DIAGNOSTIC IMMUNOHISTOCHEMISTRY WITH TISSUE MICROARRAY TECHNIQUE – A PILOT STUDY ON NON HODGKIN LYMPHOMA

Dissertation submitted to

THE TAMIL NADU DR. M. G. R MEDICAL UNIVERSITY

CHENNAI- 600032

In partial fulfillment of the requirement for the degree of

Doctor of Medicine (Branch III)

M. D. (PATHOLOGY)

DEPARTMENT OF PATHOLOGY

TIRUNELVELI MEDICAL COLLEGE

TIRUNELVELI- 11

APRIL 2015

CERTIFICATE

This is to certify that the dissertation, **''DIAGNOSTIC IMMUNOHISTOCHEMISTRY WITH TISSUE MICROARRAY TECHNIQUE – A PILOT STUDY ON NON HODGKIN LYMPHOMA'',** is a bonafide work done by **Dr.G.KUMUDHINI PRIYA**, Postgraduate student in the Department of Pathology,Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D.(Branch III) Pathology under my guidance and supervision during the academic period of 2012-2015.

The DEAN
Tirunelveli Medical College,
Tirunelveli-627011

CERTIFICATE

This is to certify that the dissertation entitled, **''DIAGNOSTIC IMMUNOHISTOCHEMISTRY WITH TISSUE MICROARRAY TECHNIQUE – A PILOT STUDY ON NON HODGKIN LYMPHOMA'',** done by **Dr.G.KUMUDHINI PRIYA**, Post graduate in Pathology (2012-2015), is a bonafide research work carried out under our direct supervision and guidance and is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, for M.D. Degree Examination in Pathology, Branch III, to be held in April 2015. This work done has not formed the basis for previous award of any degree.

Dr. J.Suresh Durai M.D. (Guide),
Professor of Pathology,
Department of Pathology,
Tirunelveli Medical College,
Tirunelveli.

Dr.K.Shantaraman M.D.,
Professor and HOD,
Department of Pathology,
Tirunelveli Medical College,
Tirunelveli.

DECLARATION

I solemnly declare that the dissertation titled **"DIAGNOSTIC IMMUNOHISTOCHEMISTRY WITH TISSUE MICROARRAY TECHNIQUE – A PILOT STUDY ON NON HODGKIN LYMPHOMA''** is an original work done by me during the academic year of 2012 – 2015 at Tirunelveli Medical College, Tirunelveli, under the supervison and guidance of Dr.J.Suresh Durai M.D.

The dissertation is submitted to The Tamilnadu Dr.M.G.R. Medical University towards the partial fulfillment of requirements for the award of M.D. Degree (Branch III) in Pathology.

Place: Tirunelveli

Date:

Dr. G.KUMUDHINI PRIYA
Postgraduate Student,
M.D Pathology,
Department of Pathology,
Tirunelveli Medical College
Tirunelveli.

TIRUNELVELI MEDICAL COLLEGE

TIRUNELVELI,

STATE OF TAMILNADU, INDIA

PIN CODE:627011

Tel: 91-462-2572733, 2572734 Fax: 91-462-2572944

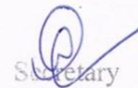
Estd:1965

Under the Directorate of Medical Education, Government of Tamilnadu.

Institutional Ethical Committee

Certificate of Approval

This is to certify that the Institutional Ethical Committee of this College unanimously approves the Thesis /Dissertation/ Research Proposal submitted before this committee by Dr. Kumudhini Priya. G, POST GRADUATE IN MD-PATHOLOGY Department of PATHOLOGY of Tirunelveli Medical College /Hospital, Tirunelveli titled **"DIAGNOSTIC IMMUNOHISTOCHEMISTRY WITH TISSUE MICROARRAY TECHNIQUE-A PILOT STUDY ON NON HODGKIN LYMPHOMA"** registered by the IEC as 272/PATH./IEC/2012 dated. 14.12.2012. The Investigator is hereby advised to adhere to all the stipulated norms and conditions of this ethical committee.



Issued on this Date

14.12.2012

Under Seal

Secretary

Secretary,
Ethical Committee,
Tirunelveli Medical College.
Tirunelveli-11.

// ACKNOWLEDGEMENT

To begin with, I thank the most powerful and compassionate, **The Almighty** for being with me always.

I take immense pleasure to acknowledge all those who have helped me to make this dissertation possible.

I am grateful to **the Dean, Tirunelveli Medical College** and **Medical superintendent of the Tirunelveli Medical College Hospita**l for permitting me to undertake this study.

I express my profound sense of gratitude to **Dr.K.Shantaraman M.D.,** my respected professor and Head of the Department of Pathology, Tirunelveli Medical College, Tirunelveli and my guide **Dr.J.Suresh Durai M.D.,** for their unstinted guidance and motivation.

I immensely thank **Dr.SithyAthiyaMunavarah M.D., Dr.S.Vallimanalan M.D., Dr.K.Swaminathan M.D., Dr.Arasi Rajesh M.D.,** Professors of pathology and all the Assistant professors for their constant support and encouragement. I profoundly thank all the other faculties for their valuable support.

I sincerely thank all the Technicians and other members of the department of pathology for their help and cooperation.

I thank all my family members for their encouragement during this study.

Turnitin Document Viewer - Google Chrome

https://turnitin.com/dv?o=455127889&u=1032402973&s=&student_user=1&lang=en_us

The Tamil Nadu Dr.M.G.R.Medical ... TNMGRMU EXAMINATIONS - DUE 15-...

Originality GradeMark PeerMark

DIAGNOSTIC IMMUNOHISTOCHEMISTRY WITH TISSUE MICRO ARRAY

BY 201213303.MD PATHOLOGY KUMUDHINI PRIYA G

13% SIMILAR

-- OUT OF 0

TITLE

DIAGNOSTIC IMMUNOHISTOCHEMISTRY WITH TISSUE MICRO ARRAY TECHNIQUE – A PILOT STUDY ON NON HODGKIN LYMPHOMA

Match Overview

	Source	Type	Match
1	www.iranpath.org	Internet source	2%
2	akramania.byethost11....	Internet source	1%
3	Faramarz Naeim. "Lym...	Publication	<1%
4	media.wiley.com	Internet source	<1%
5	Israeli, Zeev(Institut für...	Publication	<1%
6	www.ijpmonline.org	Internet source	<1%
7	"Hematopathology", La...	Publication	<1%
8	www.inmegen.org.mx	Internet source	<1%
9	Campo, Elias, and Stef...	Publication	<1%

PAGE: 1 OF 100

Navigate to text-only Originality Report

ABBREVIATIONS

AIDS	-	Acquired Immune Deficiency Syndrome.
ALK	-	Anaplastic Lymphoma Kinase.
bcl	-	b cell lymphoma gene.
CD	-	Cluster of Differentiation.
CLL	-	Chronic Lymphocytic Leukemia.
DNA	-	Deoxyribo nucleic acid.
DLBCL	-	Diffuse Large B cell Lymphoma.
EBV	-	Ebstein Barr virus.
ELISA	-	Enzyme Linked Immunosorbent Assay.
EDTA	-	Ethylene diamine tetra acetic acid.
HL	-	Hodgkin Lymphoma.
HHV	-	Human Herpes Virus.
HTLV	-	Human T cell Lymphotrophic Virus.
H&E	-	Hematoxylin and Eosin.
IHC	-	Immunohistochemistry.
Ig	-	Immunoglobulin.
NHL	-	Non Hodgkin Lymphoma.
PTLPD	-	Post Transplant Lymphoproliferative Disorder.
PCR	-	Polymerase Chain Reaction.
SLL	-	Small Lymphocytic Lymphoma.

SLE	-	Systemic Lupus Erythematosis.
TMA	-	Tissue Microarray.
Tris	-	Trisodium.
WHO	-	World Health Organisation.

CONTENTS

Sl.No.	Title	Page No.
1	AIM & OBJECTIVES	1
2	INTRODUCTION	2
3	REVIEW OF LITERATURE	4
4	MATERIALS AND METHODS	75
5	OBSERVATION & RESULTS	83
6	COLOUR PLATES	
7	DISCUSSION	94
8	SUMMARY AND CONCLUSION	101
9	BIBLIOGRAPHY	
10	ANNEXURES	
	ANNEXURE I – MASTER CHART	
	ANNEXURE II – TMA LAYOUT	

LIST OF TABLES

S.NO	CONTENT	PAGE NO
1	Comparison of histopathological examination and immunohistochemical diagnosis	83
2	Distribution of cases	85
3	Classification of B-cell lymphoma after IHC analysis	87
4	Pattern and grading of IHC staining	89
5	Quantification of IHC reagents	93

LIST OF CHARTS

S.NO	CONTENT	PAGE NO
1	Distribution of cases	86
2	Classification of B-cell lymphoma after IHC analysis	88

LIST OF COLOUR PLATES

FIGURE NUMBER	CONTENTS
Fig Ia & Ib	Diffuse large B-cell lymphoma
Fig IIa & IIb	Chronic lymphocytic leukemia/ Small lymphocytic lymphoma
Fig IIIa, IIIb & IIIc	Mantle cell lymphoma
Fig IVa & IVb	T-cell lymphoma
Fig V	Materials used for TMA construction

DIAGNOSTIC IMMUNOHISTOCHEMISTRY WITH TISSUE MICROARRAY TECHNIQUE – A PILOT STUDY ON NON HODGKIN LYMPHOMA

Abstract: Non-Hodgkin lymphoma (NHL) is a collective term for a heterogeneous group of lymphoproliferative malignancies with differing patterns of behaviour and responses to treatment. Incidence of lymphoma has doubled in the past 4 decades and continues to increase. In the context of lymphomas, immunohistochemistry is used to classify, sub-classify and predict prognosis of several classes of lymphomas. Tissue microarray facilitates the analysis of molecular alterations in thousands of tissue specimens in a massively parallel fashion. This study was conducted in Department of pathology, Tirunelveli medical college in which tissue micro array was prepared using manual method from lymph nodes of 21 cases among which 20 were diagnosed as Non-Hodgkin lymphoma by histomorphology. All the cases were subjected for immunohistochemical analysis using CD3, CD20, CD5, and CD10 from which 19 cases were taken for classification and analysis based on recent WHO classification of lymphomas and leukemias.

Key words: Non Hodgkin lymphoma, CD, Immunohistochemistry, Lymph node, Tissue microarray, WHO.

AIM & OBJECTIVES

- To apply a panel of basic IHC markers for the diagnosis and classification of Non Hodgkin lymphoma.
- To standardize the manual tissue microarray technique.
- To assess the efficacy and standardisation of Immunohistochemistry using tissue microarray technique.

DIAGNOSTIC IMMUNOHISTOCHEMISTRY WITH TISSUE MICROARRAY TECHNIQUE – A PILOT STUDY ON NON HODGKIN LYMPHOMA

Abstract: Non-Hodgkin lymphoma (NHL) is a collective term for a heterogeneous group of lymphoproliferative malignancies with differing patterns of behaviour and responses to treatment. Incidence of lymphoma has doubled in the past 4 decades and continues to increase. In the context of lymphomas, immunohistochemistry is used to classify, sub-classify and predict prognosis of several classes of lymphomas. Tissue microarray facilitates the analysis of molecular alterations in thousands of tissue specimens in a massively parallel fashion. This study was conducted in Department of pathology, Tirunelveli medical college in which tissue micro array was prepared using manual method from lymph nodes of 21 cases among which 20 were diagnosed as Non-Hodgkin lymphoma by histomorphology. All the cases were subjected for immunohistochemical analysis using CD3, CD20, CD5, and CD10 from which 19 cases were taken for classification and analysis based on recent WHO classification of lymphomas and leukemias.

Key words: Non Hodgkin lymphoma, CD, Immunohistochemistry, Lymph node, Tissue microarray, WHO.

INTRODUCTION

Lymphoid neoplasms include a diverse group of tumors of B-cell, T-cell, and NK-cell origin. In many instances the phenotype of the neoplastic cell closely resembles that of a particular stage of normal lymphocyte differentiation. Lymphomas are ranked 6thamong all cancers, worldwide [1]. Incidence of lymphoma has doubled in the past 4 decades and continues to increase. Non-Hodgkin lymphoma (NHL) is a collective term for a heterogeneous group of lymphoproliferative malignancies with differing patterns of behaviour and responses to treatment. Non Hodgkin lymphoma is the fifth most common cancer in the world with an upsurge of incidence in India. Compared with Hodgkin disease, NHL is much less predictable and has a far greater predilection to disseminate to extranodal sites. The prognosis depends on the histologic type, stage, and treatment.

Immunohistochemistry (IHC) is a method for localising specific antigens in tissues or cells based on antigen-antibody recognition. It seeks to exploit the specificity provided by the binding of an antibody with its antigen at a light microscopic level. Immunohistochemistry is applied in three circumstances: to completely phenotype the abnormal population of cells, to further characterize the abnormal population identified by flow cytometry or to screen reactive tissue for a subtle abnormal population of cells. In the context of lymphomas, immunohistochemistry is not only used

in the aforementioned circumstances, but also to classify, sub-classify and predict prognosis of several classes of lymphomas.

Since its discovery by Battifora in 1986, tissue microarray is becoming a useful tool for research and quality control in immunohistochemistry. Tissue microarray facilitates the analysis of molecular alterations in thousands of tissue specimens in a massively parallel fashion. Since the advent of high-throughput techniques like cDNA microarrays, serial analysis of gene expression (SAGE) and proteomics, the biomedical research field has changed fundamentally. Since, no single antigen is lineage or lymphoma specific, a panel of immunohistochemical markers are to be used. Hence, the use of tissue microarray allows rapid and efficient analysis of large number of paraffin-embedded lymphoma tissues by a panel of markers under standardized immunohistochemical technique.

Review of Literature

REVIEW OF LITERATURE

LYMPH NODE

Lymphoid organs constitute important component of our body. They are classified as central and peripheral lymphoid organs. The organs in which the lymphoid cells are primarily produced constitute the central lymphoid tissues while peripheral lymphoid organs are those in which the lymphoid cell differentiates, matures and processes the antigen. Bone marrow and thymus are the central lymphoid organs from which the lymphoid cells (B-lymphocytes and T-lymphocytes) are produced. Lymph nodes, spleen, MALT (Mucosa associated lymphoid tissue) constitutes peripheral lymphoid organs.

Among all the other peripheral lymphoid organs, lymph nodes are considered the most important because they actively participate in immune response. Lymph nodes are organised collection of lymphoid cells surrounded by a capsule and located in regions where they drain the lymphatic vessels. These include cervical, axillary, abdominal, pelvic and inguinal group of lymph nodes. They are bean shaped organs composed of dense collection of lymphoid cells. Normally, the lymph node measures about 2 to 20 mm with an average of 15mm in its longest dimension.(2,3)

Lymph nodes are connected to the general circulation by means of afferent and efferent lymphatic vessels. Normally, lymph nodes are non

palpable, soft, grey-pink and homogenous. They become palpable as a result of intense immune response, neoplastic transformation or metastatic deposit. The diameter of lymph node greater than 3cm with nodular whitish cut surface and firm consistency are the features suggestive of neoplastic transformation or metastatic deposit.

The characteristic features of lymph node architecture varies according to age and site based upon the exposure of lymphoid cells to different antigens. Normally, lymph nodes draining the head and neck region including the cervical and axillary lymph nodes show numerous lymphoid follicles with reactive germinal centres which are due to secondary antigenic stimulation. But, lymph nodes such as mesenteric nodes possess wide medullary cords and sinuses. The count of peripheral lymph nodes also varies significantly with no nodes in newborn, numerous in younger age groups and more numerous in the elderly(4).

Lymph nodes are organs with complex architecture where a variety of cell population are arranged in distinct compartments. This provides a favourable environment for interaction and processing of foreign antigens resulting in effective immune response. Hence, lymph nodes are not only involved in mechanical filtration of foreign bodies through the lymphatic vessels but also play a very important role in recognition and processing of antigen.

The anatomy of lymph node includes cortex, medulla and para-cortical area which are formed by lymphoid cells and plasma cells in varying stages of maturation. The supporting framework is formed by the capsule, trabeculae, reticulinfibres and network of vesicular cells. The fibroblasts along with smooth muscle cells, nerve bundles, Schwann cells and blood vessels constitute capsule which extends into the underlying parenchyma as trabeculae.

