1.0 Introduction

The cell block technique employs the retrieval of small tissue fragments from a FNA specimen which are processed to form a paraffin block. [15] A variety of cell block techniques have been in use for over a century.[15] The use of cell blocks is being increasingly advocated in the diagnostic work-up of patients with masses amenable to FNA since they provide architectural information and allow for additional investigations which complement FNA smears.[15]

1.1 Objectives of study

1.1.1 The purpose of this study was to determine the effectiveness of the cell block technique in our setting, by comparing cytomorphologic preservation and immunocytochemistry (ICC) staining reactivity on paired cell block and conventional fine needle aspiration (FNA) smear samples.

1.1.2 The aim of this study was to evaluate the cell block technique with a view to the implementing this process into routine diagnostic practice to aid cytodiagnosis and immunocytochemistry.

1.2 Importance of the problem

One of the constraints of the conventional FNA smear is the limited material available for adjuvant diagnostic investigations. This is due to many factors including the skill and experience of the aspirator to reliably obtain sufficiently cellular material.[3] As a result, only a small number of slides may be available for immunostaining.[3] The quality of smears is also variable.[3] They may contain thick clumps of cells with poor cell

distribution on slides and proteinaceous debris, which tends to contribute to high levels of background immunostaining.[3-13] Destaining conventionally prepared cytological preparations for immunocytochemical assays may result in loss of valuable material. [3-13] There are several articles in the literature that state that these dilemmas can be easily circumvented with the use of the cell block technique enabling the retrieval of small tissue fragments from an aspirate specimen which are processed to form a paraffin block.[3] The ability to obtain numerous tissue sections allows for multiple immunostains to be performed akin to paraffin sections in histopathology.[14]

1.3 Limitations of this undertaking

We obtained samples from FNA clinics held at the radiology departments at the Chris Hani Baragwanath and Charlotte Maxeke Academic Hospitals in Johannesburg, South Africa. The skill of the radiologist is paramount in obtaining adequate tissue and there may be a variation in FNA technique amongst personnel thus affecting results. [16,17,18] Some of the cell block samples contained degenerate cells. This may have been attributed to a delay in immersing the cell block specimen into fixative immediately after collection and lack of a standardised FNA technique. The absence of a dedicated needle pass for cell block preparation in a proportion of cases contributed to lower cell yield. Proper training of radiologists in FNA technique will improve and standardise sample collection. We only assessed 6 of the antibodies (CK7, CK20, TTF1, Synaptophysisn, Hepar-1 and CK-AE1/3). Some immunostains have their own idiosyncrasies and the technique may require modification for these.

1.4 Significance of this study

This study confirms the value of cell block preparation of FNA samples in cases where the diagnostic work-up requires immunocytochemical assessment. It highlights the limitations of poor cell yield and preservation. These must be addressed and remedied before this technique can be implemented routinely into the diagnostic immunocytochemistry laboratory. This study has also established the methodology for the preparation of cell blocks.

1.5 Preview of the organisation of the rest of dissertation

Study Design

This was a prospective study. Material for the preparation of FNA smears on glass slides (direct smears) and cell block samples were collected simultaneously during fine needle aspiration of 50 cases comprising of primary or metastatic adenocarcinoma of the lung and metastatic adenocarcinoma to liver or lymph nodes.

To determine the agreement between the two methods of sample preparation, analysis of the grading (on an ordinal scale) of cellularity, morphological preservation, architectural preservation, immunocytochemical staining intensity and presence of background staining was performed on paired FNA smears and cell block samples (refer to Table 2.1). Each arm of the paired analysis was performed blindly without knowledge of the grading outcome of the other. The cellularity, morphology and architecture of each FNA smear was evaluated using the Papanicoloau (Pap) stain and/or Diff-Quik stain, where the Pap stain was used for conventional immunocytochemistry (ICC) [19] and that of the cell

block samples was evaluated using the Haematoxylin and Eosin (H/E) stain as appears to be standard procedure in the literature. [3-13]

