Efficacy of utilizing chemical antibodies in histopathological studies

by

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Submitted in fulfilment of the requirements for the degree of

Master of Science

Deakin University
December, 2013
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Abstract

Immunohistochemistry (IHC) is mainly used for the diagnosis and classification of tumours in the pathological laboratories. The antibody is the reagent of choice for identifying tumour markers, which are over expressed in tumour cells and can be detected using IHC. However, the use of antibody is associated with several limitations, such as the use of animals for their production and batch-to-batch variation, resulting in inconsistent results with different batches of antibodies that recognise the same epitope. Therefore, there remains a need for an alternative detection ligand with desirable attributes superior to antibodies. Aptamers are short single stranded oligonucleotides that fold into specific three-dimensional structures, having similar properties to conventional antibodies. Aptamers have been generated against a large variety of target molecules using systematic evolution of ligands by exponential enrichment (SELEX) process. Once selected, aptamers are chemically synthesized, having the ability to fold into complex three dimensional shapes and bind to their target specifically, making them a suitable molecule for both cancer diagnostics and therapeutics.

This study aimed at exploring if our previously characterised EpCAM and CD133 RNA aptamers could be used instead of antibodies for identifying EpCAM and CD133 markers in paraffin embedded tissue sections. Both EpCAM and CD133 are considered as cancer stem cell (CSC) markers in a variety of solid cancer and are also related to prognosis and tumour recurrence. Thus, the reproducible and sensitive detection of these two markers would lead to the selection of more efficacious treatment options for the cancer patient. Utilising an immunohistochemical protocol similar to that already used in histopathology laboratories for the identification of cell specific markers, we successfully optimised the protocol for the detection of EpCAM and CD133 using paraffin embed tissue sections in a variety of tumours.

Aptamers are nucleic acid species and the optimisation process for IHC required a combination of in situ hybridisation and conventional antibody staining protocols. This involved blocking steps with animal serum and heparin to avoid non-specific background staining, as well as the use of dextran sulphate to accelerate the rate of hybridisation. When compared to traditional antibodies used for the detection of EpCAM, such as the 323/A3 or C10 antibody, the EpCAM aptamer demonstrated superior sensitivity, being able to detect the EpCAM antigen in the MDA-MB-231 xenograft, which expresses very low levels of EpCAM and is often classed as negative by conventional antibody detection methods. Following the
success of the optimisation of this protocol using fluorescently labelled aptamers, a chromogenic staining protocol was developed to allow these aptamers to be routinely used in histopathological laboratories. As with our previous results, the aptamer showed a higher level of sensitivity than the antibody. Indeed, in a number of cases, EpCAM expression in cancer cells was only demonstrated by the aptamer. These results could have important ramifications when considering the use of EpCAM immunotherapy.

As with the results with the EpCAM aptamer, similar sensitivities were observed with the CD133 aptamer. Interestingly, a peri-nuclear staining pattern was observed in one clinical case of hepatocellular carcinoma, a pattern that has been reported to lead to a poorer prognosis. This staining pattern was only observed with the aptamer and not the antibody, which demonstrated a cytoplasmic staining pattern instead. This finding will also have future potential in guiding personalised selection of most appropriate therapy and in predicting patient response to therapy.

In conclusion, we have demonstrated that the identification of EpCAM or CD133 markers by their respective aptamers were more superior than the EpCAM 323/A3 and CD133-1 (W6B3C1) antibodies used. Moreover, aptamers requires shorter incubation times (less than one hour) as opposed to the overnight incubation of both EpCAM and CD133 antibodies used in this study. We obtained consistent results with the xenograft samples used throughout this study using both fluorescent and chromogenic detection methods. Critically important is the fact that the EpCAM and CD133 aptamers have been successfully employed to detect the proteins in paraffin embedded tissues from breast cancer and liver cancer, respectively. These findings have demonstrated the distinct advantages of using aptamers as powerful probes for immunohistology and have indicated a potential demand for the use of aptamers in IHC, as these results are important from the clinical point of view. Given that the aptamers in this study were more sensitive than the antibodies tested, this study paves the way for the application of these molecular probes in future histopathological diagnosis and potentially for therapeutic applications.
**List of abbreviation**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>SELEX</td>
<td>Systematic Evolution of Ligands by Exponential Enrichment</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’- Diaminobenzidine</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HIER</td>
<td>Heat-Induced Epitope Retrieval</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating Tumour Cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
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CHAPTER 1
1. **INTRODUCTION**

1.1 Cancer

Cancer is a highly complex disease caused by genetic instability and an accumulation of multiple molecular alterations [1]. It is thought that due to our increased life expectancy, as well as an increased exposure to cancer-causing chemicals, alcohol, tobacco and an increased propensity towards obesity [2], the number of newly diagnosed cases of cancer will continue to increase on a yearly basis and remain a major cause of mortality [3, 4]. According to the American Cancer Society it is expected that approximately 1,690,290 new cancer cases will be diagnosed in 2013, with an increase in the number of cases of liver, pancreatic, thyroid and skin cancer. As well, the five-year survival rate has increased, from approximately 49% in the 1970s, closer to 68% forty years later [5]. However, there is still cause for concern in certain types of cancer, such as lung, colon, breast and prostate cancer, which are the leading causes of cancer-related deaths.

Cancers are mainly treated with conventional chemotherapy, radiation therapy and/or surgery [6, 7]. However, these treatments are unsuccessful in certain cases due to ineffective drug concentrations at the site of the tumour, non-specific targeting capacity or lack of an early diagnosis [8]. The latter is implicated due to statistics showing that the clinical outcome is strongly related to the stage at which the malignancy is detected – while cancer starts as a localised disease, it rapidly spreads to secondary organs [9]. Data has shown that at the time of clinical staging, more than 60% of patients with breast, colon, prostate, and ovarian cancer have metastases in at least one secondary site, implying that an earlier diagnosis may lead to improved survival rates [10]. Hence the opportunity to reduce cancer-related deaths depends on the early diagnosis of the disease.

1.2. Cancer diagnosis

Cancer is not necessarily an incurable disease. The success or failure in eradicating cancer is very much dependent on the timing of diagnosis [11]. An early detection of the disease offers considerably higher chances of eradicating the cancer from the body [12]. A number of attempts have been made to develop methods that will help to achieve an early diagnosis of cancer which are based on differences between the tumour cell or tumour environment and that of normal tissue [11]. When a normal cell transforms into a cancer cell, changes occur
within and on the surface of the cells that can be detected and act as a characteristic tumour marker [13].

The first tumour associated antigen, carcinoembryonic antigen was found in serum of patients with gastrointestinal malignancies [14]. These tumour markers are a biochemical facet that can be used to measure a normal or abnormal biological state or condition [15]. In other words, a trait or characteristic feature which can be particularly measured and analysed as an index of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [15, 16]. These markers could be nucleic acids, proteins, sugars, lipids, and small metabolites, as well as whole tumour cells found in body fluid which may be detected in the blood, urine or body tissue. These bodily substances are produced either by the neoplastic tissue or by the human body in response to the presence of cancer [17]. Tumour biomarkers are used to optimise treatment and hence their identification and quantification in serum, urine or tissue enhances a patient’s diagnosis and prognosis [17-19]. The aim of diagnosis is to search for direct or indirect evidence of the presence of a tumour, preferably earlier rather than later in its journey from a pre-cancerous to a malignant state. The diagnosis of cancer is generally based on symptoms that a patient presents with at their doctors surgery [20]. More commonly however, a diagnosis is made following abnormalities observed in routine blood tests. Many non-invasive tests, such as magnetic resonance imaging, computed tomography, x-rays and blood tests, such as CEA or CA199 [21], are performed to confirm the presence of a tumour. However, histopathology is generally considered as the gold standard for the diagnosis of cancer.

1.2.1. Histological diagnosis of cancer

Diagnosis of a tumour is performed following the removal of a portion of the tumour by biopsy, or the removal of the mass following surgery, which is generally sufficient to perform diagnostic procedures [22]. In most pathological laboratories, tissues are examined by pathologists whose analysis of the specimen generally delivers more clear information about the cancer staging and grading. The cancer grade describes the extent of the lesion and may also predict the tumour growth and spread to distant sites, and prognosis is predicted based on the stage and tumour grade of the tumour. An accurate diagnosis is very important to determine the optimal treatment plan [23]. Routinely, tissue sections are stained with the common dyes, haematoxylin and eosin (H & E), with these stains considered as routine in
histopathology [24]. These two stains, when used together, provide an indispensable contrast between structures of different composition, allowing for easy recognition of the cells and the cell populations, even at low microscopical magnification. The popularity of this stain is also due to it being technically simple to use on both frozen sections, as well as paraffin embedded tissues [25]. However, biopsies have some limitations in their use, depending on the type of cancer. For example, numerous biopsies may be required for a diagnosis of prostate cancer so as to ensure an accurate representation of the tumour [26]. As well, in some cases, H & E staining fails to identify tumours of unknown primary origin, especially poorly differentiated tumours, representing the failure to distinguish carcinoma, melanoma, lymphoma and sarcoma [27]. Therefore, any discrepancy in the diagnosis may lead to unnecessary or harmful therapies [28]. Moreover, given the recent advances in our understanding of cancer progression, it is important to distinguish the different cell types that make up the heterogeneity of the tumour.

1.3. Cancer Stem Cells

Despite many studies in cancer, the phenomenon of cancer initiation and development still remains uncertain. However, the clonal evolution model and the cancer stem cell model (CSC) attempt to explain this phenomenon [29]. The clonal evolution model suggests that cancerous cells may randomly have the capacity to proliferate extensively and regenerate tumour tissue [30]. On the other hand, the CSC model assumes that there exist different types of cells within the tumour including non-CSCs and CSCs [30]. A small subset of cancer cells within a tumour mass have the ability to self-renew and cause heterogeneous lineages. These are known as CSCs or tumour initiating cells [31, 32]. Heterogeneity of cancer is important for consideration of the CSC hypothesis. This phenomenon indicates that tumour cells are comprised of both CSCs with unlimited proliferation potential and differentiated cells with limited proliferation potential [30]. Recently, cancer heterogeneity was explained by a new model called “phenotypic plasticity”. Tumours may contain cells with a certain degree of plasticity that is capable of inter-converting from CSC to non-CSCs and from non-CSCs to CSCs [33]. Certainly, CSCs within a tumour might exist in different manner during initiation of tumourigenesis related to diagnosis or during therapy [34]. These studies suggest that while it is important to determine the proportion of CSCs in a tumour, it is also imperative
that all of the cells within a tumour are targeted simultaneously in order to effectively eradicate the potential for recurrence.

1.3.1. Clinical significance of CSCs

The CSC concept has important implications for cancer therapy and diagnosis [35]. If CSCs are responsible for maintaining tumour growth, then eliminating these cells should theoretically cure the patient [36]. If CSCs also express drug resistance proteins such as ABC transporters similar to normal stem cells this makes CSCs more resistant to chemotherapy [32]. Indeed, chemotherapy can destroy differentiated cancer cells but are much less effective at eradicating CSCs, which leads to tumour growth and progression. From a therapeutic point of view, if the overall CSC concept is correct, then directing cancer therapeutics against CSCs and the bulk tumour cells may eradicate the tumour [36]. Moreover, CSCs are detected by targeting the surface markers which are over expressed and differ between tumour types [37]. Hence there exists a requirement for specialised functional assays for the characterization and isolation of CSCs [38]. The answer to challenges facing CSCs may provide new options for improving cancer prevention, diagnosis, and treatment.

