td>5’ACAGTTCCACAAAGGCATCC3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin WT Forward</td>
<td>5’GACCACCGCATCTCTACATTC3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin WT Reverse</td>
<td>5’TGCCTTTATGTTCTCTATGCG3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin ΔEx3 Forward</td>
<td>5’GCTGGGAGCCAGATGACG3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin ΔEx3 Reverse</td>
<td>5’TCCGCAGTTTCTCAAATGTTT3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin 2-α Forward</td>
<td>5’GCTTTGTTTTGAACTGAGTTGCA3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin 2-α Reverse</td>
<td>5’GCAATGAGGGTGGAAGAC3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin 2-β Forward</td>
<td>5’CGGGCAGGGTGTTCA3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin 2-β Reverse</td>
<td>5’CAACCGGACGATGCTTTT3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin 3-β Forward</td>
<td>5’GAAAAATGGAAGCCAGATTCA3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin 3-β Reverse</td>
<td>5’ACAGACCGCTGCAACATCAG3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.1. Silencing of Survivin-WT and Survivin-ΔEx3 in RB cell line (Y79 cells)

2.1.1. Maintenance of Y79 cell lines

Human retinoblastoma cell line, Y79 was maintained as mentioned in section 2.6.1

2.1.2. qRT-PCR for survivin splice variants, Bax and Bcl-2 in Y79 cells

5x10⁵ Y79 cells were collected and the cells were processed for RNA isolation, cDNA conversion as mentioned in section 2.6.5 and 2.11.2 respectively. qRT-PCR was performed in Y79 cells as mentioned in section 2.11.3 with the same set of primers.
2.1.3. Transfection of Sur-WT siRNA and Sur-ΔEx3 siRNA in Y79 cells

For silencing Sur-WT and Sur-ΔEx3, 2x10^5 Y79 cells were seeded in a 6 well plate. Transfection was performed after the cells attained 50 % confluency for siRNA transfection. 100 nM of Sur-WT siRNA and Sur-ΔEx3 siRNA (Dharmacon, Inc. USA) was diluted in a serum free RPMI media separately and was incubated for 5 min at 37°C. Similarly, lipofectamine™ 2000 (Invitrogen, USA) was also diluted in a serum free RPMI media and was incubated for 5 min at 37°C. The siRNA dilution and lipofectamine dilutions were mixed gently and were incubated for 20 min at room temperature for lipofectamine/Sur-WT siRNA and lipofectamine/Sur-ΔEx3 siRNA complex to form. After the incubation period, each complex was added to the cells containing serum free RPMI media. The complexes were removed 4 h after transfection and complete RPMI media with serum was added to the cells. The transfected cells were incubated at 37°C in 5 % CO₂ incubator for 48 h until further analysis.

2.1.4. qRT-PCR for Sur-WT and Sur-ΔEx3 silenced Y79 cells

2x10^5 Sur-WT and Sur-ΔEx3 silenced Y79 cells were collected and the cells were processed for RNA isolation, cDNA conversion as mentioned in section 2.6.5 and 2.11.2. qRT-PCR was performed as mentioned in section 2.11.3 with the same set of Survivin-WT and Survivin-ΔEx3 primers.

2.1.5. Immunoblotting for Sur-WT and Sur-ΔEx3 silenced Y79 cells

5x10^5 Sur-WT and Sur-ΔEx3 silenced Y79 cells were collected and protein lysate prepared by lysing cells using RIPA buffer containing 250 µL of 1mg/mL of PIC (Sigma Aldrich, USA). Cells were then sonicated at 80 cycles/min. Protein estimation was carried out by the method of Bradford (Bradford 1976). 100 µg of each sample was run on an 18 % SDS-PAGE and then electrophoretically transferred onto nitrocellulose membrane. Nonspecific sites were blocked with 4 % nonfat dry milk. The blots were incubated with rabbit
monoclonal antibody against hSur-WT (Ab76424, Abcam) (1:500) and rabbit polyclonal antibody against hSur-ΔEx3 (Ab3731, Abcam) (1:250) overnight at 4°C. The blots were then incubated with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Sigma Aldrich, USA) at 1:2000 dilution for 2 h. After intermittent washes with TBS-T (Tris buffered saline-Tween 20), the immunoreactive bands were detected by ECL using the Supersignal West Femto-maximum sensitivity substrate (Thermo Scientific, USA). Mouse monoclonal antibody against β-actin (1:2000; Sigma Aldrich, USA) was used as the loading control and detected by Anti-mouse IgG HRP conjugated antibody (1:7000) (Sigma Aldrich, USA). The survivin band intensity was measured using a densitometer (BIORAD GS 800, USA) and was then normalized with respect to the loading control β-actin using Image J software (NIH).

2.1.6. Annexin V staining of the Sur-WT and Sur-ΔEx3 silenced Y79 cells

2x10^5 Sur-WT and Sur-ΔEx3 silenced Y79 cells were collected and washed with PBS. Five sets of samples/cells were prepared for apoptosis analysis using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, CA, USA) as shown in Table 2.8. On addition of 100 µL of the mixture to each sample, the cells were incubated for 20 min at 37°C. After the incubation period, cells were washed with PBS twice before analysing it in the FACS Calibur (BD Biosciences, CA, USA) using ‘CellQuest Pro’ software.
Table 2.8 Annexin V - PI Staining Mix

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstained</td>
<td>Untreated cells+1X Binding buffer</td>
</tr>
<tr>
<td>Annexin alone</td>
<td>Untreated cells+4 µL of Annexin</td>
</tr>
<tr>
<td>Propidium iodide alone</td>
<td>Untreated cells+2.5 µL of Propidium iodide</td>
</tr>
<tr>
<td>Control</td>
<td>Untreated cells+4 µL of Annexin+2.5 µL of Propidium iodide</td>
</tr>
<tr>
<td>Test</td>
<td>Treated cells+4 µL of Annexin+2.5 µL of Propidium iodide</td>
</tr>
</tbody>
</table>

2.1.7. Caspase 3/7 activity of the Sur-WT silenced Y79 cells

2x10⁵ Sur-WT silenced Y79 cells were collected and washed with PBS. Caspase 3/7 activity of the silenced samples was assessed by Apo-ONE® Homogeneous Caspase-3/7 Assay kit (Promega, USA). Caspase reagent was prepared by adding caspase substrate and caspase buffer in 1:100 dilution. The caspase reagent was then added to the cells in 1:1 ratio, distributed in 384 or 96 well plates and incubated at 37°C for 30 min in dark. Post-incubation, the plate was read in a Spectra Max plus 384 (Molecular devices, USA) at an excitation wavelength range of 485 ± 20nm and an emission wavelength range of 530 ± 25nm to detect caspase-3/7 activity in the silenced samples.

2.2. Statistical analysis

Statistical analysis of all data was performed using SPSS® version 21 for Windows statistical software package. Data were assessed using student $t$-test for normally distributed variables.
and Mann-Whitney U test for not normally distributed variables. In order to compare means of different subgroups, Kruskal-Wallis test was conducted for not normally distributed variables. Spearman correlation coefficient (r) was used to measure correlation of not normally distributed variables. For all tests, a two-sided P value of less than 0.05 was considered as significant.
Chapter 3

Studies on sodium iodide symporter (NIS) expression in retinoblastoma: a clinicopathological report

“One of the oldest examples of an imaging reporter gene in humans is the sodium iodide symporter (NIS) which has emerged as one of the most exciting and promising reporter genes in preclinical and translational research” – Penheiter et al, 2012
3. Chapter 3

3.1. INTRODUCTION

Retinoblastoma (RB) is a genetically determined pediatric intraocular tumor arising from the retinal cells of the infants (Xu XL et al. 2009). RB is prevalent in highly populated regions like Asia and Africa due to the lack of awareness about the disease and delay in diagnosis ultimately leading to high mortality rate in such regions (Dimaras et al. 2012). The estimated occurrence of RB in Australia is 14.8 (average number of cases per year) which covers 2.4% of all childhood cancers detected per year (Baade et al. 2010). However, in India, studies show that the estimated occurrence of RB is two-to-three times higher than the developed countries (Arora RS, Eden TO & G 2009). Also in India, human papilloma virus (HPV) infection is prevalent and the studies have shown that maternal transmission of HPV 16/18 can possibly lead to the development of sporadic form of RB (Anand Bhuvaneswari, Jayshree & Kumar 2012; Shetty et al. 2012).

Earlier, RB was considered a fatal disease among children but its survival rate has increased considerably over the past decade owing to the advancements in management and therapy (Naseripour 2012). To improve eye salvage, currently various treatments are available depending upon the size and grade of the tumors. Cryotherapy is performed on small tumors which are exposed to cold temperatures resulting in disruption of tumors (Shields, Shields & De Potter 1993) and laser photocoagulation treatment is also performed on selected small tumors where the lasers are used to coagulate the blood vessels near the tumor sites (Shields et al. 1994). Radiotherapy in RB is practised via two techniques. The first radiotherapy technique called plaque radiotherapy or brachytherapy (Shields et al. 2001) is meant for small
tumors and it uses a plaque made of gold or lead containing radioisotope which is placed near the tumor site to shrink the tumor. The second radiotherapy technique is External Beam Radiation Therapy (EBRT) which is administered on advanced tumors and is more effective than the brachytherapy (Stannard et al. 2013). EBRT in RB has its own limitations because tumors with germ line mutations are prone for secondary cancers. Its radio sensitivity could give rise to side effects and current radiation treatment practices are based entirely on location and size of the tumors (Finger PT 2002). Three main drugs used for chemotherapy in RB are carboplatin, vincristine and etoposide which are either used alone or in combination with other treatments (Bellaton et al. 2003). In order to avoid the undesirable effects of systemic chemotherapy, local chemotherapy like intra-arterial chemotherapy (Gobin et al. 2011), periocular chemotherapy (Schmack et al. 2006) and intra-vitreal chemotherapy (Buitrago et al. 2010) are practised. Apart from these conservative treatments, today there are studies aimed at various therapeutic targets in RB.

Work from our laboratory has generated various therapeutic targets in RB. Epithelial cell adhesion molecule (EpCAM) was found to be highly expressed in RB especially in invasive cases and inhibition of this molecule could be a potential treatment (Krishnakumar S et al. 2004). Another molecule known as fatty acid synthase (FASN) is also highly expressed in poorly differentiated and invasive tumors and has potential for treatment in RB (Vandhana S et al. 2011). Spleen tyrosine kinase (SYK) is extensively studied in RB with its expression involved in invasion and survival of tumor cells (Zhang J et al. 2012) and is considered to be a promising therapeutic target. Connexin 46, a gap junction protein found in hypoxic RB cell line was silenced to reveal an anti-tumor effect, thus establishing it as one of the therapeutic targets in RB (Burr DB et al. 2011). Apart from the above mentioned genes, micro RNAs (miRNAs) are also considered to be candidates for therapy. miRNA 17–92 cluster (Kandalam MM et al. 2012) has been shown to play a role in invasion and proliferation in RB and
inhibiting it would halt the cancer progression. In this context, one such novel therapeutic target that has not been studied in RB so far is sodium iodide symporter (NIS).

As discussed in chapter 1, NIS is an intrinsic plasma membrane glycoprotein which plays a pivotal role in thyroid hormonogenesis by mediating iodide transport into thyroid cells. For many years, radiotherapy has been the primary and most successful treatment for follicular cell-derived thyroid carcinoma (Dohán O et al. 2003). NIS-mediated iodide accumulation within the thyroid gland has played an important role for diagnostic nuclear imaging as well as for highly efficient molecular radionuclide-based targeted therapy of malignant thyroid diseases (E 1996a). NIS protein expression and NIS mediated radioiodide accumulation occurs not only in thyroid cells but also in other extra thyroidal tissues like salivary gland, gastric mucosa, lactating mammary gland, placenta and kidney (Alan R Penheiter, Stephen J Russell & SK 2012). NIS functions in the body in such a way that efficient adsorption of iodide from the food takes place. NIS present in salivary gland and stomach cells transports iodide from blood stream to lumen of intestinal tract (Brown-Grant 1961) and NIS present in intestines then transports iodide from lumen to circulation (Nicola et al. 2009). The transport of iodide across these organs is mainly because of NIS expression at the basolateral membrane of salivary gland and stomach (Josefsson et al. 2002) and apical membrane of intestine (Nicola et al. 2009). NIS is expressed at basolateral membrane of alveolar cells in lactating mammary gland and functions by transporting iodide from bloodstream to the milk for the feeding infants (Tazebay et al. 2000). Iodide is supplied to foetus from the mother via functional NIS expressed in the placenta (Bidart et al. 2000). $^{131}$I is used for ablation of cancer tissue after thyroidectomy, if NIS expressed in the metastatic tissues are functional (Arturi et al. 2000). Though NIS is exploited in follicular cell derived thyroid carcinoma treatment, its expression is lost in poorly differentiated thyroid tumors (Dohán O et al. 2003).
and not expressed in significant levels in non-thyroidal cancers. At present preclinical studies of radionuclide NIS gene imaging, accumulation of radionuclides in target cells at therapeutic doses are ongoing, and in future can be followed by clinical trials of NIS as an imaging agent as well as a therapeutic transgene (Baril P, Martin-Duque P & G 2010).

With relevance to breast cancer, though NIS expression was higher, only a small fraction of cells possess the ability to take up radioiodide suggesting that further augmentation of the functional activity is required (Wapnir et al. 2003). For enhancement of NIS function, various ligands and hormones were identified and one among them was a group of ligands called retinoids (Tanosaki et al. 2003). The most influential ligand of the retinoid family is all-trans-retinoic acid (tRA) which increased the NIS expression at transcriptional level and I⁻ transport up to 10 fold. MCF-7 cell xenograft study revealed that on systemic tRA treatment at 160mg/kg/day for 5 days, a 15 fold increase in NIS expression and I⁻ uptake was observed (Kogai et al. 2004). Stimulation of NIS expression at transcriptional level was shown not only in breast cancer cells but also in human follicular thyroid carcinoma cell lines like FTC-133 and FTC-238 (Schmutzler et al. 2002).

Similar to tRA’s transcriptional activity, lactoferrin (Lf) a natural molecule functions as a transcriptional trans-activator (He & Furmanski 1995). Lf is a glycoprotein which belongs to the transferrin protein family and is found in high concentrations in human milk and other epithelial secretions (Kanwar et al. 2009; Kanwar RK & JR 2013). Bovine Lf (bLf) is a multifunctional therapeutic protein with various properties like immunomodulatory, anti-cancer, anti-inflammatory, anti-microbial and anti-oxidant (Kanwar RK & JR 2013; Tomita et al. 2009). Due to its iron binding properties, it occurs in three forms, Apo, Iron and Native. Apart from the above mentioned versatile functions, it is also thought to act as a transcriptional activator once it binds to the nucleus (He & Furmanski 1995). Studies on human Lf have shown it to trans-activate transcription factors like AP-1 and NFκB which
plays a key role in cell division and differentiation (Jiang et al. 2011). Another transcriptional activity of Lf was shown in increasing gene expression of IL-1β (Son KN et al. 2002) and TGF-β1 (Liao, Jiang & Lönnertal 2012). More recently, the downregulation of survivin gene expression by bLf in colon cancer cells was reported from our laboratory (Kanwar et al. 2014).

While NIS expression has been widely detected in thyroid, breast and in various other cancers, its expression in RB has never been investigated. Since there is lack of knowledge of NIS expression in RB, the present chapter aims to unravel the NIS protein expression status in extra-thyroidal tumors, such as RB.

### 3.2. Hypothesis

NIS expression has been studied in various extra-thyroidal cancers. This study hypothesized that if NIS was expressed in RB tumors, it would be associated with clinical parameters like histopathological high risk factors and tumor differentiation. Further it was hypothesized that bLf could upregulate the NIS protein expression in Y79, an established cell line from ATCC (American Type Culture Collection).

### 3.3. Aims

The aims of this chapter are

1) To investigate for the NIS expression at protein level in Grade D and Grade E RB tumors and in an established standard RB cell line (Y79).

2) To explore if the NIS protein expression levels were correlated with the clinicopathological features of RB tumors.

3) To study if the three forms of Bovine Lactoferrin (bLf), native-bLf, apo-bLf and Fe-bLf could internalize in Y79 cells.

4) To explore whether the internalised bLf upregulated NIS expression at protein level.
3.4. MATERIALS AND METHODS

The methodology for the experiments performed in this phase of study has been described in detail in chapter 2. A brief outline of the work carried out in this chapter for NIS has been explained in Figure 3.1. Outline of the bLf work has been explained in Figure 3.2.

Figure 3.1 Schematic representation of the NIS work performed in this study
Figure 3.2 Schematic representation of the bLf work performed in this study

**Tumor specimens**

Detailed clinical information of each tumor has been provided in chapter 2. Concise details of the cohort has been provided here as Table 3.1.
Table 3.1 Clinicopathological information of all RB tumors analysed for the study.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No. of patients (N=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;5 years</td>
<td>34</td>
</tr>
<tr>
<td>≥5 years</td>
<td>7</td>
</tr>
<tr>
<td>Laterality</td>
<td></td>
</tr>
<tr>
<td>Unilateral</td>
<td>33</td>
</tr>
<tr>
<td>Bilateral</td>
<td>8</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>8</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>3</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>30</td>
</tr>
<tr>
<td>IIRC Grade*</td>
<td></td>
</tr>
<tr>
<td>Group D,E</td>
<td>41</td>
</tr>
<tr>
<td>Invasion status</td>
<td></td>
</tr>
<tr>
<td>Invasive</td>
<td>25</td>
</tr>
<tr>
<td>Non-invasive</td>
<td>16</td>
</tr>
</tbody>
</table>

*IIRC stands for International Intraocular Classification of Retinoblastoma.* No tumors belonged to Group A, B and C.
3.5. RESULTS

3.5.1. **NIS protein is localised in membrane and cytoplasm of RB tumors**

In immunohistochemistry, all the 21 RB tumors showed strong positive immunostaining for NIS expression on the cell surface and in cytoplasm. Immunopositivity for NIS was observed mostly as membranous (Figure 3.3 A, B) and cytoplasmic (Figure 3.3 C). NIS expression was predominant in the tumors invading the choroid as shown in Figure 3.3 D, E and F and was majorly observed as cytoplasmic expression. The non-neoplastic retina showed immunopositivity at the inner and outer nuclear layer (Figure 3.3 H). The primary antibody control of both non-neoplastic retina (Figure 3.3 I) and tumor (Figure 3.3 G) showed no staining revealing the specificity of the procedure. An immunoscore was computed for each tumor using the staining intensity and percentage positive cells. The immunoscore was used to analyse association of NIS expression with different clinicopathological features (Table 3.2). Though both poorly differentiated and well differentiated tumors showed NIS expression, 31.2% of the poorly differentiated tumors fell under higher immunoscore category. Additional IHC figures for NIS immunostaining are shown in Figure 3.4.
Figure 3.3 Immunostaining of NIS protein in RB tumor tissues

Immunohistostaining of representative RB tumor tissues compared with non-neoplastic retina. NIS expression was visualized with 3,3’-Diaminobenzidine (DAB) and Hematoxylin was used as counter stain. (A) The photomicrograph at 20X shows strong membrane expression of NIS (red arrow) in tumor cells close to the retinal pigment epithelium (yellow arrow). Zoomed image of a portion of the immunostaining is shown at the left hand corner. (B) The photomicrograph at 100X shows the strong membrane expression of NIS (arrows show positivity) in a RB tumor at a 100X. Zoomed image of a portion of the immunostaining is shown at the top. (C) The photomicrograph at 100X shows the tumor cells positive for NIS expression, arranged around the blood vessels (arrow) at 40X. (D) (E) (F) The photomicrograph shows strong cytoplasmic expression of NIS in tumor cells invading the choroid, 10X, 20X, 40X respectively. Zoomed image of a portion of the immunostaining is
shown at the right hand corner. (G) The photomicrograph at 20X shows the primary antibody control in a RB tumor; no non-specific staining is observed. (H) The photomicrograph at 40X shows NIS expression in a non-neoplastic retina. The staining for NIS protein observed in the inner and outer nuclear layers of the retina. (I) The photomicrograph at 20X shows the primary antibody in a non-neoplastic retina; no non-specific staining is observed.

Figure 3.4 Immunostaining of NIS protein in different sets of RB tumor tissues

Immunohistostaining of RB tumor tissues showing positivity for NIS visualized with 3,3’-Diaminobenzidine (DAB) and Hematoxylin. (A-G) Zoomed image of a portion of the immunostaining is shown at the left hand corner. (H) & (I) The photomicrograph shows strong cytoplasmic expression of NIS in tumor cells invading the choroid.
Table 3.2 Association of NIS immunoscore by IHC with clinicopathological features.

<table>
<thead>
<tr>
<th>Category</th>
<th>Total Number (n=21)</th>
<th>Immunoscore by IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Males</td>
<td>11</td>
<td>4 (36.3%)</td>
</tr>
<tr>
<td>Females</td>
<td>10</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>Laterality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unilateral</td>
<td>16</td>
<td>7 (43.7%)</td>
</tr>
<tr>
<td>Bilateral</td>
<td>5</td>
<td>2 (40%)</td>
</tr>
<tr>
<td>Differentiation*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>16</td>
<td>6 (37.5%)</td>
</tr>
<tr>
<td>Well</td>
<td>5</td>
<td>3 (60%)</td>
</tr>
<tr>
<td>Invasion Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non Invasive</td>
<td>8</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>Invasive</td>
<td>13</td>
<td>5 (38.4%)</td>
</tr>
</tbody>
</table>

*No tumor among the 21 was found to be moderately differentiated.