1.6 Past and present status of the problem: literature survey

In the past, surgical pathology was deemed to be of higher diagnostic value than cytology.[1] However, in recent years there has been a change in perception due to cytological tests displaying increased diagnostic specificity for preliminary diagnosis.[1] Radiological imaging techniques are used to guide deep biopsies and ultrasonography is helpful even when the lesion is palpable as it delineates the lesion exactly, indicates the optimal depth of the biopsy and can guide the needle to a solid portion if the lesion is partly cystic.[2] Fine needle aspirations (FNA) are safe, easy to perform, minimally invasive, inexpensive, and well tolerated by patients, the vast majority performed as an outpatient or bedside procedure. It remains an excellent diagnostic procedure for preliminary diagnosis. [2, 20, 21] An FNA is performed by inserting a fine needle (22gauge or smaller) with an attached syringe into a mass lesion and aspirating material from the lesion. The sample contained in the needle is carefully expelled onto a clean microscopy slide using air in the syringe. For the cell block sample, the same needle is rinsed in Shandon Formal-Fixx® fixative. Occasionally, for the cell block preparation, a dedicated needle aspiration is made by expelling all the material in the needle into the Shandon Formal-Fixx® fixative. In this cytology unit, four direct smear preparations are produced by steadily moving another glass slide over the sample, while exerting light pressure to achieve a thin even spread. Two of these smears are immediately fixed in alcohol for Papanicolaou (Pap) staining (refer to Appendix D), and two are air-dried for Diff-Quik

staining (refer to Appendix E). These stains facilitate the nucleic and cytoplasmic examination of the smears under the light microscope which are performed on-site (clinic) and in the cytology department. The slide containing the most representative material is selected for immunocytochemistry (ICC). This slide is split into pieces, depending on the number of ICC tests required, using a tungsten carbide knife. From the aspirate specimen, small tissue fragments are retrieved which are processed to form a paraffin block. [3] Appendix C refers. This process involves the fixation of the tissue fragments in Formal-Fixx® solution, dehydration in graded percentages of alcohol, which ultimately facilitates penetration of xylene, enabling the impregnation of the tissue fragments with paraffin wax. The processed tissue fragments are embedded in a mould of molten paraffin wax which is left to harden. From the hardened paraffin wax tissue block, numerous tissue sections are cut akin to histopathology, producing tissue sections for immunohistochemistry and other special studies.

Bibbo (1997) [22] stated that in about 85% to 90% of FNA material, a definitive diagnosis of the tumour type can be rendered by examining the material using light microscopy and referring to clinical data of the patient. The remaining 10% to 15% will benefit from immunocytochemistry (ICC), enabling tumours to be classified accurately thus facilitating appropriate selection of therapy. [22]

In a review of technical problems associated with immunostaining of cytologic preparations, Leong et al. (1999) [23] stated that the "immunostaining of cytologic preparations has not achieved the level of success attained by the same procedure

performed in paraffin sections". This was attributed to difficulties in interpretation of the immunostains due to false positivity, high background staining and inadequacy of material.[23] Colasacco et al. (2010) [24] have stated that the development of most of the commercially available antibodies used in diagnostic pathology is based on their expression in histologic tissue. As a result, the application of these antisera to cytologic material could be conducive to the false positivity and high background staining. Therefore, as proposed by Colasacco et al (2010) [24], it would more appropriate to utilize a cell block sample for immunocytochemistry instead, since it is fixed and processed in a similar manner to histologic tissue.

The limitation of rendering a definitive diagnosis using FNA material alone can be overcome when used in combination with ancillary methods such as immunohistochemistry, flow cytometry and other molecular techniques.[20] Miller(2005) [20] supports the use of cell block preparations to overcome the limited diagnostic material available in conventional FNA sample preparation.[4] This facilitates the performance of multiple immunostains since many tissue sections can be cut serially from the same block.[20]

There are several articles in the literature with investigations on the diagnostic utility of the cell block technique in cytology using the Shandon Cytoblock Kit® and other methods of preparation.[25-32] Thus far, all literature searches performed have not yielded any conflicting data on the efficacy of the Shandon Cytoblock kit utilized in any application.[25-31] These teams found that both the FNA smear and cell block contributed

and faired equally well in preservation of cytomorphologic detail with optimal immunostaining.[25-32]

This study was undertaken to determine if the cell block technique, using the Shandon Cytoblock Kit® could be successfully implemented in this cytology unit as a routine diagnostic procedure.

