Immunocytochemistry in the diagnosis of small blue cell tumours of childhood

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Summary

Objective: **This study attempts to define a limited, cost effective, reliable primary panel of antibodies for immunohistology, as an adjunct to morphological features, for the diagnosis of Small Blue Cell Tumors (SBCT) which would be convenient for use in low resource settings to improve their diagnostic accuracy. The choice of antibodies is based on the common childhood tumors in Ibadan and limited by financial constraints and availability of antibodies.**

Materials & methods: **Twenty-five representative cases of previously diagnosed small blue cell tumours of childhood were selected from the file of the Department of Pathology, University College Hospital, Ibadan. The retrieved blocks were cut and stained with antibodies to desmin, leucocyte common antigen, cytokeratin, chromogranin, neuron-specific-enolase, vimentin and neurofilament using the avidin-biotin technique as previously described.**

Results: **Of the 25 cases studied 24 (96%) gave interpretable immunostaining reaction and the immunophenotype of these were defined. The staining quality equaled that produced on the control well-fixed positive control sections. The final diagnosis of six of the 25 cases changed based on immunostaining. Four cases previously diagnosed as lymphoma were confirmed to be rhabdomyosarcoma (3 cases) and neuroblastoma, one case each of rhabdomyosarcoma and neuroblastoma were both reclassified as lymphoma.**

Conclusion: **Based on our findings, the use of a small first-line panel of antibodies to leucocyte common antigen, desmin and neuron-specific-enolase are ideal for immunohistochemical discrimination of SBCT, as an adjunct to morphology, in low-resource settings.**

Key-words: ***Small blue cell tumors, Immunocytochemistry, Childhood diagnosis, Low-resource setting.***

Résumé

Objectifs:- Cette étude essaie de définir une quantité restreinte, rentable, jury primaire fiable des anticorps pour l'immunohistologie, comme une adjointe des traits morphologiques, pour le diagnostique des tumeurs de petite cellule blue (TPCB) qui sera convenable pour l'utilisation dans un des cadres à faible revenue pour améliorer l'efficacité de leur diagnostic. Le choix des anticorps est basé sur des tumeurs d'enfance ordinaires à Ibadan et limité par des constraintes financielles et disponibilité des anticorps.

Matériels et méthodes:- Vingt cinq cas representatifs des tumeurs d'enfance de petite cellule blue diagnostiquée auparavant ont été choisies dans les dossiers du départment de la Pathologie, au Collège universitaire hospitalier, Ibadan. Les blocks, récupérés ont été coupés et tachés avec des anticorps au desmine le leucocyte antigen ordinaire, cytokeratine, chromogranine, enolase-neuron-spécifique, vimentine et neurofilament avec l'utilisation de la méthode d'avidin-biotin comme on l'avait décrit auparavant.

Résultats:- Parmi les 25 cas étudiés 24 soit 96% avaient donné une réaction immunotienture inteprétable et leur immunophénotypes ont été défini. La qualité de la tienture était de même avec celle produit chez le groupe de témoin, section de contrôle positif bien fixé. Le diagnostique final des six parmi les 25 cas avait changé attribuable au immunoteinture. 4 cas diagnostiqués auparavant comme lymphome ont été confirmés d'être rhabdomyosarcome (3 cas) et neuroblastome, un cas de rhabdomyosarcome et neuroblastome chacun était classifié comme lymphome tous les deux.

Conclusion:- D'après notre résultat, l'utilisation d'un petit panneau de première ligne des anticorps au lieu du leucocyte antigen ordinaire, desmine et neuron-spécifique-enolase sont idéals pour la discrimination immuno-histochimie de SBCT, comme un adjoint à la morphologie dans un milieu aux petites ressources