Table 3.2: Association of NIS immunoscore by IHC with clinicopathological features. Immunoscore was calculated based on the staining intensity and percentage of positive cells. 10 different fields were chosen and the score was assigned as 0, 1, 2 or 3 for staining intensity corresponding to negative, weak, intermediate and strong staining respectively. From each field, total number of cells and total number of stained cells were counted and average percentage positive was calculated. The final immunoscore of 2 to 6 was calculated as mentioned (Detre S 1995).* No tumor among the 21 was found to be moderately differentiated.

3.5.2. Quantitative analysis of NIS by flow cytometry shows elevated expression in invasive tumors

NIS was quantitatively measured by flow cytometry in another 20 RB tumor samples differing in their invasion status. Due to non-availability of normal retina tissue for flow
cytometry, analysis for normal retina could not be performed. The flow cytometric analyses of all the 20 tumors was correlated with different clinicopathological features and tabulated in the Table 3.3. Graphical representations of FACS analysis of 12 invasive tumors are shown in Figure 3.5 and graphical representations of FACS analysis of 8 non-invasive tumors are shown in Figure 3.6. The inter-tumor differences in NIS expression with respect to invasion status were clear and the overall NIS protein expression was significantly elevated in the invasive tumors than the non-invasive tumors (P<0.05) (Figure 3.7). The expression of NIS did not show any statistically significant difference between poorly differentiated and well differentiated tumors as well as between gender and laterality (Table 3.4).

Table 3.3 Association of NIS positivity with clinicopathological features on the basis of flow cytometry

<table>
<thead>
<tr>
<th>Category</th>
<th>Total Number</th>
<th>Percentage of NIS positivity by FACS (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-10%</td>
</tr>
<tr>
<td>Males</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Females</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>IIRC classification</td>
<td>Group D-E</td>
<td>20</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Poor</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Well</td>
<td>3</td>
</tr>
<tr>
<td>Choroidal Invasion</td>
<td>No invasion</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>&gt;3mm</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>&lt;3mm</td>
<td>5</td>
</tr>
<tr>
<td>Optic nerve invasion</td>
<td>No invasion</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Pre-laminar</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 3.3: Association of NIS positivity by flow cytometry with clinicopathological features.

IIRC stands for International Intraocular Retinoblastoma Classification.

Figure 3.5 Flow cytometric analysis of NIS in invasive RB tumors.

50mg of tumor was homogenized, washed with phosphate buffered saline (PBS), fixed with formaldehyde. Anti-human NIS, mouse monoclonal antibody at a dilution of 1:50 was added and incubated for 2 h. Following washing, the samples were incubated with anti-mouse IgG FITC antibody at a dilution of 1:750 for 1 h in dark. Following washing step, samples were analysed in FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA), with the CellQuest software program (BD Biosciences, USA). Isotype control was used for gating the
positive population. 10,000 events were acquired within the gating. The NIS percentage positivity in the graphs is shown.

Figure 3.6 Flow cytometric analysis of NIS in non-invasive RB tumors.

Flow cytometry was performed as described under Figure 3.5.
Figure 3.7 Computation of NIS positivity determined in flow cytometry

The average percentage positivity of NIS by flow cytometry was computed for tumors with invasion and non-invasion. * Indicates significantly increased NIS expression in the invasive tumors with respect to non-invasive tumors \((P<0.05)\). Mann-Whitney test was used to calculate the level of significance.

Table 3.4 Association of NIS protein expression analysed in flow cytometry with clinicopathological features.

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Total number (n)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NIS</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
<td>0.271</td>
</tr>
<tr>
<td>Female</td>
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<td></td>
</tr>
<tr>
<td>Laterality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unilateral</td>
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</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well Differentiated</td>
<td>3</td>
<td>0.410</td>
</tr>
<tr>
<td>Moderately Differentiated</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Poorly Differentiated</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Invasion status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-invasive</td>
<td>8</td>
<td>0.031</td>
</tr>
<tr>
<td>Invasive</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.4: Association of NIS protein expression analysed in flow cytometry with clinicopathological features in 20 RB tumors. P-values were calculated by the Mann-Whitney-test.

3.5.3. **Western Blotting reveals glycosylation status of NIS in RB**

NIS expression was semi-quantitatively assessed in 6 tumor tissues differing in invasion status by immunoblotting with two NIS antibodies. By using polyclonal anti-NIS antibody, strong partially glycosylated protein bands migrating with molecular weights of ~75kDa and ~50kDa and a faintly underglycosylated protein band migrating with a molecular weight of ~25kDa were observed (**Figure 3.8.A**). In contrast, using monoclonal antibody, the blots revealed strong intensity of band at 50 kDa (**Figure 3.8.B**). The samples were tested for uniform loading with the loading control, β-actin (**Figure 3.8.C**). Histogram was plotted to show quantitative results of Western blot analysis of all the forms of glycosylated NIS protein detected by both polyclonal and monoclonal antibodies (**Figure 3.8.D**). Due to the non-availability of normal retina tissue lysate for Western blot, analysis for normal retina could not be performed.
Western blot of NIS protein expressed in 6 RB samples differing in their tumor invasion status. Fifty microgram of protein sample was loaded and (A) anti-human NIS rabbit polyclonal antibody (1:500) and (B) anti-human NIS mouse monoclonal antibody (1:500) were used for immunoblotting. Secondary antibodies were anti-mouse IgG-HRP (1:2000) and anti-rabbit IgG-HRP (1:2000) for respective primary antibodies. The immunoreactive bands were detected by the SuperSignal West Femto Chemiluminescent Substrate (Thermo...
Scientific, USA) and developed by the X-ray method. The samples were tested for equal protein loading with β-actin. (D) The histogram depicts the NIS/β-actin ratio observed in individual lanes for all the glycosylated forms of NIS protein calculated by NIH Image J software.

3.5.4. Quantitative analysis of NIS by flow cytometry in Y79 cells

NIS was quantitatively measured by flow cytometry in Y79, a RB cell line obtained from American Type Culture Collection (ATCC, U.S.A) which was maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Sigma Aldrich, USA) supplemented with 10% foetal bovine serum. The flow cytometric analysis for NIS protein in Y79 cells showed a clear shift in population of cells that are stained positive. This shift in intensity between the secondary antibody alone and the test sample shows NIS expression at endogenous level in Y79 cell line (Figure 3.9).

![Figure 3.9 Flow cytometric analysis of NIS in Y79 cells](image-url)
1x10⁶ cells were collected, washed with phosphate buffered saline (PBS), fixed with formaldehyde. Anti-human NIS, mouse monoclonal antibody at a dilution of 1:50 was added and incubated for 2 h. Following washing, the samples were incubated with anti-mouse IgG FITC antibody at a dilution of 1:750 for 1 h in dark. Following washing step, samples were analysed in FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA), with the CellQuest software program (BD Biosciences, USA). Isotype control was used for gating the positive population. 10,000 events were acquired within the gating.

3.5.5. Immunofluorescence shows NIS positivity in Y79 cells

Y79 cells were analysed for NIS protein expression and localization by immunofluorescence technique. The results showed strong cell surface and membrane positivity for NIS in Y79 cells (Figure 3.10). The IgG1 control for NIS antibody did not show any fluorescence confirming the specificity of NIS staining.
Different fields of Y79 cells, a RB cell line showing immunofluorescence positivity for NIS expression. 1X10^6 cells were collected, washed with Phosphate Buffered Saline, fixed with 4% paraformaldehyde and blocked with fetal bovine serum. Anti-human NIS, mouse monoclonal antibody at a dilution of 1:50 was added and incubated overnight at 4°C. Following washing, the samples were incubated with anti-mouse IgG FITC antibody at a dilution of 1:60 for 1 h in dark. Staining was observed using fluorescent microscope (Carl Zeiss, Berlin, Germany). For primary antibody control, the same procedure was performed with the isotype IgG1, anti-mouse antibody (Santa Cruz). The photomicrographs at 10X and 20X show strong membrane and cell surface expression of NIS in Y79 cells.
3.5.6. *Western Blotting shows NIS expression at 75 kDa in Y79 cells*

To detect the NIS protein expression in Y79 cells, the more sensitive technique of Western blotting was performed. Seventy five microgram of the protein lysate was loaded and a single protein band of NIS migrating at 75 KDa (red arrow) was detected in the blot when probed with anti-human NIS specific antibody. GAPDH migrating at 37 kDa (red arrow) was detected for the same sample when probed with GAPDH specific antibody (Figure 3.11).

![Figure 3.11 NIS protein is expressed in Y79 cells](image)

Seventy five microgram of protein sample was loaded and anti-human anti-NIS mouse monoclonal antibody (1:500) was used for immunoblotting. Secondary antibody was anti-mouse-HRP (1:1000) used. The protein lysate was tested for loading control, GAPDH. Anti-human GAPDH rabbit monoclonal antibody (1:1000) was used as primary antibody and
anti-rabbit IgG HRP (1:80,000) was used as secondary antibody. The immunoreactive bands were detected by the ECL method.

3.5.7. Endogenous level detection of Lf receptors in Y79 cells

To detect the endogenous level of three Lf receptors, low-density lipoprotein receptor-related proteins-1 and -2 (LRP1 and LRP2) and transferrin receptor (Tfr) in Y79 cells, qRT-PCR was performed. LRP1, LRP2 and transferrin receptors specific primers were used to determine mRNA levels of the housekeeping gene as a control. Delta CT value was calculated by subtracting the CT value of receptors with CT value of GAPDH control. Based on delta CT value, LRP2 mRNA level was found to be higher followed by LRP1 and transferrin (Figure 3.12).

![Real-Time PCR for Lf receptors](image)

Figure 3.12 Y79 cells have endogenous Lf receptors mRNA expression

The mRNA expression of Lf receptors (LRP1, LRP2 and Tfr) in Y79 cells analyzed by real time quantitative reverse transcriptase PCR (qRT-PCR). 1µg of RNA was converted to cDNA. 100 ng of cDNA, specific primers and RT² SYBR® Green qPCR Mastermix was used for the analysis. The data was then analysed by Livak method (Relative Quantification). Values are expressed as mean ± SD of triplicate analyses.
3.5.8. Uptake of three forms of bLf in Y79 cells by flow cytometry

Since the Lf receptors were expressed in Y79 cells, the uptake of all three bLf forms (Native-bLf, Apo-bLf and Fe-bLf) in Y79 cells was studied at a quantitative level by flow cytometry. For the uptake studies, a range of concentration such as 12.5 nmol, 25 nmol, 37.5 nmol, 50nmol, 62.5nmol and 75 nmol of all three bLfs were chosen. Uptake study by flow cytometry was performed 48 h after the bLf treatments. In case of Apo-bLf, the percentage of positivity was approximately 75 % in all concentrations. Similar results were observed in Fe-bLf and Native-bLf which shows effective uptake of bLf forms irrespective of their iron saturation levels (Figure 3.13).
Figure 3.13 Cellular uptake of Apo-bLf, Fe-bLf and Native-bLf in Y79 cells

Y79 (RB) cells were seeded at a confluency of 1x10^6 cells/well. The cells were then treated with 12.5nmol, 25nmol, 37.5nmol, 50nmol, 62.5nmol and 75nmol of Apo-bLf, Fe-bLf and Native-bLf for 48 h. Cells were fixed with 4% paraformaldehyde for 20 mins, permeabilized with 0.1% Triton-X-100 for 2 mins. Cells were then blocked with 3% porcine gelatin for 1 h
at 37°C. Following blocking, cells were incubated with anti-bovine bLf specific polyclonal antibody raised in goat at 1:250 dilution for 1 h at 37°C. Cells were washed and then incubated with secondary anti-goat IgG- FITC antibody at 1:100 dilution for 1 h in dark at 37°C. After washing step, cells were analysed by flow cytometry (Canto II, BD Biosciences, USA). Isotype control was used for gating the positive population. 10,000 events were acquired within the gating.

3.5.9. **Cellular uptake of three forms of bLf in Y79 cells by immunofluorescence**

After the uptake of bLf was confirmed by flow cytometry, immunofluorescence followed by confocal microscopy was also performed to visualize the internalized forms of bLf. The concentration of Native-bLf, Apo-bLf and Fe-bLf used in this experiment to study the cellular internalization was 12.5 nmol. The time allowed for internalization for all the three forms of bLfs was 30 min. This short time was chosen to observe the rate of efficiency at which the bLf’s get internalized. bLf’s internalization percentage was calculated by counting the number of cells that have taken up bLf from five different fields. In all the cases, efficient internalization of bLf into the cytoplasm was observed at 30 min along with strong fluorescence signals on cell membrane indicating the membrane binding (**Figure 3.14.A**). The percentage internalization of Apo-bLf was 92%, Fe-bLf was 94% and Native-bLf was 97%. No significant difference was obtained for internalization among the different forms of bLfs (**Figure 3.14.B**).
Figure 3.14 Apo-bLf, Fe-bLf and Native-bLf bind to cell membrane and get efficiently internalized by 30 min.

(A) Y79 cells were seeded at a confluency of 1X10^5 cells/well. The cells were then treated with 12.5nmol of Apo-bLf, Fe-bLf and Native-bLf for 30 mins. Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton-X-100 for 2 min. Cells were then blocked with 3% porcine gelatin for 1 h at 37°C. Following blocking, cells were
incubated with anti-bovine bLf specific polyclonal antibody raised in goat at 1:250 dilution for 1 h at 37°C. Cells were washed and then incubated with secondary anti-goat IgG- FITC antibody at 1:100 dilution for 1 h in dark at 37°C. After washing step, cells were mounted using Vector mounting media containing DAPI which is used to stain the nucleus. Time dependent internalisation of both Apo-bLf and Fe-bLf was performed. Slides were then visualised using Leica SP5 confocal microscopy and the images were processed using LAS version software. (B) Figure shows the histogram of the immunofluorescence experiment. The experiment was performed twice independently. Data has been expressed as mean values (±SD).

3.6. Determination of NIS protein expression after bLf treatment

Following efficient internalization of three forms of bLf, NIS protein expression after the bLf treatments was analysed using Western blotting. Four different concentrations, 12.5 nmol, 25 nmol, 50 nmol and 100 nmol of all 3 bLf samples were used for treatment of Y79 cells. Forty-eight hours after the treatment, NIS expression at protein level was analysed by Western blotting. NIS protein expression when compared to control/untreated cells showed an evident increase in the apo-bLf treatment at 25 nmol and Fe-bLf treatment at 50 nmol among the other bLf treatments which showed a slight increase in NIS expression (Figure 3.15). Thus, via Western blotting, it was confirmed that bLf treatments did have a transcriptional activating effect on NIS expression in Y79 cells.
Figure 3.15 Western blot of NIS protein detected after three forms of bLf treatments in Y79 cell line

Seventy five microgram of protein sample was loaded and anti-human anti-NIS mouse monoclonal antibody (1:500) was used for immunoblotting. Secondary antibody, anti-mouse-IgG HRP (1:1000) was used. The samples were tested for equal protein loading with GAPDH. Anti-human anti-GAPDH rabbit monoclonal antibody (1:1000) was used as primary antibody and anti-rabbit IgG-HRP (1:80,000) was used as secondary antibody. The immunoreactive bands were detected by the ECL method. The histogram depicts the relative intensity of the bands observed in individual lanes for all the samples.
3.7. DISCUSSION

This study shows for the first time the presence of NIS protein in RB tumors and its correlation with the aggressiveness of tumors. Expression of NIS protein in RB tumors from patients as well as in established standard RB cell line (Y79) was determined. Earlier, NIS was found in ciliary body of the eye that can actively accumulate iodide (Dohán O et al. 2003). In this study, 41 primary RB tumors was used which belonged to advanced stage (Grade D or E) and NIS expression was observed in 41/41 (100%) tumors. By immunohistochemistry (IHC), the 13 invasive tumors which had invaded ocular coats and/or the optic nerve and 8 non-invasive tumors showed immunoreactivity for NIS protein. The adjacent retina did not show evident positivity except in the inner and outer nuclear layers. The predominant pattern of NIS immunoreactivity in RB tumors was both membranous and cytoplasmic. The expression of NIS however was also cytoplasmic staining at the invasion sites. Apart from more NIS presence in the invading front, intratumoral NIS protein expression was heterogeneous with few cells showing membrane positivity and few cells showing cytoplasmic positivity. A similar altered localization was also observed in many other cancers like thyroid cancer (Wapnir et al. 2003) and breast cancer (Tazebay et al. 2000) where the NIS was found to be expressed in cytoplasm than in the membrane which resulted in no iodide uptake activity. Recent study has shown that the intracellular NIS expressed in cancers indeed has a function in cell migration and invasion instead of transporting iodide ion (Lacoste C 2012). Since the intracellular NIS in tumors is associated with cell migration and invasion, the RB tumors expressing cytoplasmic NIS could be associated to aggression in RB tumors. This altered function of NIS has been attributed to its interaction with RhoA guanine exchange factor. In RB, Rho-like GTPase expression has been studied earlier although its role in malignancy has been reported to be minor (Adithi et al. 2006). Since the function of cytoplasmic NIS and its interaction partner has been elucidated, it clearly indicates that NIS...
expression is altered in RB and whether it may interact with Rho-like GTPase to possibly alter the function of NIS in RB needs further studies.

Flow cytometry assay further confirmed the IHC findings by showing varying levels of NIS positivity and markedly higher protein expression in tumors with invasion. The average percentage positivity of NIS by flow cytometry indicated a significantly higher NIS expression in tumors with histopathological high risk factors like invading choroid and/or optic nerve with respect to non-invasive tumors ($P<0.05$). Earlier studies in radiation therapy in RB showed that poorly differentiated RB tumors are more sensitive to radiation (Finger PT 2002). Since this study reveal that NIS is expressed modestly higher in poorly differentiated tumors, the radiosensitivity may be attributed to NIS presence. NIS expression when analysed by flow cytometry did not yield a statistically significant difference among the differentiation status of tumors, laterality and gender. Since, moderately differentiated tumors have both poorly and well differentiated areas in isolation, it was challenging to correlate the NIS expression level. However, in this study, though moderately differentiated tumors were considered, the NIS expression was not statistically significant. Out of 20 RB cases analysed for NIS expression by flow cytometry, 3 cases were chemotherapy-treated tumors and this did not show any effect of drugs on NIS expression. A recent study in breast cancer showed such non-significant relationship between NIS expression and clinical characteristics of tumors (Ryan et al. 2011). However, significant correlations between NIS expression and histopathological high risk factors have been observed in other studies in testicular and prostate cancer (Micali et al. 2013; Navarra et al. 2010). Such difference in correlation studies of NIS and clinical parameters may be primarily due to difference in tissue origin and also different methodology used to analyse the expression. The limitation of the study was smaller cohort size and dissimilar tumor cohort used for flow cytometry and IHC. Since the tumor cohorts were not similar, a conclusive association between NIS expression and
clinicopathological features could not be achieved. Nevertheless, the significant relation of NIS expression with invasion of the tumors was shown by flow cytometry and by cytoplasmic expression in IHC. Thus, the above obtained results point to a 100% NIS expression and its possible role in tumor progression and invasiveness of the tumors.

Having shown that NIS protein is expressed in RB, the next investigation was directed towards the glycosylation status of the expressed NIS protein. The monoclonal anti-NIS antibody detected only one band at 50 kDa, whereas polyclonal anti-NIS antibody detected three bands at ~75 kDa, ~50 kDa and ~25 kDa in all nine tumor samples tested. In thyroid tumors, it has been reported that fully glycosylated protein migrates at 90 kDa, partially glycosylated protein migrates with a molecular weight of 50 kDa and underglycosylated protein migrates with a molecular weight of 15 kDa (Peyrottes I et al. 2009). Earlier investigations also show that on treatment of membrane proteins from thyroid and lactating mammary gland with N-glycosidase F (an enzyme that removes N-linked carbohydrates), anti-NIS Ab recognized ~50-kDa polypeptide in membranes from both tissues. The results conclusively demonstrated that ~75 kDa and ~50 kDa immunoreactive bands detected in lactating mammary gland corresponded to glycosylated and nonglycosylated-NIS, respectively (Tazebay et al. 2000). In consistent with the published data, the Western blot results further reveal the presence of partially glycosylated and underglycosylated forms of hNIS protein in the RB tumor samples which most likely corresponds to unprocessed hNIS protein. A recent study on glycosylation showed that even hypoglycosylated NIS has the ability to locate to the cell surface and that it may function similar to mature fully glycosylated NIS protein during post translational modifications. Interestingly, a recent study on glycosylation showed that even hypoglycosylated NIS has the ability to locate to the cell surface and that it may function similar to mature fully glycosylated NIS protein (Beyer et al. 2011a). Both the IHC and Western blotting results of the current study, yielded information
that though the expressed NIS protein in RB is partially glycosylated, it was still found to be on the membrane and cell surface. Therefore, it will be interesting in the future studies to find out if the glycosylation impact the functionality of the NIS protein.

NIS is considered to be one of the primary therapeutic and diagnostic proteins because of its ability to uptake radioactive substrates and help in killing the cancer cells or in imaging the target (Kogai T & GA 2012). Our investigation on NIS protein in RB tumors showed that it was predominantly expressed in all tumors analysed. The same analysis was also performed in RB cell line, Y79, for NIS protein expression. Similar to RB tumor tissues, the NIS protein was expressed in Y79 cells which were studied by flow cytometric analysis revealing endogenous NIS expression. In addition, immunofluorescence was performed on Y79 cell line for NIS and found that NIS was expressed endogenously and displayed strong immunofluorescence for the antigen. Immunofluorescence revealed the localization of the expressed NIS protein to be primarily on the cell surface and in the cell membrane. Final confirmation of NIS protein presence was done by the sensitive technique of Western Blotting which revealed a single 75 kDa protein band revealing a glycosylated protein.