1.3.2. Evidence for the existence of CSCs

A number of studies have indicated that there is a relationship between CSCs and formation of tumour and progression, drug resistance and metastasis [39, 40]. It has been shown through numerous studies that one way to distinguish the CSC population from their non-CSC counterparts is through the examination of a set of specific markers on the cell surface [41]. The strongest evidence for the CSC theory arrives from the study of Dick and colleagues [42]. They demonstrated that only a few cells with a CD34+ CD38- phenotype in acute myeloid leukaemia (AML) were able to initiate leukaemia in immunocompromised mice. Later, CSCs were also identified from other leukaemias, colon cancer, prostate cancer, hepatocarcinomas, breast cancer, melanomas and osteosarcomas by using specific markers [43]. Studies performed in different types of cancer demonstrated that CSCs are rare, quiescent, and possess the ability to regenerate a tumour with heterogeneity [35]. Currently, there is only one standard method to define CSCs – in vivo limiting dilution assay - which involves xenotransplanting sorted cancer cells into immunocompromised mice [44]. With this method, specific surface markers expressed on cancer stem cells are used to isolate these cells
from the tumour mass, and these cells are then injected into immunocompromised mice followed by the determination of CSC frequency.

1.3.3. CSC controversies

Even though the existences of CSCs have been proven by several studies, there are still many controversies surrounding CSCs [30]. One important phenomenon which leads to the hypothesis of CSC is the heterogeneity of cancer and it is explained by clonal model and CSC model [29], but neither of these models describes how CSCs are involved in tumour progression, resistance and metastasis [45]. The CSC theory is controversial because all the evidence for the existence of CSCs relies on xenograft models in immunocompromised mice [46], which provides a different microenvironment compared to the human body [44, 47]. However, providing human stromal elements, human growth factor, or using orthotropic transplantation, has helped to overcome the disadvantages created by cross-species transplantation [48-50]. There is an ongoing debate as to whether CSCs are similar to normal stem cells and whether they possess drug resistance mechanisms [51, 52]. This last property is thought to protect them from conventional therapies and hence leads to disease relapses, even when the bulk tumour mass is eliminated [51]. Therefore, it is essential to isolate and detect CSCs to understand the acquisition of resistance of CSCs towards the chemotherapeutic drugs.

1.3.4. Detection of CSC

Tumours are normally evaluated clinically by the expression of specific cell surface markers [53]. These surface markers are often used to isolate normal cancer cells or CSCs [54]. CD24, CD29, Epithelial cell adhesion molecule (EpCAM), CD44, CD49, CD133 are some examples of markers identified within different types of cancer (Table 1.1) [37, 55].
Table 1.1 CSC markers

<table>
<thead>
<tr>
<th>TUMOUR TYPE</th>
<th>MARKER</th>
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<tbody>
<tr>
<td>Breast cancer</td>
<td>EpCAM⁺, CD44⁺CD24⁻/low Lineage⁻, CD133⁺, ALDH-1⁺ high</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>EpCAM⁺, CD133⁺, CD44⁺, CD166⁺, CD24⁺, ALDH-1⁺</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>CD133⁺, CD49f⁺, CD90⁺, EpCAM⁺, CD44⁺</td>
</tr>
<tr>
<td>Brain cancer</td>
<td>CD133⁺, BCRP1⁺, A2B5⁺, SSEA-1⁺, CD15⁺, Nestin⁺</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>CD133⁺, ALDH-1⁺</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>CD133⁺, EpCAM⁺, CD44⁺, CD24⁺, DCAMKL-1⁺, CD15⁺, CD10⁺</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>CD133⁺, integrin α2β1⁺, CD44⁺</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>CD133⁺, ALDH-1⁺</td>
</tr>
</tbody>
</table>

Common methods used for the detection of biomarkers include immunohistochemistry, cytogenetics including fluorescent in situ hybridisation, reverse transcription, ELISA and polymerase chain reaction [19]. The problem with attempting to detect CSCs in tumours is that no single marker can distinguish CSCs from non-CSCs. Indeed, not all marker expressing cells are CSCs, with some normal cells also expressing the same marker. For example, CD133 not only acts as a CSC marker, but is also used to identify hematopoietic stem cells and progenitor cells [56]. Thus, defined specific methods using multiple markers would be a good solution for the identification of CSCs and also for the differentiation of different histological subsets of a particular tumour type. Therefore, specific and distinct combinations of CSC markers have been used for the isolation of CSCs from normal cancer cells and stem cells. Among these markers EpCAM [57] and CD133 [58] are of interest because of their high level of expression in a variety of tumour cells and also in CSCs.

1.3.4.1. Epithelial cell adhesion molecule (EpCAM)

Epithelial cell adhesion molecule (EpCAM) is a 39 to 42 KDa type I transmembrane glycoprotein that functions as a homophilic, epithelial-specific intercellular cell-adhesion molecule [57]. The EpCAM protein is a 314 amino acid long chain which consists of a large extracellular domain with an epidermal growth factor like domain, a thyroglobulin domain
and a short single cytoplasmic tail [59]. It not only acts as a cell adhesion molecule but is also responsible for intracellular signalling, maintaining polarity, promoting epithelial cell migration, proliferation and EMT during organ development [60]. Hence, EpCAM is called a pleiotropic molecule [57].

EpCAM is expressed in normal epithelia except squamous epithelia, epidermal keratinocytes, gastric parietal cells, myoepithelial cells, thymic cortical epithelium and hepatocytes [61]. EpCAM was shown to be over expressed in most solid cancers, including breast, colon, prostrate, ovarian, lung and gastric [62]. Especially, EpCAM was over-expressed in metastatic breast cancer in relation to primary tumour. The difference between the expression level of EpCAM in normal epithelial cells and cancer cells is not restricted to its expression level, but also depends on the distribution across the cell membrane. In normal cells, EpCAM expression is limited to the baso-lateral membrane that makes the therapeutic EpCAM antibodies not accessible, while in cancer cells EpCAM molecules are distributed across the membrane, where therapeutic antibodies can favourably target EpCAM antigen [63].

The relationship between EpCAM and tumour progression was first validated while observing the role of EpCAM in regulation of genes involved in cell cycle and proliferation. The active EpCAM signalling pathway was observed in major cancer types including colon, lung, prostrate, breast, liver, head and neck, esophagus, liver and pancreatic [64]. EpCAM expression and activation levels are also significant in a transcriptional regulation pathway [65]. Thus, EpCAM expression is strongly related to tumour cells. In a vast majority of tumours, EpCAM over-expression is important factor for the targeted therapeutic practices.

All the current research indicates that EpCAM can be used as a diagnostic and prognostic marker and also a therapeutic target for a variety of carcinomas [66]. EpCAM has been utilised as a reliable surface binding site on cancer cells for therapeutic antibodies. EpCAM directed therapies may be particularly useful for triple negative breast cancer and targeting CSCs which are resistant to standard therapies [57]. Immunotherapy using anti-EpCAM antibodies adecatumumab and catumaxomab, have been used to treat patients with metastatic breast cancer and malignant ascites, respectively [67, 68]. However, this is only possible when cancer cells express high levels of EpCAM. Moreover, in the case of the low affinity antibody, edrecolomab, treatment is only partially effective, even when EpCAM expression is high [69]. Therefore, if EpCAM expressing cells are identified then it is possible to target
these cells with anti EpCAM therapy. It is anticipated that this strategy could lead to better therapies capable of destroying cancer cells [70].

Current research is utilising EpCAM protein to detect circulating tumour cells (CTC) in the peripheral blood. EpCAM expression is observed in most of the cancer types that makes EpCAM dependent CTC detection more reliable. It was shown that both epithelial marker EpCAM and mesenchymal markers have been observed on CTCs of advanced breast and prostate cancer. A number of studies have reported that EpCAM is a CSC marker in colon, pancreas, breast, gastric and liver cancer when compared to normal cells [71-73], and suggests that high expression of EpCAM seen in certain cancer cells could act as a suitable marker for characterising CSCs [66]. Indeed, EpCAM directed therapies may be particularly useful for targeting CSCs which are resistant to standard therapies [57]. However, further studies are required to define the role of EpCAM as a CSC marker [73].

1.3.4.2 CD133

CD133, also known as prominin-1, is a pentaspan membrane protein of about 120 KDa, located on the protrusion of cell membranes [74] and in normal cells it plays an important role in maintaining an appropriate lipid composition within the plasma membrane. CD133 is also classified as a marker of primitive hematopoietic and neural stem cells [58]. CD133 was first identified as a surface marker in brain tumours [75]. In this study, CD133 positive cells were able to form tumours in immunocompromised mice, whereas CD133 negative cells were not able to produce tumours. Subsequently, CD133 has been identified as a CSC marker in liver cancer, colon cancer, glioblastoma, melanoma and osteosarcoma [58]. It was found that CD133 co-expression was highly expressed in two out of five human pancreatic adenocarcinoma cell lines thus indicating CD133 acts as a putative CSC marker for pancreatic cancer [76].

Even though many studies have described the use of CD133 as a CSC marker, clear understanding of the pathways involved in CD133 expression does help to understand its function as a CSCs marker. Several factors have an impact on CD133 expression level in relation to change of cell type, from normal cell to cancer cell [77]. For example, hypoxia (oxygen deprived cell) induced CD133 expression is observed in pancreatic and liver cancer. In liver cancer, CD133 positive cells were associated with the interleukin-8 signalling
pathway that is responsible for tumour progression. A number of studies reported that CD133 expression was responsible for tumourigenesis in certain cell types, suggesting that CD133 could act as a tumour marker.

CD133 is also expressed in combination with other markers in a variety of tumours, for example in human prostate tumours (CD133/CD44/\(\alpha_1\beta_2\)), and human neural tumours (CD133 and nestin) [58]. Immunohistochemical studies using CD133 alone was not effective to demonstrate their role in metastasis, but both CD133\(^+\) CD44\(^+\) cells were demonstrated to play an important role in metastasis in liver cancer and also suggesting targeting these molecules could be a new approach for the treatment of liver cancer patient [78].

The hypothesis of CD133 as a marker has encouraged numerous studies to analyse the prognostic value of CD133 expression in patient with brain, liver, colon and lung cancer [79]. For example, most of studies performed before 2009 demonstrated that CD133 had no impact on brain tumour. But then, Zeppernick et al reported that CD133 expression was correlated with tumour regrowth and progression in grade II-IV gliomas [80]. To date, most studies were not able to produce consistent data due to differences in tumour subtype, different scoring systems and also due to the use of different antibodies for staining [79]. It was reported that immunohistochemical staining with different CD133 antibodies such as AC133, W6B3C1, C24B9 and ab19898 resulted in an inconsistent CD133 detection [81]. The discrepancies could be due to the difference in detection of CD133 antigen and this can be avoided by comparing the results obtained from one experiment accompanied by another method. In addition, Kemper and colleagues recently showed that a conformation change occurs to the CD133 protein during differentiation, leading to the ‘loss’ of the AC133 epitope [82]. Therefore, IHC studies need to be re-evaluated with respect to the antibody chosen to determine CD133 expression.

1.4. Immunohistochemistry

Immunohistochemistry (IHC) has made histopathology the ‘gold standard’ for diagnosis of tumours [83]. When morphological features of the tumour are not adequate for a definitive diagnosis, IHC may be of benefit [84]. IHC works on the premise that each cell type has a characteristic marker that can be used to differentially diagnose the cell of origin [83]. It
provides information that supplements the morphological characteristics seen with H & E staining [85].

Generally, IHC is a method of analysing and identifying cell surface antigen based on the binding of antibodies to the specific cell component [86]. Direct and indirect are the two principal immunohistochemistry methods (Fig. 1.1) [87]. The direct method is a one step process that involves a direct reaction between the antigen and the labelled antibody. In indirect immunostaining, the bound unlabelled primary antibody is visualized with a labelled secondary antibody. Due to higher sensitivity, an indirect method is commonly used when compared to direct method. Fluorescent dyes, biotin or enzymes are the most frequently used labelling elements.