The reticulinfibres which originate from reticulin cells are fine type 3 collagen fibrils, which form the main supporting framework of the lymph node. Reticulinfibres surround individual lymphoid cells, hence are not visualised under H&E stain. It can be well demonstrated by using silver impregnation technique (Gomori's technique).

CORTEX

The cortical region of lymph node is primarily composed of lymphoid follicles. Lymphoid follicles can either be primary or secondary. Primary follicles are usually round to ovoid nodular collection of dark staining naive B cells. They are arranged with their long axis perpendicular to the lymph node [(5)].They transform into secondary follicles after antigenic stimulation.

The secondary follicles, that are transformed primary follicles, are composed of central pale staining germinal centre, a mantle zone and a marginal zone. Germinal centres are formed by the mixture of centroblasts, centrocytes, dendritic reticulum cells, small lymphocytes which are

primarily B-cells and tingible body macrophages with few scattered T lymphocytes. The germinal centres are cuffed off by small lymphocytes which form the mantle zone. The peripheral portion of the mantle zone is composed of cells that are loosely packed forming the marginal zone. The marginal zones are distinctly made out in the reactive follicles of spleen and usually are not so evident in the lymph nodes.

PARACORTEX

Paracortical areas are the T-cell packed zones in the lymph nodes and they are located deep beneath the cortex extending in between the lymphoid follicles. These areas are composed of T-cells in various stages of maturation [(6)], admixed with post capillary venules lined by high endothelial cells. In addition, there are interdigitating dendritic cells which serve as antigen presenting cells. They play an important role in evoking an immune response.

MEDULLARY AREA

This is located deep to the cortex and is primarily composed of plasmacytoid lymphocytes, plasmablasts, mature plasma cells and cords of lymphocytes. The plasma cells lose their surface markers and synthesize immunoglobulins of varying classes with kappa and lambda chain in the ratio of 2:1[(7)]. In addition to plasma cells, the medullary area also contains monocytes and macrophages.

CELLULAR COMPOSITION

The lymph node is formed by B-cell zone and T-cell zone containing cells in various stages of activation and differentiation. Germinal centres and lymphoid follicles are the B-cell zone whereas the paracortical area forms the T-cell zone. The medullary region is predominantly formed by the plasma cells.

The dark staining naive B cells that forms the primary follicles also accounts for the mantle zone of the secondary follicles [(8)]. These are small cells with scant cytoplasm and increased nuclear cytoplasmic ratio. Centroblasts are large cells that form the dark zone in the germinal centres which express IgM on their surface. They differentiate into centrocytes which forms the light zone in the germinal centres.

Some of the centroblasts that differentiate into memory cells reside in the mantle zone whereas those that are transformed into plasma cells move into the medullary region. The histiocytes are the transformed monocytes in the tissues that accumulate in the paracortical areas and germinal centres secondary to immune activation. These are large cells that contain abundant neither strongly basophilic nor pyroninophilic cytoplasm. These can be differentiated from large lymphocytes with the help of ultrastructural and immunohistochemical study. The histiocytes that accumulate in the germinal centres form the tingible body macrophages.

The tingible body macrophages contain numerous DNA fragments formed secondary to cell death after bcl-2 gene gets shut off.

NORMAL LYMPH NODE AND IHC

The main application of immunohistochemistry in normal lymph node is to study the differential expression of the same antibody in various regions of the lymph node and assess the cellular composition and clone of the lymphoid cells. Studying the clone among various lymphoid populations helps us to differentiate reactive hyperplasia from neoplastic transformation of the lymph node, more importantly Non Hodgkin lymphoma.

B cells in the lymphoid follicles usually express pan-B cell marker such as CD19, 20, 22 and 79a. In addition, centroblasts express CD10 and cells of the germinal centre show positive expression of Bcl-6 in the nucleus. Bcl-2 which is an anti-apoptotic protein is usually shut off in the germinal centre and shows positive expression in the mantle & marginal zone. Naive B cells in the primary follicle and mantle zone expresses CD5 with surface IgM and IgD. [IgD is not expressed in the germinal centre].

The B-centroblast expresses IgM on their surface which shifts to IgD/IgA in the case of centrocytes. Immunoglobulin Kappa and lambda expression is usually seen in all the B lymphocytes but monoclonality points towards neoplastic transformation. In addition, positive expression of Bcl-2 in germinal centre is a feature of neoplastic transformation. [9,10,11]

The small lymphocytes that are scattered throughout the germinal centre show positive expression for CD3, CD5, CD7, CD43 and CD4/8 that gives a clue that they are reactive T cells. Tingible body macrophages stain with CD11b, CD35 and CD68 whereas dendritic reticulum cells stain with CD21, 35, C3b, C3d. [12]

The cells in the paracortical region stain with pan T cell markers like CD2, CD3, CD5 and CD7, CD43 and HLA-DR. Interdigitating dendritic cells show positive expression for S100, CD24, HLA- DR. Plasma cells are CD138(Syndecan 1) positive. Macrophages stain for lyzozyme, α1-antitrypsin, S100 protein and CD68. [13,14]

LYMPHOMA

Lymphomas and leukemias are the two challenging fields in medical investigation in which immunity and neoplasia interface. It is very important to identify the cases of lymphoma and differentiate it from various types of non neoplastic lymphadenopathies. Once the presence of lymphoma has been identified, it should be classified into Hodgkin and Non Hodgkin lymphoma. The Non Hodgkin lymphoma should be further subclassified morphologically, immunologically and phenotypically so as to establish the exact diagnosis for the purpose of therapy and prognosis. There are many modes of investigations to diagnose malignant lymphomas which include cytomorphology, radiology, immunology and molecular techniques. Hence,

proper selection of the investigations will facilitate correct diagnosis at a reasonable expense to the patient.

NON HODGKIN LYMPHOMA

Non Hodgkin lymphomas are clonal lymphoproliferative disorders characterised by proliferation of malignant lymphoid cells that forms discrete mass. This includes diverse group of tumor of B-cells, T-cells and natural killer cell origin. Majority of the cases of non Hodgkin lymphomas are of B-cell origin while the remainder of the cases has T-cell origin. Non-Hodgkin lymphoma very rarely involves natural killer cells. Most of the lymphoid neoplasms resemble some recognizable stage of B-cell or T-cell differentiation with neoplastic lymphoid cells recapitulating the behaviour of their normal counterpart.

In earlier days it was believed that lymphadenopathy was a secondary phenomenon occurring secondary to underlying infection or carcinoma. Thomas Hodgkin first recognised that lymph node enlargement could occur as a primary disorder rather than secondary to underlying infection or carcinomas(15). Billroth was the first one who used the term malignant lymphoma for a collection of primary lymphoid disorders(16).

During eighteenth century Sternberg(17) and Reed(18)first identified the giant cells which are considered as the characteristic feature of Hodgkin lymphoma and introduced histopathological examination as the primary method of diagnosis and classification of lymphoproliferative disorders.

Brill[19]and Symmers[20], in 1920, described the features of follicular lymphoma. In 1956, Rappaport through his classical work classified Non-Hodgkin lymphoma based upon the morphology of the malignant lymphoid cells [21]. Roulet[22] in 1930 considered reticulum cell sarcomas as a synonym for malignant lymphomas.

In 1967, Good and Finstad demonstrated the relationship of B lymphocytes and T lymphocytes to the development of lymphoid neoplasms. Later Dameshek, through his work, suggested that lymphoproliferative disorders primarily occurs due to aberrations of immunologically competent cells and also lymphoid cells has the potential to get transformed into immunoblast like cells under appropriate antigenic stimulation. Immunological origin of malignant lymphocytes was confirmed in 1972 by the presence of immunoglobulins over the surface of B lymphocytes and by the formation of sheep erythrocyte rosette with neoplastic T lymphocytes [23, 24].

Lennert, Lukes and Collins at United States in 1974, classified Non-Hodgkin lymphoma based on cell of origin into B cell and T cell lymphomas [25, 26].Later in 1980, origin of lymphoid neoplasm was confirmed at molecular level after a detailed study on immunoglobulin gene over B lymphocytes and T cell receptors over T lymphocytes [27, 28]. Working formulation in 1982 classified Non-Hodgkin lymphoma based upon the histological grade and correlated it with the patient survival. From

1994, Revised European American Lymphoma classification came into use, which classified lymphoma based upon the clinical features, morphology, immunophenotyping and genetic data which were available[29]. Finally World Health Organisation has adopted the diagnostic principle of REAL classification and now WHO classification is used as schema for the diagnosis of all hematopoietic neoplasm.

Non Hodgkin Lymphoma and non modifiable risk factors

Non-Hodgkin lymphomas are the sixth most common cancer worldwide. Frequency of Non-Hodgkin lymphoma is age dependent and has variable distribution worldwide. Non-Hodgkin lymphomas are more common in males than females and hence show male preponderance. Malignant lymphomas are most common among adults and accounts for only 10% of all childhood malignancies in developed countries. Non-Hodgkin lymphomas show a steady increase in incidence from childhood through age of 80 years[30].

Increased risk for development of Non Hodgkin lymphoma has been documented among close relatives. There is two to four fold increased incidence of Non-Hodgkin lymphoma among close relatives of patients with lymphoma[31]. Aggregations have been reported among siblings and male relatives [32, 33]. Earlier age of onset in subsequent generation has been recorded in Non-Hodgkin lymphoma[34]. Increase in incidence of lymphoproliferative disorders is seen in association with autoimmune

lymphoproliferative syndrome which includes autoimmune features, splenomegaly and chronic lymphadenopathy[35]. Malignant lymphomas are more common among people with family history of lymphoma[36].

Young age and Non Hodgkin Lymphoma

Malignant lymphomas are the third most common cause of childhood neoplasms next to brain tumours and acute lymphoblastic leukaemias[37,38]. There is increased risk for Hodgkin lymphoma among the children less than 18 years, whereas Non Hodgkin lymphomas accounts for majority of the cases below 15 years. There occur many differences in the manifestations of Non-Hodgkin lymphomas of adults and children. Lymphomas among the children are high grade with frequent extranodal manifestations, which in adults are primarily nodal and usually of low grade and intermediate grade. Non-Hodgkin lymphomas in children are more common in boys when compared to girls and occur more frequently in blacks than whites. The three most important Non Hodgkin lymphomas occurring among children are Burkitt's lymphoma, lymphoblastic lymphomas and large cell lymphomas[39]. Large cell lymphomas are characterised by neoplastic transformation of lymphoid cells which are larger than the diameter of the histiocytic nucleus. This includes diffuse large B-cell lymphomas, anaplastic large cell lymphomas and peripheral T-cell lymphomas among which anaplastic large cell lymphomas are most common followed by diffuse large B-cell lymphomas. But overall, lymphoblastic lymphomas

forms bulk of the pediatric lymph node malignancies. Follicular lymphomas and marginal zone lymphomas occurs rarely and are considered uncommon neoplasms in the childhood.

Infection and Non Hodgkin Lymphoma

Infections play a major role in lymphomagenesis. The development of lymphoma depends on various factors including environment, geography and host factors [40]. Infectious agents cause lymphoma through direct lymphocyte transformation. Most of the infectious agents that cause lymphomas are found to be viruses which include EBV, HTLV-1, HHV8. Chronic infection by hepatitis-C virus can result in B-cell Non-Hodgkin lymphoma[41]. Indolent B cell lymphoma, lymphoplasmacytic lymphoma and marginal zone lymphoma are the most common types of lymphomas that are associated with hepatitis-C virus.

Ebstein-Barr Virus plays an important role in the causation of Non Hodgkin lymphoma. It has worldwide distribution accounting for more than 80% of the people over 30years of age. Once EBV infection has occurred it remains for life time of the individual. It usually affects children, but also can involve adolescence and adulthood. EBV usually establishes asymptomatic persistent infection, but due to effective immune mechanism only few of the infected carriers develop spontaneous EBV-associated Non-Hodgkin lymphoma. EBV has unique genes that cause growth activation of infected B-cell. The lymphomas caused by Ebstein-Barr Virus include T-

cell lymphoma, post transplant Non-Hodgkin lymphoma, AIDS related Non-Hodgkin lymphoma, Burkitts lymphoma and Hodgkin lymphoma.

Human T-cell lymphotrophic virus are the first retrovirus discovered, the infection of which is strongly associated with adult T-cell leukemia/lymphoma. It is endemic in south Japan, Caribbean, South Africa and South America. Human T-cell lymphotrophic virus 1 infection increases with age and is twice as high in females when compared to males. The infection is usually asymptomatic to start with but manifests typically later in the life. The incidence of infection is found to be increased in breast fed infants than in bottle fed infants.

Hepatitis–C Virus is found to be related to lymphomagenesis. Infections usually occur through blood transfusion and needle stick injuries. It is believed that HCV causes lymphoma by chronic antigenic stimulation. Infection of Hepatits C virus is associated with increased risk of Diffuse large B-cell lymphoma, Marginal zone lymphoma and lymphoplasmacytic lymphoma. Human herpes virus-8 has been documented in the causation of Non-Hodgkin lymphoma. HHV infection is associated significantly and positively with risk of lymphoplasmacytic lymphoma.

Genetic polymorphism associated with the risk of Non Hodgkin lymphoma suggests that single nucleotide polymorphism in Tumor necrosis factor and Interleukin-10 are associated with increased risk of Non-Hodgkin

lymphoma especially Diffuse large B-cell lymphoma. Alcohol consumption appears to be inversely related to Non-Hodgkin lymphoma. Smoking does not appear to play a role in etiology of Non-Hodgkin lymphoma.

Helicobacter pylori is a gram negative rod that was discovered by Warren and Marshall in 1983 and was shown to be associated with peptic ulcer disease, carcinoma of stomach and Non Hodgkin lymphoma [42, 43]. Later on Personett recognised that Helicobacter pylori infection preceded the development of lymphoma[44] and Wotherspoon observed that there is regression of lymphoma in most of the patients after treatment with antibiotics[45]. Other infectious agents includes, campylobacter jejuni which causes small intestinal immunoproliferative diseases, Borrelia burgdoferi which causes primary cutaneous B cell lymphoma and Chlamydia psittaci which is the causative agent for ocular adnexal MALTomas[40].

AIDS and transplant related Non Hodgkin Lymphoma

Lymphomas both Hodgkin and Non Hodgkin can be secondary to infection by Human immunodeficiency virus. Non-Hodgkin lymphomas can occur in setting of underlying congenital and acquired immunodeficiencies[46]. After Kaposi sarcoma, lymphomas are the most common AIDS related cancer [47]. NHL occurs in 3% of the patients suffering from AIDS [48], with sixty time greater risk of acquiring lymphomas when compared to persons without infection. Most of the Non-

Hodgkin lymphoma represents B-cell type, among which 70% is constituted by diffuse large B-cell lymphomas and the remaining being Burkitt's lymphoma. Among patients of lymphomas found in the general population of young patients in which Hodgkin lymphomas are more common, lymphomas in AIDS patients are mostly Non Hodgkin type with frequent extranodal manifestations [49, 50]. The occurrence of lymphomas in AIDS can be attributed to florid activation of lymphoid cells which provides opportunity for translocation, mutation and deletion of tumor suppressor gene [51, 52].

Post-transplant lymphoproliferative disorders include both polymorphic and monomorphic proliferation of lymphoid cells. PTLPD are most common during first year after solid organ and bone marrow transplant. The persons with multiple organ transplants are at increased risk of developing post transplant lymphoproliferative disorders. Lymphoid cell proliferation can occur in patients both with and without EBV infection. The main cause for occurrence of both polymorphic and monomorphic proliferation can be attributed to mutation in the variable region of immunoglobulin [53]. These mutations have been found in about 75% of polymorphic PTLPD and 90% of monomorphic proliferation. Among monomorphic proliferation Burkitt's lymphomas constitute the bulk of the lymphomas, remaining being cases of Diffuse large B-cell lymphomas.

Autoimmune and other disorders

Chronic inflammation, immune hyperactivity and immunosuppression are considered to be important elements that predispose patients to lymphoma [54]. Non-Hodgkin lymphomas associated with Sjogren's syndrome and Hashimoto's thyroiditis are of B-cell origin and they usually occur in elderly females. In 1963, Bunin and Talal[55] reported Non-Hodgkin lymphoma in a case of Sjogren's syndrome. In 1957, Lindsay and Dailey [56] demonstrated the association of lymphoma with Hashimoto's thyroiditis. There is 60 to 80 fold increase in thyroid lymphomas among the cases of thyroiditis with lifetime risk of 1-2% [57]. Non-Hodgkin lymphoma has been associated with rheumatoid arthritis, SLE, and dermatomyositis.