Determination of functional activity of the endogenously expressed NIS protein in Y79 cells was performed via non-radioactive iodide uptake method (Waltz, Pillette & Ambroise 2010) but the attempt was unsuccessful primarily because of the difficulty in performing the assay in suspension cells.

Since NIS expression was observed in all tumors studied and not in their normal surrounding tissues, it may be used for targeted molecular radionuclide therapy. Though NIS was found to be present in the inner and outer layer of retina, using tumor specific promoters, NIS can be specifically overexpressed in the RB tumors, thus being functional only in the tumor areas. Diagnostic and therapeutic applications are performed on thyroid cancer cells expressing NIS using its radioactive substrates. Radiopharmaceutically, NIS has significant advantages as a
therapeutic gene as well as a reporter gene (JK 2002). Thyroid follicular cells and differentiated thyroid cancer cells have the ability to capture radioiodide more effectively and this forms the basis of radionuclide therapy in the thyroid disease (Baril P, Martin-Duque P & G 2010). Like thyroid cancer, breast cancer also majorly express NIS (Tazebay et al. 2000) (Wapnir IL et al. 2004) but unlike thyroid cancer, breast cancer does not retain functional NIS expression (Hyuk Moon et al. 2001; Wapnir et al. 2004). In breast cancer (Kogai et al. 2004; Wapnir et al. 2003) as well as in some differentiated thyroid cancers (Dohán et al. 2001; Saito et al. 1998; Wapnir et al. 2003), NIS expression is more in cytoplasm and it fails to translocate to the membrane and so reduced radioiodide uptake was observed in these cancer cells. Various epigenetic mechanisms lead to poorly differentiated cancers and studies have shown in thyroid cancer cells which are poorly differentiated, that inhibition of such mechanisms leads to re-expression of thyroid genes like NIS (Furuya F et al. 2004). The phosphatidylinositol-3 kinase (PI3K) regulates the translocation of NIS to membrane in breast cancer cells as both transient and chronic PI3K activation was shown to increase underglycosylated NIS protein levels but impair NIS cell surface trafficking, thus leading to the absence of NIS-mediated radioiodide uptake activity in MCF-7 cells (Knostman KA et al. 2007). PI3K/AKT pathway is known to be dysregulated in RB (Cohen Y et al. 2009). It would be interesting to study in future if the modulation of this dysregulated pathway has any implications for translocation of NIS to membrane for its full functionality in RB.

In the case of diagnosis of RB, CT scan or MRI is primarily used. The principle of CT scanning is that the tumor being highly dense and calcified can be easily observed after administration of contrast dye containing iodide (Beets-Tan et al. 1994). Since NIS was found to be specifically expressed at a higher level in RB tumor tissues and absent in surrounding healthy tissues, the differential contrast achieved due to the uptake of iodide
containing contrast dye in NIS expressing tumor tissues, may lead to new diagnostic avenues for RB.

In breast cancer, the localization of iodide via NIS has been attributed to the presence of insulin like growth factor (IGF). Studies have shown that breast cancer expresses IGF-1 which is associated to more dense tissues which in turn shows better radiographic contrast (Byrne et al. 2000; Diorio et al. 2005). Another study in breast cancer revealed that IGF is a promoter of NIS and iodide uptake via NIS is stimulated by IGF which leads to increased retention of iodide by NIS in the breast cancer cells (Arturi et al. 2005). In RB, a study has reported that IGF pathway involving IGF-I and IGF-II plays a role in the tumorigenesis (Giuliano et al. 1996). So, in RB, it may be suggested that IGF may stimulate NIS to retain iodide for longer period which in turn could be responsible for enhanced contrast observed during routine CT imaging.

In RB treatment, high energy radiation via external beam radiotherapy is given to cancer cells to destroy them. Since NIS protein is extensively used for radiotherapy in thyroid cancer and breast cancer treatment, the findings of this study implies that the radiotherapy for RB could be channelized via NIS. The potential for exploiting NIS mediated radioisotopic therapy is considerable especially if combined with standard therapies such as EBRT and also radiosensitizing drugs. This strategy has enormous translational potential for achieving tumor-specific escalation of radiation dose at various tumor sites. This may bring down the side effects caused by EBRT and may also make the radiotherapy be more specific.

Currently, two strategies are used to induce NIS expression in cancer cells. One is transfer of NIS gene using viral and non-viral vectors. Second strategy is to stimulate the functional activity of endogenous NIS using stimulating agents thought to be having an upregulatory effect on NIS expression along with enhancing the iodide uptake ability (Alotaibi et al. 2010). Having studied that NIS is expressed in RB at endogenous level, an attempt was made to
enhance the expression of NIS protein in Y79 cells using bovine lactoferrin (bLf), a natural transcriptional activator of genes (Gifford, Ishida & Vogel 2012). Though many studies including the one from our laboratory have shown that Lf activates genes at transcriptional level, none have shown the effect on NIS gene expression. Initially, the status of Lf receptors was analysed in Y79 cells at mRNA level in order to determine the possibility of receptor mediated cellular uptake of bLf in these cells. The findings revealed mRNA presence of three receptors, LRP1, LRP2 and Tfr in Y79 cells. Following the determination of presence of Lf receptors, three forms of bLf were used to enhance the NIS expression. The internalization studies at 30 min showed an effective internalization of all the three forms of bLf (Apo-bLf, Fe-bLf and Native-bLf) in the cytoplasm of Y79 cells along with strong presence of bLf on cell membranes. The microscopic observation of internalization was further confirmed by the flow cytometric analysis which also showed a maximum shift in cell population positive for bLf 48 h after bLf treatment. Since the internalization studies showed encouraging results, NIS protein expression after bLf treatments were analysed. Analysis of NIS protein level after treatment with various concentrations of Apo-bLf, Fe-bLf and Native-bLf revealed a dose dependent increase in NIS expression with the Apo-bLf and Fe-bLf treatments. Due to difficulty in performing the functional assay in suspension cells, the determination of the functional activity of the bLf induced NIS expression was unsuccessful. However, an increase in the NIS protein level by bLf is an encouraging outcome and future studies focussing on enhancing this expression and standardising protocol for functional assay for suspension cells could be beneficial.

Since NIS protein expression is regulated differently in different tissues (Wapnir IL et al. 2004), it also has to be seen in future, how NIS is regulated in RB tumor cells in the preclinical and clinical studies. In conclusion, using both clinical RB tumor samples and established RB cell line, Y79, this study to the best of the knowledge, demonstrates for the
first time the presence of NIS protein in RB, its association with the invasiveness of the tumors and its glycosylation status. Further detailed investigation of NIS molecule at RNA level, functional level and the fate of transported iodide are required to understand the role of NIS in RB tumor biology. In addition, the mechanistic importance of NIS in diagnosis, treatment and monitoring of RB patients can be studied by employing more number of patients using different tumor types. Functional activity of expressed NIS if proved through future experiments will be important as it may help in targeting NIS for radiotherapy in RB treatment. Thus, through this discovery of NIS in RB, a more targeted, less harmful approaches for the treatment of RB by employing natural biodrug such as bLf with clinically proven safe chemoprevention profile for colon cancer may be developed in future to achieve the goal of complete cure of this dreadly childhood disease.
Chapter 4

EpCAM specific NIS expression and delivery of NIS gene in breast cancer cells

“EpCAM is a well-established carcinoma marker that in addition to its diagnostic value for rapidly growing tumors of epithelial origin is used as a potential target for immunotherapy”

- Slanchev et al. 2009
4. CHAPTER 4

4.1. INTRODUCTION

Gene therapy is a process where exogenous genetic material is either inserted or expressed in cells for therapeutic benefits. On the basis of treatment, gene therapy can be broadly divided into three: 1) direct regulation of the disease causing gene in the case of hereditary diseases 2) indirect therapy of disease through gene modification in complex, acquired disease like Parkinson’s disease 3) immunotherapy where an introduction of a gene induces the generation of particular antigen (DNA vaccines) (Linden & Matte 2014). The idea of gene therapy was conceived in the 1960s and currently many therapeutic genes and delivery vehicles have reached till clinical trials. The success of gene therapy lies mainly on the development of recombinant DNA technology and sequencing of human genome (Lisa Li, Nakano & Hotta 2014). The transfer of genetic material can be done in vivo and with combination of other conventional treatments for higher efficiency (Linden & Matte 2014). Gene therapy promises to be an effective therapy for various diseases especially cancer (Ginn SL et al. 2013). Gene therapy has achieved progress in recent decades but the problem of successful clinical application of gene therapy still persists. Though, it is the age where optimal vector systems are in practice, there are always problem of random integration and the lack of specificity (Parker 2014).

There are many genes, expression of which will render the cells susceptible to various treatments. One such gene is Sodium Iodide Symporter (NIS), expression of which will facilitate the cells to take up radioactive iodide and thereby a therapy in itself. As described in chapter 1 and chapter 3, NIS, a transmembrane glycoprotein, responsible for accumulation of iodide in the thyroid gland is present in the basolateral side of the thyroid follicular cells (MJ 1998). Transport of iodide through NIS mediation is an active process which is inhibited
competitively by thiocyanate and perchlorate anions and occurs against the electrochemical gradient (Eskandari S et al. 1997; Weiss SJ, Philp NJ & EF 1984). Though NIS is expressed widely in various extrathyroidal tissues like mammary glands, salivary glands and gastric mucosa apart from thyroid, the accumulated iodide in these tissues are not organified (Spitzweg C et al. 1998). NIS present in the lactating mammary gland is responsible for actively transporting iodide to the milk, which helps in the iodide supply to the infant. Thus NIS has a physiological role in lactating breast unlike other non-thyroidal tissues where the function of NIS is not known (N 1993). There have been several studies which showed iodide uptake in non-thyroidal cells after the transfection of NIS cDNA (Kosugi S et al. 1996; Spitzweg C et al. 1999). Expression of NIS protein in primary RB tumors and RB cell line (Y79 cells) has been explored to a great extent in chapter 3. Since the endogenous NIS failed to show any functional activity in preliminary experiments, an attempt was further made to overexpress NIS by stably transfecting NIS plasmid in Y79 cells. However due to the difficulties in manipulating gene expression (Corson & Gallie 2007) in this cell line, the study did not materialize. As a proof of concept, breast cancer cell line, MCF-7, an ATCC cell line derived from metastatic site of a breast was used in this study. NIS based therapy is most promising as this therapeutic gene can be over expressed in non-thyroidal cancers like breast cancer enabling it to accumulate radioiodide.

Breast cancer, a complex disease with different biological characteristics and clinical behavior is the most common cancer among women. The fatality of the disease is attributed to metastasis of the disease to distant organs like liver, lungs and brain with a median survival time of 2.2 to 0.5 years (Kennecke et al. 2010). Various conventional chemotherapeutic drugs like adriamycin, cyclophosphamide and paclitaxel show promising results in treating breast cancer (Kim et al. 2011). Though the mortality rate has declined with advanced treatments (Jemal A et al. 2010), the lack of specificity, drug accumulation and bioavailability is still a
worrying factor for breast cancer therapy. The importance of gene therapy comes to the play where conventional therapy fails. However, gene therapy is never without challenges. The most challenging aspect of gene therapy is the delivery of gene itself. Targeting specific cells via a marker becomes utmost importance in gene delivery.

Multitude of cancer markers have come into the foray and functions as a perfect target for diseased states. One such biomarker is EpCAM, an epithelial cell adhesion molecule expressed in all epithelial cells (Baeuerle PA & O 2007). Even though it is expressed at a basal level in normal cells, it is overexpressed in breast cancer and other several cancers (Spizzo G et al. 2002; Spizzo G et al. 2004; Went P et al. 2006) and is correlated with high proliferation and high metastasis (Osta WA1 2004). This overexpression makes EpCAM a promising molecule for future therapies and a prognostic marker (van der Gun BT1 2010). EpCAM is an efficient molecule for cancer-specific gene delivery because it has been widely used as a biomarker in cancers (Baeuerle PA & O 2007; Münz M et al. 2010).

In this chapter, NIS gene was delivered to breast cancer cells (MCF-7 cell line) in two ways by targeting the overexpressed EpCAM protein. The first method is by employing tissue specific promoters. A tissue specific promoter is a promoter which functions only in certain tissues. The primary use of tissue specific promoter is that it can limit the unwanted transgene expression and ensures constant expression of the transgene (Zheng & Baum 2008). Maximum cytotoxicity to the target tumor tissues and minimum side effects to surrounding normal tissues can be achieved by delivering NIS gene to the malignant cells using tissue specific promoters (JK 2002). An improved approach of directing NIS specifically to the breast cancer tissues is to use a plasmid containing NIS gene coupled to a breast cancer specific promoter such as EpCAM promoter. This is the first study to target EpCAM molecule to deliver NIS gene in breast cancer cells.
The second method is by employing polymeric nanoparticle coated with specific aptamers. Though various polymers are available, the one polymer which satisfied all the criteria is Polyethylenimine (PEI). As explained in chapter 1, the advantage of polymer like PEI is that it can be easily synthesized, follows endosmolytic mechanism (Meyer M & E 2006), no immunogenicity and improved biocompatibility. The success of gene therapy highly relies on effective delivery vectors. An efficient delivery vehicle is the key to a successful gene therapy and multiple factors determine an efficient delivery vehicle. One it has to condense the DNA molecules, two it has to protect the DNA from degradation and release the molecule promptly and three it has to be less toxic and more biocompatible. Polyethylenimine, a common cationic polymer forms complex with pDNA and has high transfection efficiency in vitro as well as in vivo (Godbey WT, Wu KK & AG 1999; Kircheis R, Wightman L & E 2001). Every third atom of this organic macromolecule is an amino nitrogen group which can be protonated. Irrespective of PEI form i.e., linear or branched it forms complexes with DNA. The ability of the PEI to trap DNA is attributed to many linker amino groups which also render PEI buffering capacity (Klotz IM, Royer GP & AR 1969).

The delivery vehicle must cross several barriers to deliver its contents to the specific cells. This is done by modifying the surface of the delivery vehicle with targeting ligands which would result in overcoming undesired side effects of conventional therapy. Cell type specific surface molecules like antibodies, aptamers have been developed for cell-specific gene delivery (B 2007; Chiu SJ, Ueno NT & RJ. 2004; Germershaus O et al. 2006). Use of antibodies, specifically monoclonal antibodies as targeting ligands is widely practiced. For example, a therapeutic monoclonal antibody showed significant therapeutic effect in colorectal cancer by targeting vascular endothelial growth factor and epidermal growth factor receptor (Amado et al. 2008). However there are various limitations for monoclonal antibody based therapy for cancer. Few of the drawbacks are monoclonal antibodies of mouse origin
induce immunogenicity and monoclonal antibodies of human origin are costly and limited (Ikeda Y & K 2006; Li SD & L 2006; Lu PY, Xie F & MC 2005; Schiffelers RM et al. 2004). The limitations in monoclonal antibodies lead to the production of aptamers. Aptamers are short oligonucleotides, either RNA or DNA molecules which at nanomolar levels can bind to targets with high affinity (Bunka & Stockley 2006). Since EpCAM is overexpressed in both primary and metastatic breast cancers and considered to be a cancer biomarker (van der Gun BT et al. 2010), a RNA aptamer that targets EpCAM protein was selected in this study. Because of PEI’s highly protonated group in its surrounding (J. Suh, H.J. Paik & Hwang 1994), its surface can be easily modified with various ligands for targeted delivery of the plasmid.

Although endogenous NIS expression is detected in breast cancer cells, it is insufficient to induce iodide uptake (Kogai & Brent 2012). Moreover, earlier studies have shown that EpCAM is overexpressed in MCF-7 cell line. These two clinically important molecules have never been targeted together in breast cancer.

4.2. Hypothesis

The hypothesis of this study was that if NIS was specifically overexpressed in breast cancer cells (MCF-7) via a tissue specific promoter (EpCAM) through receptor targeted gene delivery, it would induce functional activity of NIS leading to higher cellular iodide uptake. It was also hypothesized that NIS gene could be effectively delivered in a nanoformulation (nanocomplex) decorated with EpCAM aptamer for its specific delivery in EpCAM positive breast cancer cells (MCF-7), the successful delivery would then enhance the iodide uptake.

4.3. Aims

The aims of this study were
1) To clone NIS gene in EpCAM promoter containing vector and to select stable transfectants.

2) To compare the promoter activity of universal promoter (Cytomegalo Virus promoter-CMV promoter) and tissue specific promoter (EpCAM).

3) To show the functional activity of the expressed NIS gene via CMV-NIS plasmid and EpCAM-NIS plasmid.

4) To assemble a functional nanoparticle comprising NIS plasmid within PEI polymer carriers coated with EpCAM aptamer.

5) To study the efficacy of nanocomplex in delivering NIS plasmid DNA to EpCAM positive cells and tests the functional activity of delivered NIS gene.

4.4. MATERIALS AND METHODS

The methodology for the experiments performed in this study has been explained in detail in chapter 2. A brief outline of the work carried out in this chapter has been explained in Figures 4.1-4.4.
Figure 4.1 Schematic representation of the first part of the work performed in this chapter.

Cloning mechanism has been explained in Figure 4.3 and the commercial vector obtained for cloning has been explained in Figure 4.2. qRT-PCR-Real Time PCR; MCF-CMV-NIS denotes to stably transfected cell lines using CMV-NIS plasmid and MCF-EpCAM-NIS denotes to stably transfected cell lines using EpCAM-NIS plasmid.
Figure 4.2 Vector maps of plasmids

Commercial vector map of pCMV6-AC (Origene, USA) with arrows indicating the multiple cloning sites where NIS gene was inserted. The plasmid had kanamycin resistance gene instead of ampicillin resistance gene.
Figure 4.3 Schematic representation of the cloning procedure employed in the current study to generate EpCAM-NIS clone.

Vector map of the commercial vector shown in Figure 4.2. Specific restriction enzymes like BamHI and XhoI were used for digestion of the NIS gene from the CMV-NIS plasmid and EpCAM vector backbone from the EGP2-TK plasmid. The NIS gene and the EpCAM vector backbone were ligated to generate a new clone, EpCAM-NIS.
Figure 4.4 Schematic representation of the second work performed in this chapter.

**PEI based nanocomplex containing NIS pDNA and EpCAM aptamer** was prepared and characterized using Transmission Electron Microscopy (TEM) and Zeta Sizer. Transfection efficiency of the nanocomplex was determined at mRNA level (qRT-PCR) and at protein level (Western Blotting). Functional activity of the NIS gene was determined by iodide uptake assays both radioactively and non-radioactively.
NIS plasmid DNA was initially mixed with polyethyleneimine (PEI) to form a core complex via electrostatic attraction. The synthetic RNA-based EpCAM aptamer (EpDT3) was then incorporated onto the PEI-NISpDNA complex to form the nanocomplex. This functional complex when added to the cells would selectively target EpCAM positive breast cancer (MCF-7) cells. EpCAM aptamer mediated cell binding would facilitate intracellular delivery of the nanocomplex. The NIS plasmid DNA would subsequently express its protein resulting in the uptake of radioactive iodide.

4.5. RESULTS

4.5.1. PART A: NIS gene expression via EpCAM specific promoter

4.5.1.A. EpCAM expression in MCF-7 cells

Although EpCAM expression in breast cancer cells, MCF-7 has been reported in earlier studies (Sterzyńska et al. 2012), this study again analysed the basal level of EpCAM expression before conducting further experiments. The basal level of EpCAM protein in MCF-7 cells was determined by flow cytometry. The flow-cytometric histogram of MCF-7
cells stained with anti-EpCAM antibody (Cell Signalling Technology, USA) showed a highly marked percentage (95%) of MCF-7 cells to be positive for EpCAM (Figure 4.6). This finding confirmed MCF-7 cells to be EpCAM positive and further experiments were carried out with this cell line.

Figure 4.6 Flow cytometric analysis of EpCAM expression in MCF-7 cell line.

*Top panel*- MCF 7 cells unstained; *Middle panel*- MCF 7 cells stained with anti-mouse, FITC secondary antibody alone (1:1000); *Bottom panel*- MCF 7 cells stained with anti-EpCAM antibody raised in mouse (1:1000) and anti-mouse IgG, FITC secondary antibody (1:1000.).

*M1* represents the cells that are negative for EpCAM and *M2* represents the population of EpCAM positive cells in the flow cytometric graphs.
4.5.1.B. Construction of clones and establishment of stable transfectants

The NIS gene in the pCMV6-AC vector driven by CMV promoter was excised with restriction enzymes like BamHI and XhoI. Similarly, the EGP2 vector was restricted digested with the same restriction enzymes BamHI and XhoI and the vector backbone was excised (Figure 4.3). The excised NIS gene was then ligated into the excised EGP2 vector backbone containing EpCAM promoter using ligation enzymes. The ligation was a sticky end ligation (Figure 4.3). Cloning of NIS gene into the EGP2 vector containing EpCAM promoter was confirmed by restriction-digestion (Figure 4.7.A and B). Both the plasmids used in this study had neomycin resistant genes for mammalian cell selection. MCF-7 cells were transfected with both the plasmids using lipofectamine. The stable transfectants carrying the CMV-NIS plasmids and EpCAM-NIS plasmids were generated by the addition of Geneticin (G418), an antibiotic. The MCF-7 cells transfected with the plasmids were subjected to G418 treatment for 14 days. The concentration and period of G418 treatment was established by the kill-curve experiment. After a period of 14 days, the cells that survived in the presence of G418 were isolated and screened for NIS mRNA expression by qRT-PCR. The clones that showed NIS gene expression were taken for further functional studies.
Figure 4.7 Restriction Digestion of EGP2-TK, EoCAM-NIS and CMV-NIS plasmids

(A) 1% agarose gel showing restriction digestion of three plasmids, EGP2-TK, EoCAM-NIS and CMV-NIS. Lane 1- 1kb ladder; Lane 2- digested sample of EGP2-TK with TK gene running at 700 bp (red arrow) and vector backbone at ~9kb; Lane 3- digested sample of EoCAM-NIS with EoCAM vector backbone at ~9kb and NIS gene at 3kb; Lane 4- digested sample of CMV-NIS with NIS gene at 3kb. (B) 1% agarose gel showing the undigested and digested sample of EoCAM-NIS.