![Figure 1.1- Immunohistochemical direct and indirect method.](image)

Antibodies have become an integral part of the histology laboratory in the last 40 years [88]. These reagents are capable of attaching to their target with high specificity and sensitivity and play a major part in the diagnostic procedure for a number of conditions, including cancer [89]. For example, antibodies against the antigen Ki-67 are used to evaluate malignant melanomas, breast carcinomas, and non-Hodgkin's lymphomas [90] and the tumours that have higher levels of Ki-67, indicative of a high proliferation rate [91], generally have a good response to chemotherapy [92].
IHC is used to assist in the diagnosis of various malignancies, usually in the case of tumours of unknown primary origin [93]. IHC can also help to identify the cell of origin in metastatic lesions through the use of characteristic markers on the cell surface [94]. IHC, while an integral part of the diagnostic arsenal, is also now being used to distinguish patient’s likelihood to benefit from certain directed chemotherapeutic agents. Such is the case in HER2 positive breast cancer patients who benefit from treatment with trastuzumab [95]. Detecting the expression of various receptors that may respond to drugs, such as oestrogen and progesterone, indicates a good prognosis for breast cancer patients also [96, 97]. Detection of EpCAM in tumours can be used to influence treatment decisions for immunotherapy with anti EpCAM therapeutic antibodies as well [98].

Cellular and subcellular co-localization of independent antigens in identical cells or different cell compartments can be performed by double label immunohistochemistry [99] and is performed when a single antibody is not accurate enough to identify the cell. It provides more information regarding one specific cell population, and gives valuable diagnostic information. Double antibody staining is used instead of serial sections using several single antibodies because it gives more information about a specific cell population or the tumour section due to a possible loss of the region of interest, especially if the region of interest is a small foci within the larger tumour mass [100]. The main principle of double staining involves more than one antibody being applied to the same tissue section and each antibody-antigen giving a specific staining colour due to the variety of chromogen that can be utilized for visualisation of their signals [101]. Good results are obtained when targets are localised in different cellular compartments, such as the nuclei and cell membrane.

Double staining can be achieved through a combination of immunofluorescence and IHC. Issues such as photo bleaching, fluorescent quenching, increased background staining and the fact that immunofluorescence does not result in permanent staining precludes the use of fluorescence techniques routinely [102]. As well, the majority of histology laboratories do not possess fluorescent microscopes, and therefore a combination of chromogens is preferred rather than fluorescent labels. However, this leads to a problem when targets are co-localised to the same compartment, such as two targets on the cell surface or in the nuclei. Indeed, specialised reference laboratories, such as Clarient Inc. (California, USA), are developing new diagnostic tests based on double and triple staining and are providing this service for
histological diagnoses. The problems surrounding IHC in diagnosing cancer need to be addressed in near future, since the diagnosis of cancer relies on IHC in most cases. As better techniques are developed, diagnoses are becoming more and more specific and this means the patient will receive the correct diagnosis earlier in their disease to enable them to receive treatment sooner.

1.4.1. Current issues in IHC

Although the technical advancements in immunohistochemical methods, such as heat induced epitope retrieval (HIER), have significantly enhanced the ability to detect tumour specific markers, various factors potentially compromise its specificity, interpretation and reproducibility [103]. Pre-analytical variables include tissue handling (preservation, processing, and treatment), length and type of fixation, and level of antigen expression. Analytical processing includes antibody clones, antigen retrieval, staining procedures and the detection system. Post-analytical factors include the interpreting and reporting of results. Standardization of IHC mainly involves fixation [104], variability in antibodies [105] and the interpretation of results [106].

1.4.1.1. Problems associated with fixation

The most important step in histology is fixation which is performed to avoid autolysis and degradation of the tissues [107]. Both over-fixation and under-fixation may result in inconsistent immunohistochemical results [108, 109]. Over-fixation is presumed to result in decreased antigen detection and this can be avoided by effective antigen retrieval method [110]. Tissue specimens which are fixed should undergo the antigen retrieval process (epitope demasking). This has the greatest impact on immunohistochemical results [111]. In general, different fixation procedures are followed depending on the nature of the epitope. For example, the detection of cytokeratin and vimentin in case of ovarian cystadenofibrocincoma needs different fixatives, such as ethanol and formalin [84]. Thus, suitable fixative and length of incubation time has to be optimised for each antigen. Tissue processing temperature (below 60°C) has to be retained in order to avoid loss of antigen in the tissue section [112].
1.4.1.2. Problems associated with the antibodies

Antibodies have become an integral part of the histology laboratory in the last 40 years [88]. These reagents are capable of attaching to their target with high specificity and sensitivity and play a major part in the diagnostic procedure for a number of conditions, including cancer [89]. The major problem with the use of antibodies in IHC is to select antibodies with high specificity and sensitivity [104]. The selection of the appropriate antibody type is important because it was reported that 42.1% of diagnostic inconsistencies in IHC is due to poor antibody selection [84]. In some cases weak target staining is caused by antibody cross-reactivity [113].

During immunohistochemical process, primary antibodies may produce unwanted background [114]. First, non-specific staining may be produced due to the ionic and hydrophobic forces taking place during antigen-antibody binding. Second, in some cases, the interaction between antibody conjugate and the tissue polar group can also produce unacceptable background staining. Third, the attraction of the Fc fragment of the antibody to basic groups present in the collagen fibres may generate non-specific staining. Fourth, inadequate reagent antibody storage may cause some non-specific tissue staining. Moreover, it was reported that 50% of IHC failures are due to improper reagent storage in laboratories [115]. Therefore, the use of antibodies in IHC requires a clear understanding of antibody characteristics, antibody titration for every new batch of antibodies, optimising proper antibody concentration and also incubation time. However, even with this knowledge, non-specific staining remains a problem. Thus, a suitable alternative molecule is necessary to overcome the problem surrounded with the antibodies.

1.5. Aptamers

Aptamers, also known as chemical antibodies, are short stranded DNA or RNA molecules which are selected from random pools of oligonucleotides based on their ability to bind to their target molecules [62]. The target for aptamers ranges from small organic molecules and metal ions to proteins, biological cells and tissues [89]. Although aptamers were discovered 23 years ago, the first aptamer drug, Macugen (pegaptanib sodium) was approved by Food and Drug Administration in 2004 [116]. Aptamers can be used in a variety of different fields, such as early target validation, diagnostic and therapeutic applications [117]. There is
increasing evidence supporting the notion that aptamers are suitable alternative to antibodies, but the application of aptamers in the field of diagnostics remains undeveloped. Given the wide utilization of antibodies in the diagnostic field, aptamers are also expected to prove their significance in the field of diagnostics soon.

1.5.1. Generation of aptamers

In 1990, the first RNA aptamer was synthesised by using a combinatorial technique known as SELEX (systematic evolution of ligands by exponential enrichment), which is an in vitro evolutionary selection process [118, 119]. The SELEX process starts by generating a library of different RNA species and incubating the generated RNA species with the target protein of interest [120]. This process will result in the binding of the RNA to the target protein [119]. The bound sequence is separated from the unbound sequence and further amplifications are performed using PCR. This selection and amplification process is repeated about 10-15 times until the RNA ligands with the highest affinity for the target are isolated [120]. Although SELEX is a powerful method, it requires five to twenty rounds of screening to obtain aptamers, thus it is a laborious process [121].

1.5.2. Properties of aptamers

The structure of aptamers has been determined by enzymatic or chemical probing, nuclear magnetic resonance and X-ray crystallography [122, 123]. RNA based aptamers are able to adopt a variety of structures [124, 125], possess a low molecular weight [126], and mostly present in globular form which makes the aptamer suitable for recognition of target molecule. The most desirable property of aptamers is their smaller size that makes the aptamer convenient for penetration inside the cell. Generally, the molecular weight of aptamers are in the range of 6-32 kDa and the dissociation constant is in the range of micromolar to picomolar [89, 119, 127].

1.5.3. Advantage of aptamers vs. antibody

Being clinically validated and commercially available, antibodies have been used as a tool in the diagnostic and clinical aspects of cancer diagnosis and treatment so far. However, there have been some variations to account for and in improving the techniques used; a novel method has been used to generate chemically synthesized, single stranded oligonucleotides
called aptamers. Since they are chemically synthesized, these chemical antibodies can be relied upon to be fairly similar in their activity and structure, and more importantly, without contamination from non-specific elements expected from antibody purification upon collection of the ascites fluid from an animal host [89]. The defining factor of aptamers as a worthy substitute in research lies in their similarity to antibodies. Further, aptamers are significantly smaller than the 150 KDa antibodies, also contributing to the specificity of the method. In addition, the small size aptamers increases surface coverage during immobilization on chromatography matrices or immunoassays, thus increasing sensitivity [128]. Aptamers can be stored for longer, since they remain stable, and are markedly cheaper and synthesized relatively faster [127]. Further being non-biological molecules, they show little immunogenicity when compared to antibodies.

1.5.4. Application of aptamers

1.5.4.1. Aptamers as therapeutics

Aptamers are smaller in size and therefore penetrate into tissues rapidly, and are cleared quickly from the circulation, which makes aptamers a useful molecule for therapeutic purposes. To assist aptamer stability inside the body, several base modifications are performed to overcome nuclease degradation [129]. Linking aptamers with polyethylene glycol (PEG) will improve the bioavailability and adjust the pharmacokinetics of an aptamer [117]. Indeed, the FDA approved anti-VEGF aptamer, Macugen is used for the treatment of age related mascular degeneration [130]. Initially this aptamer had a half-life of 1.4 hour, but following modification with the addition of PEG, the serum half-life was increased to 131 hour [131]. As well, aptamers are broadly used in biomedical areas such as vaccine production [132, 133], virology [117], parasitology [134, 135] and oncology [136, 137].

1.5.4.2. Aptamers in diagnostic applications

As it has been reported, aptamers possess high specificity towards its target molecule. Aptamers attached with therapeutic agents or diagnostic tools such as fluorescent molecule, MRI (magnetic resonance imaging) agents and radioisotopes could be used to detect tumour mass and also metastasised cells in vivo [138]. This is achieved by higher penetration of aptamers inside tumours than antibodies, as the latter are comparatively larger in size. Schmidt et al modified the TTA1 aptamer which binds to Tenascin-C protein, which is over
expressed in tumour tissue, and showed that this aptamer could be a promising candidate for tumour \textit{in vivo} imaging [139]. Another example for \textit{in vivo} imaging of aptamers was demonstrated against neutrophil elastase for imaging inflammation in rats [140]. Compared with antibodies, aptamers detect target molecules with high target ratio with no background signal in less time. Furthermore, aptamers are rapidly cleared from the peripheral circulation, minimising potential side effects.

The identification of tumour markers in blood and tissues is vital for both diagnosis and treatment. Aptamers play a significant role in biosensor applications [138], either as a aptamer conjugated with the fluorescent molecule producing a signal upon binding to the target molecule (as molecular beacons), or indirect method, such as aptamer attachment to the target molecule then the detection of aptamer through secondary biomolecules attached with fluorescent dyes (as aptazymes). Another important application of aptamers is in microarray detection technology for the detection of protein biomarker based on enzymatically surface plasmon resonance imaging (SPRI) [138]. In this process, aptamers are dotted on an array, where target protein attached to the aptamer, and then detection was performed through HRP attached secondary antibody that produce signal. It was shown that the aptamers were able to recognize large variety of analyte, which antibody could not able to recognize.

A microfluidic device was designed with immobilised lymphoma specific aptamers namely, Sgc8 aptamer, TD05 aptamer and sgd5 aptamer for cell sorting and detection of cancer cells from a sample [141]. By this approach, cancers cells were separated out with 96% purity that implies the specificity of aptamer towards its target molecule. Further studies were performed with the TD05 aptamer with fluorescent dyes Cy5 that was used as a molecular probe for \textit{in vivo} imaging to distinguish lymphoma inside live animals. The degradation rate of aptamers were reduced by chemical modification with polyethylenimine (PEI) thereby, half-life of aptamers was increased that makes this method more feasible for imaging tumours in live cells with high specificity and sensitivity.