Non-Hodgkin lymphoma has been observed to originate from treated cases of Hodgkin lymphoma. Krikorian [58]reported 6 cases of Non-Hodgkin lymphoma arising from Hodgkin lymphoma. First case of Non-Hodgkin lymphoma associated with AIDS has been reported in 1982 [59]**.** Majority of the lymphomas arising from AIDS patients are of B-cell origin. Unique presentation of AIDS related lymphomas include plasmablastic lymphomas of oral cavity [60] and primary effusion lymphomas [61].

Non Hodgkin Lymphoma and presentation

Most of the cases of Non-Hodgkin lymphoma presents as painless lymphadenopathy in cervical and supraclavicular region [62]. However, 40% of patients have extranodal presentation. Gastro intestinal tract is the most

common extra nodal site of presentation, in which stomach is the commonest site followed by small intestine, colon and esophagus, in that order. Rarely, patients with rectal involvement present with altered bowel habits [63]. Epistaxis and nasal obstruction are the modes of presentation in cases of Non-Hodgkin lymphoma of nasal cavity. Hepatosplenomegaly have been observed in cases of low grade B-cell lymphomas such as small B-lymphocytic lymphomas and marginal zone lymphomas. Skin is another most common site of Non-Hodgkin lymphoma presentation. Non-Hodgkin lymphoma can primarily involve CNS [64] in which diffuse large B-cell lymphomas are the most common. Other rare sites involved are eye, extraocular space including conjunctiva, eyelids & lacrimal glands [65], testis, kidney and breast. Breast lymphomas associated with pregnancies and lactation often has diffuse involvement [66]. Primary cardiac lymphomas are extremely rare and usually occur in immunodeficient state [67].

Gray zone lymphomas

Malignant lymphomas are classified into Hodgkin and NHL based on morphological pattern, cell type and more importantly based on the analysis of antigenic expression. Both should be differentiated as they require distinct treatment for regression. The new modes of investigations like immunophenotyping and genetic analysis have revealed that there exist certain lymphoid neoplasms that can neither be classified as Hodgkin lymphoma nor as NHL. These were given the name, gray zone lymphomas

[68, 69]. This includes nodular lymphocyte predominant Hodgkin lymphoma, T cell rich large B cell lymphoma, Anaplastic large cell lymphoma, peripheral T-cell lymphoma and primary mediastinal large B-cell lymphoma.

Gray zone lymphomas should be separated from conventional lymphomas as they are aggressive and require distinct treatment [70, 71].

CLASSIFICATION OF NON-HODGKIN LYMPHOMA

Currently, lymphoma diagnosis is based upon the classification of World Health Organisation, which relies upon the morphology of the node including pattern, cell size and shape, radiological features, flow cytometry and IHC for evaluating the antigenic expression and finally cytogenetics. Based upon these, lymphoma has been classified by WHO into Hodgkin and Non Hodgkin lymphoma. The Hodgkin Lymphoma has been broadly classified into nodular lymphocytic predominant HL and classic Hodgkin lymphoma. The Non Hodgkin lymphoma has been further classified into B and T cell lymphomas which are sub-classified as precursor and peripheral (mature) B cell and T cell Non Hodgkin lymphoma, respectively. The precursor B and T cell lymphomas represent the neoplastic transformation of the progenitor cells which are not exposed to antigens, hence are in an inactivated and undifferentiated stage. The peripheral lymphomas arise from the rest of the lymphoid cells in various stages of maturation.

Mature B cell neoplasm constitutes about 90% of NHL worldwide[72]. B cell represents all follicular and diffuse lymphomas in North America and Europe, whereas T cell lymphomas are most common in Asia [73]. The nomenclature, classification and differentiation of various types of lymphomas depend upon various factors including the cell of origin eg; mantle cell lymphomas, clinicopathological name eg: Burkitt's lymphoma, their location eg: mediastinal large B cell lymphomas and presumed fuction eg; MALTomas.

The grade and prognosis of Non Hodgkin lymphomas depend upon the morphological pattern, size and shape of the cell, mitosis and invasiveness. The pattern of neoplastic transformation is divided into nodular and diffuse in which the nodular growth pattern represents the differentiation of malignant lymphomas towards lymphoid follicles. The malignant lymphoma cells are classified into small, medium and large sized by comparing the nucleus of the neoplastic cells to the adjacent histiocytes or endothelial cells. On the basis of shape, it is classified into cleaved and non-cleaved in which the cleaved cells represent non-dividing cells and non-cleaved represent the dividing cells. In general, lymphomas are considered aggressive when the cells are large, non-cleaved with a diffuse pattern of growth showing increased mitosis and invasion.

T cell neoplasms are less common when compared to B cell lymphomas in western countries, representing 20% of the Non Hodgkin

lymphoma [74]. T cell lymphoma along with NK cell lymphomas account for 12% of all the NHLs [75]. They are classified similar to B cell neoplasms into precursor (or) thymic T cell lymphoma and mature (or) peripheral T cell lymphomas. The largest group which represents peripheral T cell lymphomas is the T cell lymphoma NOS type, accounting for more than 50% of the cases of T cell lymphomas. Generally, the T cell lymphomas are aggressive with a 5 year survival rate of only 20-30% which are characterised by diffuse growth pattern, with mixed population of small and large cells in an inflammatory background. More frequently, epitheloid cells and eosinophils are also made out.

MODIFIED KIEL CLASSIFICATION [76]

KIEL CLASSIFICATION	Nearest correspondence to categories of Rappaport classification	Nearest correspondence to categories of Lukes-Collins classification
LYMPHOMAS OF LOW GRADE MALIGNANCY		
Lymphocytic lymphomas		
1. Chronic lymphocytic leukemia (B-cell type)	Well-differentiated lymphocytic lymphoma	B cell lymphoma of small lymphocytes
2.Hairy cell leukemia	-	-
3.Mycosis fungoides and Sezary's syndrome	-	T-cell lymphomas:.Mycosis fungoides and Sezary's syndrome
4.Chronic lymphocytic leukemia (T- cell type)	-	-
Lymphomas of immunoglobulin -secreting cells	Apart from lymphomas-'proliferative diseases with dysprotinemia'	
1.Lymphoplasmacytic/lympho plasmacytoid lymphoma	-	B-cell lymphoma of plasmacytoid lymphocytes

2.Plasmacytic lymphoma	-	-
Lymphomas of germinal centre cells		
1.Centrocytic lymphoma	well –differentiated & poorly differentiated lymphocytic lymphomas; Mixed cell(lymphocytic-Histiocytic) lymphoma;Histiocytic lymphoma	B-cell lymphoma of follicular centre cells with cloven nucleus (diffuse type)
2.Centroblastic /centrocytic lymphoma follicular follicular and diffuse diffuse with or without sclerosis	well –differentiated & poorly differentiated lymphocytic lymphomas; Mixed cell(lymphocytic-Histiocytic) lymphoma;	B-cell lymphoma of follicular centre cells with cloven nucleus
LYMPHOMAS OF HIGH GRADE MALIGNANCY		
Centroblastic lymphomas	Histiocytic lymphoma; undifferentiated lymphoma	B-cell lymphoma of large follicular centre cells with non-cloven nucleus
1.Primary		
2.Secondary		

Lymphoblastic lymphomas (lymphoblastic sarcomas, including acute lymphoblastic leukemia)	Undifferentiated lymphoma; poorly differentiated lymphocytic lymphoma	B-cell lymphoma of small follicular centre cells with non-cloven nucleus
1.Burkitt lymphoma	Undifferentiated lymphoma	As above
2.Lymphoma of convoluted cells [i.e., cells with convoluted nucleus]	Undifferentiated lymphoma; poorly differentiated lymphocytic lymphoma	T-cell lymphoma of convoluted lymphocytes
3.Unclassified lymphoma	poorly differentiated lymphocytic lymphoma	U-cell ('undefined cell') lymphoma; unclassifiable lymphomas.
Immunoblastic lymphomas	Histiocytic lymphoma	Immunoblastic sarcoma of B cells; immunoblastic sarcoma of T cells
With plasmablastic/ plasmacytic differentiation (derived from B cells)		
Without plasmablastic/plasmacytic differentiation (derived from B cell or T cell)		
(a) Leukemic variant (immunoblastic leukemia)		

[This table represents Kiel classification modified by Lennert et al in 1977[76]. The Kiel classification and Lukes-Collins classification relates to the functional characteristics of the cells that relate to the tumors.]

A WORKING FORMULATION OF NON-HODGKIN LYMPHOMAS[77]

This classification was introduced in 1982 by National cancer institute, United States [77], as an attempt to provide a morphologic classification scheme that had a prognostic relevance.

LOW- GRADE

Malignant lymphoma

- Small lymphocytic
  - Consistent with chronic lymphocytic leukemia
  - Plasmacytoid

Malignant lymphoma, follicular

- Predominantly small cleaved
  - Diffuse areas;sclerosis

Malignant lymphoma, follicular

- Mixed, small cleaved and large cells
  - Diffuse areas;Sclerosis

INTERMEDIATE-GRADE

Malignant lymphoma, Follicular

- Predominantly large cell

Diffuse areas; Sclerosis

Malignant lymphoma, Diffuse

Small cleaved

Sclerosis

Malignant lymphoma, Diffuse

Mixed, small and large cell

Sclerosis; Epithelioid cell component

Malignant lymphoma, Diffuse

Large cell

Cleaved, non cleaved; Sclerosis

HIGH GRADE

Malignant lymphoma

Large-cell, Immunoblastic

Plasmacytoid; clear cell; polymorphic; epithelioid cell component

Malignant lymphoma

Lymphoblastic

Convoluted; non convoluted

Malignant lymphomas

Small noncleaved

Burkitt; Follicular areas

MISCELLANEOUS

Composite

Mycosis fungoides

Histiocytic

Extramedullaryplasmacytoma

Unclassifiable

Others

WORLD HEALTH ORGANISATION CLASSIFICATION OF LYMPHOID NEOPLASMS (INTERNATIONAL AGENCY FOR RESEARCH ON CANCER, LYON, 2008) [78]

This classification is based on the collaborative project of European Association for Haematopathology and society for Hematology. This classification is based on principles initially defined in the "Revised European-American Classification of Lymphoid Neoplasms" (REAL), from the International Lymphoma Study Group (ILSG) [29].

PRECURSOR B- AND T-CELL NEOPLASMS

Precursor B-lymphoblastic leukaemia/lymphoblastic lymphoma

Precursor T-lymphoblastic leukaemia/lymphoblastic lymphoma

MATURE B-CELL NEOPLASM

Chronic lymphocytic leukaemia/ Small lymphocytic lymphoma

B-cell prolymphocytic leukaemia

Lymphoplasmacytic lymphoma

Splenic b-cell marginal zone lymphoma

Hairy cell leukaemia

Plasma cell myeloma

Solitary plasmacytoma of bone

Extraosseous plasmacytoma

Heavy chain disease

MALT-lymphoma

Nodal marginal zone B-cell lymphoma

Follicular lymphoma

Mantle cell lymphoma

Diffuse large B-cell lymphoma, NOS

DLBCL associated with chronic inflammation

Lymphomatoid granulomatosis

Mediastinal large B-cell lymphoma

Intravascular large B-cell lymphoma

ALK positive large B-cell lymphoma

Plasmablastic lymphoma

Primary effusion lymphoma

Burkitt lymphoma

B-cell lymphoma, unclassifiable with features intermediate between DLBCL &Burkitt lymphoma

B-cell lymphoma, unclassifiable with features intermediate between DLBCL and classic Hodgkin lymphoma

MATURE T-CELL AND NK-CELL NEOPLASM

T-cell prolymphocytic leukaemia

T-cell large granular lymphocytic leukaemia

Aggressive NK-cell leukaemia

Adult T-cell leukaemia/lymphoma

Systemic EBV positive T-cell lymphoproliferative disease of childhood

Hydroa vacciniforme-like lymphoma

Mycosis fungoides

Sezary syndrome

Primary cutaneous anaplastic large cell lymphoma

Primary cutaneous CD30 positive T-cell lymphoproliferative disorder

Primary cutaneous gamma-delta T-cell lymphoma

Lymphomatoid papulosis

Extranodal NK/T-cell lymphoma, nasal type

Enteropathy-type T-cell lymphoma

Hepatosplenic T-cell lymphoma

Subcutaneous panniculitis- like T-cell lymphoma

Angioimmunoblastic T-cell lymphoma

Peripheral T-cell lymphoma

Anaplastic large cell lymphoma

HODGKIN LYMPHOMA

Nodular lymphocyte predominant Hodgkin lymphoma

Classical Hodgkin type

- Nodular sclerosis classical Hodgkin lymphoma
- Mixed cellularity classical Hodgkin lymphoma
- Lymphocyte rich classical Hodgkin lymphoma
- Lymphocyte depleted classical Hodgkin lymphoma

CELLS OF ORIGIN OF NON HODGKIN LYMPHOMA

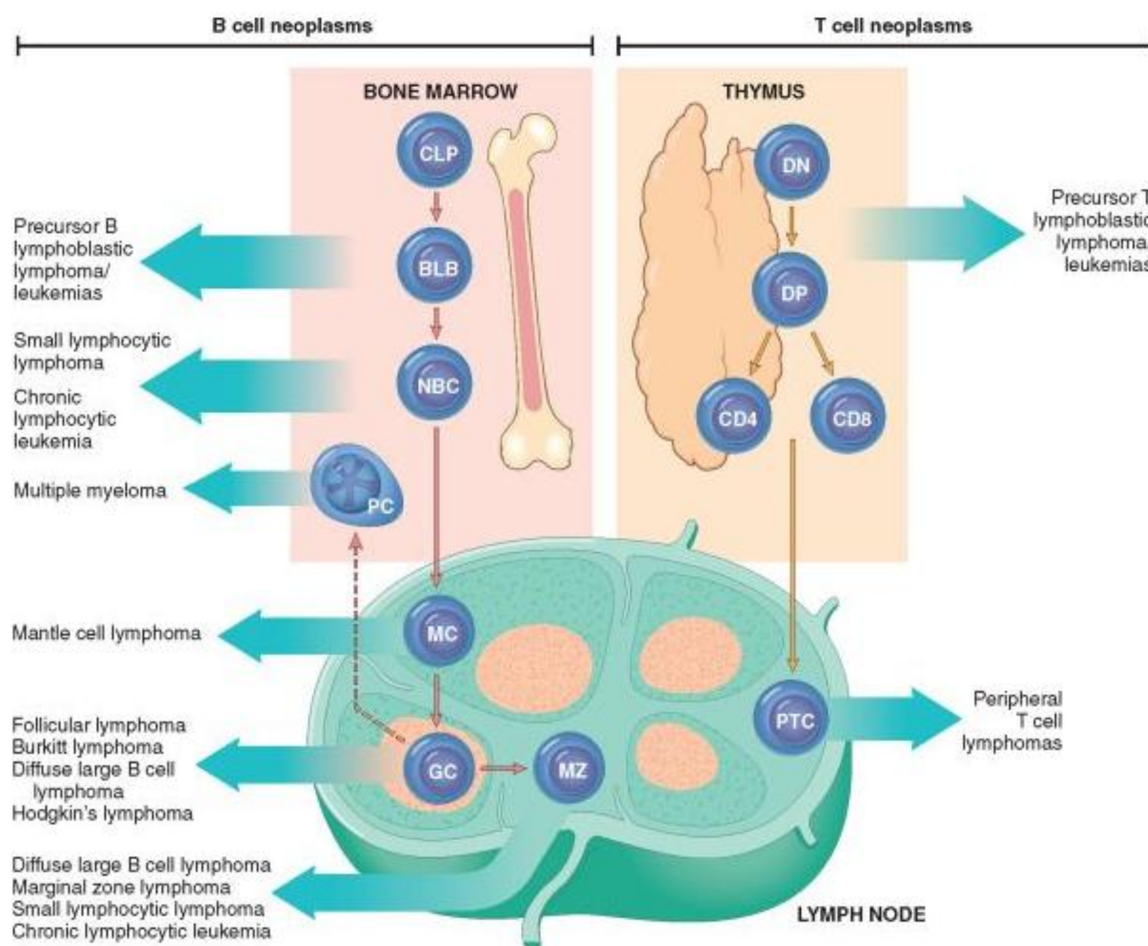


Figure 1[79]: Origin of lymphoid neoplasms. Stages of B- and T-cell differentiation from which specific lymphoid tumors emerge are seen.