Introduction

Small blue cell tumours of childhood are undifferentiated neoplasms characterized histologically by sheets of primitive cells with hyperchromatic nuclei and minimal cytoplasm, and most often not reflecting their histiogenetic origin. Tumours that fall in to this category include lymphoma, rhabdomyosarcoma, neuroblastoma, nephroblastoma, Ewing's sarcoma/peripheral neuroectodermal tumour, hepatoblastoma, retinoblastoma and medulloblastoma. These tumours are composed of cells with histological characteristics that reflect their embryonic and primitive features. They pose diagnostic problems on routine haematoxylin and eosin (H & E) stained paraffin sections, especially if the primary origin is unknown or ill-defined. The differentiation of one unique primitive tumour from the other would therefore depend on the expression of specific intracytoplasmic intermediate filament proteins or ultrastructural features which reflect their cell of origin. These features often remain unchanged in even in neoplastic cells.

Lymphoma, particularly Burkitt's lymphoma (BL) is the most common childhood malignancy in Nigeria and most

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parts of tropical Africa while rhabdomyosarcoma (RB) is the most common soft tissue sarcoma.[1,2] These tumours have common presenting sites and patients often present with advanced extensive disease, which in some cases are difficult to localize to specific primary sites. Biopsy diagnosis in this environment is based mainly on morphological criteria seen on H&E stained sections and special histochemical staining techniques, as necessary. Ancillary investigations such as immunohistochemistry (IHC) and electron microscopic are not available. The routine histological diagnosis of these tumours is therefore sometimes difficult and inconclusive rendering treatment protocols in such cases questionable and inappropriate.

In the developed countries immunohistochemistry has been established as important diagnostic modality in tumor pathology with a significant role in the diagnosis of these childhood primitive tumours.[3,4,5] When employed with an appropriately selected diagnostic panel of antibodies, immunohistochemical studies can provide accurate diagnosis in about 75% of undifferentiated tumours. [3,4] Financial constraint is a major limiting factor to the use of immunohistochemistry as an adjunct to morphological criteria in the diagnosis of small blue cell tumours in Ibadan. However the development of a carefully selected small panel of antibodies can be employed to improve the diagnostic accuracy of these tumours.

This study therefore aims at defining a minimum suitable screening panel of antibodies for the diagnosis of SBCT when necessary, which should be cost –effective, reliable and therefore convenient for our situation in Nigeria and other developing countries with limited resources.

Materials and methods

Cases of childhood tumours in the files of the Department of Pathology University College Hospital, Ibadan Nigeria seen between 1992 and 1997 were retrieved. Twenty –five cases representative of the common childhood tumours in Ibadan, were selected to reflect the range and different types of tumour from different sites. All cases were previously diagnosed on routine H&E-stained sections. The retrieved slides were screened and representative tissue blocks were selected for immunostaining. For this study, fresh 3-micron thick sections were cut and stained with routine H&E and immunostained with different primary antibodies, at the stated appropriate dilutions after initial titration, using the avidin-biotin peroxidase complex technique as previously described, with diaminobenzidine as chromogen.[6,7] The details of the immunohistochemical procedure used are as detailed and described by Hsu et al.[6] The panel of seven available antibodies used is: leukocyte common antigen (LCA), desmin, neuron-specific enolase (NSE), cytokeratin, vimentin, neurofilament and chromogranin. Information regarding the antibodies used including their working dilutions and sources are summarized in Table 1. Appropriate positive and negative tissue controls were included and stained along side tissue sections from the cases. The positive tissue controls used for the different antibodies were: tonsil (LCA); skeletal muscle (desmin); skin (pancytokeratin); known positive case of carcinoid tumour (NSE). For the negative control sections of each case tissue was stained without the primary labeling antibody. The antibodies used in this study were donated from previous work done by one of the authors. The most suitable working dilutions for best tissue staining of each of the antibody were previously determined by prior staining of known positive and control tissue sections.