1.5.4.3. Aptamers as immunohistochemical agents

Since aptamers mimic the properties of antibodies, it can be substituted in IHC for the detection of antigens. To date, a limited number of experiments have been reported on substituting aptamers for antibodies in IHC. Zeng \textit{et al} worked on demonstrating the
efficiency of a synthetic aptamer as a specific probe for the immunohistochemical study of paraffin embedded tumour tissues, which includes classical Hodgkin lymphoma and anaplastic large cell lymphoma [142]. They used CD30 antibody as a control. The CD30 aptamer specifically recognized and immunostained the target cells, the tumour cells of Hodgkin lymphoma and anaplastic large cell lymphoma, but did not cross-react with cells that did not express CD30 or the cell debris from surrounding necrotic tissue. Particularly, the CD30 aptamer optimally immunostained the target cells retrieved at a lower temperature (37°C for aptamer vs. the 96°C for antibody). CD30 aptamer significantly utilized shorter length of time (20 minutes for aptamer vs. 90 minutes for antibody) for the detection of CD30 antigen.

Another study utilised DNA aptamer against heparanase, an enzyme associate with tumour cell penetration to other organs [143]. In this study, paraffin embedded placental tissue section was stained with both aptamer and antibody (HPSE1). Both IHC and IF staining experimental results indicated that aptamers were superior to the antibody. They also demonstrated that the staining pattern of aptamer was consistent with the level of expression of the enzyme in the tissue section. Although aptamer staining was superior to antibody staining, the aptamer recognizes a different binding site of the enzyme relative to the antibody recognition. This study validated that the aptamer against heparanase could act as a potential therapeutic and diagnostic agents.

In summary, merging evidence show that further laboratory and clinical studies may open the door for aptamers as an ideal diagnostics reagent.

1.6. Aim of the project

Traditional IHC relies on antibodies for the detection of markers that are expressed on cancer cells as well as CSCs - a subpopulation of cancer cells that are considered to be the root of cancer development. There are some limitations to antibodies, such as large size, batch to batch variation, and the production of antibodies, which is a time consuming and complicated process. These drawbacks can be overcome by utilizing nucleic acid based molecules known as aptamers which have smaller size, lower molecular weight, and are easier and less expensive to produce, compared to that of antibodies. This project focuses on utilizing
aptamers instead of antibodies for the immunostaining of paraffin-embedded tumour tissue. The main aims of this project are:

1. To develop a suitable staining procedure for the use of aptamers in diagnostic pathology laboratories.
2. To develop an IHC staining procedure for detection of cells that express EpCAM and CD133 markers in formalin fixed paraffin embedded tissue.

1.7. Hypotheses

1. Aptamers are effective at identifying marker-positive cells in formalin fixed paraffin embedded tissue.
2. Aptamers are capable of identifying marker-positive cells at lower concentrations, and shorter incubation times than antibodies.

In order to achieve the aims and test these hypotheses, aptamers used in immunostaining need to be examined. Current cancer treatment has not progressed to the stage of cure due to late diagnosis, lack of effective chemotherapy and targeted drug delivery [144]. Indeed, early diagnosis of cancer is important in order to improve the survival rate and quality of life of patients with cancer. Current cancer treatments are not efficient because these therapies fail to destroy CSCs and in many cases cancer cells acquire drug resistance mechanisms [145]. Modern technologies are focused on developing simple, faster and cost effective methods to more effectively treat cancer and one such method is finding cell surface antigens that can act as biomarkers and will permit the identification and/or isolation of CSCs within a tumour mass. These biomarkers are highly expressed in CSCs when compared with normal cancer cells. In order for these biomarkers to be used effectively to treat cancer, a sensitive detection method is required. In routine practices, various antibodies are used for detecting these markers using immunohistochemistry, but different antibodies against the same antigen may show different results. Hence, it is necessary to increase the specific recognition of antibodies to markers, which can be attained by an alternative solution – the use of chemical antibodies (aptamers). It is hoped that this project will show that aptamers, because of their smaller size, are superior to antibodies in detecting tumour markers in paraffin embedded tissue sections.
CHAPTER 2
2. MATERIALS AND METHODS

2.1. Ethics statement

Human ethics approval was obtained in October 2011 from Human Research Ethics Committee. Human ethics approval: Deakin University Human Research Ethics Committee EC00213.

2.2. Sample material

2.2.1. Cell lines and cell culture

The colon cancer cell line (HT-29), glioblastoma cell line (U118), breast cancer cell lines (T47D, MCF-7, MDA-MB-231) used in this study were purchased from American Type Culture Collection (ATCC, Manassas, USA). Cells were grown and maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Victoria, Australia) supplemented with 10% fetal calf serum and incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

2.2.2. Tumour xenograft samples

All xenograft tumours were obtained from our laboratory under the approval and guidance of the Institutional Animal Care and Use Committee, School of Medicine, Deakin University. The samples were fixed at 10% neutral buffered formalin, processed and embedded in paraffin in order to retain their shape and architecture, as well as for their long term storage.

2.2.3. Clinical samples

Clinical samples for this study were obtained from breast cancer patient specimens randomly selected from archived cases at Second Affiliated Hospital of Dalian Medical University, Dalian, China. Samples had already been formalin fixed, paraffin embedded, sectioned and mounted on glass slides.
### 2.3. Antibodies

Antibodies used in this study and their optimal concentrations are provided below.

**Table 2.1 List of antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalogue Number and supplier</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EpCAM- Immunofluorescence staining</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal mouse anti-human EpCAM antibody (323/A3)</td>
<td>AB8601, Abcam (Cambridge, MA)</td>
<td>10 µg/mL</td>
</tr>
<tr>
<td>Mouse monoclonal EpCAM antibody (C10)</td>
<td>SC25308, Santa Cruz Biotechnology. (Texas, US)</td>
<td>5 µg/mL and 10 µg/mL</td>
</tr>
<tr>
<td>Goat anti-mouse-Alexa Fluor 647 secondary antibody</td>
<td>A21240, Life Technologies (Victoria, Australia)</td>
<td>10 µg/mL</td>
</tr>
<tr>
<td><strong>EpCAM- Chromogenic staining</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal mouse anti-human EpCAM antibody (323/A3)</td>
<td>AB8601, Abcam (Cambridge, MA).</td>
<td>20 µg/mL</td>
</tr>
<tr>
<td>Goat anti-mouse anti-fluorescein HRP secondary antibody</td>
<td>AB6656, Abcam (Cambridge, MA).</td>
<td>10 µg/mL</td>
</tr>
<tr>
<td>Goat anti-mouse HRP secondary antibody</td>
<td>31430, Thermo Fisher Scientific (Victoria, Australia).</td>
<td>1.6 µg/mL</td>
</tr>
<tr>
<td><strong>CD133- Chromogenic staining</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD133-1 (W6B3C1) pure human</td>
<td>Miltenyi Biotec, (Victoria, Australia).</td>
<td>20 µg/mL</td>
</tr>
<tr>
<td>Goat anti-mouse anti-fluorescein HRP secondary antibody</td>
<td>AB6656, Abcam (Cambridge, MA).</td>
<td>4 µg/mL</td>
</tr>
<tr>
<td>Goat anti-mouse HRP secondary antibody</td>
<td>31430, Thermo Fisher Scientific (Victoria, Australia).</td>
<td>3.2 µg/mL</td>
</tr>
<tr>
<td><strong>Western blot</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal mouse anti-human EpCAM antibody (323/A3)</td>
<td>AB8601, Abcam (Cambridge, MA)</td>
<td>1.5 µg/mL</td>
</tr>
<tr>
<td>CD133-1 (W6B3C1) pure human</td>
<td>Miltenyi Biotec, (Victoria, Australia).</td>
<td>0.2 µg/mL</td>
</tr>
<tr>
<td>Goat anti-mouse HRP secondary antibody</td>
<td>31430, Thermo Fisher Scientific (Victoria, Australia).</td>
<td>0.08 µg/mL</td>
</tr>
<tr>
<td>Monoclonal mouse β-actin antibody</td>
<td>AB8226, Abcam (Victoria, Australia).</td>
<td>0.02 µg/mL</td>
</tr>
</tbody>
</table>
2.4. Aptamers

The EpCAM-DT3, EpCAM-23 and CD133 aptamers were generated and characterized as previously described [62, 146] (Table 2.2). EpCAM aptamer DT3 is 19 nucleotides long with a DY647 attached to its 5’prime end whereas aptamer 23 is a 19 nucleotide long aptamer with a TYE 665 conjugated to its 5’ prime end. The control aptamer has the same nucleotide sequence as DT3 but it has 2’-O-methyl modifications instead of 2’-fluoropyrimidines. CD133-A15 aptamer is 15 nucleotides long with a fluorescent tag FITC attached to its 5’ prime end.

Table 2.2 Aptamers sequences

<table>
<thead>
<tr>
<th>APTAMER</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>EpCAM-23 aptamer</td>
<td>5’-TYE665-ACGUAUCCUUUUUGCGUA-dT-3’ or 5’-FITC-ACGUAUCCUUUUGCGUA dT-3’.</td>
</tr>
<tr>
<td>EpCAM-DT3 aptamer</td>
<td>5’-DY647-GCGACUGGUUUACCCGCGGUCG-dT-3’.</td>
</tr>
<tr>
<td>Control aptamer</td>
<td>5’-DY647-mGCmGACUmGUUuACCCmGuACmGuACm-dT-3’.</td>
</tr>
<tr>
<td>CD133 aptamer</td>
<td>5’-FITC-CCCUCUUACAUAGGG-dT-3’.</td>
</tr>
</tbody>
</table>

Prior to incubation of aptamers with sample slides, EpCAM and CD133 aptamers were prepared in PBS containing 5 mM and 2.5 mM MgCl₂ respectively, and then folded by denaturation at 85°C for 5 mins, followed by 10 mins incubation at room temperature and refolding at 37°C for 15 mins. Then 10% dextran sulphate and heparin 500 μg/mL was added to the aptamer (EpCAM and CD133 aptamer) before incubation with the slides.

2.5. Western blot

Cells were lysed in RIPA lysis buffer. The cell debris was pelleted by centrifugation at 21,000 x g at 4°C for 30 min, and the supernatant was retained and termed as total cell lysate. Fifty micrograms of total protein from each sample (MCF-7, T47D, MDA-MB-231, HT-29 and U118) was mixed with a sample buffer (12 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 2.9 mM 2-mercaptoethanol, 0.02% bromophenol blue) and loaded into wells of 10% SDS-Polyacrylamide gel and run at 25 mA/gel for 50 min. After the resolving process, the proteins in the gel were transferred to a nitrocellulose membrane for 1 h at 300 mA. The
Chapter 2

nitrocellulose membrane was blocked by incubating in 5% non-fat milk in TBST for 3 - 4 h at room temperature. The membrane was then incubated at 4°C overnight with the primary antibodies: monoclonal mouse anti-human EpCAM antibody (323/A3) and CD133-1 (W6B3C1) antibody prepared at a concentration of 1.5 μg/mL and 0.2 μg/mL respectively in blocking buffer. Blots were washed 5 times for 5 min each, followed by incubation at room temperature for 1 h with goat anti-mouse HRP secondary antibody at a concentration of 0.08 μg/mL. β-actin antibody at a concentration of 0.02 μg/mL was prepared in blocking buffer and was used as the internal control. This was applied to the membrane and incubated for about 1 h at room temperature. The membrane was then washed 3 times, 5 min each and incubated with secondary antibody for about 1 h. Detection was carried out using an ImageQuantTM LAS 4000 Biomolecular Imager (GE Healthcare, NSW, Australia).