4.5.1.C. Analysis of NIS overexpression in the stable MCF-7 transfectants at mRNA level

As explained earlier, overexpression of NIS in breast cancer is necessary as the endogenously expressed NIS is non-functional. So, in order to check the NIS overexpression at mRNA level
in the generated stable transfectants, qRT-PCR was performed. Figure 4.8 shows that when compared to the vehicle control MCF-7 cells, both the MCF-CMV-NIS and MCF-EpCAM-NIS transfectants showed significantly higher (P<0.001) NIS expression at mRNA level, 10.3 and 8.4 fold up regulation respectively. This confirms the successful overexpression in both the clones. In addition, a significant difference (P<0.005) in NIS expression was also observed between the transfectants with MCF-CMV-NIS showing higher expression than the MCF-EpCAM-NIS.

Figure 4.8 qRT-PCR analysis of the mRNA transcripts of the hNIS gene in the stable transfectants.

The stable transfectants, CMV driven NIS gene and EpCAM driven NIS gene show significant increase in NIS mRNA when compared to the vehicle control cells. Significant difference is also seen between the transfectants. The error bar represents standard deviation of
experiments performed in triplicates. P-value less than 0.05 are considered as significant. 

****P<0.001and ***P<0.005. P-values were calculated by the Student’s t-test.

4.5.1.D. Analysis of NIS overexpression in the stable MCF-7 transfectants at protein level

Following the analysis of NIS overexpression in the stable transfectants at mRNA level, further investigation was done to confirm the overexpression at protein level. The protein level study was performed by Western blotting technique. Mouse monoclonal, anti-human NIS antibody (Abcam, USA) was used to examine the NIS protein expression. The Figure 4.9.A shows that stable transfectants, MCF-CMV-NIS and MCF-EpCAM-NIS overexpressed NIS at protein level when compared to the vehicle control MCF-7 cells. The NIS protein band was detected at 75 kDa in all the samples including Nthy-ori 3-1(Normal human primary thyroid follicular epithelial cell line) which was used as a positive control. β-actin protein band detected at 43 kDa was used as a loading control. The image J analysis after normalising the NIS expression with the β-actin revealed higher expression in the stable transfectants when compared to the control cells. Between the stable transfectants, the MCF-CMV-NIS transfectant showed higher NIS protein expression than the MCF-EpCAM-NIS transfectant (Figure 4.9.B). These results were in agreement with the aforementioned results of qRT-PCR analysis.
Figure 4.9 Western blot analysis in stable transfectants

(A) Succeeding stable transfection of MCF-7 cell line with CMV-NIS and EpCAM-NIS plasmid DNA, NIS protein expression was analysed in the stable transfectants. Immunoblotting analysis using anti-NIS mouse monoclonal antibody revealed a 75 kDa protein. Lane 1: Nthy-ori 3-1 cell line lysate was used as a positive control; Lane 2: MCF-7 vehicle control; Lane 3 and Lane 4: overexpression of NIS protein in MCF-CMV-NIS and MCF-EpCAM-NIS transfectants respectively. Bottom panel: β-actin as loading control, detected at 43 kDa using anti-human anti-β-actin mouse monoclonal antibody. HRP
conjugated, anti-mouse IgG secondary antibody was used and the blot developed using enhanced chemiluminescence (ECL) method. (B) Histogram plotted for the NIS protein after normalization with the β-actin protein expression.

4.5.1.E. Radioiodide uptake assay shows higher functional activity of EpCAM promoter driven NIS gene

Following the expression studies, the stable transfectants were subjected to functional studies. The iodide uptake assay was necessary in order to show that the expressed NIS protein was a functional protein. Along with the uptake studies, efflux of iodide also was studied. Radioactive iodide uptake was measured by incubating the NIS transfected cells with 4µCi/mL Na\textsuperscript{125}I for 2 h. Both the stable transfectants showed significant iodide uptake compared to the vehicle control MCF-7 cells (P<0.05). Between the stable transfectants, MCF-EpCAM-NIS concentrated more iodide than the MCF-CMV-NIS. The vehicle control cells though expresses NIS endogenously concentrated radioiodide only at a basal level. Iodide uptake was blocked in the presence of 1mM Sodium perchlorate, an inhibitor of NIS which was important to show the iodide uptake is specifically via the expressed NIS (Figure 4.10.A). Efflux of iodide was rapid with over 75% of the total cellular iodide released within 15 mins in both CMV-NIS and EpCAM-NIS (Figure 4.10.B). The above results demonstrate significantly higher and perchlorate inhibiting radioactive iodide uptake in stable transfectants of MCF-7 cell line, a cell line of non-thyroidal origin.
Figure 4.10 Radioactive iodide uptake assay, inhibition assay and efflux assay for stable transfectants

(A) NIS dependant iodide uptake was measured after treating the control and stable transfectants with 4µCi/mL of Na\textsuperscript{125}I for 2 h. For the inhibition studies, cells were treated with 1mM of NaClO\textsubscript{4} along with the 4µCi/mL of Na\textsuperscript{125}I. Uptakes are expressed as counts per min (cpm). The error bar represents standard deviation of experiments performed in duplicates. (B) Efflux of iodide was faster in both CMV-NIS and EpCAM-NIS with over 75% released in 15 mins. Data are expressed as a percentage of the maximum uptake. P-value less than 0.05 were considered significant. *P<0.05 and ***P<0.005. P-values were calculated by the Student’s t-test.
4.5.1.F. Non-radioactive iodide uptake assay proves the functional activity of *EpCAM* promoter driven NIS gene

Subsequent to assessment of functional activity of the stable clones via the standard radioactive iodide uptake method, a novel colorimetric assay which is fast and sensitive was used to measure the cell-trapped iodide in the stable transfectants. The cell trapped iodide in vehicle control MCF-7 cells and both the stable transfectants was assayed after 30 min of iodide incubation. The iodide concentrations in all the cells (MCF-7 vehicle control, MCF-CMV-NIS & MCF-EpCAM-NIS) were determined after linear regression of the calibration curve (Figure 4.11.A). The uptake in both the stable transfectants was significantly higher than the control cells. Maximum uptake of 349 nM of NaI by MCF-CMV-NIS (P<0.01) and 438 nM of NaI by MCF-EpCAM-NIS (P<0.005) was observed at 30 min time period. The vehicle control MCF-7 cells failed to show any uptake of iodide (Figure 4.11.B). This proves the specific iodide uptake activity of the stable transfectants expressing NIS protein.
Figure 4.11 Non-radioactive iodide uptake assay for the stable transfectants

*NIS* gene functional activity as measured by non-radioactive iodide method. (A) Logarithmic conversion of the means of A420 at 30 min \((n = 3)\) vs. iodide standards. (B) Cell-trapped iodide determined by the As/Ce method (explained in methods chapter) and the iodide uptake expressed in nM concentration. Both the stable transfectants showed significantly higher iodide uptake compared to the control cells. The error bar represents standard deviation of experiments performed in triplicates. *P* value less than 0.05 were considered significant.

***\(P<0.005\) and **\(P<0.01\). *P*-values were calculated by the Student’s *t*-test.
4.5.1.G. *Stable transfectants showed increased migration ability*

After confirming the functional activity of the stable transfectants, the migration ability was studied. Overexpression of NIS by the stable transfectants confers higher migration ability than the endogenous NIS expressing untransfected MCF-7 cells. DMEM medium supplemented with 10% FBS was added to the cells to support migration. Wounds were observed to close at 24 h after wound formation. However there was little or no difference in the migration rate between the stable transfectants, MCF-CMV-NIS and MCF-EpCAM-NIS (Figure 4.12).
Figure 4.12 Migration assay for the stable transfectants

Cell migration assay in the stable transfectants and control MCF-7 cells. (A) Control MCF-7 at 0 h (B) MCF-7-CMV-NIS at 0 h (C) MCF-7-EpCAM NIS at 0 h (D) Control MCF-7 at 24 h (B) MCF-7-CMV-NIS at 24 h (C) MCF-7-EpCAM NIS at 24 h. The dotted yellow colour circles shows the point of closure of the wound. Photomicrographs were taken at a magnification of 20X.
4.5.2. **Part B: NIS gene delivery via EpCAM targeting nanocomplex**

4.5.2.A. **EpDT3 aptamer displayed increased affinity towards EpCAM positive cells**

The basal level of EpCAM protein expression in MCF-7 cells was determined in the first part of the study. Although EpCAM aptamer’s (EpDT3) affinity to EpCAM positive cells have been analysed by our lab in earlier studies (Subramanian et al. 2012) this study again analysed the affinity of the EpDT3 aptamer in MCF-7 cells before conducting further experiments. To optimize the cell specific binding efficiency of the EpDT3 aptamer to the EpCAM positive cells, various concentrations of the aptamer were incubated with MCF-7 (EpCAM positive) and MIOM-1 (EpCAM low) cells (Danda R et al. 2013). The resultant cell binding by the EpDT3 aptamer was monitored by flow cytometry. The population of bound aptamer to the MCF-7 cells increased as the concentration of the aptamer increased (Figure 4.13.A, B). In MIOM-1 cells, only 2% of the aptamer was bound to the cells at 200nM concentration (Figure 4.13.B, C) whereas 50% of the MCF-7 cells exhibited aptamer binding (Figure 4.13.A). This concentration of the aptamer was used for PEI/NIS/EpDT3 synthesis. This confirms the specificity of the aptamer. Similar concentration of the scrambled aptamer (Scr-EpDT3) whose non-specificity was determined in our lab (Subramanian et al. 2012) was used for further studies.
Figure 4.13 EpDT3 aptamer binding affinity studies

(A) EpCAM aptamer (EpDT3) binding affinity in MCF-7 cells (EpCAM positive cells) (B) EpDT3 aptamer binding affinity in MIO-M1 cells (EpCAM low cells). Increasing concentration of Cy3 labelled EpDT3 aptamer was incubated in the cells for 1 h at 37°C. Cells were washed post incubation period and subjected to flow cytometric analysis. Isotype control was used for gating the positive population. 10,000 events were acquired within the gating. (C) Representative picture showing aptamer binding affinity towards EpCAM +ve and EpCAM low cells.
4.5.2.B. Nanocomplex formation

The complex formation between the positively charged PEI and negatively charged NIS plasmid (NISpDNA) at different charge ratios was assessed by agarose gel electrophoresis. In Figure 4.14.A, free NISpDNA was observed in lane 1. The NISpDNA concentration was kept constant and PEI concentration was increased steadily. A fraction of NISpDNA still migrated in the gel at NISpDNA: PEI ratio of 1:1.25, 1:2.5 and 1:3.75 (Figure 4.14.A, lane 2-4) whereas the NISpDNA band disappeared at 1:5 and above ratio (lane 5-7), indicating PEI-NISpDNA complex formation. The PEI/NISpDNA/EpCAM aptamer nanocomplex (PEI/NIS/EpDT3) formation was also observed by agarose gel electrophoresis (Figure 4.14.B). The NISpDNA is smaller than (Figure 4.14.B, lane 1) NISpDNA/PEI complex (Figure 14.B, lane 2). Though complete complex formation with EpDT3 aptamer was observed at 50nM, 100nM and 200nM concentration (Figure 4.14.B, lane 3-5), further study proceeded with 200nM concentration because it was found to be optimal in the aptamer affinity studies. Naked fluorescent EpDT3 aptamer ran as a small band with low molecular weight of 3 kDa (Figure 4.14.B, lane 6). The band for residual aptamer at 3 kDa was not observed at 200nM concentration (Figure 4.14.B, lane 5) while the PEI/NIS/EpDT3 nanocomplex of higher molecular weight in the same lane indicated maximal coating of the aptamer. This nanocomplex was characterized for size, shape and serum stability.
Figure 4.14 Complex formation: Agarose gel electrophoresis of NISpDNA forming complexes at different concentration of PEI.

(A) Lane 1 shows naked pDNA, lane 2-7 shows complexes of NISpDNA: PEI in various ratios. Complete encapsulation of NISpDNA observed from the 1:5 ratio. (B) Coating of fluorescent EpDT3 aptamer on the NISpDNA:PEI complex. Lane 1 shows naked NISpDNA, lane 2 shows NISpDNA:PEI complex and lane 3-5 shows increasing concentration of EpDT3 aptamer forming complexes with NISpDNA:PEI. Lane 6 shows fluorescent EpDT3 aptamer alone (red arrow) as a low molecular weight band.

4.5.2.C. Characterisation of the nanocomplex (PEI/NIS/EpDT3)

The DLS measurements revealed the size of the nanocomplex (PEI/NIS/EpDT3) when suspended in water to be at around 100 nm (z-average diameter). However, the number distribution of the PEI/NIS/EpDT3 was between 25-45 nm (Figure 4.15.B). The TEM investigation showed spherical particles with an average size of 30 nm (Figure 4.15.A). DLS, unlike TEM also measures the hydrodynamic diameter of the particle which leads to difference in size measurements (Jie Zheng et al. 2002). The Zeta potential was found to be
positive for the PEI alone and when complexed with NISpDNA, the potential drops but still remained positive. But when the PEI/NISpDNA was coated with EpDT3 aptamer, the potential changes to negative suggesting a concentrated distribution of the anionic ligand over the nanocomplex (Kurosaki T et al. 2009) (Table 4.1).

Figure 4.15 Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) of the nanocomplex.

(A) TEM picture of PEI/NIS/EpDT3 showing spherical shape. The PEI/NIS/EpDT3 nanocomplex was measured in a scale of 100nm. The analysis was carried out at an accelerating voltage of 80 kV using JEOL JEM1400 microscope. (B) PEI/NIS/EpDT3 characterised by Dynamic Light Scattering- Size measurement by number distribution. The PEI/NIS/EpDT3 was suspended in water for the analysis. The parameters used in the measurements included viscosity of 0.34 cP, reflective index of 1.054, and temperature of 25°C by the Zetasizer Nano ZS.
Table 4.1 Zeta potential of the PEI, PEI/NISpDNA complex and PEI/NIS/EpDT3 nanocomplex prepared in water determined by Zetasizer Nano ZS.

<table>
<thead>
<tr>
<th>Zeta potential (mV)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PEI alone</td>
<td>+9.31</td>
</tr>
<tr>
<td>PEI/NISpDNA</td>
<td>+4.01</td>
</tr>
<tr>
<td>PEI/NIS/EpDT3</td>
<td>-14.2</td>
</tr>
</tbody>
</table>

4.5.2.D. Biostability of the PEI/NIS/EpDT3:

Biostability of the PEI/NIS/EpDT3 in the presence of serum was monitored over a period of 72h (Table 4.2). Size and zeta recorded every 24 h of the PEI/NIS/EpDT3 showed that the nanocomplex maintained its characteristics until 72 h in the presence of the serum. The nanocomplexes aggregated beyond 72 h with the serum treatment.

Table 4.2 Biostability of the nanocomplexes

<table>
<thead>
<tr>
<th>PEI/NIS/EpDT3</th>
<th>Size (d.nm)</th>
<th>Zeta (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>29.85</td>
<td>-11</td>
</tr>
<tr>
<td>24 h</td>
<td>24.8</td>
<td>-10.1</td>
</tr>
<tr>
<td>48 h</td>
<td>27.1</td>
<td>-11.5</td>
</tr>
<tr>
<td>72 h</td>
<td>25.7</td>
<td>-7.8</td>
</tr>
</tbody>
</table>

Table 4.2: Biostability of the nanocomplexes over a period of 3 days determined by Zetasizer Nano ZS. PEI/NIS/EpDT3 was prepared and incubated in serum and size and zeta of the nanocomplex was recorded once every 3 days.
4.5.2.E. NIS gene delivery via PEI/NIS/EpDT3 leads to increased expression of NIS protein

Following the nanocomplex preparation and characterisation, PEI/NIS/EpDT3 was used to deliver NIS gene in MCF-7 cells. In order to determine whether the gene construct delivered via the nanocomplexes resulted in overexpression of NIS protein, Western blotting was performed (Figure 4.16.A). When compared to the vehicle control, the overexpression of the NIS protein was observed after the NIS pDNA was delivered by the lipofectamine transfection reagent and PEI polymer. However on comparing the expression of NIS pDNA between lipofectamine reagent and PEI polymer alone, NIS expression was higher when delivered with the commercial transfection reagent. But when compared to vehicle control, lipofectamine and PEI alone, NIS expression was remarkably higher when delivered via PEI/NIS/EpDT3 nanocomplex. This is because of the targeted and specific delivery of NIS plasmid in EpCAM positive MCF-7 cells and active overexpression expression of NIS gene translating into NIS protein. β-actin protein band detected at 43 kDa was used as a loading control. The image J analysis after normalising the NIS expression with the β-actin revealed higher expression in the PEI/NIS/EpDT3 nanocomplex (Figure 4.16.B).
Figure 4.16 Western blot image showing the overexpression of NIS protein in MCF-7 cells after the delivery of NIS plasmid using various transfer vehicles.

(A) 50 μg of protein sample was loaded and anti-human NIS, mouse monoclonal antibody (1:250) was used as primary antibody, HRP-conjugated anti-mouse IgG (1:1000) was used as secondary antibody; (B) β-actin as loading control, detected at 43 kDa with anti-human
β-actin mouse monoclonal antibody (1:4000) was used as primary antibody, HRP-conjugated anti-mouse IgG (1:7000) was used as the secondary antibody. The blot was developed using enhanced chemiluminescence (ECL) method. (B) Histogram plotted for the NIS protein after normalization with the β-actin protein expression using NIH’s Image J software.

4.5.2.F. Radioactive iodide uptake by the nanocomplexes

Following the NIS expression studies at protein level, radioactive iodide uptake was measured to determine if the synthesized PEI/NIS/EpDT3 nanocomplex could be used for targeted NIS gene therapy by incubating the NIS nanocomplex treated cells with 4µCi/mL Na^{125}I for 2 h. Iodide accumulation by the MCF-7 cells transfected with NIS pDNA using lipofectamine as transfection reagent was higher than the vehicle control cells which had an endogenous NIS expression (Figure 4.17). Furthermore, the PEI/NIS/EpDT3 nanocomplex delivered NIS pDNA showed a significant higher uptake (P<0.05) when compared to the vehicle control cells. The iodide uptake in the PEI/NIS/Scr-EpDT3 nanocomplex (Scrambled aptamer) transfection was similar to that of the control cells, thus confirming that EpCAM based targeted delivery of NIS plasmid by the PEI/NIS/EpDT3 nanocomplex could lead to functional NIS.
Radioactive iodide uptake was measured after treating the cells with 4μCi/mL of Na\textsuperscript{125}I for 2 h in MCF-7 cells transfected with various forms of NIS pDNA complexes. The radioactive iodide activity is expressed in counts per min (cpm). The error bar represents standard deviation of experiments performed in duplicates. *P<0.05. P-values were calculated by the Student’s t-test.
4.5.2.G. Non-radioactive Iodide uptake by the nanocomplexes

Apart from radioactive uptake assay, the nanocomplex functionality was also assessed by non-radioactive iodide uptake assay. The iodide uptake was studied to assess the functional capability of the overexpressed NIS protein by the PEI/NIS/EpDT3 nanocomplex. The iodide uptake decreased linearly with increasing concentration of iodide (Figure 4.18.A). The standard curve obtained using the As/Ce assay was used for estimating iodide concentration in the cells. A significantly increased uptake of iodide was observed in the MCF-7 cells transfected with NIS pDNA using lipofectamine transfection reagent compared to control (untransfected) cells (P<0.001) (Figure 4.18.B). A lower uptake of iodide was observed in MCF-7 cells transfected with NIS pDNA using PEI polymer alone compared to the lipofectamine delivered NIS pDNA but a significantly higher uptake compared to the control cells (P<0.01). However, the highest uptake of 271 nM of iodide was observed in cells transfected with PEI/NIS/EpDT3 nanocomplex (P<0.001) reflecting the increased NIS protein overexpression by the nanocomplex. This was because of the targeted delivery of NIS pDNA to EpCAM positive cells mediated by the EpDT3 aptamer.
Figure 4.18 Functional studies using non-radioactive iodide uptake assays

(A) Logarithmic conversion of the means of A420 at 30 min (n = 3) vs. iodide standards. (B) Cell-trapped iodide determined by the As/Ce method and the iodide uptake expressed in nM concentration. NIS gene delivered by all the complexes showed significant iodide uptake when compared to the control cells. The error bar represents standard deviation of
experiments performed in triplicates. ****P<0.001, **P<0.01. P-values were calculated by the Student’s t-test.