Reactivity of immunostained sections were reviewed under the light microscope and semi-quantitatively evaluated on a 4-point scale based on the number of positive cells:

Negative staining (–) = no tumour cell stained
Minimal positive staining (+) = less than 10% of tumour cells positive
Moderate positive staining (++) = 10-50% of tumour cells are positive.
Marked positive staining (+++) = greater than 50% of tumour cells positive

Table 1 Monoclonal antibody profile used for immunohistochemical analysis

Antibody	Species sources	Reported specificity	Antigen location	Antibody concentration used	Trypsinization	Source
Anti-Neurofilament	Mouse	Neuron	Cytoplasmic	1/250	Nil	Dako®
Anti-Neuron Specific Enolase	Rabbit	Neurons, reactive astrocytes	Cytoplasmic	1/250	Nil	Dako®
Anti-Cytokeratin (Pancytokeratin)	Mouse	Epithelial cells	Cytoplasmic	1/250	Yes	Organon® Technik
Anti-Leukocyte common antigen	Mouse	Leukocytes	Membrane	1/250	Nil	Dako®
Anti-Desmin	Mouse	Skeletal & smooth muscle	Cytoplasmic	1/250	Nil	Dako®
Anti-Chromogranin	Mouse	Neuroendocrine cells	Cytoplasmic	1/250	Nil	Dako®
Anti-Vimentin	Mouse	Mesenchymal tissues	Cytoplasmic	1/200	Nil	Dako®

Table 2 Immunohistochemical profile of the individual cases studied.

Cases	Age	Sex	Site	Previous haematoxylin & eosin diagnosis	DES	LCA	VI	CK	CG	NSE	NF	Final Diagnosis
1	3	M	Facial mass	Burkitt's lymphoma	-	+++	-	-	-	-	-	Lymphoma
2	9	M	Intestinal tumor	Malignant lymphoma	-	+	-	-	-	-	-	Lymphoma
3*	14	F	Nasopharynx	Burkitt's lymphoma	++	-	++	-	-	-	-	Rhabdomyosarcoma
4*	10	M	Cervical	Malignant lymphoma	+	-	+++	-	-	-	-	Rhabdomyosarcoma
5	6/12	F	Right trapezium	Rhabdomyosarcoma	+	-	-	-	-	-	-	Rhabdomyosarcoma
6	10	M	Testes	Malignant lymphoma	-	+	-	-	-	-	-	Lymphoma
7	14	F	Ovarian tumor	Burkitt's lymphoma	-	+++	-	-	-	-	-	Lymphoma
8	7	M	Right axillary lymph node	Burkitt's lymphoma	-	++	-	-	-	-	-	Lymphoma
9	11	F	Liver	Hepatoblastoma	-	-	+	+++	-	-	-	Hepatoblastoma
10*	9	M	Abdominal tumor	Neuroblastoma	-	+++	-	-	-	-	-	Lymphoma
11	3	M	Renal biopsy	Nephroblastoma	-	-	++	+++	-	-	-	Nephroblastoma
12	8	M	Right orbital mass	Embryonal Rhabdomyosarcoma	+	-	++	-	-	-	-	Rhabdomyosarcoma
13*	3	F	Nasopharynx	Embryonal Rhabdomyosarcoma	-	++	-	-	-	-	-	Lymphoma
14	41/2	F	Renal mass	Nephroblastoma	-	-	++	+++	-	-	-	Nephroblastoma
15*	9	F	Fibrofatty tissue	Burkitt's lymphoma	++	-	-	-	-	-	-	Rhabdomyosarcoma
16	4	M	Intraocular tumor	Retinoblastoma	-	-	+	-	-	++	-	Retinoblastoma
17	3	M	Intraocular tumor	Retinoblastoma	-	-	+	-	-	+	-	Retinoblastoma
18	4	F	Cervical lymph node	Metastatic retinoblastoma	-	-	-	-	-	+	+	Metastatic retinoblastoma
19	10	F	Brain	Unknown	-	-	+	-	-	+	-	Medulloblastoma
20	6	M	Right/left nasal mass	Malignant lymphoma	-	+	-	-	-	-	-	Lymphoma
21	7	F	Upper lid	Embryonal rhabdomyosarcoma	+	-	-	-	-	-	-	Rhabdomyosarcoma
22	8	F	Left upper lid	Embryonal rhabdomyosarcoma	-	-	++	-	-	-	-	Mesenchymal tumor – likely Embryonal Rhab-domyosarcoma
23	11	M	Ventricular tumor	Medulloblastoma	-	-	-	-	-	-	-	Non-reactive
24	7	F	Left orbital mass	Embryonal rhabdomyosarcoma	+	-	++	-	-	-	-	Rhabdomyosarcoma
25*	10	M	Abdominal mass	Burkitt's lymphoma	-	-	-	-	-	+++	+	Neuroblastoma