2.6. Immunostaining of tissue sections

2.6.1. Preparation of tissue sections

Sections at a desired thickness of approximately 4 μm were prepared from blocks of formalin-fixed paraffin-embedded tissues. Excess paraffin was trimmed away prior to slicing. Sections were cut and removed with clean tweezers from the microtome, and placed in a water bath at around 40°C. Sections were then transferred to poly-l-lysine coated glass slides which have a permanent positively charged surface that facilitates the adherence of the tissue sections to the glass slide. Slides were then allowed to dry overnight at 37°C.

2.6.2. Deparaffinising process and antigen retrieval

Slides loaded into plastic slide holders were deparaffinised with histolene twice for 5 mins each to remove the paraffin. Rehydration of sections was then accomplished by immersing through 100% alcohol for 3 mins, 95% and 75% alcohol for 2 mins and final wash in distilled water for 30 sec.

Protease digestion was carried out using protease solution (0.5% in distilled water, pH 7.8) for 10-20 mins at 37°C. Heat-induced epitope retrieval (HIER) was performed by transferring the slides loaded rack into approximately 400 mL pre-warmed (95°C–100°C) target retrieval solution in a glass container for 20 mins. Antigen retrieval solutions used for detecting EpCAM and CD133 markers were Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 0.05%
Tween, pH 9.0) and citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0), respectively. The slides immersed in the buffer were allowed to cool down for 20 to 30 mins and then washed with PBS/0.1% Tween for 2 mins prior to incubation with aptamers / antibodies.

### 2.6.3. Immunofluorescence staining protocol

#### 2.6.3.1. EpCAM antibody staining

Following antigen retrieval, slides were removed from PBS/0.1% Tween and wiped around the section to create an obvious area onto which 100 μL blocking buffer containing 1 mg/mL bovine serum albumin (BSA) and 10% normal goat serum was carefully added for 1 h incubation. Following a wash with PBS/0.1% Tween, slides were wiped and the mouse monoclonal anti-human EpCAM antibody (clone 323/A3) at a concentration of 10 μg/mL was applied overnight at 4°C. Following the primary antibody incubation and three five min washes in PBS/0.1% Tween, 100 μL goat anti mouse IgG Alexafluor 647 conjugate secondary antibody at a concentration of 10 μg/mL was added to the slides and incubated for approximately 2 h prior to washing thrice for 5 mins each. Finally, the section was incubated with Bisbenzimide Hoecht 33342 (Sigma, New South Wales, Australia) (3 μg/mL) for 10 mins and a cover slip applied using Vectashield (Vector Laboratories, Burlingame, CA) before visualization under a Fluoview FV10i laser scanning confocal microscope (Olympus, NSW, Australia).

#### 2.6.3.2. EpCAM aptamer staining

Following antigen retrieval, slides were removed from PBS/0.1% Tween, blocked with 100 μL blocking buffer containing 1 mg/mL BSA, 10% normal goat serum and 0.1 mg/mL tRNA for 20 mins at room temperature. Washes were followed by the addition of aptamers at a concentration of 100 nM to the slides for 15 mins incubation at 37°C. Following the wash with PBS/0.1% Tween thrice, for 5 mins each, slides were cover slipped as previously described prior to visualisation using confocal microscopy.

#### 2.6.4. Chromogenic staining protocol

For chromogenic staining of the EpCAM marker, procedures of deparaffinization, rehydration and antigen retrieval were carried out as the same protocol described above. After two washes with PBS/0.1% Tween, tissue sections were incubated in 0.3% hydrogen
peroxide for 20 min prior to blocking with BSA and 10% normal goat serum as previously described. Tissue sections were incubated with 100 nM FITC labelled EpCAM aptamer for 15 mins at 37°C, or EpCAM antibody (323/A3, 20 μg/mL) at 4°C overnight. After primary antibody/aptamer incubation, slides were incubated with 100 μL either goat anti-mouse anti-fluorescein HRP secondary antibody (AB6656, 10 μg/mL) or goat anti-mouse horseradish peroxidase (HRP) secondary antibody (31430, 1.6 μg/mL) for about 2 h. After two washes with PBS/0.1% Tween, tissue sections were treated with 100 μL DAB peroxidase substrate solution (Vector Laboratories, Burlingame, CA) for 5-10 mins at room temperature for colour development, followed by a single wash under running tap water. Counterstaining of tissue sections was performed by immersion in haematoxylin solution for 5 mins followed by washing under running tap water for 3-5 mins. Slides were then differentiated in 1% acid alcohol for 30 sec and washed under running water for 1 min prior to bluing with scott’s solution for 1 min. Slides were washed under running water for 5 mins and then dehydrated with 95% and 100 % alcohol serially. Finally, slides were cleared using two changes of histolene for 5 mins each, and then mounted using DPX. Stained sections were examined under a light microscope equipped with an Olympus SC20 camera (Victoria, Australia).

For chromogenic staining of the CD133 marker, deparaffinization and rehydration were performed as previously described. Following the previously described blocking process, slides were incubated with 400 nM FITC labelled CD133 aptamer for 30 mins at 37°C or CD133/1 antibody (W6B3C1, 20 μg/mL) at 4°C overnight. The slides were then incubated with 100 μL either goat anti-mouse anti-fluorescein HRP secondary antibody (AB6656, 4 μg/mL) or goat anti-mouse HRP secondary antibody (31430, 3.2 μg/mL) for about 2 h, respectively. Following incubation of primary and secondary antibodies, the slides were treated with DAB peroxidase substrate and counterstained as mentioned above.

2.7. Haematoxylin and Eosin staining

Tissue sections were deparaffinised using histoclear twice for 5 mins each, followed by rehydration with a series of graded alcohols (100%, 95%, and 70%). Slides were then immersed in the haematoxylin solution for 5 mins followed by washing under running tap water for 3-5 mins. Slides were then differentiated in 1% acid alcohol for 30 sec and washed under running water for 1 min prior to bluing within Scott’s solution for 1 min. Slides were washed under running water for 5 mins and then rinsed in 95% alcohol and counterstained
with eosin for 5 mins before the slides were dehydrated with 95% and 100 % alcohol serially. Finally slides were cleared using two changes of histolene for 5 mins each, and then mounted using DPX. Stained sections were examined under a light microscope equipped with an Olympus SC20 camera (Victoria, Australia).
CHAPTER 3
3. RESULTS

3.1. EpCAM Marker

3.1.1. Evaluation of EpCAM expression in cancer cell lines by Western blot

Before proceeding to immunohistology, the level of EpCAM expression on cells used in this study was analysed by Western blot. As shown in Fig 3.1, EpCAM expression was strong in HT29 cell line, whereas moderate expression was observed in T47D and MCF-7. Although MDA-MB-231 is considered to be weakly positive for EpCAM by flow cytometry, no expression was observed by Western blot. No EpCAM was detected in U118MG cells and thus it was used as a negative control in the demonstration of the specificity of the aptamer in this study.

Figure 3.1 - Evaluation of EpCAM expression in HT29 cells. Images are representative of three independent experiments. β-actin served as internal loading control.

3.1.2. Morphological study of xenograft tumours by haematoxylin and eosin staining

To confirm the tumour diagnosis of samples, haematoxylin and eosin staining was performed. The tumour sections were assessed by a qualified histopathologist and confirmed to be ductal carcinoma (T47D), breast adenocarcinomas (MCF7 and MDA-MB-231), colorectal adenocarcinomas (HT-29) and glioblastoma (U118MG) (Fig 3.2).
3.1.3. Immunofluorescence staining

3.1.3.1. Optimisation of antigen retrieval

Following an extensive review of the literature, two different methods were chosen for the development of an efficient antigen retrieval method using aptamers against EpCAM and were tested using the HT-29 cell line xenograft. The first method, protease digestion, did not show any positive staining following aptamer incubation. The second method, heat-induced epitope retrieval (HIER) was tested at 37°C and 95-100°C with both Tris-EDTA and citrate buffer. Antigen retrieval at 37°C did not yield any positive results with either buffer. HIER at 95-100°C was effective at unmasking the EpCAM epitope in HT-29 xenograft tissue sections and the results with the Tris-EDTA buffer at pH 8 showed staining of a superior quality to those obtained with the citrate buffer at pH 6. For subsequent experiments, Tris-EDTA was used as the antigen retrieval buffer for both EpCAM antibody and aptamer staining.

3.1.3.2. Optimisation of antibody concentration and incubation time

Once the antigen retrieval step had been optimized, the optimisation of the primary and secondary antibody concentrations, incubation time and temperature followed. Initially, two different primary antibodies (C10 and 323/A3 EpCAM antibody) were used to determine the optimal antibody concentration and to select the most sensitive antibody. Several experiments were carried out with these two EpCAM primary antibodies along with different concentrations of secondary antibody. Only the result of final optimisation experiments were shown in Fig 3.3. The two primary antibodies showed positive staining to a degree, with the 323/A3 antibody showing optimal results, which was then used in all subsequent experiments to compare the staining with aptamers. The optimal length of incubation time with EpCAM primary antibody (323/A3) was studied with HT29 paraffin embedded sections at different
points: 20 minutes and 1 h at room temperature or overnight incubation at 4°C. Moderate strong staining was observed at a concentration of 10 μg/mL, suggesting that the most effective incubation time for the primary antibody was overnight incubation at 4°C, and this antibody concentration and incubation time were adopted for rest of the studies (Fig 3.3). Secondary antibody was tested at different concentrations such as 2 μg/mL, 5 μg/mL and 10 μg/mL. As shown in Fig 3.3, a secondary antibody concentration at 10 μg/mL was found to be most effective, and this was used for all subsequent experiments.

**Figure 3.3 - Optimisation of EpCAM antibody concentration and incubation time.** (A) Represents EpCAM 323/A3 and C10 antibody (5 μg/mL) incubated at 20 mins with a secondary antibody concentration of 2 μg/mL. (B) Represents EpCAM 323/A3 and C10 antibody (10 μg/mL) incubated for overnight at 4°C with a secondary antibody concentration of 10 μg/mL. Images were taken at 60× magnification. Scale bar: 50μm.

### 3.1.3.3. Immunofluorescence staining using EpCAM antibody

After the optimisation of antibody concentration and incubation time with the colon cancer xenograft tumour sections, immunofluorescence staining with xenograft breast cancer, colon cancer and glioblastoma were performed. As expected, the staining intensity was highly dependent on the level of the EpCAM expression. Good staining was seen in HT29 when compared to other xenograft samples such as MDA-MB-231, MCF7 and T47D (Fig 3.4).
Moreover, staining was highly specific as there was no staining seen in the U118MG xenograft tissue.

Figure 3.4 - Immunofluorescence staining of xenograft tumours using EpCAM antibody 323/A3. Blue colour indicates nucleus staining, whereas red colour indicates EpCAM antibody staining. Images are representative of three independent experiments. All fluorescent images were taken at 60 × magnification. Scale bar: 50 μm.

In addition, to determine specific staining of the antibody, a study of the staining of each tumour section without the use of primary antibody was performed. As shown in Fig 3.5, no non-specific staining was observed in all xenograft samples, indicating the positive staining with EpCAM antibody in this study was specific.

Figure 3.5 - Control slides with omission of primary antibody. There was no non-specific staining seen in all the tissue sections. All fluorescent images were taken at 60 × magnification. Scale bar: 50μm.