4.6. DISCUSSION

In the light of an urgent need of an effective and targeted treatment for cancer, the current study embarked upon a NIS based therapeutic strategy for breast cancer by addressing the issue of specificity. Firstly, NIS was expressed in breast cancer cell line using EpCAM specific promoter and analyzed the feasibility of specific expression. Secondly, NIS was delivered in breast cancer cell line using nanocomplex and the possibility of NIS targeted expression and function was then evaluated.

The breakthrough in radionuclide gene therapy occurred when NIS gene of rat and human origin was cloned and characterized (Dai G, Levy O & N 1996; Smanik PA 1996). Various factors such as rate of iodide uptake, iodide retention, iodide recirculation, iodide organification and rate of iodide decay determine the therapeutic radiation dose (Scholz IV et al. 2005). NIS substrates are determined by anion size and monovalency which results in several anions being transported by NIS such as Technetium pertechnetate and Rhenium perrhenate (Maheshwari YK et al. 1981). NIS has a pivotal role consisting radioiodide treatment in the therapeutic management of thyroid cancer (Schmutzler C & J 1998). The ability of the NIS to accumulate radioactive iodide has been studied in various non-thyroidal tumors (Scholz IV et al. 2005; Spitzweg C et al. 2001; Spitzweg C et al. 2000; Spitzweg C et al. 1999). In order to concentrate iodide from plasma, NIS gene transfer and its functional expression is necessary and this in turn would enable radioiodide therapy (Spitzweg C et al. 2001).

NIS protein expression and its correct translocation to the plasma membrane are necessary for proper function of NIS. To address this issue, various attempts to re-activate NIS function
have been ongoing. Primary approach has been to induce the endogenous NIS expression and restore it using various molecules (Kogai T et al. 2000). Alternative method for achieving functionally active NIS was to introduce the NIS gene exogenously (Yu Yan et al. 2008). In breast cancer, an altered location of NIS protein results in lost capacity to concentrate radioiodide (Wapnir IL et al. 2003).

It is necessary to influence NIS expression so that it is expressed only in cancer cells because its universal expression can cause undesirable effects during treatment. This specific expression can be done by cancer tissue specific promoters which will minimise non-specific uptake and maximise tumor specific accumulation of radioiodide. Tissue specific expression of the therapeutic gene is obtained as a result of the transcriptional targeting of therapeutic genes which in turn leads to reduction in extratumoral toxicity (IR 1996). Cancer tissue specific promoters for specific NIS gene expression has been used in prostate by prostate specific antigen promoter (Kakinuma H et al. 2003; Spitzweg C et al. 2000) and in colon by carcinoembryonic antigen promoter. Only a limited number of investigations have analyzed tissue specific promoter mediated NIS gene delivery in breast cancer.

The approach of the current study was to address the issue of non-targeted delivery of therapeutics. For this, in the first part of the study, a new vector was cloned by placing the therapeutic gene, NIS under the control of tissue specific promoter like EpCAM. Although EpCAM is present in the normal epithelial cells of breast tissue, its high expression in breast cancer is associated with poor prognosis and decreased survival rate in patients (Spizzo G et al. 2004). Such high expression of EpCAM in cancer tissues than the normal cells makes it an attractive target in the treatment of breast cancer (Soysal et al. 2013). In this study, MCF-7 cell line was used and its high EpCAM expression was confirmed by flow cytometry. In addition to the NIS gene under the tissue specific promoter like EpCAM, universal promoter, CMV driven NIS gene was also used for efficacy comparison studies. The expression and
functional studies of NIS gene by both the vectors were analysed after establishing stable cell lines. Transient NIS gene expression curtails options for radioiodide therapy (Trujillo MA et al. 2009) and this lead to studies being performed in stably transfected cell lines. The feasibility of targeted delivery was then analysed by evaluating the expression and function of NIS gene in MCF-7 transfectants. Overexpression of NIS at mRNA level was observed in both the stable transfectants with MCF-CMV-NIS showing significantly higher NIS expression than MCF-EpCAM-NIS. The reason behind this increased expression in CMV-NIS is because of the activity of universal promoter in all the cells unlike EpCAM-NIS which is specific.

Both the stable transfectants, MCF-CMV-NIS and MCF-EpCAM-NIS showed iodide uptake which demonstrates correct membrane translocation of the overexpressed NIS protein following NIS gene delivery. Between the stable transfectants, there was a difference in the uptake of iodide determined via both non-radioactive and radioactive iodide uptake assays. In both the assays, MCF-EpCAM-NIS showed evidently higher uptake of iodide than the MCF-CMV-NIS indicating an active EpCAM promoter operating in EpCAM positive cells. The high uptake observed in MCF-EpCAM-NIS transfectant than the control cells is also the characteristic of the promoter used. It should be noted that even though the mRNA expression level of MCF-EpCAM-NIS was significantly lower than that of MCF-CMV-NIS, it resulted in a robust iodide uptake. To assess whether the accumulation of iodide was explicitly brought about by the functional activity of the NIS gene product, iodide uptake was analysed in the presence of NaClO₄. Iodide uptake was significantly inhibited, revealing NIS mediated transport of iodide in the stable transfectants.

However, the efflux studies in both MCF-CMV-NIS and MCF-EpCAM-NIS showed no difference in the rapid flushing out of iodide. Rapid efflux indicated that no organification of the accumulated iodide materialized. Various studies in prostate cancer have shown that even
in the absence of iodide organification, significant therapeutic effect of radionuclides have been achieved by expressing NIS gene using tissue specific promoters (Scholz IV et al. 2004; Spitzweg C et al. 2000; Willhauck MJ et al. 2007). A study found that because the microenvironment of in vivo tumor has more iodide content, the half-life of radioiodide was longer in tumors than in vitro cell lines (Schipper ML et al. 2003). This occurrence enhances NIS based gene therapy. It is understood that specific promoter exhibit lesser activity compared to universal promoters like CMV promoter. But in the current study, it was found that EpCAM promoter driven NIS gene showed higher functional activity. These results are consistent with previous studies which showed a higher functional activity of NIS expressed via a specific promoter, human telomerase reverse transcriptase (hTERT) promoter (Zhang M et al. 2014). Thus, the EpCAM promoter may act not only as a specific promoter but also as a promoter with strong transcriptional activity.

The migration ability of the stable transfectants was further analysed. Migration studies revealed an increase in migration ability in the stable transfectants overexpressing NIS. This enhancement in migration may due to the interaction of NIS with leukemia-associated RhoA guanine exchange factor (LARG) to activate RhoA which inturn escalates the cell invasion and migration (Lacoste C 2012 ). However, no difference in migration ability was observed between the stable transfectants when observed through wound healing assay. Since, NIS overexpression is enhancing the migration ability of the cancer cells, it has to be counteracted with agents to suppress the invasive quality of the overexpressed NIS. So, in future studies, nanoparticle could be loaded with molecules exhibiting anti-metastatic property such as curcumin and bovine lactoferrin (Chatterjee et al. 2003; Kanwar, Mahidhara & Kanwar 2012; Tsuda et al. 2010) which may arrest the migratory ability of NIS thus could enhance the NIS mediated radiotherapy.
Clinical potential of establishing a stable transfectant should be assessed after *in vivo* gene delivery in breast cancer models. In that case, ablation of thyroid has to be performed before radioiodide treatment for breast cancer to prevent the uptake of radioiodide by the thyroid cells. Furthermore, the benefit of NIS gene is that, apart from its radioiodide uptake ability, it can also be used as a reporter gene by positron emission tomography or single photon emission computed tomography to track the organs which had taken up iodide (Shin JH et al. 2004).

To further optimize the tumor selectivity, in the second phase of the study, a ligand-mediated NIS gene delivery to the tumor sites was studied. Therapeutic gene delivery holds great future and currently emerges as a great promise to be one of the major strategies for the treatment of cancers and various other diseases. For NIS gene delivery, various methods like liposomes, viral vectors, electroporation were used earlier (Boland A et al. 2000; Schipper ML et al. 2003; Shimura H et al. 1997). A recent study showed delivery of NIS gene using pseudo dendritic polymers into neuroblastoma mouse model which achieved significant iodide accumulation thereby, a therapeutic effect (Klutz K et al. 2009).

Non-viral gene delivery technique also termed as “artificial viruses” is considered to be less potent inspite of it mimicking viral systems with less clinical risks (AP 1998; FD 1995; Roy K et al. 1999). As a further step towards delivery of NIS gene in this study a polycation, PEI was used. PEI has been used as an efficient nonviral vector for gene delivery *in vitro* as well as *in vivo* (Clamme JP, Azoulay J & Y 2003 ; Fischer D et al. 1999 ; Kichler A et al. 2001; Tang MX & FC 1997 ). Though PEI condenses DNA to form complexes, for improved retainability the PEI-DNA complex has to be in the nanoformulation. The downside of using PEI alone to deliver pDNA is that it non-specifically binds to negatively charged cell membrane and thus making use of PEI/pDNA complex difficult for specific gene delivery system (Demeneix B & JP 2005; Lemkine GF & BA 2001). So, this study embarked on
aptamer technology by employing a ligand which is small enough not to elicit immunogenicity but efficient enough to bind to specific targets.

Studies have reported development of EpCAM aptamers and their high specificity for the target (Shigdar S1 2011). However, there are few reports about using EpCAM aptamer to deliver a gene. Thus a novel nanocomplex of NIS pDNA/PEI coated by the EpCAM aptamer (EpDT3) was prepared and tested. EpDT3 aptamer affinity and its specificity along with its optimum concentration for the nanocomplex were first determined. The affinity studies revealed an effective binding of EpDT3 aptamer to EpCAM positive MCF-7 cells but at a higher aptamer concentration, MIOM-1 with low EpCAM expression cells showed a faint affinity towards the EpDT3 aptamer. The low EpCAM expression in MIOM-1 cells has already been studied (Danda R et al. 2013) and thus the optimum concentration of the aptamer was fixed at 200nM at which there was insignificant binding for MIOM-1 cells. Complex formation occurred between the components of the nanocomplex based on electrostatic interactions. Various ratios of PEI:NISpDNA:EpDT3 were experimented and further analysed on agarose gel electrophoresis. Following the confirmation of complex formation by agarose gel electrophoresis, size and zeta potential of the nanocomplexes were determined. Addition of the anionic EpDT3 aptamer affected the zeta potential which was evident from the clear shift of potential from positive to negative. This proved the binding of the aptamer with NIS pDNA/PEI complex.

One of the important properties of a nanoparticle is its stability in the presence of serum. Serum has the inhibitory effect and they function by disrupting the PEI/pDNA complex or obstruct the binding of the complex to the cell membrane (Guo W & RJ 2001). Therefore the PEI:NISpDNA:EpDT3 nanocomplexes was subjected to serum stability check for a period of 5 days. It maintained its size and potential till 72 h in the presence of serum which proves its
bio-stability and availability. However, beyond 3 days, the nanocomplexes started to aggregate.

NIS gene delivery by different vehicles such as lipofectamine, PEI and finally the nanocomplex was studied in MCF-7 cells by determining the NIS protein level through Western blotting. Overexpression of NIS protein was observed in all the samples, however difference in NIS protein expression delivered by different vehicles also was evident. The NIS protein expressed by the targeted nanocomplex was higher compared to NIS expressed via other vehicles. This could be the result of EpDT3 aptamer binding to EpCAM positive MCF-7 cells. The increased NIS protein level also reflected in the functional studies where the cells with NIS delivered by the nanocomplex had significantly higher iodide uptake compared to the cells transfected with other vehicles like lipofectamine and nanocomplex coated with scrambled aptamer (PEI/NISpDNA/Scr-EpDT3). This shows that the NIS pDNA released from the PEI/pDNA/EpDT3 nanocomplex formulation retained functional integrity at a higher rate.

The high iodide uptake by the cells transfected with PEI/pDNA/EpDT3 nanocomplex could be attributed to the recognition of EpCAM aptamer on the nanocomplex surface by the EpCAM positive cells. These results suggest that the nanocomplex with a negative charge is taken up by cells through EpCAM-specific receptor mediated pathway (Winkler J et al. 2009).

Thus, in this experiment, a nanocomplex to deliver a therapeutic gene specifically to cancer cells was developed. Following EpCAM aptamer mediated NIS gene delivery complexed with PEI, in future studies, by applying different radionuclides such as $^{188}$Re and $^{231}$At, the therapeutic efficacy of NIS can be enhanced for non-thyroidal cancers (Dadachova E et al. 2002; Willhauck MJ et al. 2008).
The present study though require improvements to achieve much higher accumulation of iodide and more retention of iodide, application of this specific radiotherapy can also be applied to various other cancers where EpCAM is overexpressed. In addition, the current study has been performed in \textit{in vitro}, in future it can be considered for \textit{in vivo} studies in breast cancer models which would validate at pre-clinical levels the benefit for cancer treatment by avoiding the undesirable effects of therapy to surrounding normal tissues.

Thus the current study achieved the goal of showing the feasibility and specificity of EpCAM specific promoter to express NIS gene. In addition to comparing the promoter activity between universal and tissue specific promoter, the current study also showed the functional activity of NIS gene expressed by both the promoters. This study also assembled a functional nanocomplex which was successful in targeting EpCAM positive cells. Furthermore, the nanocomplex retained the functional activity of the NIS plasmid it carried. In conclusion, the above study evaluated the potentials of EpCAM receptor targeted therapeutic NIS gene expression in breast tumor cells and proved it to be an effective approach.
Chapter 5

Study of survivin splice variants, Bcl-2 and Bax expression profiles in retinoblastoma by Real Time PCR

“PCR technique is highly original and significant, virtually dividing biology into the two epochs of before P.C.R. and after P.C.R”

5. Chapter 5

5.1. INTRODUCTION

Cancers evade cell death by overexpressing anti-apoptotic proteins resulting in tumor progression and poor prognosis of the disease. Retinoblastoma progression includes various genetic changes like triggering of oncogenes and inactivation of tumor suppressor genes which leads to loss of balance between cell proliferation and apoptosis (Thompson 1995). Aberrations at the genetic level contribute to cancer progression by disturbing cellular mechanisms like apoptosis. In RB though, apoptosis and its inhibition has not been extensively studied. Apoptosis is a programmed cell death and a genetically organised process that occurs under both normal and pathological conditions (E 1996b). Apoptosis is one of the three forms of programmed cell death, the other two being autophagy and necrosis. Apoptosis is triggered by numerous death and damage signals arising physiologically and pathologically (Gross A, McDonnell JM & SJ 1999). As shown in Figure 5.1, a number of morphological changes like cell shrinkage, DNA fragmentation and nuclear condensation which are the characteristics of a cell undergoing apoptosis (Nishida, Yamaguchi & Otsu 2008). As described in chapter 1, Figure 1.10, extrinsic pathway containing cell surface death receptors and intrinsic pathway exploiting mitochondria are the two main pathways of apoptosis (Tang D et al. 2011). The death receptors that are involved in the extrinsic pathway include members of the TNF (Tumor Necrosis Factor) family. Two of the main ligand and death receptors combination are TNFα/TNFR1, FasL/FasR (Ashkenazi, AviDixit & M 1998). When the ligand binds to their corresponding receptors, it stimulates the recruitment of adapter proteins. FADD adapter protein is recruited when Fas ligand binds with Fas receptors, similarly TRADD adapter protein along with FADD and RIP are recruited when
TNF ligand is bound to TNF receptors (Hsu, Xiong & Goeddel 1995; Wajant 2002). This results in activation of procaspase-8 to caspase-8 which finally triggers apoptosis. A protein called c-FLIP can inhibit apoptosis by binding to caspase-8 (Scaffidi et al. 1999). The intrinsic pathway is free of receptors and the signals act directly on the targets. Some of the signals that trigger intrinsic pathway are loss of hormones, cytokines or exposure to toxins and radiation (Elmore 2007). Such signals stimulate the opening of the mitochondrial membrane resulting in the cytosolic release of cytochrome c, Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein (Smac/DIABLO), apoptosis-inducing factor (AIF) and caspase-activated DNAse (CAD) (Du et al. 2000; Garrido et al. 2006; Joza et al. 2001). Defects in these pathways lead to disturbance in the balance between proliferation and apoptosis which ultimately leads to cancer development and treatment resistance (Afford S & S 2000; Hanahan D & RA 2000).

![Figure 5.1 Schematic illustration of process of apoptosis](image-url)
Apoptosis is a natural cellular response to any physical damage and occurs both at normal and pathological conditions. The process starts with shrinkage of the cell, followed by blebbing of the membrane, nuclear condensation and formation of apoptotic bodies. The cell death ends with lysis of the formed apoptotic bodies. The entire mechanism is called “programmed cell death”. Image adapted from http://www.microbiologybytes.com/virology/kalmakoff/baculo/baculohostinteract.htmL.

Apoptosis is regulated mainly by two families. One family is the B-cell lymphoma 2 (Bcl-2) family which plays a key role in regulating intrinsic pathway of apoptosis. Few of the members of this family functions as repressors/anti-apoptotic (Bcl-2, Bcl-xl, Mcl-1) whereas others functions as promotors of apoptosis (Bax, Bad, Bid) (Adams JM & S 1998). Apoptosis via the pro-apoptotic molecules is initiated by the death signals which in turn stimulates post-translational modifications leading to translocation of these pro-apoptotic molecules to the mitochondria (Shamas-Din et al. 2011). Bcl-2 is considered as one of the vital genes in suppressing apoptosis. Its abnormal overexpression has absolute null effect on cell proliferation and in turn prolongs the survival of the tumor cells and abolishes the apoptotic effect (Llambi & Green 2011). Bcl-2 has a key role in cancer therapy as its increased expression leads to resistance to drugs whereas its reduced expression results in better response to anticancer drugs. In the case of cutaneous B-cell lymphoma, rituximab treatment was ineffective and the rituximab treated cells showed high expression of Bcl-2 suggesting its role in therapy resistance (Wobser et al. 2007). Since majority of anti-cancer drugs works by promoting apoptosis in cancer cells, the Bcl-2 opposes this action thus increasing drug resistance. Bcl-2 allows drug accumulation intracellularly and so its mechanism of drug resistance induction is different from other resistance genes (Geng, Wang & Li 2013). Bcl-2 has been considered a biomarker and has reached clinical trials with two drugs, ABT-263 and
ABT-199 directly targeting Bcl-2 (Roberts et al. 2012; Tse et al. 2008). However, recent study with the drugs, ABT-263, ABT-199 along with ABT-737 (all structural analogues) showed the hazardous effects of them on cancer patients with viral infections by targeting non-cancerous cells (Kakkola et al. 2013).

The first pro-apoptotic members of Bcl-2 family to be discovered were Bcl-2 associated X (Bax) protein (Oltvai ZN, Milliman CL & SJ 1993). Bax plays a vital role as a facilitator of apoptosis and its function is always kept in check by the anti-apoptotic member of the Bcl-2 family, Bcl-2 (Reed 1996). The pro-apoptotic activity of Bax is induced by functional p53 which results in increased cell death induced by chemotherapeutic drugs (Fulda & Debatin 2006). Sensitivity to drugs increases when more Bax/Bax homodimers are present in the mitochondria (Reed 2006) and its reduced concentration renders the cell resistant to apoptosis induced by chemotherapy even in a p53-independent manner. Various cancers like breast, head and neck and ovarian have reported low expression of Bax to be a prognostic factor in chemotherapy treated malignancies (Kang et al. 2005; Krajewski et al. 1995; Kupryjańczyk et al. 2003). This confirms the prominence of Bax in the control of apoptotic cell death (Pietrantonio et al. 2013).

Among the other apoptotic and anti-apoptotic molecules that facilitate the progression of cancer, is the main family of apoptotic molecules are Inhibitors of Apoptosis (IAP) family. The role of the IAP family in cancer has been associated with cell protection from anti-cancer drugs and also to block apoptosis induced by radiation (Jäättelä 1999). In humans, there are eight IAPs and one among them is survivin (BIRC5) known to be hot and validated cancer target (Ambrosini, Adida & Altieri 1997; Kanwar, Kamalapuram & Kanwar 2011). The primary mode of action of IAPs against apoptosis is by inhibiting caspases (Salvesen & Duckett 2002) and other alternative modes are by activating JNK signalling or TGF-β mediated signalling (Reffey et al. 2001; Sanna et al. 2002). Although, many studies have
shown interaction between survivin and caspases, few studies have indicated that the interaction does not really inactivate caspases thus bringing in the possibility of caspase independent inhibition of apoptosis (Banks et al. 2000). Apart from the cell protecting ability of survivin, it also regulates the cell division by forming complexes with the chromosomal passenger proteins which in turn regulates the checkpoint of spindle bodies, chromosomal movements during mitosis (Noton et al. 2006). The expression of survivin was reported to be high in the fetal period and trivial in the normal differentiated tissues. However, recent reports have suggested expression of survivin in normal tissues like prostate, stomach, colon, skin and various other organs (Yamada et al. 2003). In cancer, survivin overexpression overrides the checkpoint resulting in abnormal progression via mitosis and high expression has been observed in multitude of cancers such as breast, prostate, lung, ovarian and pancreatic cancers (Kanwar, Kamalapuram & Kanwar 2013). Survivin plays an important role in progression of various cancers and so it is considered to be a potent anti-cancer target (Kanwar, Kamalapuram & Kanwar 2011). In addition to its therapeutic potential, survivin expression has prognostic and diagnostic value because of its expression in circulating tumor cells and patients serum (Rodel et al. 2012). Studies from our laboratory have targeted survivin using bLf in a nanoformulation in colon epithelial cancer cells which showed a significant lapse in survivin levels (Kanwar et al. 2014).