KEY: DES – *Desmin*, LCA – *Leukocyte common antigen*, VI – *Vimentin*, CK – *Cytokeratin*, CG – *Chromogranin*,

NSE – *Neuron specific enolase*, NF - *Neurofilament*. * *Cases with a change in diagnosis on immune staining*

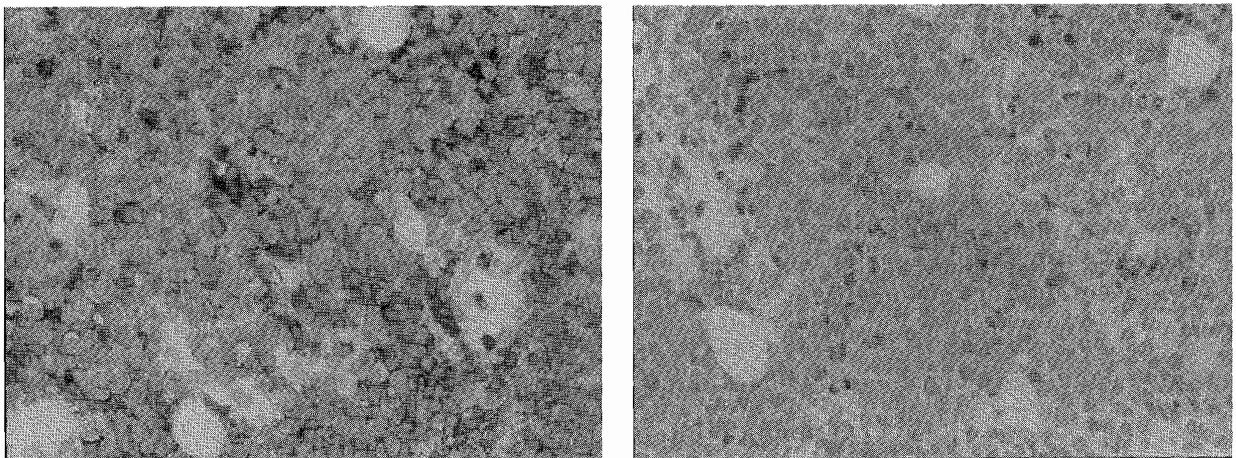
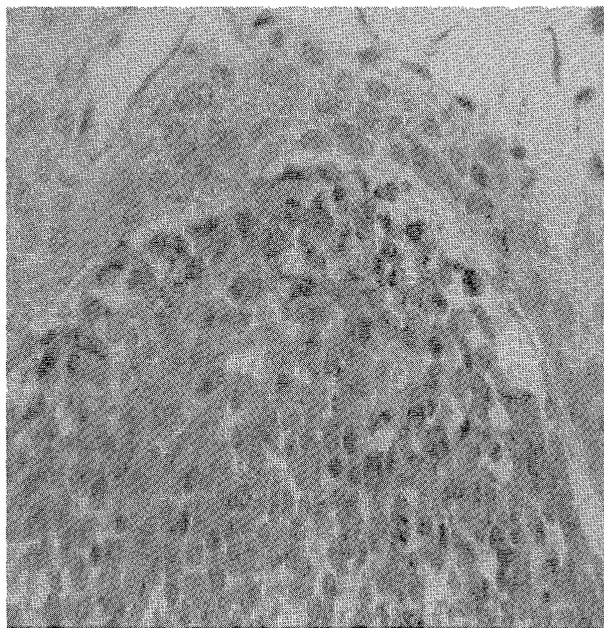