As seen in the above illustration, the immature B- cells and T-cells derived from the bone marrow are the cells of origin for precursor B-cell and T-cell lymphomas/leukemias, respectively. The germinal centre B cell gives rise to burkitt lymphoma, follicular lymphoma and diffuse large B-cell lymphoma. DLBCL also arises from the post germinal centre B-cells. The post germinal centre memory B-cells give rise to hairy cell leukemia, extranodal marginal zone lymphoma and CLL/SLL. The peripheral T cell lymphomas of nodal origin arise from the T cells in the paracortical area of the lymph node.

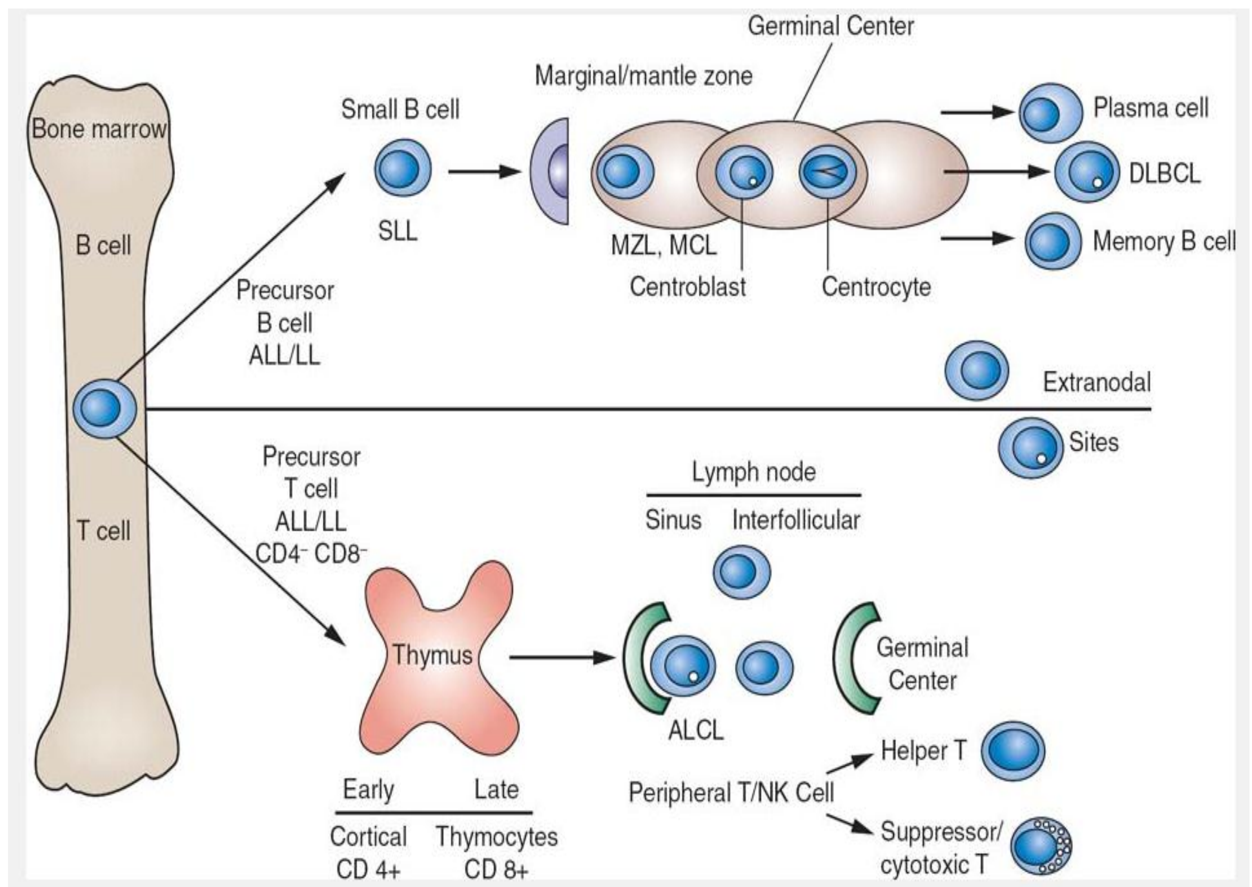


Figure 2[80]: cellular origin of Non Hodgkin Lymphoma based on B cell and T cell differentiation

The above illustration shows the different classes of lymphoma based on Lukes-Collin classification. The B cell lymphoma of small lymphocytes constitutes CLL/SLL, whereas the B-cell lymphoma of follicular centre cells with cloven nucleus (diffuse type) constitutes the centrocytic lymphoma. B-cell lymphoma of large follicular centre cells with non-cloven nucleus constitutes centroblastic lymphoma , whereas B-cell lymphoma of small follicular centre cells with non-cloven nucleus constitute lymphoblastic lymphoma.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a technique which is used to detect the specific antigens over the tissues or cells based on the pattern of antigen-antibody reaction. Coons [(81)] in 1940 introduced immunoflorescence to localise corresponding antigen in frozen section. The enzymatic label horseradish peroxidase developed by Avrameas[(82)] and by Nakane and colleagues[(83)] provided the new path for the visualisation of the labelled antibody by light microscopy in the presence of a appropriate colorigenic substrate system.

The aims of immunohistochemistry are akin to those of histochemistry. Indeed, immunohistochemistry builds on the foundations of histochemistry. Immunohistochemistry does not replace histochemistry but rather serves as the valuable adjunct that greatly extends the variety of tissue components that can be demonstrated specifically with tissue sections. The

main aim of performing IHC is to recognize microchemically the existence and distribution of substances which we have been made aware of macrochemically[84]. Immunohistochemistry can provide a tissue based immunoassay with the reproducibility and quantitative characteristics of an ELISA test.

Demonstration of antigen in routinely processed formalin-fixed paraffin embedded tissues[85] was done for the first time in 1974 by Taylor and Burns in Oxford.Then as the immunohistochemistry evolved, its utilisation in diagnostic pathology steadily expanded such that more than one immunohistochemistry markers were used in routine surgical pathology especially with respect to tumour diagnosis and classification. The introduction of hybridoma technique[86] facilitated in the development of immunohistochemistry and the manufacture of many highly specific monoclonal antibodies.

The phenomenon of enzyme digestion was then introduced by Huang as the pretreatment to immunohistochemistry staining to unmask some antigens that had been altered by formalin fixation [87]. The main disadvantage of using enzyme digestion was that it proved difficult to control the optimal digestion conditions for different tissue sections when stained with different antibodies. Hence, to overcome the difficulties encountered in enzyme digestion Shi and his associates in 1991[88, 89, 90, 91, 92, 93] introduced antigen retrieval technique, based on series of studies by

Fraenkel and co-workers [94, 95, 96]. Various modifications of antigen retrieval has been described, among which majority of those used different buffer solution in the place of metal salt solution, which may have serious toxic effect[97, 98].

Antigen and antibody reaction

An antibody is a molecule that has the property of combining specifically with the antigen. Antigen-antibody recognition is based on three dimensional structure of the protein. Antibodies are immunoglobulin molecules containing pair of light chain and pair of heavy chain. An epitope is an antigenic determinant on which the antibody combines. For a protein, the term epitope corresponds to a cluster of aminoacid residues that binds specifically to the paratope of an antibody [99].

Antigen-antibody reaction depends on two factors that are related to antibody which includes sensitivity and specificity. Antibodies are primarily of two types monoclonal and polyclonal. Comparitive studies on sensitivity and specificity among monoclonal and polyclonal antibodies proves that polyclonal antibodies are more sensitive and less specific than monoclonal antibodies. The reason may be polyclonal antibody which is a composite of many antibodies may recognise several binding epitope whereas monoclonal antibody recognise single type of epitope.

Most of the monoclonal antibodies in current use are derived from murine clones. Recently a number of rabbit derived monoclonal antibodies

have appeared on the market. Some of the rabbit derived antibodies offer advantage over murine clones for the detection of antigens by immunohistochemistry.

Enzyme labels in immunohistochemistry

Enzymes are the widely used labels used in immunohistochemistry. Incubation with the chromogen using proper histochemical method produces stable colour reaction end product that can be easily read by using light microscopy. Variety of enzymes and chromogens are available which produces many number of coloured end products. Horseradish peroxidase is the most commonly used enzyme label. When used in conjunction with most favoured chromogen like Diaminobenzidine tetrachloride yield insoluble, stable brown coloured end product. Horseradish peroxidase is most preferred as they are very small in size, and can be very easily obtained in pure form. It is very stable and does not undergo alteration during manufacture, storage and during application.

Various other chromogens are also used based on the choice of end product coloured reaction. Graham in 1965 used 3-amino 9-ethyl carbazole which produces red colour. Later in 1968, Nakane used 4-chloro 1-napthol produces blue colour. Hanker in 1977, used Hanker- Yates reagent, that produces a dark blue colour. Commercially chromogens are available in Kit form such as vector laboratories which used vector red and vector blue. Alternatives to Diaminobenzidine tetrachloride are more commonly used in

multilabelling technique. Rarely colloid metal label such as gold conjugates are used. When used alone it appears pink under microscope. A silver precipitation reaction can be used to amplify the visibility of gold conjugates. The use of radioisotopes as the tracer requires autoradiographic facilities.

Antigen retrieval

Antigen retrieval is the process of reversal of chemical reaction that has occurred between formalin and protein. Heat induced antigen retrieval technique is now widely used in pathology[(100)]. It has certainly brought a very good improvement in the quality of immunohistochemistry. The optimal result of antigen retrieval in immunohistochemistry depends on heating temperature, period of heating and P_H of antigen retrieval solution.

Various methods are used in the process of antigen retrieval which includes microwave antigen retrieval, pressure cooker antigen retrieval and retrieval using streamer, water-bath, autoclave and combined microwave and trypsin digestion antigen retrieval. The use of conventional heating at 100^0c achieves results similar to those obtained by microwave. Distilled water could be used as the antigen retrieval solution with slight less effect [(84)].

Shi et al in 1991 introduced the use of microwave in the technique of antigen retrieval, but the use of heavy metals posed significant difficulties and problems. Later in 1992 Gerdes performed antigen retrieval with non

toxic citrate buffer. Cottoretti in 1993 argued that microwave antigen retrieval is an alternative to proteolytic enzymes. Various problems including uneven heating and hot spot production are the important problems with the use of microwave.

In 1994, Norton suggested the use of pressure cooker method for antigen retrieval. It is considered as one of the suitable method as it produces even heating and has no disadvantages which includes hot spot and cold spot production which often occur with the use of microwave. Streamer antigen retrieval is less efficient when compared to microwave and pressure cooker. Kawai in 1994 showed that water bath set at 90^0C is adequate for antigen retrieval. However, quality of antigen retrieval improves with increase in the temperature to 95-98^0C.

Evers and Nylings[101]in his study found that antigen retrieval depends on both p_H and temperature. They concluded that it is not important what kind of solution is used as long as the p_H is appropriate. Chemical composition and the molarity of the antigen retrieval solution may act as a cofactor for effectiveness of antigen retrieval and hence immunohistochemistry.

Non specific background staining

Non specific staining in immunohistochemistry is attributed to non specific antibody binding and action of endogenous enzymes. Non specific staining is more common with usage of polyclonal antibody because of

multiple unwanted antibodies. Blocking of endogenous enzymes activity is important. The degree of susceptibility of an enzyme to denaturation and inactivation varies from tissues to tissues. Any residual activity of endogenous enzymes must be abolished. Endogenous peroxidase activity is seen in neoplastic cells, erythrocytes, neutrophils, eosinophils, and hepatocytes. Usage of various substances for blocking endogenous peroxidase is suggested.

Some investigators thought that use of methanol-hydrogen peroxide may cause denaturation of antigen. Strans[102,103] advocated the use of phenylhydrazine by combination of phenyl hydrazine, nascent hydrogen peroxide and sodium azide. Mixture of hydrogen peroxide and sodium azide was found to be very effective[104]. More recently cyclopropane hydrate was shown to inhibit endogenous activity without adverse effect[105]. Endogenous alkaline phosphatase activity is usually blocked by addition of Levamisole to the substrate. Levamisole selectively inhibits certain types of alkaline phosphatase but not intestinal and placental. 20% glacial acetic acid is a better alternative to levamisole.

IMMUNOHISTOCHEMISTRY AND NHL

The use of antibody panels in immunohistochemistry is well accepted. Single antibody directed against the malignant cells will not serve for the diagnosis and is insufficient. We need to use panel of markers to differentiate one form of Non-Hodgkin lymphoma from the others [106,107].

Much of the early work in diagnostic hematopathology required frozen section or acetone fixed frozen section as available antibodies failed to recognize the antigens fixed by cross linking fixatives. Partial enzyme digestion gives good result[108].

Understanding of the development, maturation and migration of lymphocytes provides rationale for immunophenotyping because stages in lymphocyte maturation are defined in part by the macromolecules they produce. Malignant cells expresses antigen that resembles the antigens of lymphocytes in different developmental stages. Lymphomas are thought of as being composed of cells arrested in development of certain stages[109]. Hence the panel of markers directed against the malignant cells will be of more help.

Immunohistochemical markers are selected based upon the morphology. Morphological assessment takes into account the architectural alteration in B-cell zone and T-cell zone. The markers include leucocyte common antigen, B-cell markers such as CD20 and CD79a, T-cell markers like CD3 and CD5 and other markers including CD23, bcl-2, CD10, cyclinD1, CD15, CD30, ALK-1 and CD138. Currently there are more than 50 antibodies which are used in the diagnosis of Non-Hodgkin lymphoma. Explosion of antigens and reagents to detect them needs judicious use of selected panel of markers. It should always be noted that no antigens is

totally specific and therefore needs careful selection of panel of markers to arrive at proper diagnosis.

LYMPHOMA DIAGNOSIS

The diagnosis of lymphoma is based on the World health organisation classification [110](2008). Further classification is based on the stage of maturation and cell of origin. Other features which help in the diagnosis are anatomic architectural alteration in the lymphoid compartment, determination of cell size, nuclear character, and character of nucleoli.

B-cell markers

CD20 is the most common antibody used as the pan-B cell marker and is expressed by naive B cell until the final stages of maturation just prior to plasmacytic differentiation. Chronic lymphocytic lymphoma/small lymphocytic lymphoma rarely shows weak positivity[111] to CD20. In cases of prior rituximab therapy, CD79a should be added. Early stage including naive cells and other B-cell expresses Pax-5 but not the plasma cells[112]. Few cases of precursor T-cell acute lymphoblastic lymphoma expresses CD79a and rare cases of AML and merkel cell carcinoma shows positive expression of Pax-5.

When B-cell follicles are not well made under histopathology the immunohistochemical markers like CD21, CD23 and CD35 may be helpful in highlighting follicular dendritic cells. Staining the sections for kappa and lambda light chains may be useful in detecting the abnormal clonal

population, especially plasma cells. Immunohistochemistry although less sensitive in the detection of immunoglobulin expression, can be more valuable when used along with histopathology. Plasma cells and immunoblast shows strong expression of immunoglobulins which can be detected by paraffin immunoperoxidase method. Calorimetric insitu hybridisation is an alternate and most sensitive method used in the detection of immunoglobulin.

T-cell markers

CD3 is the most commonly used pan-T cell marker which detect the antigenic expression over the malignant and normal T-cell at the second stage of thymic differentiation and beyond. Sometimes there may be loss of pan-T cell marker over the malignant lymphoid cells as in case of anaplastic large cell lymphoma(113). T cells are divided into CD4 and CD8 subsets. Antibodies directed against these antigens are used as basic panels to detect abnormal distribution of lymphoid cells. CD5 is another pan-Tcell marker which is lineage non-specific and is not expressed on Natural killer cells. CD5 is present over small subset of B-cells especially among the cases of chronic lymphocytic lymphoma, mantle cell lymphoma.

Atypical cells and Lymphoma

Histopathological examination of some lymphomas may show many large atypical lymphoid cells admixed with other similar looking lymphoma cells. When there is presence of large atypical cells, the possibilities of

carcinomas, Hodgkin lymphoma and anaplastic large cell lymphoma should be ruled out. This is done by using a panel of markers including CD45, CD15, CD30 and HMB45. The usage of antibody directed against ALK-1 may prove more valuable.