3.1.3.4. Optimisation of EpCAM aptamers

Initial experiments with the EpCAM aptamer were performed using HT-29 xenograft tumour. A faint positive signal was observed when tissue sections were incubated with 100 nM EpCAM aptamers. Incubation times were increased in an attempt to enhance the positive signal from 15 min to 30 min but did not improve the staining intensity. Interestingly, the addition of 10% dextran sulfate and 500 μg/mL heparin to the aptamer staining solution prior to incubation with the tissue sections enhanced staining at the 15 min incubation.
3.1.3.5. Immunofluorescence staining using EpCAM aptamer

To confirm specific staining of the EpCAM aptamers, aptamer fluorescent staining of breast cancer, colon cancer and glioblastoma xenografts were performed. Aptamer-DT3 and -23 showed highly sensitive staining at a concentration of 100 nM for 15 minutes at 37°C. Aptamer 23 showed high specificity to T47D and HT-29 samples, especially when compared to that of aptamer DT3. The staining intensity was seen to be correlated with the reported level of EpCAM expression, which was demonstrated by means of strong staining pattern observed in the breast cancer xenograft T47D compared to weak staining in the MDA-MB-231 xenograft section. Immunofluorescent staining of EpCAM aptamers was shown to be highly specific, with no staining observed in the U118MG xenograft sections, or the control aptamer with all xenograft samples (Fig 3.6).

![Immunofluorescent staining of xenograft tumours using EpCAM aptamer](image)

**Figure 3.6 - Immunofluorescent staining of xenograft tumours using EpCAM aptamer.** Staining performed in breast cancer (MDA-MB-231, T47D and MCF7), colon cancer (HT-29) and glioblastoma (U118MG) xenografts by EpCAM aptamer DT3 and 23 and control aptamer. Blue colour indicates nucleus staining, whereas red colour indicates EpCAM aptamer staining. Images are representative of three independent experiments. All fluorescent images were taken at 60 × magnification. Scale bar: 50 μm.
3.1.4. Chromogenic staining

3.1.4.1. Optimisation of chromogenic staining

As fluorescence detection is not used in routine pathological diagnosis, chromogenic staining using aptamer is therefore highly desirable to translate aptamer-based staining into clinical practice. Therefore, the protocol for immunofluorescence staining using aptamer was further adapted and modified for chromogenic staining. This involved synthesizing an aptamer with a fluorescein label that could be detected with a horseradish peroxidase (HRP)-conjugated anti-fluorescein antibody. Given that EpCAM aptamer 23 showed superior staining to EpCAM aptamer DT3, the EpCAM aptamer 23 was chosen for chromogenic staining. In addition, HT29 xenograft tissue was chosen for subsequent experiments because the colon tumour sections showed superior staining compared to all other xenograft tissues using the fluorescence detection system (Fig 3.6).

Chromogenic staining of EpCAM aptamer was tested at different concentrations (100 nM, 150 nM and 200 nM) with various secondary antibody concentrations 3.3 μg/mL, 5 μg/mL, and 10 μg/mL in HT-29 xenograft tissue sections. Good staining of the xenograft tumour sections was observed at a concentration of 100 nM with EpCAM aptamer 23 and with the secondary antibody concentration at 10 μg/mL. For EpCAM antibody staining, either EpCAM 323/A3 or C10 primary antibody was used at a concentration of 20 μg/mL and tested with different secondary antibody concentrations such as 8 μg/mL, 3.2 μg/mL or 1.6 μg/mL. The best staining was observed for the EpCAM antibody 323/A3 at a concentration of 20 μg/mL with the optimal secondary antibody concentration at 1.6 μg/mL. As shown in Fig 3.7, positive staining (using both aptamer and antibody) was observed in HT-29 and no non-specific staining was observed with U118MG xenograft tumour section. The optimised aptamer and antibody concentration was maintained for the subsequent staining analysis performed in clinical samples.
3.1.4.2. Comparison of aptamer staining with antibody staining

To ascertain the clinical utility of the aptamer staining, one of the EpCAM antibodies used in pathology laboratories for staining EpCAM, BerEP4, was included in this study (Fig 3.8). Following the routine protocol for this antibody used in pathology diagnostic practice, we obtained similar staining of all xenograft tumours to those achieved using the 323/A3 antibody (Fig 3.3B, 3.4 and 3.7), indicating that our aptamers are much more sensitive than the standard antibodies in use in pathology laboratories.

The quantification of EpCAM staining in different xenografts was performed using DT3, 23 and control aptamers and 323/A3 antibody by immunofluorescence and immunohistochemistry using BerEP4 antibody (Table 3.1). These data indicate that the
aptamers are much more sensitive than the standard antibodies used in pathology laboratories.

Table 3.1 Quantification of anti-EpCAM staining in cell lines.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>T47D</th>
<th>MCF7</th>
<th>MDA-MB-231</th>
<th>HT-29</th>
<th>U118MG</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT3 Aptamer</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23 Aptamer</td>
<td>3+</td>
<td>2+</td>
<td>1+</td>
<td>3+</td>
<td>-</td>
</tr>
<tr>
<td>Control Aptamer</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>323/A3 Antibody</td>
<td>1+</td>
<td>1+</td>
<td>-</td>
<td>2+/3+</td>
<td>-</td>
</tr>
<tr>
<td>BerEP4 Antibody</td>
<td>-</td>
<td>1+</td>
<td>-</td>
<td>3+</td>
<td>-</td>
</tr>
</tbody>
</table>

‘-’ no staining; ‘1+’ faint incomplete staining in >10% of cells; ‘2+’ moderate complete membrane staining; ‘3+’ strong complete membrane staining.

3.1.4.3. Chromogenic staining of clinical samples

Following the successful evaluation of chromogenic staining in xenograft tissue sections, the EpCAM-23 aptamer sensitivity was compared to EpCAM antibody in clinical samples. Archived FFPE histopathology samples from eight breast cancer patients with matched tumour and lymph node metastasis sections were independently evaluated for aptamer and antibody staining intensity. In 14 of the 16 sections, the aptamer staining intensity of the tumour cells was superior to that seen with the antibody (Table 3.2, Fig 3.9). Indeed, in a number of breast tumours, no obvious staining was observed with the EpCAM antibody (Fig. 3.9L, J), while the aptamer showed a moderate positive signal (Fig. 3.9K, L). One lymph node could not be assessed as only 1% of the lymph node showed tumour and this area was missing from the section for evaluation. This case was used as a negative control (Fig. 3.9Y, Z) showing that both the antibody and aptamer stained specific EpCAM positive cells, and were not prone to non-specific staining. Interestingly, only one breast ductal carcinoma showed a slightly superior staining with the antibody compared to the aptamer (Fig 3.9Q, R). The specificity of our EpCAM aptamer was demonstrated by no cross-reactive staining of the normal cells or lymphocytes within the tissue sections (Fig. 3.9Z, AA). To confirm that non-specific binding was not the cause of the increased sensitivity seen with our aptamer, normal human liver (EpCAM negative) sections were also evaluated for staining and all showed no
discernible DAB signals (Fig. 3.9AB). Staining of both positive and negative control slides showed appropriate reactivity indicating the correct optimization of both antibody and aptamer staining protocols. As a negative control, aptamers were omitted from the staining reaction and no non-specific staining was observed.

Table 3.2 Clinical details and immunostaining results of breast tumour cases.

| Patient | Pathological diagnosis | Breast Tumour | Lymph Node | | | |
|---------|-----------------------|---------------|------------| T | N | M |
| 1       | invasive ductal carcinoma | 2+ | 3+ | 1+/2+ | 2+ | 3 | 1 | 0 |
| 2       | invasive ductal carcinoma | 0 | 2+ | 0 | 1+/2+ | 2 | 2 | 0 |
| 3       | invasive ductal carcinoma | 0 | 2+/3+ | 1+ | 2+ | 4 | 3 | 0 |
| 4       | mucinous carcinoma | 0/1+ | 2+ | No Tumour | 1+/2+ | 3 | 1 | 0 |
| 5       | invasive ductal carcinoma | 0 | 2+/3+ | 0 | 1+/2+ | 1 | 3 | 0 |
| 6       | invasive ductal carcinoma | 0 | 2+/3+ | 1+ | 1+/2+ | 3 | 3 | 0 |
| 7       | invasive ductal carcinoma | 3+ | 2+ | 1+ | 1+/2+ | 3 | 0 | 1 |
| 8       | invasive ductal carcinoma | 0 | 2+ | 0 | 1+/2+ | 1 | 3 | 0 |

*'- no staining; ‘1+’ faint incomplete staining (negative); ‘2+’ moderate complete membrane staining (equivocal); ‘3+’ strong complete membrane staining (positive) [147].
Figure 3.9 - Chromogenic staining of EpCAM aptamer and antibody in breast cancer. A – H: EpCAM antibody immunostaining was weaker in both the breast tumour (A (× 40), B (× 400)) and lymph node (E (× 40), F (× 400)) in comparison to EpCAM aptamer immunostaining in patient 1 (Breast tumour C (×40), D (×400); and lymph node G (× 40), H (× 400)); I – P No immunostaining was observed with the EpCAM antibody in the breast tumour (I (× 40), J (× 400)) or the lymph node (M (× 40), N (× 400)) while the EpCAM aptamer showed a strong positive signal in both the breast (K (× 40), L (× 400)) and lymph node (O (× 40), P (× 400)) in patient 5; Q – X Immunostaining with EpCAM antibody was stronger in the breast tumour (Q (× 40), R (× 400)) but not the lymph node (U (× 40), V (× 400)) than the EpCAM aptamer (Breast tumour S (× 40), T (× 400); and lymph node W (× 40), X (× 400)) in patient 7; Y – AB Representative images of negative control tissues. Patient 4 showed negative areas of lymphocyte staining within the lymph node by EpCAM antibody (Y (× 400)) and EpCAM aptamer (Z (× 400)); Patient 2 showed normal regions of lymph node that was negative by EpCAM aptamer (AA (× 400)); Normal liver sample negative for EpCAM by EpCAM aptamer (AB (× 400)). All pictures were taken under a light microscope with × 40 magnifications and × 400 magnifications (taken from the centre of the × 40 magnification).
3.2. CD133 marker

3.2.1. Evaluation of CD133 expression in cancer cell lines by Western blot

The level of CD133 expression in cell lines to be used in this study was analysed by Western blot analysis. As shown in Fig 3.10, HT-29 colon cancer cells showed strong expression of CD133, while no signal for CD133 was discernible in U118MG glioma cells.

![Figure 3.10 - Evaluation of CD133 expression in HT-29 cells. Images are representative of three independent experiments. β-actin served as internal loading control.](image)

3.2.2. Chromogenic staining

As a chromogenic staining protocol using EpCAM aptamer-substrate system was successfully optimized for light microscopy evaluation, the same protocol was followed and optimised for the FITC-labelled CD133 aptamer.

3.2.2.1. Optimisation of antigen retrieval

For chromogenic staining, HT-29 and U118MG were used as a positive control and negative control, respectively. HIER was performed at 95-100°C using citrate buffer (pH 6), sodium citrate buffer (pH 6) and Tris EDTA buffer (pH 8). The CD133 aptamer was used to test these different antigen retrieval buffers and it was found that the citrate buffer at pH 6 showed staining of a superior quality to those obtained with the sodium citrate buffer at pH 6 or Tris-EDTA buffer at pH 8.

3.2.2.2. Optimisation of antibody concentration and incubation time

As citrate buffer was shown to be effective at unmasking the CD133 epitope, it was used for the subsequent experiments with the CD133/1 antibody and the same staining protocol was followed as described in section 2.6.4. The staining was effective when the primary antibody was incubated overnight at 4°C at a concentration of 20 μg/mL, followed by incubation with a goat anti-mouse HRP secondary antibody for about 2 h at room temperature. Initial
experiments showed some staining in U118MG, when the slides were blocked with normal goat serum and BSA for about 20 mins. However, there was no staining observed in U118MG xenograft tissue sections when the blocking incubation time was increased to 1 h (Fig 3.11).

**Figure 3.11 - CD133 antibody staining in HT-29 and U118MG xenograft tissue.** All images were taken at 40× magnification. Scale bar: 50 μm.