Figure 1 ***Case of Burkitt's lymphoma with diffuse sheets of lymphoblastic cells (a) positively immunostained with antibody to LCA (b) negative control.***

Results

Twenty-five cases of childhood tumours from various anatomical sites were examined and studied. The age-range of the patients was from 6 months to 14 years. All the selected cases had previous H&E stained sections and had been diagnosed on routine microscopy as either lymphoma (10), rhabdomyosarcoma (6), retinoblastoma (3), nephroblastoma (2), neuroblastoma (1) hepatoblastoma (1) and medulloblastoma(1) and SBCT, unclassified (1).

Table 2 shows the immunostaining pattern of the 25 cases against the various antibodies used. In one case (4%) the sections did not show reactivity with any of the antibodies. Of the 25 cases studied 24 (96%) gave interpretable immunostaining reaction and the immunophenotype of these were defined. The staining quality of these cases equaled that produced on the well-fixed positive control sections.



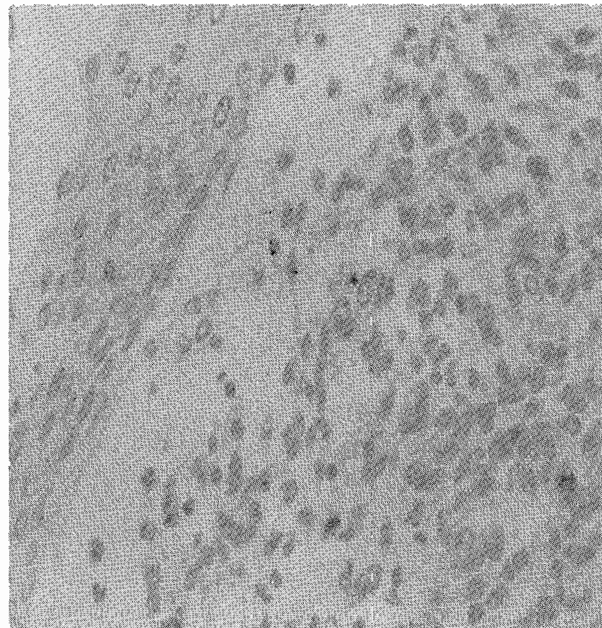


Figure 2 ***Section shows sub-epithelial proliferation of primitive undifferentiated cells of embryonal rhabdomyosarcoma (a) positively immunostained with anti-desmin (b) negative control.***

Immunostaining for LCA was detected in 8 cases (32%) and were therefore sub-typed as lymphomas. In 5 of these cases, there was marked to moderate surface membrane staining pattern (Fig 1) while 3 showed mild focal membrane staining of the tumour cells. Two of these 8 cases had previous morphologic diagnosis of rhabdomyosarcoma and neuroblastoma and had presented as nasopharyngeal and abdominal masses respectively. Only 6 (60%) of the 10 cases previously diagnosed as lymphoma stained positively with LCA, retaining the final immunohisto-chemically confirmed diagnosis of lymphoma. The positive predictive value of morphologic diagnosis of lymphoma on routine H&E stained sections was 60% with a negative predictive value of 86.7%. Of the 4 cases negative with LCA, 3 showed positive labeling with anti-desmin (Fig 2). The fourth case, reclassified as neuroblastoma, showed marked diffuse cytoplasmic positivity of tumour cells for NSE with co-expression of neurofilament.

On the whole 6 cases (24%) had significant change in their diagnosis and would have required different therapeutic approaches and management protocol. The wrong diagnosis was usually in tumours presenting with abdominal (2), soft tissue (2) and nasopharygeaal masses (2). Accurate diagnoses were made in tumours arising in specific organ sites. Lymphomas, rhabdomyosarcoma and neuroblastoma were often misdiagnosed, 3 cases initially diagnosed as Burkitt's lymphomas turned out to be rhabdomyosarcoma.