Other antibodies

Several other markers have found application in the diagnosis of Non-Hodgkin lymphoma. These include CD10, Bcl-6, cyclin D1, Bcl-2, CD56 and CD57. CD10 and Bcl-6 are the markers that show positive expression in normal and neoplastic follicular centres. CD10 also show positive expression on granulocytes, T-cells in angioimmunoblastic lymphadenopathy, precursor B-cells and T-cells, epithelial cell neoplasms like renal cell carcinomas, hepatocellular carcinomas and endometrial stromal cells. Bcl-6 is expressed in follicular centres and few T-cells. The presence of CD10 positive lymphocytes along with Bcl-6 positive cells outside the follicles strongly suggests neoplastic proliferation. But rare cases of follicular lymphoma which shows negative expression of CD10 have been recorded.

Bcl-2 is an antiapoptotic molecule which is normally expressed over pre-B cells, and resting B-cells of normal mantle zone and in rare cases with proliferating B-cells. Most of the neoplasms composed of small lymphoid cells usually show positive expression of Bcl-2[(114)] and expression of Bcl-2 is downregulated in normal follicles. B-cells in reactive follicles show

negative expression of Bcl-2 and there is strong expression of Bcl-2 in lymphoid cells of mantle zone and cells of primary follicle[115]. The interpretation of Bcl-2 should be carried out in conjunction with CD3 staining for T-cells as T-cells normally show positive expression of Bcl-2. Most of the cases of marginal zone lymphoma shows Bcl-2 expression but reactive hyperplastic marginal zones of spleen and lymph nodes of abdomen also show Bcl-2 positivity. CyclinD1 is another immunohistochemical marker which has gained its importance in the diagnosis of Mantle cell lymphoma. Cyclin D1 is normally expressed in endothelium, nucleus of histiocytes, and fibroblast. It is usually expressed in the nucleus of lymphoid cells of mantle cell lymphoma. Cytoplasmic expression is considered non diagnostic. Although nuclear expression of CyclinD1 is almost diagnostic of mantle cell lymphoma many cases of CyclinD1 negative mantle cell lymphomas are on the records and even CyclinD1expression has been recorded in many cases of small lymphocytic lymphoma. Immunohistochemical markers like CD56 and CD57 play important role in the diagnosis of natural killer cell proliferation and neoplasms arising from natural killer cells.

PANEL OF MARKERS FOR NON HODGKIN LYMPHOMA

Lynette K Tumwine et al [116], studied the expression of B cell antigens in 119 Non Hodgkin lymphoma patients using a panel of CD3,5,10,20,23,30,38,79a,138, bcl-2, MUM1/IRF4, cyclinD1 and Ki-

67/Mib1 antibodies. They found 100% positivity for CD20 in DLBCL and Burkitt lymphoma patients. Among DLBCL patients, 10.5% positivity was seen for bcl6, 22.2% positivity for MUM1/IRF4 and 66.7% for CD138. According to I.Satish Rao[1] distinction of monomorphic small B cell lymphomas are done by basic panel of markers such as CD5, 23, 10, cyclin D1 and bcl2, intermediate cells with diffuse pattern by markers such as CD20,10, Tdt, CD99 and Mib1 and large cells with diffuse pattern by CD20, CD79a, CD138 and EBER. Nirmala Ajit Jambhekar et al [117] suggests a panel of markers for Non Hodgkin lymphoma diagnosis which includes CD20 which is a B-cell marker showing membrane staining, CD3 is a T-cell marker, CD21/CD35 stains follicular dendritic cells and hence are useful to demonstrate germinal centers within the lymphoid proliferation, CD5 which detects B-cell CLL/SLL and mantle cell lymphomas. CD23 shows positivity for B-CLL/SLL, whereas it is negative in mantle cell lymphoma. Mantle cell lymphoma shows immunoreactivity to Cyclin D1. Alk-1 is specific for anaplastic large cell lymphomas (ALCL) which also shows CD30 positivity and focal EMA positivity. Bcl-2 is useful in distinguishing reactive follicles (negative) from follicles of follicular NHL (positive) whereas CD10 is positive in Burkitts lymphoma.

Kwak LW and Wilson M [118] in their study found out that among all the cases of DLBCL, 65% to 85% are of B cell type and 15%-35% are T cell type. T cell rich variant of DLBCL may show membranous staining of

pan T cell marker (CD3). Hans et al [119] in their study on 152 cases of DLBCL demonstrated the expression of CD10 in 28% of cases, bcl-6 in 56%, MUM1 in 47%, bcl-2 in 50%, cyclin D2 in 13% and FOXP1 in 61% of cases. Ting Li et al [120] studied immunohistochemical features in 63 cases which resulted in expression of CD10 in 30% of cases, bcl6 in 35% and MUM1 in 51% of cases.

Strauchen JA and Mandeli JP [121] in their study of 345 cases of B cell NHL found Ig expression in 59% cases of large cell lymphoma and 100% of small cell lymphoma. Picker LJ et al [122] documented that immunoblastic variant especially those with plasmablastic differentiation showed cytoplasmic Ig expression. Piris M et al [123] through their study suggested that most of the anaplastic variant showed positive membrane staining for CD30. Fang JM et al [124] found that 20-30% of DLBCL showed CD10 positivity.

Follicular lymphoma cells show positive membrane staining for CD 19, 20, 22 & 79a with typical surface monoclonal Ig expression. Jaffe ES et al [29] through their study found out that follicular lymphoma cells do not express CD3, 5 and 23. Both follicular and inter follicular zones contain κ or λ clonally restricted B cell. Lai R et al [125] in their study found that malignant cells were negative for CD43. Gaulard P et al [126] demonstrated overexpression of nuclear positivity for bcl-2 in germinal centre among the cases of low grade follicular lymphoma.

Robert R. Lorsbach et al [127] through their study on 19 paediatric follicular lymphoma patients showed CD20 and bcl6 positivity in all cases and CD10 positivity in 80% of cases. Andrew J Davies et al [128] observed through their study that among the follicular lymphoma patients assessed for transformation to DLBCL, 89% showed germinal centre phenotype with $CD10^{+}$, $bcl6^{+}$, and $MUM1^{-}$. Follicular lymphoma in addition to mimicking the growth pattern of reactive follicles, contain non neoplastic T cells and follicular dendritic reticulum cells. According to Said JW et al [129] follicular dendritic reticulum cells in follicular lymphoma and reactive follicular hyperplasia express CD21 and CD35 whereas follicular dendritic reticulum cells in follicular lymphoma but not in reactive follicular hyperplasia, lack the expression of fascin.

In mantle cell lymphoma, the neoplastic cells show membranous positive expression of CD19, 20, 22, 79a and CD5. Vasef MA [130] and Cheuk W [131] through their study showed that almost all the cases of mantle cell lymphoma showed strong nuclear expression for cyclin D1. In case of CLL/SLL, the cells are B cells with positivity for surface Ig light chain, IgM, IgD, pan B cell antigens, CD5 and 23 antigens.

According to Williamze R et al [132] the cells of mycosis fungoides and Sezary syndrome have a mature T cell phenotype and are of T helper cell lineage. These cells express pan T cell antigens CD2,3,5 and CD45 RO. Picker LJ et al [133] in their study showed that PTCL cells express

CD2,3,CD5 and 7,43 and CD45RO but are negative for B cell antigens. Through their study Rudiger et al [134] showed that AITL express antigens like CD10, bcl6, CXCL13, SAP, programmed death-1 and activation induced cytidinedeaminase. In cases of anaplastic large cell lymphoma, ALK^{+} anaplastic large cell lymphoma by definition shows ALK positivity and CD30 expression in a distinctive pattern along with expression of T cell antigens.

According to Afaf Abdel-Aziz, Abdel-Ghafar[135] the grading of immunohistochemical staining can be done using percentage of positive cells which include four grades, 0: no reaction, 1+: $<5\%$, 2+: 5–9%, 3+: 10–20% and 4+: $\geq 20\%$.

OTHER MODALITIES OF DIAGNOSIS OF LYMPHOMA

Ultrastructural examination is not routinely done in the identification of lymph node lesions, but it is inexpensive to fix the sample in glutaraldehyde which can be used later, if needed. Electron microscopy is useful in rare disorders which include storage disorder like Gaucher’s disease, lymphoblastic lymphoma, mycosis fungoides and amyloidosis. Immune electron microscopy can be helpful to study the cell surface markers and immunoglobulins after applying peroxidase labelled antibodies. Ultrastructure is more useful in identifying metastatic deposits in lymph nodes especially in the cases of malignant melanomas demonstrating

melanosomes[136], Ewing's sarcoma with abundant glycogen[137] and small cell carcinomas with neurosecretory granules[138].

Chronic lymphocytic lymphomas are characterised by increased synthesis of immunoglobulin light chain when compared to heavy chain. Ultrastructural examination in such cases reveals Ig light chain in both perinuclear space and rough endoplasmic reticulum whereas heavy chains are limited to rough endoplasmic reticulum.

Lymphoblastic lymphoma under electron microscope shows round cells with a high nuclear to cytoplasmic ratio with convoluted nucleus showing deep indentation and fine chromatin with peripheral nucleolus [139]. The examination of angioimmunoblastic lymphoma shows nuclear indentation, speckled heterochromatin and prominent nucleolus [140].

The diagnosis of lymphoma, as proposed by WHO, now primarily depends on imunophenotyping. Immunophenotyping is usually performed by using IHC and flow cytometry. Flow cytometry has many advantages over IHC and is usually performed in higher centres. It is a qualitative analysis of antigen expression which determines the density of more than one antigenic expression simultaneously by using many fluorochromes per test tube. Hence, it can be easily standardised and also has a rapid turnover time. The main disadvantage of flow cytometry is it can be performed only in fresh living tissues.

Molecular diagnosis is considered as gold standard [141,142] in assessing monoclonality and chromosomal translocations. Unlike immunohistochemistry which detects proteins, molecular diagnosis detects DNA of genes encoding immunoglobulins and T cell receptor. Various techniques used in routine practice are conventional cytogenetics, southern blot technique, polymerase chain reaction and fluorescence insitu hybridisation. Polymerase chain reaction is a versatile technique used in the analysis of clonality, chromosomal translocation, genetic mutation, infectious agents and minimal residual disease. The main disadvantage is the occurrence of false positivity because of its high sensitivity.

Chromosomal translocation plays an important role in the occurrence of many lymphomas and hence identification of such translocation proves the diagnosis. This is carried out often using conventional cytogenetics or florescence insitu hybridisation technique. This can identify wide range of translocations which includes myc translocation in Burkitt's lymphoma[143], t(11;14) involving cyclinD1 in mantle cell lymphoma[144], t(14;18) involving Bcl2 gene in follicular lymphoma, Bcl6 in case of diffuse large B-cell lymphoma and subset of follicular lymphoma[145] , ALK translocation as in the case of anaplastic large cell lymphoma, t(1;14) (11;18)(14;18) in MALT lymphomas and t(11;14)(4;14) as in case of myeloma.

CONDITIONS MIMICKING LYMPHOMA

Reactive lymphoid hyperplasia is defined as a benign reversible process characterised by proliferation of reactive lymphoid cells in various pattern secondary to antigenic stimulation. Clinically, this condition is manifested as enlarged lymph nodes in multiple sites including cervical nodes which are often affected in infectious mononucleosis, posterior cervical group as in the case of toxoplasmosis, axillary node in cat- scratch disease, parotid, submaxillary and epitrochlear group as in the case of HIV infection. When there is involvement of supraclavicular lymph nodes careful evaluation of the patient including age, sex, family history, symptoms and signs are performed. This is because these nodes are associated with either primary or metastatic malignancy in 25% of patients younger than 40 years and 90% of patients older than 40 years. [146]

Reactive lymphoid hyperplasia can be caused by various bacterial and viral infections, exposure to chemical substances and environmental pollution, drugs and numerous allergens. Among the drugs phenytoin, penicillins, gold and quinidine play a major role[147]. Reactive lymphoid hyperplasia, occur most commonly in children and younger age group. Hyperplastic nodes are less common among the elderly because of the decreased humoral immune response [148] and hence the proliferation of germinal centre is less apparent than in young patients [149].

Most of the hyperplastic nodes show nonspecific pattern. But characteristic morphological pattern can be seen in reactive follicular hyperplasia secondary to activated humoral immune response, diffuse paracortical hyperplasia as in the case of viral lymphadenitis and sinus histiocytosis. Histopathology of reactive follicular hyperplasia is characterised by numerous reactive secondary follicles of varying size and shape distributed in the cortex and medullary region, with well demarcated mantle cell zone. The germinal centre comprises mixture of small and large lymphoid cells.

The presence of numerous follicles throughout the cortical and medullary zones makes morphological distinction of exaggerated follicular hyperplasia from follicular lymphoma. However, according to Nathwani(150), numerous back to back follicles with even distribution of the cortex and medulla with scant inter-follicular area in addition with cytological features helps in the distinction. But in more difficult cases, immunohistochemistry may prove useful.

Diffuse paracortical hyperplasia is characterised by expansion of paracortical area due to increase in number of reactive T cells containing mixture of small cells and activated large cells, the immunoblast. Immunoblasts are larger cells with scant cytoplasm, large nucleus and prominent nucleoli resembling Hodgkin and RS cell (151) and rarely

metastatic deposit from amelanotic melanoma. In difficult cases like this, immunohistochemistry solves the problem.

Atypical lymphoid hyperplasia is a group of disorder that is neither benign nor malignant which occurs in cases of primary immunodeficiency. This disorder occupies an intermediate position between reactive lymphoid hyperplasia and malignant lymphoma. The morphology is characterised by nodular or diffuse proliferation of lymphoid follicles admixed with many atypical large cells with large nucleus and prominent nucleoli. These atypical cells can be either singly scattered or in sheets, and when so can cause difficulties in differentiating it from malignant lymphomas and metastatic deposit. In such cases immunostaining to demonstrate monoclonal light chain (either κ or λ) is necessary.

Some of the drugs that are used for therapy, either in shorter term or after a longer period of time, can produce generalised lymph node enlargement mimicking malignant lymphoma. But these can be differentiated from true cases of malignant lymphoma by the regression of the lymph nodes after discontinuation of the drugs. This is called as anticonvulsant hypersensitivity syndrome as such cases can occur after the use of phenytoin and carbamazepine. This syndrome is found to have genetic predisposition which can be attributed to inherited defects in the epoxide dehydrolase [(152)] which detoxifies epoxide hydrolase formed as a result of metaboloism of phenytoin and carbamazepine by cytochrome

p450. Gennis estimated that pseudolymphoma after phenytoin therapy occurs in the range of 1:1000. Anticonvulsant hypersensitivity syndrome (pseudolymphoma) occurs in association with haematological abnormalities such as eosinophilia, leucocytosis/ leucopenia and atypical lymphocytosis.

This is manifested as acute increase in the size of the lymph nodes with effaced nodal architecture but preservation of atleast portion of lymph node can be usually seen. Histopathologically, this is characetrised by paracortical expansion with rarely follicular hyperplasia. Paracortical expansion is characterised by proliferation of mixed inflammatory cell infiltrates composed of eosinophils, small lymphoid cells and large atypical looking immunoblast. Hence it should not be confused with malignant lymphomas including Hodgkin and Non-Hodgkin lymphomas especially T-cell lymphomas like anaplastic large cell lymphoma and angioimmunoblastic lymphomas. In case of difficulties immunohistochemistry, flow cytometry, PCR or molecular diagnostic approaches will be useful. The absence of monoclonal gene rearrangement favors drug associated lymphadenopathy over true malignant lymphomas. (153,154)

DRUGS CAUSING LYMPHADENOPATHY

Phenytoin	Primidone
Carbamazepine	Methimazole
Phenobarbitol	Quinidine
Gabapentin	Tetracycline
Lamotrigine	Ethosuximide
Penicillin	Halothane
Gentamicin	Mexiletine
Allopurinol	Abacavir
Methyldopa	Ivermectin
Aspirin	Gold
Phenylbutazone	Iron dextran

LYMPHNODE AND NECROSIS

Necrosis in the lymph node can be focal or diffuse replacing the nodal architecture. It can be of any type which includes coagulative, caseous and fibrinoid type. Caseous necrosis most commonly occurs in infections like tuberculosis in which the necrotic areas appear granular and eosiniphilic surrounded by epithiloid cell granulomas, lymphocytes and fibroblasts. In addition, the disease causing organisms can be demonstrated in such kind of lesions.