1.7 Benefits of the cell block technique

Accurate FNA diagnoses often require immunocytochemistry in addition to morphology. Quality, reliability and reproducibility of the immunohistochemistry assay performed is essential.[24] Since the development of most commercially available antibodies used in diagnostic pathology is based on their expression in formalin fixed paraffin embedded tissue (FFPE), IHC performed on cytological material is less reliable.[24] The diagnostic utility and feasibility of the cell block preparation to overcome this limitation has been evaluated, and found to greatly improve diagnostic accuracy.[8, 9,11,12,13] An important diagnostic decision, once cancer is evident in organs like lung, liver and lymph node, is the determination of the primary site of cancer.[33] This decision often requires the use of immunocytochemical (ICC) investigations. Immunocytochemistry is increasingly being used for prognostication and treatment in patients e.g. oestrogen receptor status, progesterone receptor status and HER2-neu in breast carcinoma.[34]

FNA of most large masses yield large numbers of tumour cells and tissue fragments which may be partially obscured by necrosis and inflammation.[33] Since the cellularity of these samples is high, it is possible to produce paraffin sections of cell blocks. This

facilitates ancillary investigations, namely immunocytochemistry, special stains, electron microscopy, molecular studies, etc, enabling a rapid and accurate diagnosis thus improving patient management.

1.8 Benefits of IHC / ICC in cytopathology

The direct and indirect immunoperoxidase staining methods have been in use since the 1940's.[35] Both demonstrate specific cellular or tissue constituents (antigens) by means of highly specific antigen-antibody interactions.[35] However, the indirect method is a more sensitive technique than the direct procedure due the ability to demonstrate both tissue morphology and antibody localisation.[35] There are many commercial reagent kits available for both the direct and indirect staining methods. For this study, the indirect two-step procedure using the Dako REAL™ Envision™ Detection Sytem (product number K5007) was used. Since this detection system is a peroxidase – conjugated polymer it is does not contribute to additional background staining like the avidin-biotin based systems. [35, 36] This is especially beneficial for cytological smear preparations since the FNA samples are mostly likely to contain proteinaceous debris and predominantly necrotic or very thickly smeared areas all of which result in background staining.[19, 37] This detection system is also ideal for use with normal and neoplastic cells namely hepatocytes, erythrocytes, granulocytes and eosinophils that contain endogenous peroxidase activity which contributes to background and anomalous staining.[37]

As with routine histological sample processing, during the fixation and processing of cell block samples for paraffin embedding, some antigenic epitopes (binding sites for the

respective antibody) are masked or destroyed.[35] Many masked antigens in processed tissue can be revealed using one of the following enhancement techniques: heat induced epitope retrieval (HIER) eg. microwave antigen recovery or pressure cooker antigen retrieval or proteolytic enzyme digestion.[35] Appendix G and Table 2.2 refers. Each antibody is identified by its unique catalogue number as allocated by the manufacturer (source). HIER involves the boiling of dewaxed formalin-fixed paraffin sections in the respective buffer solutions [35] as recommended by the manufacturer (source) of the antibody. Thereafter tissue sections, obtained from the cell block by microtomy (Appendix C refers), are incubated with the specific optimally diluted monoclonal or polyclonal primary antibody. A guideline for optimal antibody dilutions is supplied by the manufacturer to facilitate optimal immunostaining. Following a stringent wash in Dako Wash Buffer™ (product number S3002) the tissue sections are incubated with Dako REAL™ Envision™ / HRP reagent. This reagent carries antibodies to rabbit and mouse immunoglobulins, and is a peroxidase – conjugated polymer thereby functioning as the secondary and tertiary antibody respectively.[35, 36] The reaction is visualised by Dako REAL™ DAB+ Chromogen which is included in the detection system. This reagent gives the brown colour at the site where the target antigen was recognised by the primary antibody. These sites are either in the cytoplasm, cell membrane or nucleus of a specific cell /s, resulting in cytoplasmic, membranous or nucleic staining / colouring pattern. [37] The expected localisation of the target antigen on the positive control tissue section is also stained brown. Any non-specific staining is evident as brown staining on the negative control comprising the test section that is subject to all procedural steps except incubation with the primary antibody.