Alternative splicing is a regulatory process which produces distinct protein isoforms from the common mRNA precursor and a variety of apoptotic factors undergoes this process (Schwerk & Schulze-Osthoff 2005). Studies have reported six isoforms of survivin gene in humans. The wild type survivin consists of 4 exons, containing 426 bp of coding transcript which translates into 16.5 kDa protein (Caldas, Honsey & Altura 2005). Apart from the mature wild type survivin, the other alternative splice variants are Survivin-2β which derived its name because of addition of 69 bp from intron 2. Survivin ΔEx3 is created by deletion of 102 bp of
exon 3 (Mahotka et al. 1999) and addition of 165 bp from intron 3 gives rise to Survivin-3β (Badran et al. 2004). The fifth variant known as Survivin-2α is generated by the introduction of 197 bp from intron 2 (Caldas, Honsey & Altura 2005) and the final variant Survivin-3α is also produced by the addition of 209 bp from intron 2 (Huang et al. 2011b).

In cancer, abnormal expression of both apoptosis regulating protein families has been associated with proliferation and resistance to drugs. While apoptosis regulatory genes have been widely detected in various cancers, to date, endogenous expression of the apoptotic regulatory proteins in RB has not been explored. Earlier publication from our laboratory (Sudhakar et al. 2013) showed survivin protein expression in advanced stages in RB. Similarly, in another recent study from our laboratory (Beta et al. 2013), it was found from miRNA profiling of RB patient’s serum that Bax was one of the potential target genes involved in RB progression. Thus, in continuation to above studies performed by our research groups, the current study on Bcl-2, Bax and survivin splice variants apoptotic role in RB tumors was warranted. Since there is lack of knowledge of endogenous expression of survivin splice variants, Bcl-2 and Bax in primary RB tumors, the study aims dealt in this chapter are to unravel the mRNA expression status Bcl-2, Bax and survivin splice variants molecules in RB.

### 5.2. Hypothesis

The hypothesis of this study was that the expression of the apoptotic regulatory genes would be associated with clinicopathological features of the RB tumors.

### 5.3. Aims

The aims of the work performed in this chapter were

1) To investigate the mRNA expression of survivin splice variants like anti-apoptotic, Survivin-WT, Survivin-3β and Survivin-ΔEx3 and pro-apoptotic variants like
Survivin-2α, Survivin-2β, as well as pro-apoptotic molecule, Bax, anti-apoptotic molecule, Bel-2 in 20 RB tumors and in an established standard RB cell line (Y79) using qRT-PCR.

2) To investigate if the expression levels of these apoptotic molecules could be correlated with the clinicopathological features of RB tumors.

3) To study the apoptotic effects on silencing the Survivin-WT and one of the anti-apoptotic splice variant, Survivin-ΔEx3 in Y79 cells.

5.4. MATERIALS AND METHODS

Figure 5.2 Schematic representation of the research work performed in this phase of study
Table 5.1 Histopathological features of RB tumor tissues analysed

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No. of patients (N=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;5 years</td>
<td>19</td>
</tr>
<tr>
<td>≥5 years</td>
<td>1</td>
</tr>
<tr>
<td><strong>Laterality</strong></td>
<td></td>
</tr>
<tr>
<td>Unilateral</td>
<td>20</td>
</tr>
<tr>
<td>Bilateral</td>
<td>Nil</td>
</tr>
<tr>
<td><strong>Differentiation</strong></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>5</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>5</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>10</td>
</tr>
<tr>
<td><strong>IIRC Grade</strong></td>
<td></td>
</tr>
<tr>
<td>Group A-C</td>
<td>Nil</td>
</tr>
<tr>
<td>Group D,E</td>
<td>20</td>
</tr>
<tr>
<td><strong>Invasion status</strong></td>
<td></td>
</tr>
<tr>
<td>Invasive</td>
<td>12</td>
</tr>
<tr>
<td>Non-invasive</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 5.1: Tumor cohort and its clinical parameters. IIRC stands for International Intraocular Retinoblastoma Classification. Group A-C is the early stage tumors and group D & E are the advanced stage tumors. Invasive tumors include both choroidal invasion cases and optic nerve invasion cases.
5.5. RESULTS

5.5.1. Expression of survivin variants, Bax and Bcl-2 in RB tissues

The transcriptional expression of survivin variants was evaluated in 20 different fresh-frozen RB tumors by quantitative real time PCR. The mRNA level expression in tumor was quantified by Relative Standard Curve method initially, and then compared with the expression in normal retina. The obtained results suggest that the survivin variants showed higher expression in majority of the tumors irrespective of sex and age when compared to normal retina. The relative mRNA expression of all the survivin variants was plotted onto a graph (Figure 5.3.A). Overall expression of Survivin-WT was detected in 90% (18/20) tumors, Survivin-2α in 85% (17/20), Survivin-2β in 80% (16/20), Survivin-3β in 85% (17/20) and Survivin-ΔEx3 in all 20 tumors (100%). All the five survivin isoforms were present in 16 tumor samples (80%). Although all the survivin variants were highly expressed in RB, Survivin-WT in terms of overall mRNA expression was significantly highly expressed (P<0.05) when compared to Survivin-2β (Figure 5.4.A). The mRNA level expression of Bax and Bcl-2 was also evaluated in 20 different fresh-frozen RB tumors and then compared with the expression in normal retina by quantitative real time PCR. Higher expression of Bax and Bcl-2 was observed in tumors irrespective of clinical parameters and the relative expression were plotted onto a graph (Figure 5.3.B). Overall expression of Bax was detected in 80% (16/20) and Bcl-2 was detected in all 20 tumors (100%). Both Bax and Bcl-2 were present in 16 tumor samples (80%) out of 20 tumors. Between Bax and Bcl-2 expression, Bcl-2 was highly expressed in RB with a high statistical significance of expression (P<0.001) (Figure 5.4.B).
Figure 5.3 Expression level of Survivin splice variants, Bax and Bcl-2 in RB tumors

(A) mRNA levels of Survivin-WT (Sur-WT), Survivin-2α (Sur-2α), Survivin-2β (Sur-2β), Survivin-3β (Sur-3β) and Survivin-ΔEx3 (Sur-ΔEx3). (B) Bax and Bcl-2 mRNA expression levels in RB tumors relative to non-neoplastic retina by Real Time Quantitative Reverse Transcriptase PCR (qRT-PCR). Relative fold expression levels are expressed as mean ± SD of triplicate analyses.
Figure 5.4 Relative mRNA level expression of survivin splice variants, Bax and Bcl-2

(A) Relative mRNA levels of survivin variants with respect to normal retina in RB. Survivin-WT is the dominant transcript among the variants and showed significant difference of expression ($P<0.05$) with Survivin-$2\beta$. (B) Bcl-2 and Bax were statistically significant ($P<0.001$). P-values were calculated by the Wilcoxon-test.

5.5.2. Association with the clinicopathological characteristics of tumor

Association of the survivin variants with invasion status, sex and differentiation of the RB tumors was further analysed using non-parametric tests like Mann-Whitney and Kruskal Wallis test due to the data not following normal distribution. The Shapiro-Wilk test was initially used to determine the normality of the data. Since the significance value was below 0.05, the data deviated from normal distribution. The association of the clinical parameters such as gender and invasion status and the variables with its P-value have been shown in the Table 5.2. The association of survivin variants expression with the invasion status of the tumors was analysed, and the expression level of the survivin variants did not significantly change between the invasive and non-invasive cases (Figure 5.5). Similarly, as shown in
Figure 5.7, all the survivin variants did not show a statistically significant association with differentiation status of tumors. However, only Survivin-2α, a pro-apoptotic protein showed a statistically significant (P<0.05) higher expression in moderately differentiated tumors than poorly differentiated tumors and well differentiated tumors with 30.9% and 35% variability respectively in rank score for differentiation. The other variant, Survivin-2β, a pro-apoptotic protein also showed a statistically significant (P<0.05) higher expression in moderately differentiated tumors than poorly differentiated tumors and well differentiated tumors (P<0.05) with 34.7% and 53.4% variability in rank score respectively. Survivin-ΔEx3, an anti-apoptotic protein also showed a statistically significant (P<0.05) lower expression in well differentiated tumors compared to moderately differentiated tumors with 43.7% variability in rank score for differentiation. Surprisingly, an association of gender with expression of proteins showed a significant increase (P<0.05) of anti-apoptotic proteins, Survivin-WT and Survivin-3β in males compared to females (Figure 5.9).

Similar to survivin splice variants, association of the Bcl-2/Bax with invasion status, sex and differentiation of the RB tumors was further analysed using non-parametric tests like Mann-Whitney and Kruskal Wallis test. The association of the clinical parameters such as gender and invasion status and the variables with its P-value have been shown in the Table 5.2. Unlike the survivin splice variants, Bax, a pro-apoptotic protein, showed a statistically significant (P<0.05) higher expression in invasive tumors than the non-invasive tumors (Figure 5.6). In the case of differentiation, the Bcl-2/Bax ratio showed an increased expression in poorly differentiated tumors compared to well differentiated and moderately differentiated tumors (Figure 5.8.A). However, the statistical analysis did not show any significant association with any of the differentiation stages either in Bax or in Bcl-2 (Figure 5.8.B). No significant association of gender with Bcl-2 and Bax expression was observed (Figure 5.10).
Table 5.2 Association of mRNA expression of survivin splice variants, Bcl-2 and Bax with gender and invasion status in RB tumors

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Total number (n)</th>
<th>P-value Sur-WT</th>
<th>P-value Sur-2α</th>
<th>P-value Sur-2β</th>
<th>P-value Sur-3β</th>
<th>P-value Sur-ΔEx3</th>
<th>P-value Bax</th>
<th>P-value Bcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
<td>0.048</td>
<td>NS</td>
<td>NS</td>
<td>0.013</td>
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<td>NS</td>
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<tr>
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<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
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<tr>
<td>Female</td>
<td>6</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td>NS</td>
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<tr>
<td><strong>Invasion status</strong></td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Non-invasive</td>
<td>20</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.045</td>
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<td>Invasive</td>
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<td>NS</td>
<td>NS</td>
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<tr>
<td>Invasive</td>
<td>12</td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

*Table 5.2: Association of mRNA expression of survivin splice variants, Bcl-2 and Bax with gender and invasion status in 20 RB tumors. P-values were calculated by the Mann-Whitney-test.*
Figure 5.5 Relative mRNA levels of all survivin variants in RB in relation to invasiveness of the RB tumors.

*P*-values were calculated by the Mann-Whitney-test.
Figure 5.6 Relative mRNA levels of Bcl-2 and Bax in RB in relation to invasiveness of the RB tumors.

Significant difference in expression \((P<0.05)\) was observed in Bax. \(P\)-values were calculated by the Mann-Whitney-test.
Figure 5.7 Relative mRNA levels of survivin variants in RB in relation to differentiation of the RB tumors.

Significant difference in expression ($P<0.05$) was observed in Survivin-2α, Survivin-2β and Survivin-ΔEx3. $P$-values were calculated by the Kruskal-Wallis-test.
Figure 5.8 Ratio of Bcl-2/Bax among differentiated RB tumors

(A) Real-time PCR analysis for the ratio of Bcl-2/Bax among different differentiated RB tumors. (B) Relative mRNA levels of Bcl-2 and Bax in RB in relation to differentiation of the RB tumors. P-values were calculated by the Kruskal-Wallis-test.
Figure 5.9 Relative mRNA levels of survivin variants in RB in relation to gender of the RB tumors.

Significant difference in expression ($P<0.05$) was observed in Survivin-WT and Survivin-3β. $P$-values were calculated by the Mann-Whitney-test.
5.5.3. Correlation among the survivin variants, Bcl-2/Bax in RB

The five survivin isoforms showed a significant positive correlation to each other in the tumor samples (Table 5.3). The mRNA expression of Survivin-WT showed a significant positive correlation (P<0.05) with the expression of Survivin-2α with a correlation coefficient of 0.740 (Table 5.4). The mRNA expression of Survivin-WT demonstrated a statistically significant positive correlation (P<0.05) with the expression of Survivin-2β with a correlation coefficient of 0.537. Similarly, the mRNA expression of Survivin-WT exhibited a statistically significant positive correlation (P<0.05) with the expression of Survivin-3β and Survivin-ΔEx3 with a correlation coefficient of 0.889 and 0.534 respectively. As shown in Table 5.3, the correlation among all the variants was significant and yielded to a strong positive correlation. Unlike, the ratios of survivin variants, the Bcl-2/Bax ratio did not yield to a statistically significant correlation. But a significant correlation

P-values were calculated by the Mann-Whitney-test.
was observed among all the survivin variants and Bax. The mRNA expression of Survivin-WT, Survivin-2α, Survivin-2β, Survivin-3β and Survivin-ΔEx3 demonstrated a statistically significant positive correlation (P<0.05) with the expression of Bax with a correlation coefficient of 0.690, 0.483, 0.668, 0.595 and 0.444 respectively. There was a significant (P<0.05) positive correlation between Survivin-2β and Bcl-2 with a correlation coefficient of 0.608.

Table 5.3 Correlation among the expression levels of survivin variants, Bcl-2 and Bax in RB tumors

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sur-WT</th>
<th>Sur-2α</th>
<th>Sur-2β</th>
<th>Sur-3β</th>
<th>Sur-ΔEx3</th>
<th>Bax</th>
<th>Bcl-2</th>
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<tbody>
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<td>0.015</td>
<td>0.001</td>
<td>0.450</td>
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<tr>
<td>Sur-2α</td>
<td></td>
<td>0.007</td>
<td>0.000</td>
<td>0.002</td>
<td>0.031</td>
<td>0.289</td>
<td></td>
</tr>
<tr>
<td>Sur-2β</td>
<td></td>
<td></td>
<td>0.024</td>
<td>0.001</td>
<td>0.001</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Sur-3β</td>
<td></td>
<td></td>
<td></td>
<td>0.001</td>
<td>0.006</td>
<td>0.164</td>
<td></td>
</tr>
<tr>
<td>Sur-ΔEx3</td>
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<td></td>
<td></td>
<td></td>
<td>0.050</td>
<td>0.102</td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.076</td>
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</tr>
</tbody>
</table>

*Table 5.3: Correlation among the expression levels of survivin variants, Bcl-2 and Bax in RB tumors (n=20). P-values were calculated by the Spearman correlation test.*
Table 5.4 Spearman correlation coefficients of variables like survivin variants, Bcl-2 and Bax in RB tumors

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sur-WT</th>
<th>Sur-2α</th>
<th>Sur-2β</th>
<th>Sur-3β</th>
<th>Sur-ΔEx3</th>
<th>Bax</th>
<th>Bcl-2</th>
</tr>
</thead>
<tbody>
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<td>Sur-WT</td>
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<td>0.537</td>
<td>0.889</td>
<td>0.534</td>
<td>0.690</td>
<td>0.179</td>
<td></td>
</tr>
<tr>
<td>Sur-2α</td>
<td></td>
<td>0.579</td>
<td>0.776</td>
<td>0.657</td>
<td>0.483</td>
<td>0.250</td>
<td></td>
</tr>
<tr>
<td>Sur-2β</td>
<td></td>
<td></td>
<td>0.504</td>
<td>0.695</td>
<td>0.668</td>
<td>0.608</td>
<td></td>
</tr>
<tr>
<td>Sur-3β</td>
<td></td>
<td></td>
<td></td>
<td>0.663</td>
<td>0.595</td>
<td>0.323</td>
<td></td>
</tr>
<tr>
<td>Sur-ΔEx3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.444</td>
<td>0.376</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.406</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.4: Spearman correlation coefficients of variables like survivin variants, Bcl-2 and Bax in RB tumors (n=20).

5.5.4. Survivin and its variants mRNA expression in Y79 cells

Y79, a retinoblastoma cell line endogenously expressed Survivin-WT and its variants at a higher level. The increased level of all the variants was similar to the RB tissue samples. Statistically significant difference (P<0.001) was observed between all survivin variants and a significance of P<0.05 was observed between Sur-WT and Sur-ΔEx3. No statistically significant difference was observed between Sur-2α and Sur-3β. Altogether, the anti-apoptotic variants like Survivin-WT, Survivin-3β and Survivin-ΔEx3 were significantly
higher in the Y79 cells when compared to the pro-apoptotic variants like Survivin-2α and Survivin-2β (Figure 5.11).

![Figure 5.11 mRNA levels of survivin splice variants in Y79 cell line](image)

**Figure 5.11** mRNA levels of survivin splice variants in Y79 cell line

Quantification of mRNA levels of Survivin-WT (Sur-WT), Survivin-2α (Sur-2α), Survivin-2β (Sur-2β), Survivin-3β (Sur-3β), Survivin-ΔEx3 (Sur-ΔEx3) mRNA expression levels in RB cell line (Y79) relative to the non-neoplastic retina by real time quantitative reverse transcriptase
PCR (qRT-PCR). Relative fold expression levels are expressed as mean ± SD of triplicate analyses. **** (P<0.001) and * (P<0.05). P-values were calculated by the Student’s t-test.

5.5.5. In vitro silencing of Survivin-WT and Survivin-ΔEx3 in Y79 cells

The Survivin-WT siRNA at a concentration of 100 nM efficiently knocked down the target transcripts at 48 h after transfection. A time related analysis determined the optimal time for gene knockdown. The downregulation of Survivin-WT at the mRNA level was significant (P<0.001) when compared to its endogenous level (Figure 5.12.A). This downregulation of Survivin-WT was also observed at the protein level by Western blotting (Figure 5.12.B & C). Similarly, the Survivin-ΔEx3 siRNA at a concentration of 100 nM efficiently knocked down the target transcript at 48 h after transfection. A time related analysis determined the optimal time for gene knockdown. The downregulation of Survivin-ΔEx3 at the mRNA level was significant (P<0.005) when compared to its endogenous levels (Figure 5.13.A). However, this downregulation was not translated at the protein level (Figure 5.13.B).
Figure 5.12 Downregulation of Survivin-WT in Y79 cells after silencing with Sur-WT siRNA

(A) A Real-Time Quantitative PCR (qRT-PCR) shows that 100 nM of Sur-WT siRNA treatment in Y79 cells showed a significant downregulation (P<0.001) compared to the control Y79 cells. (B) Immunoblot showed an effective silencing of Survivin in Y79 cells transfected with Sur-WT siRNA at 100 nM compared to the control Y79 cells. (C) Histogram analysis plotted with relative intensity determined by Image J analysis shows the silencing effect of Sur-WT siRNA in Y79 cells. P-values were calculated by the Student’s t-test.
Figure 5.13 Downregulation of survivin-ΔEx3 in Y79 cells after silencing with Sur-ΔEx3 siRNA

(A) A Real-Time Quantitative PCR (qRT-PCR) shows that 100 nM of Sur-ΔEx3 siRNA treatment in Y79 cells showed a significant downregulation ($P<0.005$) compared to the control Y79 cells. (B) Immunoblot showed no effective silencing of Sur-ΔEx3 in Y79 cells transfected with Sur-ΔEx3 siRNA at 100 nM compared to the control Y79 cells (Image shown after cropping of empty lane). Histogram analysis plotted with relative intensity determined...
by Image J analysis shows the silencing effect of Sur-ΔEx3 siRNA in Y79 cells. P-values were calculated by the Student's t-test.

5.5.6. Inhibition of Survivin-WT and Survivin-ΔEx3 leads to significant induction of cell apoptosis

The silencing effect of Survivin-WT and Survivin-ΔEx3 on cell viability was analysed by Annexin-V/PI staining after 48 h of transfection. The proportion of viable cells notably decreased in the Survivin-WT silenced cells as compared to the control cells. A higher proportion of cells were also observed in late apoptotic phase in the silenced cells than the control cells (Figure 5.14.A). However, no apoptotic effects were observed in the Survivin-ΔEx3 silenced samples. In order to determine whether the decrease in the cell viability shown by apoptosis is induced via caspases, caspase 3/7 activity was measured 48 h after Sur-WT siRNA transfection. An increased activity of caspases 3/7 was observed in the silenced cells than the control cells (Figure 5.14.B).
Figure 5.14 Annexin V-PI staining and caspase 3/7 activity in Sur-WT silenced cells

(A) Annexin V and PI staining of 100 nM of Survivin-WT siRNA transfected Y79 cells. 48 h following transfection, Y79 cells were stained with Annexin V/PI and analyzed by flow cytometry. The difference in the percentage of viable and apoptotic cells between the control Y79 cells and Sur-WT siRNA treated cells is shown in a histogram. (B) Y79 cells were transfected with Survivin-WT siRNA at 100nM concentration; 48 h later, caspase-3/7 activity was examined and normalized to number of viable cells. Fold induction is indicated compared to the control cells. Data are shown as the percentage of total caspase-3 activation. Experiments were performed in duplicates and values are shown with ± Standard Deviation.
5.5.7. Effects of Survivin-WT silencing on the splice variants

The silencing of the Survivin-WT led to effective knockdown of mRNA expression of all the splice variants. Although there was a significant downregulation (P<0.05) of all the variants (Figure 5.15) on silencing the Survivin-WT alone, a higher downregulation was observed in both the pro-apoptotic variants, Survivin-2β and survivin-2α.

![Figure 5.15 mRNA levels of survivin splice variants on silencing Survivin-WT](image)

Figure 5.15 mRNA levels of survivin splice variants on silencing Survivin-WT

A Real-Time Quantitative PCR (qRT-PCR) of Survivin-WT silenced Y79 cells shows a significant downregulation of all survivin variants compared to the control Y79 cells.* P<0.05 and *** P<0.005. P-values were calculated by the Student’s t-test.