Fibrinoid necrosis can occur in the cases of vasculitis involving the lymph node. Apart from this, histiocytic necrotising lymphadenitis also called Kikuchi-fujimotos lymphadenopathy is characterised by patchy or confluent areas of fibrinoid material with necrotic debris surrounded by histiocytic proliferation. Some cases rarely show scattered cells with karyorrhexis and pyknosis[155]. The periphery of the necrosis is characterised by thrombosed vessels, and nests of plasma cells, monocytes and immunoblast. Some of these cells are atypical and can be mistaken for lymphoma. The histiocytes which phagocytosed nuclear debris have peripherally placed crescentic nucleus and hence can be mistaken for signet ring cell carcinoma deposit. But importantly, absence of neutrophils and eosinophils are the distinct morphological feature of Kikuchi-Fujimoto disease.

Lymph nodes are organs with rich vascularity and hence rarely infracted. However, cases of coagulative necrosis involving most of the nodal parenchyma, sparing only a narrow subcapsular rim have been recorded [156, 157]. The causes include occlusive vascular thrombosis, thrombophlebitis, localised trauma secondary to fine needle aspiration and as a secondary complication to surgical procedure. Coagulative necrosis can occur secondary to malignant lymphomas. This may be due to expansion of lymphomas causing pressure effects over the vessels or due to direct invasion of the hilar vessels. Hence massive infarction should always be viewed with a suspicion of underlying malignancy. The demonstration of preserved antigen in the necrotic area through immune histochemistry is useful in such situations.

Castleman's disease is defined as the large benign asymptomatic lymphadenopathy usually involving mediastinal groups. The cause for Castleman lymphadenopathy is not known, however HHV-8 is considered to be a known etiological agent. Primarily two variants has been described namely hyaline vascular and plasma cell variant which can be unicentric or multicentric. The hyaline vascular variant is by far the most common variant [158,159]. Histologically it is characterised by changes in the follicular and interfollicular area. Within the follicles there may be twinning of germinal centres with reduced number of small lymphoid cells. The blood vessels are arranged radially and there may be deposition of hyaline material in the

centre of the vessels. The expansion of mantle zone is observed with small lymphoid cells arranged concentrically in an onion-skin fashion. The interfollicular area shows mixed population of cells.

The plasma cell variant is characterised by sheets of plasmablasts and mature plasma cells expanding the interfollicular area. Immunohistochemical evaluation is needed when there is florid proliferation of these cells leading to confusion with lymphocytic lymphoma and plasma cell neoplasm.

TISSUE MICROARRAY

Tissue microarray is a method which is used to evaluate numerous samples in a short period of time. Tissue microarray allows for high-throughput molecular profiling of tissue specimens by a number of techniques including immunohistochemistry[(160)]. Tissue microarray is very useful in investigating a large number of different molecules involved in solid tumour development[(161)]. TMA are very useful tools for rapid and efficient analysis of large number of paraffin embedded tissue sections[144] and a revolutionizing approach to allow multiplex histological analysis. This technique is now widely used in the analysis of a many number of biomarkers [(162)] and are used as a tool in the diagnosis of a variety of infections[(163)]. However, the use of Tissue microarray as a tool of quality control of diagnostic staining has been advocated in recent years.

The origin of Tissue microarray technique is attributed to Dr. Hector Battifora's sausage block technique[164] which he introduced in 1986 in which a number of tissues from various organs are thrown into the same block and tissue distribution of particular antigen was processed. The idea of Battifora was later redefined by Wan et al[165] who produced a library of paraffin embedded cores and used it to determine various staining patterns of many number of monoclonal antibodies. Wan et al in his technique used 16 gauge needle to manually bore cores from tissue blocks and array them in recognisable pattern. Later this was modified by Kononen et al[160] who introduced the term 'Tissue microarray' in 1998 which is widely used nowadays, which includes the usage of 4mm skin biopsy punch.

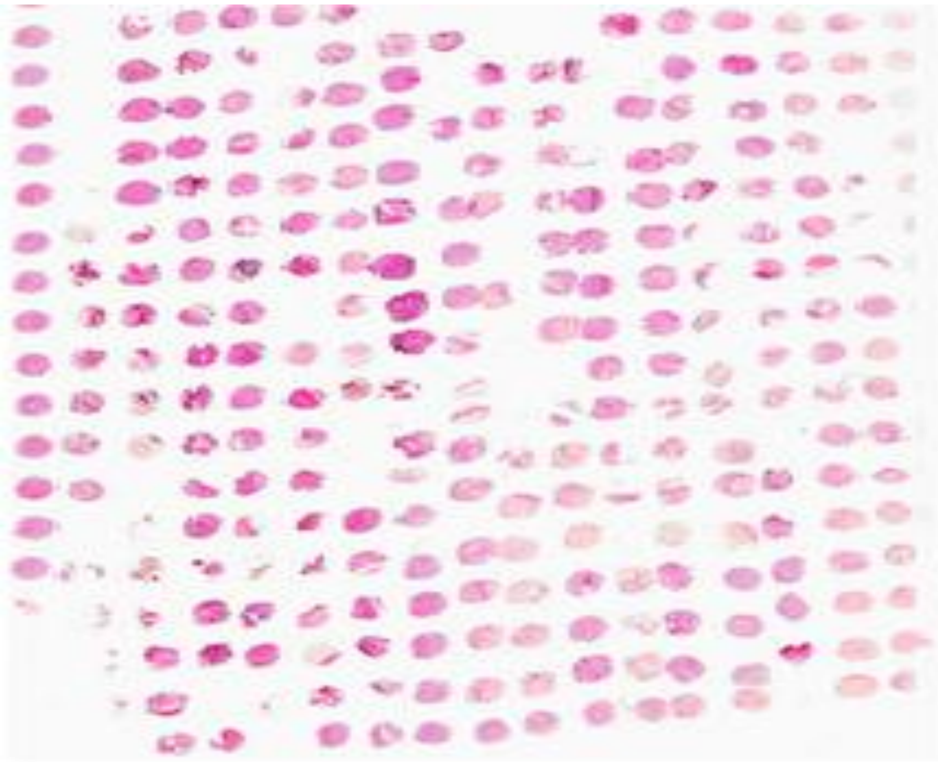


Figure 3[166]: Low magnification picture of an H&E stained TMA section that was cut from a TMA block

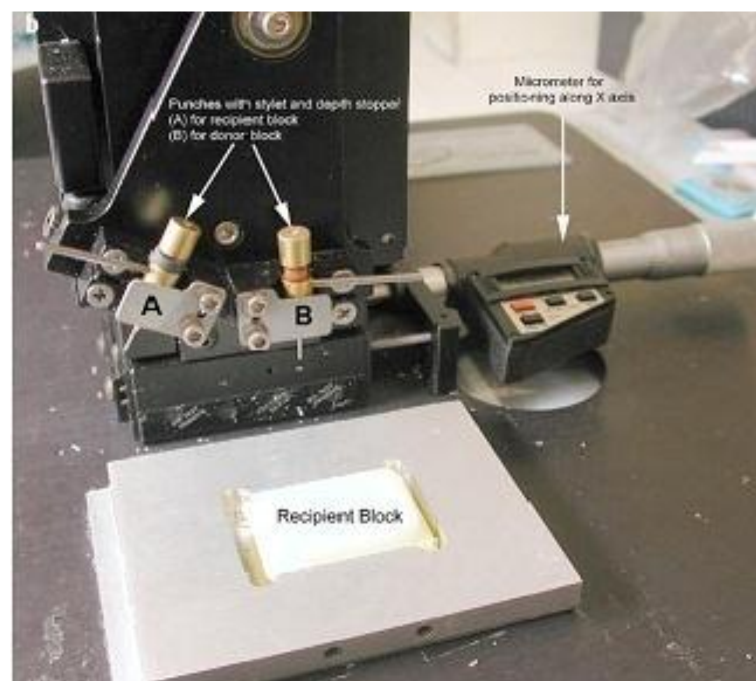


Figure 4[167]: Beecher Instruments Tissue Arrayer

This picture shows the view of the front of the machine with recipient block in block holder which is attached to the rest of the machine via a magnet.

Materials for TMA

The source of the material for construction of Tissue microarray varies widely. Tissue microarrays are categorised based on material of origin. They are called simply as Tissue microarray when they are constructed from paraffin embedded materials. Tissue microarrays can also be constructed using resin as recipient block, if very thin sections are required. But the construction of resin Tissue microarray is very difficult when compared to paraffin Tissue microarray(168).Tissue microarrays can also be constructed using frozen sections in which case they are called as Cryoarrays(169)or can be constructed using paraffin embedded cell lines and by using cell blocks(170).

TMA categorisation

Tissue microarray is categorised based upon the usage of instruments and purpose of tissue microarray. Based upon the usage of the instruments and microarrayer used it has been classified into manual, semi-automated and automated tissue microarray. There are many instruments which are available nowadays which includes, the manual and automated tissue arrayer from beecher instruments, the semi-automated tissue arrayer from veridiam, the quick ray manual and the automated tissue arrayer from unitma and the manual arraymold tissue arrayer. Apart from this many number of home- made tissue arraying methods have been published(171).

The common feature between all these devices is that they use hollow needles or punches and they adopt the technique of skin biopsy to extract tissue cores from a donor block and make a new paraffin recipient block. After cutting all the tissue cores appear as circular samples arranged in grid like fashion.

The initial step which is considered most important in construction of Tissue microarray is to clearly define the purpose of Tissue microarray construction and to decide the number and size of the tissue core which should be taken from the donor block. Then blocks and slides of the selected cases are carefully reviewed to mark the area of interest. The method of tissue sampling varies widely from tissue to tissue. If task is to compare the expression pattern of a marker from tumour centre and periphery, the cores from the particular location may be compatible. However the method of tissue sampling is entirely different when the task is to characterise the overall expression of the protein in a tumour. Targeted sampling technique is followed in the cases of comparing the expression patterns of tumour centre and periphery whereas Random sampling technique is best suited to study the overall expression patterns[(172)].

Tumour heterogeneity, which results in differential expression of in different tumourcell has been recognised as the potential problem in tissue sampling for the construction of Tissue microarray [(173)]. Taking multiple samples from each tumour or area of interest appears to be the best

technique to overcome this problem. Although no standard and universally agreed sampling methods are in the records, it is intuitive that the more samples are taken it becomes the representative area for donor tissue. The concordance of Tissue microarray technique with full section eventually depends upon the number of cores obtained. Most of the studies seem to indicate that the results from triplicate Tissue microarray cores have upto 98% concordance with the results from full section[(174)]. However Goethal through his study suggests that atleast four cores are needed to achieve greater accuracy, whereas others achieved 95% accuracy with only two cores [(175)].

It should be also noted that there are also technical reasons which increases number of cores taken from each tissue block. The reasons are tissue folding and complete loss of tissues during processing and section cutting. Total number of lost cases accounts for as high as 23% in the Tissue microarray construction study by using tissue cores from the cases of renal cell carcinoma[(176)]. There are controversial data regarding the size of the cores that should be used in Tissue microarray technique and their influence over the technique.

The next critical step included in tissue microarray is designing the layout of the tissue microarray. There are no general agreements regarding the designing of optimal layout of a tissue microarray. As there are problems of staining artifacts when performing immunohistochemistry in

the full sections, the use of protection wall in the Tissue array technique is recommended as introduced by Hoose et al, which uses a row of tissue cores which will not be analysed and can be any tissue that is available in plenty in the laboratory. Any confusion in identifying the origin of the cores after Tissue microarray construction makes the staining and analysis very difficult and hence orientation of the tissue cores needs to be perfect. Many use orientation cores in the specific position, usually outside the geometric margin of the array. Using the intentionally left empty core position, it is possible to identify and orient the position of the cores macroscopically as well as to orient cut section microscopically. In addition insertion of control tissue array may be of more value in orientation of the tissue cores. Thus the control core serves as an orientation control and also as both internal positive and negative control.

Recipient blocks are nothing but the empty paraffin blocks that are prepared by pouring the soft molten wax into existing metal moulds of varying size. The major difficulty encountered in using metal moulds is the formation of air bubbles within the recipient blocks during the cooling procedure. The air bubbles which are formed during the process of cooling will not be evident apparently and not be identified till the section cutting. The subsequent sectioning of Tissue microarray causes severe distortion of constructed array and leads to difficulties during the steps of interpretation and analysis. To minimise this kind of problem and as a quality control

measure, all the recipient blocks are subjected to X-ray by using Faxitron machine before Tissue microarray construction and if air bubbles are found the recipient blocks are melted for reuse. The use of moulds which are made from paper and plastic may reduce the problem of bubble formation which is commonly encountered when paraffin recipient blocks are used.

Tissue Arrayers

Both automated and manual tissue arrayers are available for the construction of Tissue microarray. Automated tissue arrayers are easy to use. The instrument usually marks, edits and saves punch co-ordinates by using an on screen display and software tools. Automated tissue arraying instruments are commonly used in laboratories with high volume of Tissue microarray and can punch upto 180 cores/hour.

Automated HT-1tissue microarray

Automated Tissue microarray is a highly potential and efficient technique which can be used to study gene expression. At present TMA has become a standard research platform for the histopathological analysis .This technique involves the acquisition of multiple core biopsies from area of interest from the donor block and transferring it into the recipient paraffin blocks with the help of special instrument. This includes collection and selection of donor blocks to be analysed, identifying the area of interest and representative areas, preparation and making array pores in the recipient paraffin blocks, punching tissue cylinders from the area of interest from the

donor block, insertion of cylinders containing tissues into the recipient blocks and embedding and cutting of newly formed Tissue microarray. However most important of all these steps is the construction of tissue microarray blocks using commercially available instruments. The key steps in automated tissue microarray are punching array pores in recipient blocks and embedding multi-tissue cylinders into the recipient blocks.

Recipient block formation

This method uses three types of recipient block maker which are called as recipient block-molding machine that can accommodate 24, 42, 56 tissue cylinders . The spacing between the cores is usually fixed. Recipient paraffin blocks can be made within several seconds by using block-molding machine. The instrument is composed of an array pores forming metal tamp, series of metal embedding boxes and punch needles with corresponding inner cores. The array pores-metal consist of metal plates and a bracket. The lower plate is fixed with hollow cores while upper plate is fixed with inner cores. The inner diameter is designed from 0.5 to 2.5mm.The instrument for making array recipient blocks is fixed in the manipulator with the help of the bracket. The lower plate with the hollow array can move up and down by controlling the handle. The residual paraffin in array hollow punch is automatically removed by the piston.

Negative-pressure embedding

Negative pressure embedding instrument is designed to re-embed the multi-tissue cylinders into the recipient block for the construction of Tisssue microarray. It is composed of negative pressure room, vacuum pump and a embedding box. The embedding box is situated in the negative pressure room and is connected to the vacuum pump through the tube. The bottom of the embedding box consists of metal mesh, which can effectively adjust the negative pressure so that the air bubbles which are formed can be drained out of recipient paraffin blocks. Screen mesh can also be used to adjust the temperature for the embedding media.

TMA without prefabricated recipient block

Array construction done with the help of automated tissue arrayers proved to be very costly and hence not suitable in developing laboratories. Hence efficient microarray system which is cost effective was designed using manual tissue arrayer technique. Most of the manual methods used preconstructed paraffin recipient block into which holes are punched followed by insertion of tissue cores. The use of paraffin blocks proved difficult because of block breakage during punching, non alignment of holes, and mismatch between the size of the recipient hole and the tissue core. With the desire to overcome these difficulties, TMA construction was done without the use of prefabricated paraffin blocks.

This technique followed the method which was initially modified by Kononen et al. A disposable skin punch biopsy needle of 2mm diameter was used for punching cores from the donor blocks. A long steel wire with a blunt end was modified into a stylet. The skin biopsy needle has the sharp cutting edge and a uniform cylindrical core. Double sided adhesive tapes, pair of stainless steel forceps, paper cutters, embedding mould and plastic cassettes are the requirements of this technique. A piece of double sided adhesive tape is cut according to the size of embedding mould and the top surface is exposed to receive the tissue cores. The site for the attachment is marked with the ruler and the felt pen. The area to be cored is marked on the block by superimposing the marked area over the slides. After obtaining the core from the donor block, the core is transferred to adhesive tape using the forceps. Then it is transferred to stainless steel mould and after which melted paraffin wax is poured. Later after uniform setting of the block the adhesive tape is peeled off to expose the cutting surface. This method can give rise upto 20sections from each core. This is reliable, readily reproducible and does not need any specialisation.