Immunohistochemical and immunocytochemical analyses should not be interpreted in isolation since they serve as an adjunct to morphological classification. Optimal interpretation of results requires a comprehensive knowledge of expression characteristics of antibodies applied to a type of lesion. [35] For example, the immunohistochemical reaction of carcinomas is demonstrated by the specific interaction of antibodies to the epithelial cytokeratin.[35] Similarly, in the immunohistochemical demonstration of lymphomas, a membranous staining pattern is displayed for the specific interaction of the antibody directed against the leucocyte common antigen. [35]

The most common diagnostic dilemmas in assessment of FNAs involve poorly differentiated tumours including carcinomas which are composed of cells that cannot be readily classified by light microscopy.[22] By employing broad-specificity pan keratin antibodies, carcinomas can be detected immunohistochemically since they are keratin positive.[22] A diagnostic dilemma also exists in the diagnosis of papillary carcinoma of the thyroid in cytomorphological equivocal cases.[12] In this instance the invaluable role of ICC is demonstrated using the cytokeratin 19 antibody as a diagnostic aid in confirming the diagnosis of papillary carcinoma of the thyroid.[12]

Other instances where ICC plays a pivotal role in the differential diagnosis of major tumour categories include [22]: carcinoma versus Non-Hodgkin's lymphoma versus melanoma versus sarcoma, primary hepatocellular carcinoma versus metastatic cancer, primary lung adenocarcinoma versus ductal breast carcinoma metastatic to the lung, reactive mesothelial cells versus metastatic adenocarcinoma, reactive mesothelial cells

versus mesothelioma, mesothelioma versus adenocarcinoma, reactive lymphoid cells versus Non-Hodgkin's lymphoma. ICC is also useful in the analysis of small round cell tumours especially in the paediatric population.[22] In patients with an unknown primary tumour, the use of FNA ICC as a diagnostic tool, together with clinical data and optimal interpretation of the morphology of the tumour cells allows for the identification of the site of origin of the tumour and allows for rapid and accurate patient management.[22] In the current study, samples were collected from lung, liver and lymph nodes that had an initial on-site diagnosis on direct FNA smear of either adenocarcinoma , neuroendocrine carcinoma or metastatic adenocarcinoma.

The panel of immunostains applied in this study were: CK7, CK20, TTF1, Synaptophysin, AE1/3, Hepar-1.

1.9 FNA in tumours metastatic to the lung and lymph nodes

Since the advent of the FNA technique, patients with a suspected metastatic lung tumour can be spared from major surgical procedures (thoracotomy) or to being treated on the basis of radiological and clinical findings.[33] Using FNA, it is possible to sample pulmonary nodules of metastatic disease, and render a definitive diagnosis.[33] "In patients with multiple primary cancers, additional information may be provided regarding which primary cancer has metastasized" with the use of immunocytochemical investigations.[33]

1.10 Biomarkers / immunocytochemical studies of FNA in tumours metastatic to the lung and lymph nodes

The expression of keratin in human tissue and neoplasms was studied comprehensively in a review by Chu and Weiss (2002).[38] This team described the CK 20/7 immunophenotypic staining profile for the different tumour subtypes and proposed the use of cytokeratins (CK7 and CK20) to identify the primary sites of metastatic disease since the keratin profile of epithelium usually remains constant during malignant transformation.[38] Similarly, Tot (2002) [39] advocated the use of CK 20/7 phenotyping of adenocarcinomas as an useful aid in determining likely sites of the primary tumour.

Table 1.1: Histological distribution of CK7 and CK20 in hepatocellular carcinoma (HCC)
(Chu & Weiss, 2002): [38]

CK 7 / CK20 Staining Profile	**Cases positive (%)**
CK7+ / CK20+	(5% of cases)
CK7+ / CK20-	(15% of cases)
CK7- / CK20+	(2% of cases)
CK7- / CK20-	(78% of cases)

Table 1.2: Histological distribution of CK7 and CK20 in lung adenocarcinoma (Chu & Weiss, 2002): [38]

CK 7 / CK20 Staining Profile	Cases positive (%)
CK7+ / CK20+	(10% of cases)
CK7+ / CK20-	(90% of cases)
CK7- / CK20+	(0 % of cases)
CK7- / CK20-	(0 % of cases)

Table 1.3: Histological distribution of CK7 and CK20 in small cell carcinoma of lung (Chu & Weiss, 2002): [38]

CK 7 / CK20 Staining Profile	Cases positive (%)
CK7+ / CK20+	(0% of cases)
CK7+ / CK20-	(24% of cases)
CK7- / CK20+	(0% of cases)
CK7- / CK20-	(76% of cases)

1.11 Cytokeratin (CK 7): [38] Epithelial neoplasms positive for CK7: the majority of adenocarcinoma of lung, ovary, uterus, breast, pancreas, salivary gland and thyroid tumours, cholangiocarcinoma and transitional cell carcinoma. Only a small percentage of colorectal and gastric carcinoma is positive for CK 7. [38] The cells labelled by CK7 antibody displays a cytoplasmic and membranous reactivity / staining pattern.[40]