Discussion

Several studies have attested to the usefulness of and the complementary role of immunohistochemistry in routine morphological diagnosis of tumours, including SBCT of childhood[3-5] and immunophenotyping is now considered a routine technique. Unfortunately because of financial constraints and difficulty in obtaining antibodies, this technique is not established in most laboratories in the developing countries including Nigeria. The antibodies used in this study were obtained from institutions where one of the authors had previously worked. Using this technique, it was possible to subtype 96% of the cases in this study. This demonstrates the feasibility of the technique, the sensitivity and reliability of the panel of antibodies used and the suitability of the archival tissue specimens in our laboratory for immunostaining. Archival tissues, from the same laboratory, have been shown previously to be suitable for immunostaining technique.[8]

Based on the reactivity of these tumours to the panel of antibodies, a change was made in the diagnosis of 24% of the cases reflecting a significant improvement in the diagnostic accuracy of these undifferentiated tumours with

immunohistochemistry. This indicates that if immunostaining were routinely available, accurate diagnosis and institution of appropriate chemotherapeutic protocols would be possible.

Lymphomas, including BL are the most common childhood tumours in Ibadan constituting 45.4% of childhood neoplasms while rhabdomyosarcoma accounts for 61% of childhood sarcomas.[1] These tumours are often misdiagnosed as shown in this study. Morphologic diagnosis of lymphoma and rhabdomyosarcoma based on routine H&E stained sections showed a sensitivity of 75% and 62.5% and a specificity of 76.5% and 94.1% respectively. Final diagnosis of lymphoma and rhabdomyosarcoma was made in 8 and 7 cases respectively, both tumours constituted 15 (60%) of 25 cases studied. It is imperative that these 2 common tumours should be accurately differentiated as their management protocols are distinctly different. This study demonstrates the importance of LCA and desmin as part of any panel of antibodies to be used in the diagnosis of small blue cell tumors in Ibadan.

Desmin expression was observed in 7(28%) of the 25 cases and these tumours were finally sub-typed as rhabdomyosarcoma. More intense staining was observed in rhabdomyoblasts, with abundant eosinophilic cytoplasm and cross-striations, consistent with the finding that the expression of desmin is related to their state of differentiation. One of the tumours initially diagnosed as rhabdomyosarcoma on H&E sections did not show any reactivity with anti-desmin but showed positive labeling with vimentin. In view of this staining and the overall morphology it was delineated as a mesenchymal tumour, likely rhabdomyosarcoma. Though desmin is classically considered a useful marker of myogenous differentiation, its utilization however suffers some limitations.[9] Primitive rhabdomyoblasts may lack desmin which is related to the degree of differentiation of the tumours cells,[10] therefore the non-reactivity of this tumour with desmin does not negate its final diagnosis. Other limitations include the presence of desmin in smooth muscle tumours and other non-myogenous cells. However, childhood leiomyomas and leiomyosarcomas are extremely rare. Though vimentin is a highly sensitive marker for mesenchymal tissues it is rather non-specific. It was expressed in 11 cases (44%) which included rhabdomyosarcoma (4 cases), nephroblastoma (2 cases) and retinoblastoma (2 cases) and one case each of hepatoblastoma and medulloblastoma. Immunostaining with anti-vimentin was therefore not very useful in delineating SBCTs. Vimentin is of limited value as a diagnostic tool, however when used in a panel it is useful in sub-classification of a given tumour. Vimentin is useful as a tissue process control, as over fixation in formalin often masks the epitopes and block immunoreactivity.[11]

Neuron-specific enolase which was expressed in 5 cases (20%) showed cytoplasmic positivity in retinoblastoma, neuroblastoma and medulloblastoma. Only 2 cases classified as neuroblastoma showed co-expression of neurofilament with patchy staining in the more differentiated areas of the tumour. An important observation in this study was the lack of antigen discrimination between the studied neuroectodermal tumours – retinoblastoma, neuroblastoma and medulloblastoma. This is probably due to the common histiogenetic origin shared by this group of tumours. Antibody to NSE is therefore useful in delineating this group of tumours from others and should be included in the panel for phenotyping. Clinical correlation and site of tumour can help distinguish between these tumour types