3.2.2.3. Optimisation of CD133 aptamer

With antigen retrieval using citrate buffer at pH 6, initial experiments with the CD133 aptamer at 200 nM did not show positive staining, while moderate staining was observed when the concentration of the aptamer was increased to 400 nM. As the pH in the environment for aptamer can affect the folding and thus the 3-D structure of aptamer, the staining of the HT-29 xenograft tumour sections with CD133 was investigated under different pH incubation buffers (PBS containing 2.5 mM MgCl₂ with pH of 6.0, 6.2, 6.4, 6.8 or 7.0). The pH of PBS with 2.5 mM MgCl₂ at 6.6 showed the best staining in comparison to the other pHs (Fig 3.12).
3.2.3. Chromogenic staining of xenograft tumour samples

Ten different HT-29 and U118MG xenograft tissue sections were selected, and the staining was performed using both the aptamer and the antibody as previously described. CD133 aptamer at a concentration of about 400 nM were prepared using PBS/2.5 mM MgCl₂ with a pH of 6.6 or CD133 antibody at a concentration of 20 μg/mL. A goat anti-mouse anti-fluorescein HRP secondary antibody (4 μg/mL) or goat anti-mouse HRP secondary antibody (3.2 μg/mL) was used to develop the signal from the CD133 aptamer or antibody for 2 h, respectively. Both aptamer and antibody staining was moderate in four HT-29 tissue sections (Fig 3.13c, d, k, m). In Fig 3.13 o, antibody staining appeared to be negative, though remarkably there was strong positive signal seen in the cytoplasm with aptamer staining. In Fig 3.13a, b, e, l, n, antibody staining appeared to be weak but strong positive staining was observed with the aptamer. Overall, the aptamer staining was superior compared to antibody staining. To demonstrate the staining intensity exactly, all the images were taken in the same area from the respective different tissue block. There was no staining observed in the U118MG, either when stained with aptamer or antibody and therefore, indicated the specificity of the staining.
Figure 3.13 - Chromogenic staining of HT-29 and U118MG xenograft tissue section. Images are representative of ten different HT-29 and U118MG xenograft tissue sections. Images a-e and k-o represents HT-29 xenograft tissue sections and images f-j and p-t represents U118MG xenograft tissue sections. Images a, b, n, e, l, o represents strong positive staining of CD133 aptamer. Images m and c represents moderate staining of CD133 aptamer. Images k, d represents weak staining of CD133 aptamer. All the images were taken in the same area from the respective different tissue block at 40× magnification. Scale bar: 50 μm.
3.2.4. Chromogenic staining of clinical samples

Archived FFPE histopathology samples from liver cancer patients were evaluated for aptamer and antibody staining intensity (Table 3.3). Following the successful chromogenic staining of xenograft tissues, the same protocol was followed in the staining of CD133 in three different hepatocarcinoma tissue samples. One tissue section from each sample was stained with either hematoxylin and eosin to confirm the morphology of the tumour, or anti-CD133 antibody or CD133 aptamer. In all three samples, aptamer staining was superior to antibody (Fig 3.14). In sample 1, the aptamer incubation showed perinuclear staining while the antibody mostly stained the cytoplasmic region. Both the aptamer and antibody staining in sample 2 and 3 showed extensive staining in the cytoplasm. To confirm specificity of aptamer staining in clinical samples, HT-29 and U118MG cell line xenografts were included as a positive and negative control, respectively.

Table 3.3 Clinical details and immunostaining results of liver tumour cases.

<table>
<thead>
<tr>
<th>Patient Sample</th>
<th>Patient details</th>
<th>Liver tumour</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sex</td>
<td>Age</td>
<td>Antibody Staining</td>
<td>Aptamer Staining</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Male</td>
<td>43</td>
<td>1-2+</td>
<td>3-4+/2-3+</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>61</td>
<td>1-2+</td>
<td>2-3+</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>56</td>
<td>1-2+</td>
<td>3-4+/2-3+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Score (1-4+) based on the cytoplasmic staining intensity. 1-2+ weak staining, 2-3+ moderate, 3-4+ strong.
Figure 3.14 - Chromogenic staining of CD133 aptamer and antibody in liver cancer tissue section. To compare antibody and aptamer staining, all the images were taken from the same area in respective different slides. All images were taken at 40× magnification. Scale bar: 50 μm.
4.1. DISCUSSION

Cancer diagnosis is very important to the prognosis and well-being of the patient. Various diagnostics tests are available to identify the specific type of cancer, but determining the cellular or molecular distinguishing features of the cancer is important for initiation of the appropriate treatment at the right time [149]. Cancer cells are usually detected with an over expression of certain molecules known as tumour markers which are used to help diagnose cancer, predict prognosis, and also assist with monitoring response after treatment [18]. An accurate identification of tumour markers is also important to isolate and identify CSCs from tumours [150]. Currently, a large number of tumour markers are being identified due to the advancement in the area of bioinformatics and proteomics [149].

Immunohistochemistry (IHC) is considered to be an important technique that is applied in pathology and research laboratories for the identification of tumour markers [104, 151]. For the past several decades, antibodies have been utilised for the identification of these markers as they can detect antigens with high affinity and specificity. However, there are certain limitations associated with antibodies. Some of the main issues associated with the antibodies are their size, batch to batch variation, and sensitivity to temperature [89]. These issues prompt the use of alternative classes of diagnostic molecules, such as aptamers, that were first described more than two decades ago [152].

Aptamers have the potential to revolutionise the field of diagnostic agents [128]. However, while aptamers show great promise compared to antibodies, there have been limited reports on their use as probes for IHC. The very first use of aptamers in IHC was performed by Zeng et al, who demonstrated that the CD30 aptamer was superior when compared to staining with the CD30 antibody in FFPE tissues [142]. One of the important advantages in this study was that they showed that aptamers required less incubation time, about 20 mins, when compared to antibody incubation time of about 90 mins. Simmons and colleagues performed a similar study where they demonstrated that aptamers were able to detect heparanase superior to the HPSE1 antibody control [153]. They performed both IHC and immunofluorescence studies and found that the aptamer was able to produce consistent staining results when compared to the antibody. Following the success of aptamers in IHC by Zeng and Simmons group, we sought to determine if our previously characterised aptamers directed against EpCAM [62]
and CD133 [146] were also capable of being used as diagnostic agents for the detection of cancer stem cell markers in pathology laboratories.

EpCAM is a tumour marker with its overexpression documented in both primary and metastatic breast cancer [66]. Increasing EpCAM expression is associated with adverse clinical outcomes in these patients, especially in metastases [72, 154, 155]. Following these immunohistochemical studies and the results of certain EpCAM immunotherapy trials, it has been suggested that the level of EpCAM expression be tested for in cancer patients, given the increased use of immunotherapy and the use of anti-EpCAM antibodies for the treatment of tumours that express high levels of EpCAM [69, 72]. Indeed, it has even been suggested that the failure of some trials of anti-EpCAM immunotherapy could be related to the lack of prior knowledge of EpCAM expression in the tumours to be treated [69]. Therefore, a sensitive and reliable method for evaluating EpCAM expression is essential for successful immunotherapy in personalised medicine [69]. Here we demonstrate that aptamers are not only suitable but also superior to antibodies for histological diagnosis.

To confirm the sensitivity of our aptamers, we initially chose several breast cancer cell lines that have a known level of EpCAM expression on their cell surface, as well as a colon cancer cell line that has a known high level of EpCAM expression. It has been reported that HT-29 has 2.3 million EpCAM binding sites on the cell surface [156], while T47D and MCF-7 have similar levels of expression (0.22 million binding sites per cell) [157, 158]. These results were confirmed by Western analysis (Fig 3.1). MDA-MB-231 has a very low level of EpCAM expression, with a reported 1.7 thousand EpCAM binding sites per cell [157]. In this study, our aptamer was able to detect low levels of EpCAM expression on the surface of breast cancer MDA-MB-231 cells, even though this cell line is regarded as EpCAM negative due to the inability of antibodies to detect its expression. Conceivably, low levels of EpCAM expression may be missed during diagnosis using conventional antibody staining.

However, when fully optimized, our aptamer staining protocol was able to stain this weakly positive EpCAM expressing xenograft tissue as well as HT-29 xenograft tissue with a dynamic range of 3 orders of magnitude, thus confirming the high sensitivity of our detection system. In this study, aptamer DT3 did not show any staining of HT-29 xenograft sections, while the other aptamer tested, aptamer 23, showed strong positive staining. One possibility is that the particular epitope that DT3 binds to on the EpCAM molecule may be lost or
remains inaccessible due to other antigens on the cell surface masking that epitope on this particular cell line. However, given the positive reactivity seen in the three breast cancer xenografts, it is unlikely that a loss of epitope is the cause. Therefore, it is more likely that this particular epitope remains masked on this cell line following antigen retrieval. As well, the lower affinity that aptamer DT3 has for this cell line may also play a role and it could be a combination of these two factors that lead to a lack of immunostaining.

The chromogenic staining protocol was optimized using EpCAM aptamer 23, as it showed a superior fluorescence staining result. As expected, the EpCAM aptamer staining in all breast cancer patient samples was superior to antibody staining except in one case. Traditional negative controls, such as our glioblastoma xenograft and normal human liver, were incorporated into each staining experiment. An additional negative control, the omission of the aptamer from the staining reaction, was performed for all tumour cases to confirm the enhanced sensitivity of our aptamer was not due to non-specific binding of the chromogen. As well, staining of normal cells was assessed in all lymph node cases. Specificity was confirmed by the lack of staining of lymphocytes within each assessed section with positive staining. The inclusion of positive and negative control cancer cell line xenografts provided additional evidence on the specificity of both the aptamer and antibody. The reliability and reproducibility was demonstrated by the consistent staining patterns observed with the positive and negative xenograft tissue sections throughout the entire study.

On the whole, our EpCAM aptamer showed superior staining results when compared to the antibody in both human xenograft tumours via fluorescently labelled aptamer and in primary human breast cancers using chromogenic staining. These results are consistent with findings by others that aptamers display a high level of sensitivity and low background staining [62, 142]. Given the results from the present study, it is possible that the level of EpCAM positivity is underestimated in primary and metastatic tumours using conventional antibodies. It has been well documented that antigen density is a critical factor in the effectiveness of the detection of antigens by monoclonal antibodies [159]. Therefore, it is possible that low levels of EpCAM expression may be missed during diagnosis using conventional antibody staining. This phenomenon was reported for the oestrogen receptor in an external quality control scheme (UK-NEQAS-ICC), where tumours expressing low levels of oestrogen receptors were often falsely reported as negative [160]. The staining intensity and sensitivity of our aptamer would improve detection in these cases and would lead to more accurate and
quantitative assessment of the level of EpCAM expression, thus better informing the clinician when deciding on targeted treatment options. Considering that our EpCAM aptamer staining was much more superior when compared to both 323/A3 and Ber-EP4 antibody, which regrettably produced weaker or inconclusive results in most cases, additional aptamer staining will more likely reduce these diagnostic pitfalls.

CD133 is also considered to be an important marker for identifying CSCs, along with other CSC markers [161]. Until now, the specific function of the CD133 molecule has not been fully elucidated, though it is considered to be an important marker for identifying CSCs, alone or in combination with other CSC markers [162]. A number of studies have confirmed that CD133 expression correlates with metastasis, overall survival rate, recurrence, and resistance to drugs in patient samples [77, 161, 163]. However, some studies have suggested that there was no correlation between CD133 expression and survival rate. The following table describes the relationship between CD133 expression and survival rate in colon and liver cancer patients using IHC (Table 4.1).

Table 4.1 Relationship between CD133 expression and survival rate.