1.12 Cytokeratin (CK 20): [38] In contrast to CK 7 expression, CK 20 expression is restricted to a few organ systems. Epithelial neoplasms that are positive for CK 20: 90% colon carcinomas, 86% Merkel cell tumours of the skin, 68% transitional cell carcinomas, 56% gastric and 52% pancreatic adenocarcinoma.[38] The cells labelled by CK20 antibody displays a cytoplasmic and membrane reactivity / staining pattern.[41] Su et al. (2006) [42] used a panel of three antibodies: thyroid transcription factor 1 (TTF1), CK 7 and CK 20 in differentiating primary from metastatic pulmonary adenocarcinoma. An increase in diagnostic accuracy was observed with this application. In metastatic tumours to the lung, TTF1 staining is usually negative.[43]

1.13 TTF1: [42,44] TTF1 nuclear expression has been demonstrated by IHC in carcinomas of the lung and thyroid. TTF1 expression was not found in the majority of other tumours tested. The cells labelled by TTF1 antibody display nuclear reactivity / staining pattern.

1.14 Synaptophysin [43, 45, 46]

Synaptophysin detects neuronal and neuroepithelial differentiation in primary and metastatic tumours. [45, 46] A diffuse cytoplasmic staining pattern is observed.[45] In small cell carcinoma a granular cytoplasmic reactivity reflects neurosecretory granules.[43]

1.15 Cytokeratin (CK) AE1/3 (pankeratin) [43, 45, 47]

Most carcinomas are cytokeratin AE1/3 immunoreactive [43, 45] and display a cytoplasmic staining pattern.[47] The versatility of this antibody is displayed by its many uses. It is particularly useful to confirm or rule out the epithelial nature of tissue and tumours,

identify metastatic carcinoma in lymph nodes, identify residual tumour post-treatment and assess depth of invasion. [43]

1.16 Hepar-1[43, 48]

This antibody recognises mitochondrial antigen of hepatocytes.[43] Hepar-1 positive immunostaining is displayed in most hepatocellular carcinomas, some cholangiocarcinomas[23] and some adenocarcinomas metastatic to the liver.[43] This antibody displays a distinct granular cytoplasmic staining pattern.[48]

1.17 FNA in tumours metastatic to the liver and lymph nodes

One of the difficulties with rendering a definitive diagnosis on FNA of the liver is to differentiate hepatocellular carcinoma (HCC) from metastatic carcinoma (MC) and to identify the site of primary carcinoma that has spread to the liver.[49] There are some instances in which it is impossible to predict the origin of a metastatic tumour on morphological grounds alone. [33] Onofre et al. (2007) [49] applied specific panels of immunocytochemistry markers to distinguish the above and found that it served as a useful diagnostic tool.

1.18 Biomarkers / immunocytochemical studies of FNA in tumours metastatic to the liver and lymph nodes: [43, 49]

Table 1.4: The most likely immunocytochemistry panel in hepatocellular carcinoma for distinguishing from metastatic carcinoma [43]

Positive immunostains	**Negative immunostains**
HepPar1	AE1/3
CEA (polyclonal)	CK7
Alpha-fetoprotein (AFP)	CK13
Alpha-1-antitrypsin	CK19
CD10	CK20
CAM5.2	Keratin 903
CK8/18	EMA
	CEA(monoclonal)
	CD15
	MOC31
	BerEP4

Table 1.5: The most likely ICC panel in hepatocellular carcinoma for the identification of the unknown primary tumour metastatic to the liver: [43]

Positive immunostains	**Negative immunostains**
AFP	CK7
CK8	HepPar1
CK19	TTF1
CK20	

1.19 Conclusion to introduction

Cytology is a valuable investigation as it is minimally invasive, relatively cost effective and rapid. [1] In order to render an accurate cytologic diagnosis, patient's clinical data and cytomorphology need to be interpreted together with the judicious use of ancillary investigations such as appropriate immunocytochemistry.[15] Immunocytochemical tests such as CK7, CK20, TTF1, Synaptophysin, AE1/3 and Hepar-1 are very useful in establishing a primary diagnosis and documenting recurrent or metastatic disease. Cell block may be useful in assessing cytomorphology and allow for more immunocytochemical tests to be performed than a conventional cytology smear.