Simple manual tissue microarray

This is the modification of conventional manual tissue microarray which used skin punch biopsy needle. This technique used bone marrow aspiration needle for the construction of manual tissue microarray. Both 14 gauge and 16 gauge needles are used. After the selection of donor block, the

area of interest is marked and empty recipient paraffin block is made using the mould. By using 16gauge needle pores are made in the recipient block. Then with the help of 14 gauge needle tissue cores are obtained from the area of interest and then inserted into the recipient block. The smaller diameter (16G) of the needle used to punch the blank paraffin wax block allowed the bigger tissue cores (14G) to fit exactly into the blank [177]. Once the array is complete hot liquid paraffin is poured over the array surface and the tissue cylinders are levelled with the blocks using a glass slide. Then the array is incubated at 60^0c for 15 minutes after which array is chilled on ice.

Types of TMA based on Application

The technique of tissue microarray can be classified based on the purpose of Tissue microarray construction. Random arrays contain tissue from multiple sites including both tumoral and non-tumoral tissues. This is most widely used for monitoring the efficacy of the existing antibodies. Cell line arrays consist of normal and cancer cell lines that are grown in cultures. The main purpose of using this array is to analyse the utility of an antibody in detecting the proteins. Outcome based arrays are the most valuable and the most difficult to construct as they involve the collation of tissues from the patients of same disease, those who were exposed to similar pattern of treatment and have been followed up for significant period. Progression arrays are used in analysis of role of proteins in the progression of cancer.

Tumor characteristic based array is constructed based on the given characteristics such as patient age and tumor grade.

The use of tissue microarrays has its own advantage and disadvantage. Tissue microarray allows the performance of tissue based array such as immunohistochemical analysis, histochemistry and in situ hybridisation on a very large number of sample in a cost effective manner. Several different tissues from a number of patients can be examined in short period using TMA. Automated Tissue microarrayers are very quick and manual tissue arrays are simple to construct and cost effective. The major disadvantage of using Tissue microarray is that each tissue core obtained from the selected donor block represents only the fraction of the lesion. But this is overcome by taking multiple cores usually from various sites. Hence Tissue microarray can be considered as one of the superior and more advantageous technique used in studying tissue biology.

Materials and Methods

MATERIALS AND METHODS

Study material includes 21 cases of Non Hodgkin lymphoma diagnosed in the Department of Pathology of Tirunelveli Medical College during a period of 3 years from 2012 to 2014.

Inclusion criteria

All the cases of Non-Hodgkin lymphoma that were diagnosed by using Hematoxylin and Eosin stain and cases with a differential diagnosis of Non Hodgkin Lymphoma and cases where an IHC confirmation was requested.

Exclusion criteria

i. Inadequate lymph node sample.
ii. Poorly processed material.
iii. All the cases of Hodgkin lymphoma
iv. All cases of extra-nodal Non Hodgkin Lymphoma

Materials required

1. Lay out for constructing Tissue microarray.
2. Metal moulds and molten wax for preparing empty recipient paraffin block.
3. Donor blocks which contain formalin fixed paraffin embedded tissue obtained from all the cases of Non-Hodgkin lymphoma.

4. Hematoxylin and eosin stained tissue sections made from the donor blocks.
5. Black glass marking pen for marking area of interest.
6. 16gauge bone marrow aspiration needle for making punches in the recipient block and 14gauge needle for obtaining core from the donor block.
7. Microtome and incubator for obtaining tissue sections from area of interest and for baking the sections, respectively
8. Postively charged slides for holding tissue sections for IHC.
9. Chemicals for preparing antigen retrieval solutions and for wash buffers.
10. Pressure cooker for antigen retrieval.
11. Kit for performing immunohistochemistry which includes primary antibody (CD3, CD5, CD10, CD20) and universal kit. Microscope used for interpretation and grading of IHC.

METHODOLOGY

The method of performing immunohistochemistry over the paraffin tissue microarray includes the following steps.

1. Designing the layout for TMA construction.
2. Collection of the donor blocks.
3. Preparation of the recipient paraffin blocks.
4. Immunohistochemistry and analysis.

Designing the lay out

Before constructing the array proper, the layout of the tissue microarray defining the geometric position of each tissue core in the recipient block was made. The grid was constructed in such a way that there were minimum of two cores from each case and maximum of three cores on the recipient blocks except for two cases which had single representative core. All the cores from each case were placed in the same block in different positions. The grid was constructed in such a way that there were blank cores in between the cores from the cases which helped in determining the position of the cases on the immunohistochemistry performed slides.

Collection of the donor blocks

The hematoxylin and eosin stained sections which were prepared from formalin fixed paraffin embedded blocks of all the cases of Non-Hodgkin lymphoma in the Department of pathology during the study period

were retrieved. The corresponding formalin fixed paraffin embedded tissues were also obtained which constituted the donor block. Then the hematoxylin and eosin stained slides which contained full sections were examined and the area of interest was marked by using black glass marking pen. The area of interest is nothing but the area of tumor containing well preserved and well stained malignant cells. Then these marked areas on the slides were matched with the donor blocks and the corresponding areas over the donor blocks were also marked with the help of black glass marking pen. This area was used as the site for obtaining cores for the recipient blocks.

Preparation of the recipient paraffin blocks

The empty paraffin recipient blocks with minimum size of 25mm x 25mm were first prepared by freshly poured molten wax in the metal moulds. Then it was allowed to cool. Later using 16gauge needle, paraffin wax cylinders of 2mm diameter were punched from the recipient blocks. Each block contained 3x3 cylinder matrix at a distance less than 2mm. Seven such blocks were prepared.

Then using 14gauge bone marrow aspiration needle, tissue cylinders were obtained from the area of interest which were previously marked over the donor blocks, after which it was injected into the recipient blocks into the corresponding empty cylinders with the help of predesigned layouts so that each recipient blocks contained three cases. After the recipient block was embedded with the tissue cores, the block was incubated at 40^0c for 15

minutes and then it was allowed to cool for few minutes at room temperature and then the array was chilled on the ice for few minutes. Finally the 5microns sections were taken over the surface of the APES (3-aminopropyltriethoxysilane) coated slides using a microtome.

IMMUNOHISTOCHEMISTRY

Section cutting

Sections were taken at 5microns thickness after tissue microarray construction along with a control section on the surface of the APES (3-aminopropyltriethoxysilane) coated slides. This was followed by incubation of slides at 58-60^{0}c for one hour.

Antigen retrieval solution

We used two antigen retrieval solution and a wash buffer as prescribed by the manufacturer (DAKO).

1. Citrate buffer at a P_H of 6.2 for CD3, CD5, CD10.
2. Tris EDTA at a P_H of 9 for CD20.
3. Tris wash buffer at P_H of 7.6 for both.

Antigen retrieval

Many methods have been used for antigen retrieval which includes Microwave method, and water bath, autoclave, proteolytic enzyme and pressure cooker method. In our institution we followed antigen retrieval by using pressure cooker as it produces even heating with lesser disadvantages as compared to other methods.

Procedure for immunohistochemistry as given by manufacturer

1. Section cutting and incubation is followed by Xylene wash (3 changes) for 10minutes each.
2. Rehydrated in graded alchohol containing 100%, 80%, 70% for five minutes each.
3. Rinsed in distilled water for 2minutes.
4. Antigen retrieval.
5. Cooling for 15minutes.
6. Washed in TBS wash buffer- 3changes 5minutes each.
7. Treated with peroxide block for 10minutes.
8. Washed in TBS wash buffer- 3changes 10minutes each.
9. Kept in protein block for 10minutes.
10. Application of primary antibody (CD3/CD5/CD10/CD20) – 1 Hour.
11. Washed in TBS wash buffer- 3changes 10minutes each.
12. Amplifier application for 15minutes.
13. Washed in TBS wash buffer- 3changes 10minutes each.
14. Application of secondary antibody (HRP POLYMERASE).
15. Washed in TBS wash buffer- 3changes 10minutes each.
16. Application of Diamino-benzidine tetrachloride(DAB) chromogen.
17. Washed in distilled water – 2 changes.
18. Counterstaining with Mayer’s Hematoxylin – 1dip/30seconds to impart background staining.

19. Wash in running tap water.
20. Place in xylene – 2 changes 5 minutes each.
21. Dehydrate in 100% alcohol – 5minutes.
22. Mount the section with Dextrene phthalate xylene
23. Observation and grading under light microscope.

Grading of IHC stained sections

After immunohistochemistry was done, the slides were examined under all the magnification with the help of light microscopy and grading is done. This is done by following the method as opted by Afaf Abdel- Aziz, Abdel-Ghafar[(135)] in his study on "Immunophenotyping of chronic B-cell neoplasms". First the tumour cells are observed for positive membrane/cytoplasmic staining pattern after which it is graded based upon the percentage of tumour cells which shows positive expression of antibody used as follows,

No Reaction: 0.

Less than 5% of the tumour cells showing positive membranous/cytoplasmic expression: 1+.

5-9% of the tumour cells showing positive membranous/cytoplasmic expression: 2+.

10-20% of the tumour cells showing positive membranous/cytoplasmic expression: 3+.

More than 20% of the tumour cells showing positive membranous/cytoplasmic expression: 4+.

After IHC grading, the cases are re-classified as per WHO classification of lymphoid neoplasms (International agency for research on cancer, Lyon, 2008)[78] and results are tabulated for analysis.

Observation & Results

OBSERVATION AND RESULTS

TABLE 1: Comparison of Histopathological and immunohistochemical diagnosis

S no	Histopathology no	Age	Sex	Site	Histopathological diagnosis	Diagnosis after immunohistochemistry (based on who classification-2008)
1	1567/11	65	F	Femoral	Large cell anaplastic lymphoma	Diffuse large b cell lymphoma
2	1593/11	62	F	Axilla	Diffuse non hodgkin lymphoma	Cll/sll / mantle cell lymphoma
3	1813/11	65	F	Cervical	Diffuse small & large cell lymphoma	Diffuse large b cell lymphoma
4	2857/11	15	M	Cervical	Follicular lymphoma	Nodal marginal zone lymphoma
5	2860/11	60	M	Cervical	Diffuse small cell lymphoma	Cll/sll / mantle cell lymphoma
6	2426/11	78	M	Cervical	Non hodgkin lymphoma	Cll/sll / mantle cell lymphoma
7	2551/12	60	M	Cervical	Diffuse mixed cell type of nhl	Diffuse large b cell lymphoma
8	2072/12	11	M	Cervical	Reactive follicular hyperplasia with progressive transformation of germinal centre.	Progressive transformation of germinal centre
9	3143/12	11	M	Cervical	Non hodgkin lymphoma with rdd changes	Cll/sll / mantle cell lymphoma
10	2444/13	65	M	Cervical	Non hodgkin lymphoma, diffuse type	Diffuse large b cell lymphoma
11	1883/13	53	F	Mesentric	Non Hodgkin lymphoma,mixed type	Diffuse large b cell lymphoma
12	2607/13	45	F	Mesentric	Mantle cell lymphoma	Mantle cell lymphoma

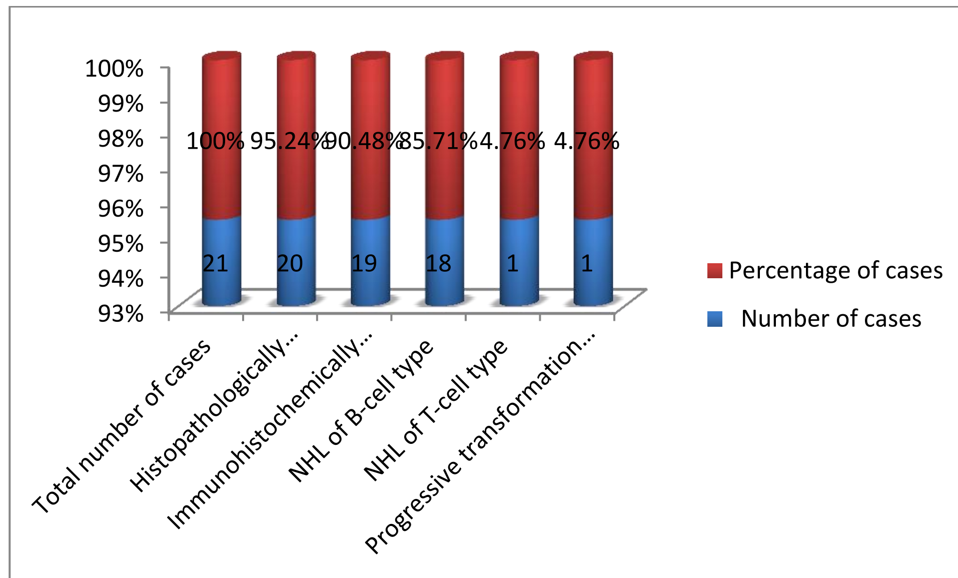
13	0026/11	55	M	Axilla	Large cell anaplastic lymphoma	Diffuse large b cell lymphoma
14	2928/13	60	F	Cervical	Non hodgkin lymphoma	Cll/sll / mantle cell lymphoma
15	1438/13	65	F	Cervical	Nhl, follicular and mixed cell type	Diffuse large b cell lymphoma
16	1104/14	65	M	Axilla, inguinal	Angioimmunoblas tic lymphadenopathy	T cell lymphoma
17	980/14	53	F	Axilla	Non hodgkin lymphoma	Cll/sll / mantle cell lymphoma
18	0095/14	44	M	Omentum	Small cell carcinoma/lympho ma	Mantle cell lymphoma
19	1998/14	60	M	Inguinal	Non hodgkin lymphoma	Diffuse large b cell lymphoma
20	1428/14	75	M	Axilla	Lymphoproliferati ve disorder	Not possible
21	768/14	60	M	Cervical	Non hodgkin lymphoma	Diffuse large b-cell lymphoma - nos

All the cases diagnosed histopathologically as Non Hodgkin lymphoma are confirmed to be of the same class by immunohistochemistry except for one case of lymphoproliferative disorder in which the diagnosis was not possible. So the concordance rate of immunohistochemical analysis with hisopathological examination was 95%.

TABLE 2: DISTRIBUTION OF CASES

Case	Number of cases	Percentage of cases
Total number of cases	21	100%
Histopathologically diagnosed NHL	20	95.24%
Immunohistochemically proven NHL	19	90.48%
NHL of B-cell type	18	85.71%
NHL of T-cell type	1	4.76%
Progressive transformation of germinal centre(PTGC)	1	4.76%

CHART-1

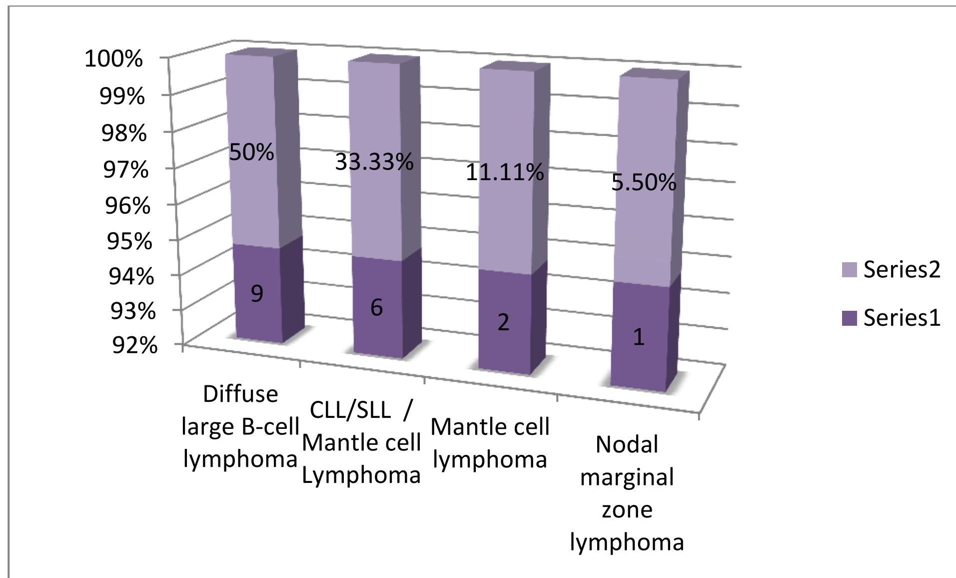


Out of the 19 cases that were classified by immunohistochemistry, B cell lymphoma constituted 18 cases and there was a single case of T cell lymphoma. Thus B cell lymphoma type formed the majority of Non Hodgkin lymphoma.