A conclusive diagnosis of epithelial blast tumours was made in 3 cases which included nephroblastoma, (2 cases) and hepatoblastoma (1 case). Cytokeratin expression in these biopsies was observed mainly in the areas showing definite tubular differentiation in the Wilm's tumours. However these diagnoses were also accurately made on routine H&E sections. The high diagnostic accuracy observed may have been influenced by the small sample size of the cases studied, sources of tumour biopsy and evidence of epithelial differentiation. Thus a limited primary panel consisting of antibodies to LCA, cytokeratin, desmin, NSE could be used successfully, as a first-line investigative panel, to assist in the discrimination of the common small undifferentiated tumours in our environment. Additional panel of antibodies may however subsequently be employed as considered appropriate to eliminate or confirm specific diagnosis thus making the technique cost-effective.

Limitations encountered in this study included prohibitive cost of reagents, poor storage facility and preservation of reagents particularly the antibodies. Irregular electricity supply for effective refrigeration, limits the shelf-life of these reagents. Immunohistochemistry, is an expensive technique, even in centres where it is done routinely, it costs a minimum of $50.00 per antibody testing. Notwithstanding, the cost effectiveness of the technique in terms of patient outcome is enormous. This relates to increased diagnostic certainty and the increased ability to predict patient prognosis more accurately.[5,12] The procedure can be simply modified without altering the principle of the technique to limit total cost. Cost reduction can be effected by using small sections and incorporating control and test sections on the same slide. Another possible cost saving technique is the use of multi-block or tissue micro-array (using the "sausage tissue" block) that allows the simultaneous staining of multiple tissue samples on the same slide with single drop of antibody.[13]

Tissue micro-array is a tissue conserving, high through –put technique that utilizes the multiple tumour core biopsies of 0.6–1.0 mm in diameter that are transferred to a new paraffin block, which allows for multiple tumours to be analyzed simultaneously.[14]

One of the 25 cases in this study did not show labeling of the neoplastic cells with any of the antibodies. Its non-reactivity may be attributed to inherent factor of the neoplastic cells, quality of tissue fixation or processing. Improper or prolonged tissue fixation which may result in denaturation or masking of antigens, an important limita

tion [15,16] can be addressed by proper education of laboratory staff and physicians. Fortunately, several fixatives are compatible with immunostaining. In order to improve antigen retrieval, microwave and wet heat processing are being used as alternative methods to trypsinization that was utilized in this present study.[17,18] However the fact that many of the tissue sections were stained indicates that this was not a major limitation in this study.

Excessive background staining which was observed in few slides may be misinterpreted as false positive staining in inexperienced hands. This may can be reduced by careful selection of appropriate sections, increasing dilution of antibodies and ensuring adequate removal of endogenous peroxidase. In this study sections were incubated in methanol/hydrogen peroxide solution for 15 minutes to reduce endogenous peroxidase activity. It must be noted also that false negative staining can occur when tissue is poorly fixed, the antigens are denatured or in low density, or when antibody is used at inappropriate dilution, however the use of positive control section should identify this. In view of these limiting factors, it must be emphasized that an apparently negative immunohistochemical result should not be used to rule out a diagnosis, if the diagnosis is strongly suggested by clinical and morphological features. However, with increasing use and experience with the technique, these problems can be reduced significantly. Finally the fact that immunophenotyping of most of the cases was possible demonstrates the usefulness and reliability of the technique and the antibody panel used in this study.

That the diagnosis of the 24% of cases had to be changed demonstrates the relevance of the use of the technique in making accurate diagnosis and institution of appropriate chemotherapeutic protocols in the management of SBCT in our institution. The application of this panel of antibodies in the routine diagnosis of SBCT will bring into perspective the exact prevalence of these tumours in this environment in relation to age and sex which is of extreme importance for continued research.

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