5.6. DISCUSSION

Biallelic RB1 inactivation leads to development of retinal tumor and unlike other malignancies, RB is strongly predisposed to germline RB1 mutations (Gombos 2012). In a recent study, it was revealed that cone precursors are sensitive to Rb protein exhaustion. It
was shown that these Rb depleted cone precursors proliferated in intact retina and formed tumors in orthotopic xenografts which was similar to human RB at histological and molecular cytogenetic levels. Thus, the above study established a link between loss of Rb protein and cone precursor specific circuitries (Xu et al. 2014). In RB, apoptotic paradox is a phenomenon where percentage of apoptotic cells are higher than the dividing cells but the unique feature is that these abundant apoptotic cells does not prevent the tumor from growing. According to an earlier study, there was an association between apoptotic index and mitotic index in RB revealing a close correlation between cell proliferation and apoptosis. However, the tumor favoured proliferation because of net growth. It was also found from a small cohort of RB tumors that there are no inclination towards a single apoptotic pathway (Sitorus, Gumay & Van Der Valk 2009). Apoptosis is regulated by a network of molecules, involved either in promoting or inhibiting apoptosis. IAP and Bcl-2 family has been studied extensively in various cancers for their role in tumorigenesis and disease progression (Elmore 2007).

This study shows for the first time that the mRNA levels of endogenous expression of survivin splice variants, Bcl-2 and Bax in RB tumors and its correlation with the aggressiveness of tumors. Expression of the apoptosis related molecules in RB tumors from patients as well as in established standard RB cell line (Y79) was determined. The expression of two apoptosis regulating proteins from the Bcl-2 family, Bcl-2 and Bax in RB was analysed as several studies suggest that Bax is a pro-apoptotic molecule while Bcl-2 is an anti-apoptotic molecule and higher Bcl-2/Bax ratio leads to inhibition of apoptosis in cancer. One study have shown that overexpression of Bcl-2, an antiapoptotic protein in endometrium signals the initiation of cancer cell overgrowth (Mitselou et al. 2002). In cancers like colorectal cancer, head and neck cancer, Bcl-2 family members have been shown to possess prognostic potential (Hector & Prehn 2009). On the other hand, overexpression of Bax, a pro-
apoptotic protein stimulates apoptosis of cancer cells leading to arrest of progression of cancer. Bcl-2 and Bax coexist as a heterodimer in vivo and the balance between the two determines whether inhibition or induction of apoptosis has to occur (Pietrantonio et al. 2013). Apoptotic inhibition occurs in the cell when the Bcl-2/Bax ratio is tilted towards Bcl-2 and apoptotic induction prevails in the cell when the ratio of Bcl-2/Bax is tilted towards Bax (Sakuragi et al. 2002). In order to determine the status of this ratio in RB, expression of these two proteins were studied by real-time PCR. The expression level of Bcl-2 was significantly higher in RB compared to normal retina and it was also remarkably higher compared to Bax. When Bcl-2/Bax ratio was determined, it was found to be more than 1 favouring Bcl-2 dominance in RB. We next analysed the Bcl-2/Bax ratio with respect to differentiation of tumors and found that Bcl-2/Bax ratio to be remarkably higher in poorly differentiated tumors. However this difference did not yield to a significant difference when statistical analysis was done among the differentiated tumors. This may be due to the limitation in the sample size analysed for Real-Time PCR (Porichi et al. 2009). Also, in moderately differentiated tumors compared to well differentiated tumors, a decrease in Bcl-2/Bax ratio was observed. However, such difference in expression level either for Bax or for Bcl-2 was not observed between males and females, thus implying the fact that Bcl-2/Bax expression in RB is independent of gender of the tumors.

Since, Bcl-2 was higher in both invasive and non-invasive tumors, the statistical analysis did not yield a statistically significant difference between invasive and non-invasive tumors. Bcl-2 association with invasive tumors have been earlier studied in invasive bladder cancers where it is also related to reduced survival in patients (Kong et al. 1998). Another study in breast cancer showed Bcl-2 to possess prognostic value because of its high expression in invasive tumors (Yang et al. 2003). Surprisingly, in nasopharyngeal carcinoma, Bcl-2 positivity showed a better outcome clinically (Vera-Sempere et al. 1997). In the case of Bax,
though its mRNA expression level was lower compared to Bcl-2, its expression was significantly higher in invasive tumors compared to non-invasive tumors. Higher expression of Bax signifies higher apoptotic activity followed by apoptosis of tumor cells. However, in RB this study shows that higher anti-apoptotic activity of Bcl-2 nullifies this apoptotic activity of Bax and only anti-apoptotic activity of Bcl-2 appears to prevail. Studies in colorectal cancer showed that Bax expression gradually decreased from well differentiated tumors to poorly differentiated tumors pointing an association of Bax to tumor differentiation (Jansson & Sun 2002). Studies related to Bax correlation with clinical parameters such as reduced expression in advanced stage tumors were shown in nasopharyngeal tumors (Kontos et al. 2013). In the case of nephroblastoma, a round cell tumor and a pediatric malignancy of the kidney, alterations of the Bcl-2/Bax balance deregulates apoptosis and heavily influences the clinical outcome of the nephroblastoma patients (Ghanem et al. 2001). Surprisingly, in RB tumors, Bcl-2 and Bax did not show any significant correlation with each other in terms of expression level. The logical reasons behind difference in findings with other studies could be due to the varied nature of the tumors and also different methods used to analyse the expression. Thus, we could suggest that this strong overexpression of anti-apoptotic protein, Bcl-2, may play a role in the carcinogenesis of RB.

Survivin is considered to be one of the primary cancer associated gene because of its differential expression in normal as well as cancer cells, its role in cell division and inhibition of apoptosis (Altieri 2003b; Kanwar, Kamalapuram & Kanwar 2011). Therefore, the expression of members of survivin family in RB tumors was analysed. Survivin was highly expressed in RB tumor tissues than in normal tissues. Expression of survivin at protein level in RB has already been shown by a study from our group, which revealed that 86% of tumors expressed survivin and its expression is associated with advanced stages of RB (Sudhakar et al. 2013). In concurrent with the previous study from our lab, the current study also observed
90% of Survivin-WT positivity at mRNA level. The current study also observed an increased expression of survivin and its variants in poorly differentiated tumors which are associated to high risk factors (Kashyap et al. 2012) in the RB tumors analysed.

RB belong to a group of tumors called malignant round cell tumor (MRCT), which is a group composed of round, small undifferentiated tumors (Rajwanshi, Srinivas & Upasana 2009). IAPs like survivin has been shown to play a major role in some of the pediatric MRCT’s. In neuroblastoma, a pediatric extracranial cancer, showed high survivin expression which correlated with poor prognosis and advanced tumor stages (Altieri 2001). Hepatoblastoma, a pediatric liver neoplasm also overexpresses survivin and involved in the survival of the tumor cells (Uehara et al. 2013) whereas in non-Hodgkin lymphoma, survivin expression is closely associated to advanced stages and prognosis (Li & Wu 2006). In Wilm’s tumor, a pediatric kidney cancer, survivin is involved in tumorigenesis and its increased expression in the nucleus is considered to be favourable for prognosis of the disease (Basta-Jovanovic et al. 2011). But in another round cell tumor like Ewing sarcoma, a pediatric bone or soft tissue cancer, although survivin is over expressed in tumors and plays a key role in viability, it serves as a poor prognostic marker (Hingorani et al. 2013).

When compared to the normal retina, survivin and its isoforms were expressed at a higher percentage in the RB tumors. This suggests that survivin along with its alternatively spliced variants has an important role in tumorigenesis of RB and corroborates the earlier widely acknowledged findings about cancer specific overexpression of survivin. The predominant form was Survivin-WT followed by Survivin-3β and Survivin-ΔEx3. Among the pro-apoptotic variants, the dominant form was Survivin-2α and the least expressed was Survivin-2β, although its expression level was 3.7 fold higher than the normal retina. Given the higher expression of all the anti-apoptotic variants of survivin over the pro-apoptotic variants of survivin in RB, it could be inferred that these anti-apoptotic molecules drive the progression
of RB tumors. The increased expression of anti-apoptotic Survivin-3β in breast cancer was associated with tumor development and correlated inversely to pro-apoptotic genes (Végran et al. 2011). Similarly, in this study, Survivin-3β was observed to be highly expressed and could be suggested to have a role in tumor progression. Since in the current study, all the 20 tumors are of high grade (Grade D or E), there is an agreement between this study and earlier studies on breast cancer samples where it was shown that high mRNA concentrations of the anti-apoptotic molecules like Survivin-WT and Survivin-ΔEx3 are associated to high grade phenotype (Span et al. 2006).

In this study, not all the variants of survivin were found to be associated to all the clinical parameters of the tumors analysed. Only Survivin-2α, Survivin-2β and Survivin-ΔEx3 showed correlation in the differentiation of the tumors. The Survivin-2α was found to be significantly decreased in poorly differentiated tumors when compared to moderately differentiated tumors based on the mean rank. The decrease in Survivin-2α, a pro-apoptotic molecule might reduce the strength of its pro-apoptotic activity in poorly differentiated tumors, giving rise to the generation of potent anti-apoptotic variants as the differentiation level of RB tumors deteriorates. Similar to Survivin-2α gene expression downregulation in poorly differentiated tumors, another main pro-apoptotic variant, Survivin-2β was also found to be significantly downregulated in poorly differentiated tumors based on the mean rank. The decline in the expression of two pro-apoptotic molecules in such advanced poorly differentiated tumors may thus result in progression and development of RB. The decreased mRNA expression of these pro-apoptotic variants might be related to an early event in the onset of RB. Such downregulation of Survivin-2β in advanced clinical stages of cancer has also been observed in gastric carcinoma and renal cell carcinoma (Mahotka et al. 2002a; Meng et al. 2004). Survivin-ΔEx3 has been shown to possess anti-apoptotic property and it was found to be highly detectable in chronic or advanced cancer samples (Nakagawa et al. 2002b).
2004). The anti-apoptotic property of Survivin-ΔEx3 is counteracted by the antagonist property of Survivin-2β, thus the ratio of both these variants in cancer cells determines the progression of cancer. The ratio of Survivin-ΔEx3/Survivin-2β in RB was higher towards the anti-apoptotic molecule. Since Survivin-2β was decreased in poorly differentiated tumors, Survivin-ΔEx3 was expected to be increased in the poorly differentiated tumors. Surprisingly, Survivin-ΔEx3 did not show a statistically significant increase in poorly differentiated tumors though it showed a significant increase in the expression in moderately differentiated tumors, compared to well differentiated tumors based on mean rank. None of the other survivin variants showed any significant association with the differentiation of the tumors. Among the gender parameter, Survivin-WT and Survivin-3β showed statistically significant increase (P<0.05) in expression in males compared to females. Employing more clinical samples and analysing with respect to gender might throw light on the actual effect of gender on the expression of survivin splice variants.

Another important clinical parameter that was considered for the expression study was invasiveness of the RB tumors. In concurrence with the earlier study in RB from our laboratory, this study also did not show any significant association with the invasion status of the tumors not only for Survivin-WT but also for all the variants. Correlation among the five variants was positive and highly significant which implies the fact that survivin family members are closely related to each other and work in tandem. The correlation of three of the alternative splice variants of survivin with the clinical parameter like differentiation of the tumor shows an active involvement of this hot cancer target in the development of RB.

The current investigation on survivin and its variants in RB tumors showed that Survivin-WT was the predominant form expressed although its variants are also highly expressed. The same mRNA analysis was also performed in RB cell line, Y79, for Survivin-WT and all of its variants. Similar to tumor tissues, the survivin and all its variants were highly expressed in
Y79 cells. Among the variants, the anti-apoptotic proteins were evidently higher than the pro-apoptotic proteins in the Y79 cells. Thus the results obtained from the RB cell line is concurrent with the results obtained from the RB tumors (Sudhakar et al. 2013). So, the Y79 cell line mimics the tumor environment and further experiments were carried out with this cell line. Apart from Survivin-WT, the most important variant is anti-apoptotic variant, Survivin-ΔEx3 because of its role in proliferation and apoptosis (Altieri 2003a). Thus along with Survivin-WT, targeting of Survivin-ΔEx3 would be interesting from the point of therapeutic intervention. Since Survivin-WT along with Survivin-ΔEx3 was overexpressed in both the clinical samples as well as in RB cell line, hence the study was focused on targeting Survivin-WT and Survivin-ΔEx3 via RNAi mediated inhibition in RB.

For siRNA transfection experiments, siRNA targeting Survivin-WT was used at 100nM after efficient inhibition at 48 h was observed at this particular concentration. Significant downregulation of Survivin-WT was observed both at mRNA level as well as at protein level. This effective mRNA reduction and protein reduction resulted in remarkable pro-apoptotic effects in the silenced samples. In various cancer cells, it has been studied that on silencing survivin, the caspase-3 and caspase-7 gets activated (Shin et al. 2001). This activated caspase-3/7 functions by degrading the key intracellular substrates within the cell thus leading to apoptosis (Xu & Shi 2007). Also, survivin is known to inhibit caspase-3 and caspase-7, so in this study, it is shown that the apoptosis in the survivin silenced samples was associated with increased level of caspase-3/7 activity which are the executioner/mediator of apoptosis. The results obtained are concurrent with the previous study in bladder cancer where the inhibition of Survivin-WT exhibited similar increase in apoptosis (Wuttig et al. 2007). Apoptosis induction on effective inhibition of Survivin-WT was observed not only in bladder cancer, but also in other cancers like colon and breast cancers (Li et al. 2005; Williams et al. 2003).
For siRNA transfection experiments with Survivin-ΔEx3, the siRNA showed significant downregulation at 100nM after 48 h incubation period. Though the downregulation of Survivin-ΔEx3 was observed at the mRNA level, a decrease in the corresponding protein was not observed by Western blotting using specific antibody against Survivin-ΔEx3. Such observation was seen in a similar study in bladder cancer where the Survivin-ΔEx3 inhibition did not result in protein reduction (Wuttig et al. 2007). The possible reasons for such a result is that Survivin-ΔEx3 inhibition at mRNA level does not produce noticeable difference at protein level and for such a noticeable inhibition, it may require more time than the 48 h of transfection. In addition, regulatory mechanism of alternative splice variants tends to bring the expression of other variants when one of the variants is inhibited (Lai & McCobb 2006). The inhibition of Survivin-ΔEx3 did not also induce noticeable apoptosis in the silenced samples. But a recent in vitro study in breast cancer on the inhibition of Survivin-ΔEx3 by plasmid expressing antisense Survivin-ΔEx3 showed significant anti-proliferative effects (Zheng et al. 2011) indicating the survivin’s role in cell division. Thus the results in the current study for Survivin-ΔEx3 inhibition in Y79 cells, confirms that inhibition of one of the variants alone does not lead to effective increased response in apoptotic induction, at least when it was silenced via the siRNA.

In order to check whether the variants were silenced when Survivin-WT was silenced, a qRT-PCR was performed on the Survivin-WT silenced sample and it was observed that all the variants were significantly downregulated. This downregulation of gene expression was observed because the Survivin-WT siRNA used in this study targets a region in exon 2 which is common to all the variants. This complete downregulation of all variants was effective in inducing apoptosis. In concurrence with the previous published work (Wuttig et al. 2007), the current study results also showed that inhibition of one of the variants alone did not yield to desired results of apoptosis induction in RB. Nevertheless, inhibition of Survivin-WT
silenced all the alternative splice variants resulting in a desired pro-apoptotic effect in RB cells.

Correlation between survivin and Bcl-2 has been studied in various cancers and positive correlation between the two has been observed in invasive ductal carcinoma of breast (Al-Joudi, Iskandar & Imran 2007) in and non-small cell lung cancer (Han et al. 2009). However, in this study, Bcl-2 showed no such significant positive correlation with any of the survivin variants except Survivin-2β. Survivin-2β’s pro-apoptotic activity may be masked/suppressed by the increased anti-apoptotic activity of other survivin variants and Bcl-2. Such non-correlation between survivin and Bcl-2 was also observed in various other studies like prostatic adenocarcinoma (Kaur et al. 2004), urothelial carcinoma (Jeong et al. 2009) and cervical carcinoma (Zhu et al. 2010). However, the correlation between survivin variants and Bax was positive and highly significant. This correlation implies that there could possibly be interplay between the variants and Bcl-2/Bax and the dominant player emerges and exerts its activity for the progression of RB tumor. Since Bcl-2 is highly expressed in RB than Bax, Bcl-2 exerts its activity by maintaining the integrity of the mitochondrial membrane and thus preventing the release of cytochrome c for the apoptosis to take place via Bax (Kirkin, Joos & Zörnig 2004). On the other hand, highly expressed survivin and its anti-apoptotic variants exert their anti-apoptotic function in the downstream pathways of Bax by targeting caspase-3 (Tamm et al. 1998). In addition, survivin was shown to suppress the cell death induced by Bax (Tamm et al. 1998). Thus, the upregulation of Bcl-2 and anti-apoptotic variants of survivin in RB can suppress even the upregulated activity of Bax and thereby may partly promote the aggressiveness of RB. Therefore, considering the high expression of Bcl-2 and survivin in RB, a targeted therapy towards these molecules could be promising in the near future. By targeting Survivin-WT and Survivin-ΔEx3 at in vitro level in RB, the current study demonstrates that targeting induces apoptosis and that this is a viable option.
The limitation of the current study is that the expression of survivin splice variants, Bcl-2 and Bax in RB tumors were studied only at the mRNA level and not at the protein level. The major impediment was the non-availability of antibodies for all the splice variants of the survivin. Thus the future studies needs to explore whether the mRNA level expression of the survivin splice variants, Bcl-2 and Bax investigated in this study translates into protein level. Nevertheless, the current study with its modest cohort size with respect to tumor samples was therefore able to produce statistically significant correlation between the expression of apoptotic genes and few of the clinical parameters of the tumors. Future studies focussing on the precise role of each survivin variant and each member of Bcl-2 family in the recurrence and patient survival in a larger cohort of RB tumors are necessary for a conclusive report.
CONCLUSION AND FUTURE PERSPECTIVES

CONCLUSION

Therapy for cancer has eluded medical and scientific community for centuries. Many newer and complicated therapies have been introduced but only few established. One of the reasons for many failed attempts at achieving cure for cancer is lack of targeted treatments and also unwarranted side effects. Treatments for breast cancer and RB have such similar problems and this needs to be addressed. RB, though a rare form of pediatric cancer is highly fatal if left untreated. Our laboratory has studied various therapeutic targets for RB which has potential in targeting cancerous cells and arresting its growth. In line with those findings, Sodium Iodide Symporter (NIS) expression at protein level was explored in RB. Although basal level NIS is present in breast cancer, the possibility of EpCAM directed NIS overexpression was explored. Apart from studying NIS expression in RB, important apoptosis related genes such as survivin splice variants, Bcl-2 and Bax were also studied.

Main conclusions from this study are:

1) NIS protein is expressed at a higher percentage in all 41/41 tumors (100%) analysed and the expression is associated to invasiveness of the tumors.

2) Overexpression of NIS gene in breast cancer cells via EpCAM specific promoter showed higher iodide uptake than the CMV promoter.

3) PEI/NIS pDNA/EpDT3 nanocomplex delivered NIS pDNA specifically to EpCAM positive cells and retained the functional activity of the NIS plasmid.

4) Apoptotic regulatory genes such as Survivin-WT, Survivin-2α, Survivin-2β, Survivin-3β, Survivin-ΔEx3, Bcl-2 and Bax are expressed at a higher fold in RB when compared to normal retina. The expression of proapoptotic genes Bax, Survivin-2α
and Survivin-2β showed correlation to histopathological features of RB which means that it may play a role in tumorigenesis if not masked by the strong anti-apoptotic effects of the antiapoptotic genes.

5) Silencing of Survivin-WT in Y79 cells silenced all the splice variants and also induced apoptosis.

More detailed inferences from each study are summarized below:

**NIS expression in primary RB tumor**

In chapter 3, the NIS protein expression in primary RB tumors and RB cell line has been studied. This is the first study to explore NIS expression in a non-thyroidal tumor like RB. Immunohistochemically, NIS expression was found to be cytoplasmic and membranous which is consistent with results reported in other non-thyroidal cancers. NIS, being a membrane protein when expressed in cytoplasm takes up a different role other than iodide uptake. Cytoplasmic NIS is involved in cell migration and invasion and thus its expression in RB tumors could be associated to invasive quality of the tumor. Confirming the IHC findings, flow cytometric analysis showed significant association between increased expression of NIS protein and invasiveness of the tumors. One of the post-translational modifications of NIS protein is glycosylation and the NIS protein was found to be fully, partially and under glycosylated in RB tumors. Since recent studies have shown that glycosylation doesn’t affect the functionality of the NIS protein, the expressed NIS could be functionally active. Findings obtained in RB tumor were concurrent with RB cell line confirming the NIS expression. Thus, this study explored the expression of NIS in primary RB tumors and proved the proposed hypothesis that when NIS is expressed in RB, it is related to the histopathological high risk factors of the tumor. In the context of NIS’s role in RB, it can be considered as a
molecule capable of enhancing diagnosis. In the case of therapy, when combined with EBRT and drugs that lead to radiosensitivity, NIS can be a potent tool in RB.