<table>
<thead>
<tr>
<th>Colon cancer</th>
<th>Hepatocellular carcinoma</th>
<th>References</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patient samples</td>
<td>Association of CD133 expression with survival rate</td>
<td>Number of patient samples</td>
<td>Association of CD133 expression with survival rate</td>
</tr>
<tr>
<td>523</td>
<td>No: overall survival</td>
<td>136</td>
<td>Yes: overall survival</td>
</tr>
<tr>
<td>233</td>
<td>Yes: overall survival</td>
<td>[164]</td>
<td>[163]</td>
</tr>
<tr>
<td>77</td>
<td>Yes: cancer-specific survival</td>
<td>63</td>
<td>Yes: overall survival</td>
</tr>
<tr>
<td>104</td>
<td>Yes: overall survival</td>
<td>[165]</td>
<td>[166]</td>
</tr>
<tr>
<td>160</td>
<td>Yes: overall survival</td>
<td>12</td>
<td>No: overall survival</td>
</tr>
<tr>
<td>140</td>
<td>No: recurrence free survival</td>
<td>[167]</td>
<td>[168]</td>
</tr>
<tr>
<td>104</td>
<td>Yes: overall survival</td>
<td>24</td>
<td>No: overall survival</td>
</tr>
<tr>
<td>160</td>
<td>Yes: overall survival</td>
<td>[169]</td>
<td>[170]</td>
</tr>
<tr>
<td>140</td>
<td>No: recurrence free survival</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>104</td>
<td>Yes: overall survival</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
The discrepancy in these results might derive from analysis of different subgroups of patients with the same tumour, variation in the protocols followed, the use of commercial antibodies with varied background and variations in the interpretation of results. Thus, the use of different antibody clones or different batch of antibodies could be one of the reasons for the inconsistent study conclusions. Indeed, it was recently shown that there was inconsistent immunostaining results using different CD133 antibody clones, such as AC133, W6B3C1, C24B9 and ab19898 for the detection of CD133 antigen in glioblastoma [81]. From this study, it was reported that detecting CD133 antigen using CD133 antibodies might produce different staining results and also indicates the importance of utilising other detection methods or using different antibodies to confirm the results [79].

It has been suggested that the inconsistent results observed with different CD133 antibodies may be due to (i) the binding of the antibodies dependent on the glycosylation status of the CD133 molecule, such as the epitope recognised by the AC133 antibody being masked due to differential glycosylation [82] or (ii) the absence of corresponding epitope during evaluation. The various problems behind the detection of the CD133 antigen highlights the need for a standardized method for identifying this molecule, which may answer the question as to whether this antigen is indeed associated with a poor prognosis, as some studies suggest. Therefore, it is important to detect the expression of CD133 in patient samples in the hope that it may enhance treatment options either alone or alongside with other CSC markers.

Having already optimised the protocol to achieve consistent staining results with the EpCAM aptamer 23 and devised a method for chromogenic staining, we sought to determine if the same protocol could be used for aptamers directed against different cell surface markers. Therefore, ten different colon cancer xenografts were selected and staining was performed using aptamer or antibody. In the majority of the xenograft tissue sections, the staining results of our aptamer were consistently more sensitive than antibody staining. One of the notable results was the CD133 aptamer showing strong positive staining in one xenograft sample, where antibody staining was negative. The specificity of the staining could be due to the difference in the recognition of the epitope by aptamer or antibody, in a similar manner to that observed when different antibody clones directed against the same antigen are used.

It has been shown that there is a correlation between HCC patient and CD133 positive cells, particularly for overall patient survival with stage III and IV HCC [163]. Therefore, we
sought to determine if the CD133 aptamer might be superior in detecting CD133 in this
patient cohort. We analysed three liver tumour patient samples using both CD133 aptamer
and antibody and found there was a large difference between the CD133 aptamer and
antibody staining. Indeed, the CD133 aptamer staining was consistently superior to that of the
antibody.

One of the most remarkable results was that peri-nuclear staining was observed with the
CD133 aptamer, whereas the antibody showed cytoplasmic staining in samples derived from
liver cancer patients. The first subcellular localisation of CD133 antigen in HCC patients was
proposed by Sasaki et al and they showed that there was a positive correlation between
CD133 expression and hepatocellular carcinoma patient survival (Table 4.2) [163]. Our
aptamer showed peri-nuclear staining in sections of liver cancer which was consistent with
this previous study in identifying the subcellular expression of CD133 antigen, whereas
antibody staining was more diffuse. Due to the fact that there were different antibodies used
and different protocol were followed in various laboratories, peri-nuclear staining may not be
observed in some patient samples, which could lead to negative results being reported. Given
that peri-nuclear staining has been linked to poor prognosis, a better, more sensitive method
of detection for this antigen is required.

Thus, we have demonstrated that EpCAM aptamers or CD133 aptamers were suitable for
histological diagnosis. The following table shows the comparison of our EpCAM and CD133
aptamer with its respective antibodies to the use in histological slides (Table 4.2).
Table 4.2 Comparison of EpCAM/CD133 immunostaining of aptamer vs. antibody.

<table>
<thead>
<tr>
<th>APTAMER</th>
<th>ANTIBODY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Easy membrane penetration because of its smaller size, and binds to larger number of target molecules.</td>
<td>Difficult penetration and decreases its access to smaller target molecules.</td>
</tr>
<tr>
<td>Incubation time of aptamer is 15-30 mins.</td>
<td>Longer incubation period for primary antibody.</td>
</tr>
<tr>
<td>Less time consuming protocol.</td>
<td>Time consuming protocol (in case of overnight incubation of primary antibody)</td>
</tr>
<tr>
<td>Good staining sensitivity.</td>
<td>A range of sensitivities depending on the antibody used.</td>
</tr>
<tr>
<td>A low concentration is sufficient.</td>
<td>A high concentration is needed.</td>
</tr>
</tbody>
</table>

While extensive optimization was required in the attempt to use these ligands as chemical antibodies, the realization that these probes are nucleic acids and therefore might require a combinational approach of both conventional antibody staining, and methods similar to those used for in situ hybridization, led to successful results [172-174]. Several optimisation procedures, such as the addition of dextran sulphate accelerate the rate of nucleic acid hybridization [172], thus reducing the time required for aptamer binding, while heparin reduces background binding in the hybridization procedure [174]. When either of these two reagents was omitted from the hybridization buffer, positive staining was not achieved, even when incubation time was increased. Indeed, this approach worked for all aptamers used in this study. It is anticipated that this improved protocol will prove to be highly effective for staining other biomarkers in paraffin embedded tissues using aptamers.

The CD133 aptamer required some additional optimisation as some inconsistencies were observed with initial optimisation protocols. The variation in staining results between the CD133 aptamer with different pH in buffer was also investigated in this study. It is
anticipated that pH could have an effect on hydrogen bonding between bases in the aptamer, which in turn might affect the folding of the aptamer and ultimately the binding to the epitope [175]. However, the influence of pH on the CD133 aptamer binding to the antigen needs to be further studied. In addition, as with antibodies where different concentrations of different clones are required to achieve equivalent staining positivity, a similar situation was observed with the EpCAM and CD133 aptamers, most probably due to the difference in binding affinities of these aptamers to their respective targets.

It has been suggested that there might be non-specific staining due to electrostatic attraction of polyanion nucleic acid aptamers to positively charged sites, such as histones present in the nucleus [176]. This could be a reason for the smaller number of published data on the use of aptamer in histology. In our study, to confirm the specific staining of either aptamer or antibody, we used glioblastoma xenograft tissue (U118MG) as a negative control that is known to be devoid of the tumour marker of interest (EpCAM and CD133). In addition, the lack of expression of these two markers in the U118MG cell line was confirmed by Western blot assay. Traditional negative control such as omission of aptamer or primary antibody from the staining reaction was also performed for both EpCAM and CD133 markers. The use of negative control slides indicates that the specificity of our aptamer was not due to non-specific binding. Moreover, uses of these proper controls are important to avoid false positive or false negative results and also help in interpretation of results. According to the staining results of both EpCAM and CD133 aptamers used in this study, it is anticipated that the different aptamers possess different properties. Therefore, the protocol for the use of individual aptamers need to be optimised independently.
4.2. CONCLUSION

In regards to all the above mentioned facts, it could be anticipated either additional aptamer staining could be performed to confirm the staining results or antibodies could be replaced by aptamers in pathological laboratories. One recent editorial “Where are all the aptamers?” clearly described that only a few laboratories have utilized aptamers, and suggested that it may take another few years to establish aptamers in clinical applications [177]. The paragon to describe this is the fact that the first antibody was produced in 1975, a single monoclonal antibody was approved by FDA in 1986, though it took a further 10 years to reach the market [178]. Currently, aptamers are in the development phase, from pre-clinical to clinical trials and one therapeutic aptamer was approved by the FDA in 2004. The notion of replacing aptamers instead of antibodies in immunohistochemistry (IHC) is mainly due to the fact that the antibodies possess limitations such as they are larger in size, have batch-to-batch variation, and also requires in vivo production. So the use of aptamers for the detection of markers in paraffin embedded tissues could be a new approach for better diagnosis. Indeed, the results from this study suggest that aptamers can provide a robust and cost-effective tool to translate discoveries from biomarker and cancer stem cell research in to pathology diagnostic practice to better stratify patients for personalised medicine. Our EpCAM and CD133 aptamer staining was found to be superior in terms sensitivity for detecting cancer stem cell markers in FFPE specimen when compared to that using EpCAM 323/A3 and CD133-1 (W6B3C1) antibodies. Moreover, our EpCAM aptamer results indicate the possibility that low level of EpCAM expression may be missed during diagnosis with currently available EpCAM antibodies. Similarly, peri- nuclear staining has been shown to be associated with the prognosis of the patient and this was observed with only CD133 aptamer but not with antibody staining in our studies. These findings have demonstrated the distinct advantages of using aptamers as powerful probes for immunohistology and indicated a potential demand for the use of aptamers in IHC, as these results are important from the clinical point of view. Given that the aptamers in this study were more sensitive than the antibodies tested, this study paves the way for the application of these molecular probes in future histopathological diagnosis and potentially for therapeutic applications.
4.3. FUTURE DIRECTION

This research has been the groundwork for the use of aptamers to detect EpCAM and CD133 markers in paraffin embedded tissues. Recent findings suggest that there is a high level of expression of EpCAM and CD133 in CSCs [55] compared to that in normal cells. To successfully identify the CSC population in paraffin embedded tissue sections, a double or triple staining method is required, since the identification of CSCs mainly depends on the reliable detection of multiple markers. Due to the technical advancement of immunohistochemistry (IHC) techniques, it is possible to detect multiple markers using IHC. This project showed that aptamers, by virtue of their small size, are superior to antibodies in terms of sensitivity. Therefore, it is possible to use our aptamer for the identification of CSCs by double or even triple staining.

Even though many methods such as flow cytometry and side population assays have been applied for the detection of CSCs, each method has its own advantages and disadvantages. Thus, an additional study is required to increase the specificity and sensitivity of the existing method. Furthermore, for solid tumours, the amount of tissues available is usually limited, and thus often precludes the use of flow cytometry. A recent study has demonstrated the use of IHC for the identification of CSCs using two markers that implies that CSCs can be identified using IHC techniques. However, the use of antibodies for double staining protocols has some intrinsic limitations due to the potential steric hindrance caused by the large (150 kDa) size of the antibodies. In contrast, aptamers have several advantages, including its small size (~6 kDa), as mentioned in section 1.5.3. Therefore, the use of aptamers for the identification of CSCs could represent one of the future methods to enhance the capability of identification of CSCs in IHC. The further development of aptamer-based IHC technology in identifying CSC in pathology specimens will provide pathologists with a highly sensitive and reproducible method to assess the nature and extent of CSCs in a biopsy sample, which in turn will provide invaluable information on the propensity of the tumour to respond to a particular treatment regimen and the risk of metastasis and relapse. The work presented in this thesis has set a solid foundation for the development of a new aptamer-based molecular diagnostics enabling a new era of personalised cancer care.
CHAPTER 5
5. REFERENCE