**EpCAM targeted NIS overexpression in breast cancer**

Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein strongly expressed in breast cancer supporting the disease progression. NIS being a therapeutic gene though endogenously expressed in breast cancer is insufficient for radiotherapy. In chapter 4, NIS is specifically overexpressed in breast cancer cells via two ways by targeting overexpressed EpCAM protein. Cloning of EpCAM specific promoter driven NIS gene was successful and stable transfectants in MCF-7 was established. Although the universal (CMV) promoter induced higher NIS mRNA and protein expression, the NIS functional activity was remarkably higher in stable MCF-EpCAM-NIS transfectant than the stable MCF-CMV-NIS transfectant. Thus the superior promoter activity of EpCAM over CMV was established in this study. Similarly, NIS pDNA was delivered in a nanoformulation by targeting EpCAM via EpCAM aptamer. The nanocomplex consisted of PEI the polymer, NIS the plasmid DNA and EpDT3 the EpCAM aptamer. The synthesized nanocomplex (PEI/NISpDNA/EpDT3) were smaller in size (30-40nm) and spherical in shape with high serum stability. The nanocomplex successfully delivered NIS pDNA which was analysed by NIS expression at mRNA and protein level. The PEI/NISpDNA/EpDT3 delivered NIS gene showed significantly higher iodide uptake. This ability to specifically deliver the NIS pDNA to EpCAM positive cells by retaining the complete functional activity proves the potential of the nanocomplex. The nanocomplex coated with scrambled aptamer could not deliver NIS pDNA in a targeted manner and therefore no iodide uptake activity was observed in EpCAM positive cells. In conclusion, this study proved the proposed hypothesis that targeting EpCAM would indeed lead to specific overexpression of NIS gene in EpCAM positive breast
Conclusions and Future Perspectives

cancer cells. Thus, in the context of NIS and EpCAM in breast cancer, a combination of therapeutic molecule, NIS and an ideal targeting molecule like EpCAM is bound to strengthen the breast cancer treatment.

Apoptotic genes expression in RB

Apoptosis in other words programmed cell death is a natural process in a healthy cell which occurs to balance the cell division. The status of the endogenous apoptotic regulatory molecules in RB has never been explored. In chapter 5, the study shows for the first time the endogenous mRNA expression of Bcl-2, Bax, Survivin-WT, Survivin-2α, Survivin-2β, survivin-3β and Survivin-ΔEx3 in 20 RB tumor cohorts. The expression studies of Bcl-2 and Bax mRNA showed anti-apoptotic molecule Bcl-2 to be significantly higher than Bax. Though Bax expression is also higher in invasive tumors compared to normal retina, Bcl-2 appears to be the dominant player masking the pro-apoptotic activity of Bax.

Real time PCR studies on RB tumors revealed a higher expression of wild type survivin and all survivin splice variants. However, the predominant form was Survivin-WT. Overall, the anti-apoptotic variants were highly expressed as compared to the pro-apoptotic variants. This tilt towards anti-apoptotic variants enhances the progression of RB. Individually, Survivin-2α expression was lower in poorly differentiated tumors and this could enhance the anti-apoptotic variants. Survivin-2β also showed similar expression patterns to Survivin-2α. The main variant of all is Survivin-ΔEx3 because of its strong anti-apoptotic activity. The expression of Survivin-ΔEx3 was interesting mainly because of the ratio of Survivin-ΔEx3/Survivin-2β which is tilted towards Survivin-ΔEx3. This conforms that Survivin-ΔEx3 is dominant; however its mRNA expression in poorly differentiated did not turn out to be significant. Since Survivin-WT and Survivin-ΔEx3 expressed significantly high in tumors, these molecules were silenced in RB cell line which mimics the RB tumor. Silencing the
Survivin-ΔEx3 alone did not yield to any apoptotic effects. However silencing Survivin-WT lead not only to downregulation of all the variants but also induction of apoptosis. This confirms the role of survivin in inhibiting apoptosis in RB. In conclusion, this study proved the proposed hypothesis that all the survivin and its splice variants, Bax and Bcl-2 genes could be related to tumor progression of RB and that survivin silencing could lead to induction of apoptosis. Thus, the above molecules’s expression in RB not only shows its role in tumor progression but also helps in elucidating the apoptotic pathway involved in RB.

**FUTURE PERSPECTIVES**

1) Since NIS protein expression is regulated differently in different tissues, in future studies, it can be seen how NIS is regulated in RB tumor cells.

2) It would be interesting to study the impact of glycosylation on the functionality of NIS protein expressed in RB.

3) In future, exploitation of NIS in diagnosis, treatment and monitoring of RB patients can be studied by employing more number of patients using RB tumors with different grading.

4) Interestingly, bLf receptors were found to be expressed in Y79 cell line and bLf was rapidly internalized into these cells. bLf as a transcriptional activator upregulated the endogenous NIS expression. Future studies focussing on further enhancing this expression and standardising techniques for functional assay for suspension cells could be beneficial.

5) Since survivin and Bcl-2 is highly expressed in RB, in future it can be studied whether bLf targets survivin and Bcl-2 in RB.

6) The future study needs to explore whether the mRNA level expression of the survivin splice variants, Bcl-2 and Bax investigated in RB tumors translates into protein level.
7) Future studies can also focus on finding out the precise role of each survivin variant and each member of Bcl-2 family in the recurrence and patient survival in a larger cohort of RB tumors.

8) A combinatorial nano formulation targeting of anti-apoptotic molecules of survivin variants and Bcl-2 may be a promising therapy for RB.

9) The PEI/NISpDNA/EpDT3 nanocomplex study was performed in *in vitro*, in future it can be considered for *in vivo* studies in breast cancer models which would validate at pre-clinical levels the benefit for cancer treatment by avoiding the undesirable effects of therapy to surrounding normal tissues.
## APPENDIX

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Chemical</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1</strong></td>
<td>0.1% HCl</td>
<td>- 1mL of concentrated Hydrochloric acid&lt;br&gt;- 999mL of milliQ</td>
</tr>
<tr>
<td><strong>2</strong></td>
<td>4% (APES)</td>
<td>- 4mL of aminopropyl ethoxy silane&lt;br&gt;- 96mL of milliQ</td>
</tr>
<tr>
<td><strong>3</strong></td>
<td>10mM citrate buffer, pH 6.0</td>
<td>- 0.1M trisodium citrate-41mL&lt;br&gt;- 0.1M citric acid-9mL&lt;br&gt;- Adjust the pH to 6.0 with conc.HCl and Made upto 500mL with milliQ</td>
</tr>
<tr>
<td><strong>4</strong></td>
<td>1X Tris Buffered Saline (TBS), pH 7.6</td>
<td>- 1.517gm of Tris buffer&lt;br&gt;- 2.175gm of Sodium Chloride&lt;br&gt;- Adjust the pH to 7.6 and Made upto 250mL with milliQ</td>
</tr>
<tr>
<td><strong>5</strong></td>
<td>FACS buffer</td>
<td>- 1X phosphate buffered saline&lt;br&gt;- 10% fetal bovine serum&lt;br&gt;- 1% sodium azide</td>
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<tr>
<td><strong>6</strong></td>
<td>RadioImmunoPrecipitation assay (RIPA) lysis buffer</td>
<td>- 50 mM of Tris HCl (pH 7.6)&lt;br&gt;- 1% NP40&lt;br&gt;- 0.28% of Deoxycholate&lt;br&gt;- 0.1% SDS&lt;br&gt;- 1 mM of EDTA&lt;br&gt;- 150 mM of sodium chloride&lt;br&gt;- 1% PMSF&lt;br&gt;- 250 μL of 1 mg/mL Protease Inhibitor Cocktail</td>
</tr>
<tr>
<td><strong>7</strong></td>
<td>1X Tris buffered saline-Tween 20 (1XTBS-T)</td>
<td>- 1.517gm of Tris buffer&lt;br&gt;- 2.175 gm of Sodium Chloride&lt;br&gt;- 0.1% tween-20&lt;br&gt;- Made upto 250mL with milliQ</td>
</tr>
<tr>
<td>Appendix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>8</strong> 10X Running buffer</td>
<td>- 144gm of Glycine</td>
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</tr>
<tr>
<td></td>
<td>- 30gm of Tris buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 10gm of Sodium Dodecyl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sulphate (SDS)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Made upto 1000mL</td>
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</tr>
<tr>
<td><strong>9</strong> 10X Transfer buffer</td>
<td>- 144gm of Glycine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 30gm of Tris buffer</td>
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<tr>
<td></td>
<td>- 20% of Methanol</td>
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<td></td>
<td>- Made upto 1000mL</td>
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</tr>
<tr>
<td><strong>10</strong> Sample loading buffer</td>
<td>- 2.0 mL 1M Tris-HCl, pH 6.8</td>
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</tr>
<tr>
<td>(4X, 10mL)</td>
<td>- 0.8 g SDS</td>
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<tr>
<td></td>
<td>- 4.0 mL 100% glycerol</td>
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<tr>
<td></td>
<td>- 0.4 mL 14.7 M β-mercaptoethanol</td>
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<td></td>
<td>- 1.0 mL 0.5 M EDTA</td>
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</tr>
<tr>
<td></td>
<td>- 8.0 mg bromophenol blue</td>
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<td></td>
<td>- 2.6 mL H2O</td>
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<td><strong>11</strong> 30% acrylamide (100mL)</td>
<td>- 29gm of acrylamide</td>
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</tr>
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<td></td>
<td>- 1gm of bisacrylamide</td>
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<td></td>
<td>- Made upto 100mL with milliQ</td>
<td></td>
</tr>
<tr>
<td><strong>12</strong> 1.5M Tris, pH 8.8 (250mL)</td>
<td>- 45.43gm of Tris base</td>
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<td>- Adjust the pH to 8.8 and Madeupto 250mL</td>
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</tr>
<tr>
<td><strong>13</strong> 1M Tris, pH 6.8 (50mL)</td>
<td>- 6.06gm of Tris base</td>
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<td></td>
<td>- Adjust the pH to 6.8 and Madeupto 50mL</td>
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<tr>
<td><strong>14</strong> 10% SDS (50mL)</td>
<td>- 5gm of Sodium Dodecyl Sulphate</td>
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<tr>
<td></td>
<td>- Made upto 50mL</td>
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</tr>
<tr>
<td><strong>15</strong> 5% skimmed milk (50mL)</td>
<td>- 2.5 gm of skimmed milk</td>
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<td></td>
<td>- Made upto 50mL with 1XTBS-T</td>
<td></td>
</tr>
<tr>
<td><strong>16</strong> Stripping buffer</td>
<td>- 100mM Glycine, pH 2</td>
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<td><strong>17</strong> 0.1M CaCl₂ Solution</td>
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<td></td>
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<td>---</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------</td>
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<tr>
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<td>1X TE Buffer, pH 8.0</td>
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<tr>
<td></td>
<td></td>
<td>- 1mM EDTA</td>
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<tr>
<td>19</td>
<td>Incubation buffer for RAIU assay</td>
<td>- 4gm NaCl</td>
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<tr>
<td></td>
<td></td>
<td>- 0.1gm KCl</td>
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<td></td>
<td></td>
<td>- 0.72gm Na₂HPO₄</td>
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<tr>
<td></td>
<td></td>
<td>- 0.12gm KH₂PO₄</td>
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<tr>
<td></td>
<td></td>
<td>- 0.5gm Bovine Serum Albumin</td>
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<tr>
<td></td>
<td></td>
<td>- Made upto 500mL with milliQ, pH-7.4</td>
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<td>20</td>
<td>Lysis Buffer for RAIU assay</td>
<td>- 0.1M NaOH</td>
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<tr>
<td></td>
<td></td>
<td>- 1% SDS</td>
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<tr>
<td>21</td>
<td>Uptake Buffer</td>
<td>- HBSS</td>
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<td></td>
<td>- Hepes (10mM final)</td>
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<td>Cerium IV Sulphate, 42mM</td>
<td>- 1.25 gm of Ammonium cerium (IV) sulphate hydrate</td>
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<td>- 20mL of MilliQ</td>
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<td></td>
<td></td>
<td>- 5mL of Conc.H₂SO₄</td>
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<td></td>
<td></td>
<td>- Made upto 50mL with MilliQ</td>
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<td>23</td>
<td>Sodium Arsenite (III) solution, 96mM</td>
<td>- 475mg of Arsenic III oxide</td>
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<td>- 2.4gm of Sodium Chloride</td>
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<td>- 5mL of 2M Sodium Hydroxide</td>
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<td></td>
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<td>- Made upto 50mL with MilliQ</td>
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<td>24</td>
<td>Buffer P1</td>
<td>- 50 mM Tris-HCl pH 8.0</td>
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<td></td>
<td></td>
<td>- 10 mM EDTA</td>
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<tr>
<td></td>
<td></td>
<td>- 100 μg/mL RNaseA</td>
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<td>25</td>
<td>Buffer P2</td>
<td>- 200 mM NaOH</td>
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<td></td>
<td></td>
<td>- 1% SDS</td>
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<td>26</td>
<td>Buffer N3</td>
<td>- 4 M guanidine hydrochloride</td>
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<td></td>
<td></td>
<td>- 0.5M Potassium acetate, pH 4.2</td>
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<td>27</td>
<td>Buffer PB</td>
<td>- 5 M Gu-HCl</td>
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<tr>
<td></td>
<td></td>
<td>- 30% isopropanol</td>
</tr>
<tr>
<td>28</td>
<td>Buffer PE</td>
<td>- 10 mM Tris-HCl pH 7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- 80% ethanol</td>
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<td>29</td>
<td>Buffer EB</td>
<td>- 10 mM Tris-HCl pH 8.5</td>
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<tr>
<td>30</td>
<td>Buffer QBT</td>
<td>- 750 mM NaCl</td>
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<td>Buffer QC</td>
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</table>
| 31 | 1.0 M NaCl | - 50 mM MOPS, pH 7.0  
- 15% isopropanol  
- 0.15% Triton-X 100 |

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<th>Buffer QF</th>
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</table>
| 32 | 1.25 M NaCl | - 50 mM Tris-HCl, pH 8.5  
- 15% isopropanol |

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<th>Buffer QG</th>
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### List of Instruments

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<td>SDS-PAGE setup</td>
<td>Powerpac Hc</td>
<td>Bio-Rad</td>
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<td>Biosafety Cabinet</td>
<td>Safemate 1.2</td>
<td>Laftechnologies</td>
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<td>5% CO₂ incubator</td>
<td>Heracell 150i</td>
<td>Thermo Scientific</td>
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<tr>
<td>4</td>
<td>Chemidoc XRS</td>
<td>XRS</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>5</td>
<td>Gel electrophoresis setup</td>
<td>Major Science</td>
<td>MP 300N</td>
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<td>6</td>
<td>pH meter</td>
<td>-</td>
<td>Labchem pH</td>
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<td>7</td>
<td>Confocal Microscope</td>
<td>LASaf</td>
<td>Leica</td>
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<td>8</td>
<td>Inverted Microscope</td>
<td>-</td>
<td>Prism Optical</td>
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<td>Sonicator</td>
<td>Vibra-cell</td>
<td>Sonics</td>
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<td>Microplate Reader</td>
<td>SH-1000</td>
<td>Corona electric</td>
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<td>11</td>
<td>Fluorescent Reader</td>
<td>MTO-601F</td>
<td>Corona electric</td>
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<td>12</td>
<td>Flow cytometer</td>
<td>CantoII</td>
<td>BD biosciences</td>
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<td>Centrifuge</td>
<td>5804</td>
<td>Eppendorf</td>
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<td>14</td>
<td>Protein transfer system</td>
<td>Trans-blot turbo</td>
<td>Bio-Rad</td>
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<tr>
<td>15</td>
<td>Real Time PCR</td>
<td>AB 2500</td>
<td>Applied Biosystems</td>
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<td>16</td>
<td>Sample Heater</td>
<td>HB-2</td>
<td>Wealtec Corp</td>
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<td>17</td>
<td>Dynamic light scattering</td>
<td>Nano-2S</td>
<td>Malvern Instruments</td>
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<tr>
<td>18</td>
<td>UV transilluminator</td>
<td>3UV</td>
<td>UVP company</td>
</tr>
<tr>
<td>19</td>
<td>Shaking incubator</td>
<td>LT</td>
<td>Orbitek</td>
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### List of Chemicals

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<th>S.no.</th>
<th>Reagent/Chemical/Antibody/Buffers</th>
<th>Company</th>
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<tbody>
<tr>
<td>1.</td>
<td>Acetone</td>
<td>Chem Supply</td>
</tr>
<tr>
<td>2.</td>
<td>Acetonitrile</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>3.</td>
<td>Acrylamide</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>4.</td>
<td>Agarose</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>5.</td>
<td>Ammonium bicarbonate</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>6.</td>
<td>Ammonium persulfate (APS)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>7.</td>
<td>Annexin V staining kit</td>
<td>Roche</td>
</tr>
<tr>
<td>8.</td>
<td>Anti-goat IgG-FITC</td>
<td>(F7367,Sigma Aldrich)</td>
</tr>
<tr>
<td>9.</td>
<td>Anti-mouse IgG-FITC</td>
<td>(F0257,Sigma Aldrich)</td>
</tr>
<tr>
<td>10.</td>
<td>Anti-rabbit IgG-FITC</td>
<td>(F0382, Sigma Aldrich)</td>
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<td>11.</td>
<td>Anti-mouse IgG- horse radish peroxidise (HRP)</td>
<td>(A9044, Sigma Aldrich)</td>
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<tr>
<td>12.</td>
<td>Anti-rabbit IgG-horse radish peroxidise (HRP)</td>
<td>(A8275, Sigma Aldrich)</td>
</tr>
<tr>
<td>13.</td>
<td>Anti-goat IgG-horse radish peroxidise (HRP)</td>
<td>(A3682, Sigma Aldrich)</td>
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<tr>
<td>14.</td>
<td>Bisacrylamide</td>
<td>Sigma Aldrich</td>
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<tr>
<td>15.</td>
<td>Bradford-Coommassie plus protein assay reagent</td>
<td>Thermo Scientific</td>
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<td>16.</td>
<td>Bovine serum albumin (BSA)</td>
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<tr>
<td>17.</td>
<td>Bromophenol blue</td>
<td>Sigma Aldrich</td>
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<tr>
<td>18.</td>
<td>Chloroform</td>
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<tr>
<td>19.</td>
<td>Dithiothreitol (DTT)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>20.</td>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma Aldrich</td>
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<td>Dulbecco Modified Eagles’ Media (DMEM) high glucose</td>
<td>Sigma Aldrich</td>
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<td>Description</td>
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<td>Ethylenediamine tetra acetic acid (EDTA)</td>
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<td>Fluoro shield 4’,6-diamidino-2-phenylindole (DAPI)</td>
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<td>Sigma Aldrich</td>
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<td>30</td>
<td>Goat anti-bLf (A10-126, Bethyl Scientific)</td>
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<tr>
<td>31</td>
<td>Haematoxylin</td>
<td>Hi Media Laboratories</td>
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<td>35</td>
<td>Mouse anti-EpCAM monoclonal antibody</td>
<td>Santa Cruz USA</td>
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<tr>
<td>36</td>
<td>Mouse anti-NIS monoclonal antibody</td>
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<td>37</td>
<td>Mouse, FP5A, anti-NIS monoclonal antibody</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>38</td>
<td>Mouse anti-β-actin monoclonal antibody</td>
<td>Sigma Aldrich</td>
</tr>
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<td>39</td>
<td>Paraformaldehyde</td>
<td>Sigma Aldrich</td>
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<tr>
<td>40</td>
<td>Polyvinylidene difluoride (PVDF) membrane</td>
<td>GE healthcare</td>
</tr>
<tr>
<td>41</td>
<td>Protein marker, wide range</td>
<td>Bio Rad</td>
</tr>
<tr>
<td>42</td>
<td>Propidium Iodide (PI) stain</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>43</td>
<td>Protease inhibitory cocktails</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Supplier</td>
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<tr>
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<tr>
<td>44.</td>
<td>Rabbit anti-NIS polyclonal antibody</td>
<td>Abcam</td>
</tr>
<tr>
<td>45.</td>
<td>Rabbit anti-Survivin monoclonal antibody</td>
<td>Abcam</td>
</tr>
<tr>
<td>46.</td>
<td>Rabbit anti-Survivin ΔEx3 polyclonal antibody</td>
<td>Abcam</td>
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<tr>
<td>47.</td>
<td>Rabbit anti-GAPDH polyclonal antibody</td>
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<tr>
<td>48.</td>
<td>RNase OUT</td>
<td>Life Technologies</td>
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<tr>
<td>49.</td>
<td>Roswell park memorial institute (RPMI) media</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>50.</td>
<td>Sodium azide</td>
<td>Sigma Aldrich</td>
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<tr>
<td>51.</td>
<td>Sodium Chloride</td>
<td>Sigma Aldrich</td>
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<td>Sodium citrate</td>
<td>Sigma Aldrich</td>
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<tr>
<td>53.</td>
<td>Sodium do-decyl sulphate (SDS)</td>
<td>Sigma Aldrich</td>
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<td>Superscript III</td>
<td>Life Technologies</td>
</tr>
<tr>
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<td>SYBR green premix</td>
<td>Life Technologies</td>
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<td>Tris base</td>
<td>Sigma Aldrich</td>
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<td>57.</td>
<td>Triton X-100</td>
<td>Sigma Aldrich</td>
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<td>TRIzol reagent</td>
<td>Life Technologies</td>
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<td>59.</td>
<td>Trypan Blue</td>
<td>Sigma Aldrich</td>
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<tr>
<td>60.</td>
<td>Trypsin/EDTA (0.25%)</td>
<td>Life Technologies</td>
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<tr>
<td>61.</td>
<td>Tween-20</td>
<td>Sigma Aldrich</td>
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