TABLE 3: CLASSIFICATION OF B-CELL LYMPHOMA AFTER IMMUNOHISTOCHEMICAL ANALYSIS

B-CELL NHL	NUMBER OF CASES	PERCENTAGE
Diffuse large B-cell lymphoma	9	50%
CLL/SLL / Mantle cell Lymphoma	6	33.33%
Mantle cell lymphoma	2	11.11%
Nodal marginal zone lymphoma	1	5.5%

CHART-2



Diffuse large B-cell lymphomas are the most common type of B-cell lymphomas encountered. CLL/SLL / Mantle cell lymphoma stand next to DLBCL.

TABLE4: PATTERN AND GRADE OF STAINING IN NON HODGKIN LYMPHOMA

Histopathology no	Diagnosis after immunohistochemistry	CD3	CD20	CD5	CD10
1567/11	DIFFUSE LARGE B CELL LYMPHOMA	0	Diffuse 4+	Diffuse 2+	Diffuse 1+
1593/11	CLL/SLL / MANTLE CELL LYMPHOMA	0	Diffuse 4+	Diffuse 2+	0
1813/11	DIFFUSE LARGE B CELL LYMPHOMA	0	Diffuse 4+	0	Diffuse 1+
2857/11	NODAL MARGINAL ZONE LYMPHOMA	Focal-interfollicular 1+	Diffuse 4+	Diffuse 2+	0
2860/11	CLL/SLL / MANTLE CELL LYMPHOMA	Focal follicular 2+	Diffuse 4+	Diffuse 3+	0
2426/11	CLL/SLL / MANTLE CELL LYMPHOMA	0	Diffuse 4+	Diffuse 2+	0
2551/12	DIFFUSE LARGE B CELL LYMPHOMA	Focal 2+	Diffuse 3+	Diffuse 3+	Diffuse 1+
2072/12	PROGRESSIVE TRANSFORMATION OF GERMINAL CENTRE	Focal-interfollicular 2+	Follicular 3+	Focal-follicular 3+	0
3143/12	CLL/SLL / MANTLE CELL LYMPHOMA	0	Diffuse 3+	Diffuse 3+	0
2444/13	DIFFUSE LARGE B CELL LYMPHOMA	Focal 1+	Diffuse 3+	0	Diffuse 1+
1883/13	DIFFUSE LARGE B CELL LYMPHOMA	0	Diffuse 3+	0	Diffuse 1+
2607/13	MANTLE CELL LYMPHOMA	Focal-follicular1+	Diffuse 3+	Diffuse 2+	0

0026/11	DIFFUSE LARGE B CELL LYMPHOMA	0	Diffuse 3+	Diffuse 1+	Diffuse 2+
2928/13	CLL/SLL / MANTLE CELL LYMPHOMA	0	Diffuse 3+	Diffuse 1+	0
1438/13	DIFFUSE LARGE B CELL LYMPHOMA	Focal 2+	Diffuse 3+	Diffuse 3+	Diffuse 1+
1104/14	T CELL LYMPHOMA	Diffuse 2+	0	Focal 2+	0
980/14	CLL/SLL / MANTLE CELL LYMPHOMA	0	Diffuse 1+	Diffuse 2+	0
0095/14	MANTLE CELL LYMPHOMA	0	Diffuse 1+	Focal-follicular 3+	0
1998/14	DIFFUSE LARGE B CELL LYMPHOMA	0	Diffuse 1+	Diffuse 1+	Diffuse 2+
1428/14	NOT POSSIBLE	Loss	loss	Diffuse 1+	Loss
768/14	DIFFUSE LARGE B-CELL LYMPHOMA - NOS	0	Diffuse 2+	Loss	Loss

All of the 9 cases of diffuse large B cell lymphoma showed diffuse pattern of staining with CD 20 and CD10, 5 cases showed diffuse pattern of staining for CD5 and 3 cases showed focal non specific staining for CD3. Out of the 6 cases of CLL/SLL / Mantle cell lymphoma, all of them showed diffuse pattern of staining for CD20 and CD5 and one case showed focal

staining in the interfollicular area with CD3. Out of the 2 cases of mantle cell lymphoma, one case showed diffuse staining of CD20 and CD5 with focal staining in the follicular area with CD3 while the other showed diffuse staining with CD20 and focal follicular staining with CD 5. The single case of nodal marginal zone lymphoma showed diffuse staining pattern with CD20 and CD5 and interfollicular area was positively stained with CD3. The one case that was diagnosed as T cell lymphoma showed diffuse grade 2+ positivity for CD3 and focal grade 2+positivity for CD5 while CD20 was negative.

Out of the 9 cases of diffuse large B cell lymphoma, 5 of them showed grade 4+ membrane positivity, 2 of them showed grade 3+ membrane positivity and one case showed grade 1+ membrane positivity with CD20. As far as CD10 was concerned in diffuse large B cell lymphoma cases, 2 of them showed grade2+ membrane positivity and the rest had grade 1+ membrane positivity. Out of the 6 cases of CLL/SLL / Mantle cell lymphoma, 3 cases showed grade 4+ membrane positivity, 2 were grade 3+ membrane positive and rest showed grade 1+ membrane positivity with CD20. As far as CD5 was concerned in CLL/SLL / Mantle cell lymphoma cases, 2 cases showed grade 3+ and 3 of them showed grade2+ membrane positivity while one case showed grade 1+ membrane positivity. Out of the single case of nodal marginal zone lymphoma there was grade 2+ positivity for CD5, grade 4+ positivity for CD20 and grade

1+ positivity for CD3. Mantle cell lymphoma cases, one had grade 3+ positivity for CD20 and grade 2+ positivity for CD5 while the other had grade1+ for CD 20 and 3+ for CD5.

TABLE5: QUANTIFICATION OF IHC REAGENTS USED IN CONVENTIONAL VS TMA SECTIONS

IMMUNOHISTOCHEMICAL REAGENT	TMA	CONVENTIONAL	CONSUMPTION RATIO
Primary antibody	.13 IU	.8 IU	1:6
Secondary antibody	.13 IU	.8 IU	1:6
Chromogen	.13 IU	.8 IU	1:6

The conventional immunohistochemistry using full section consumes 0.8 IU of the chemical reagents whereas only one sixth of the reagent is consumed by tissue microarray.

Colour Plates

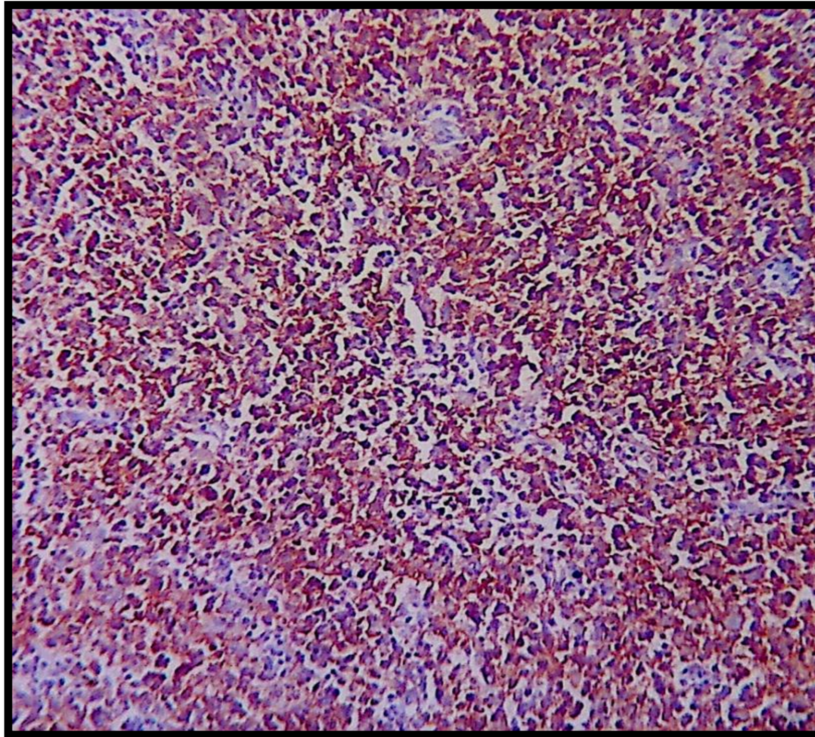


Fig.Ia. A case of diffuse large B-cell lymphoma showing diffuse positive expression for CD 20 (IHC, x100).

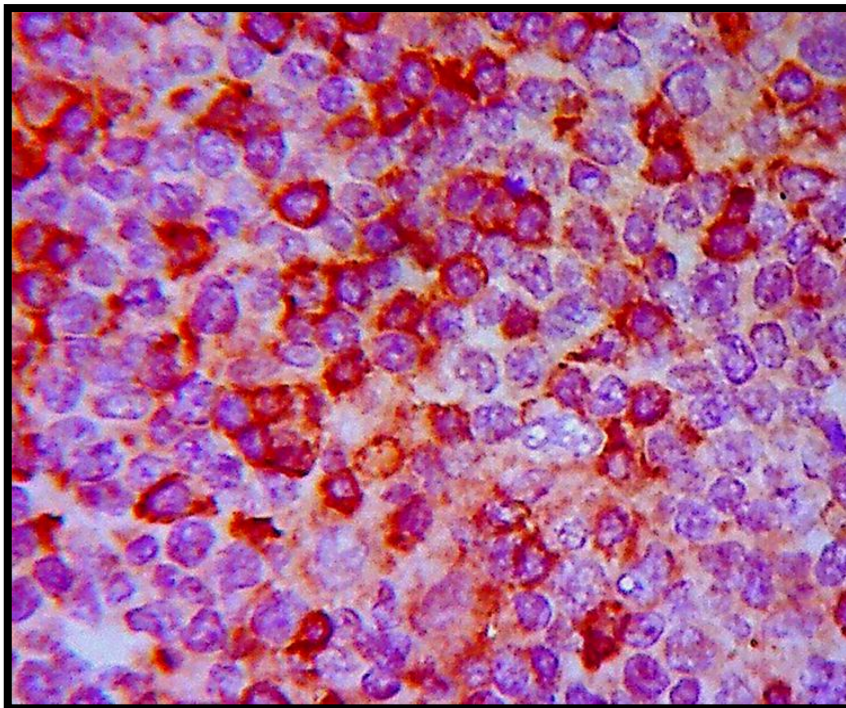


Fig.Ib. A case of diffuse large B-cell lymphoma showing positive membrane expression for CD20 (IHC, x400).

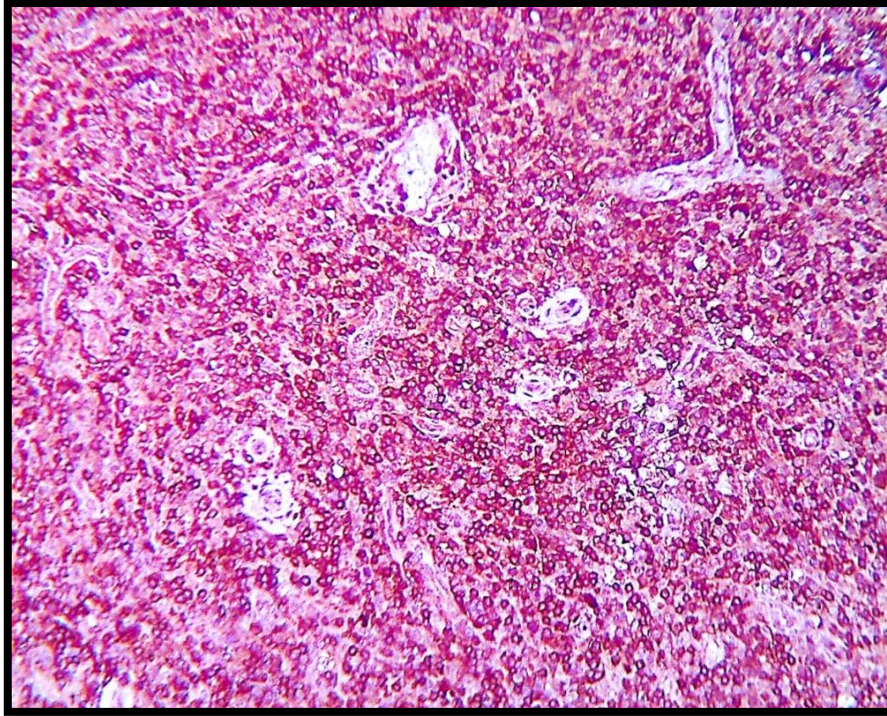


Fig.IIa. Diffuse CD5 expression in a case of CLL/SLL (IHC, x100).

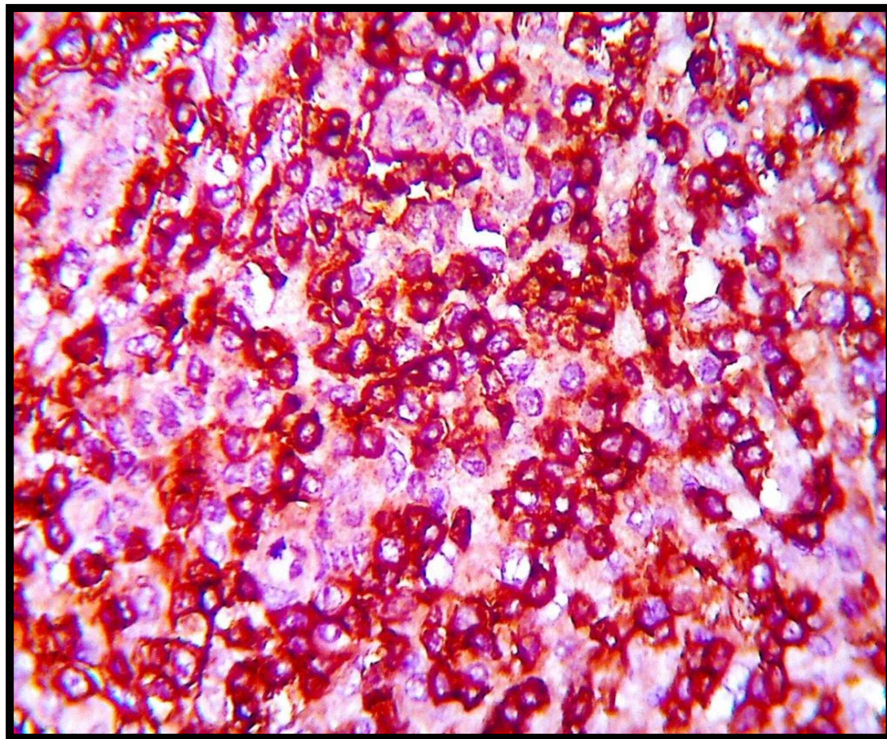


Fig.IIb. Section showing positive cytoplasmic membrane expression for CD5 (IHC, x 400).

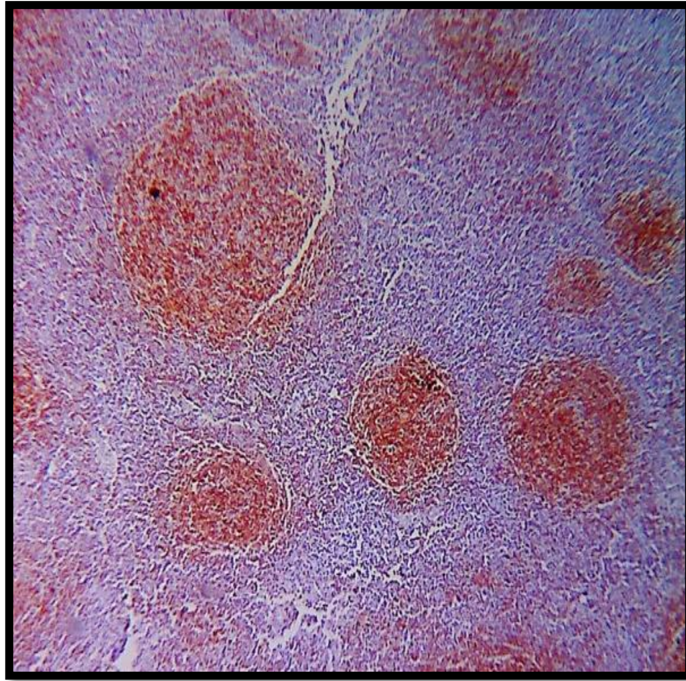


Fig.IIIa. Positive expression for CD5 in a case of mantle cell lymphoma (IHC, x40).

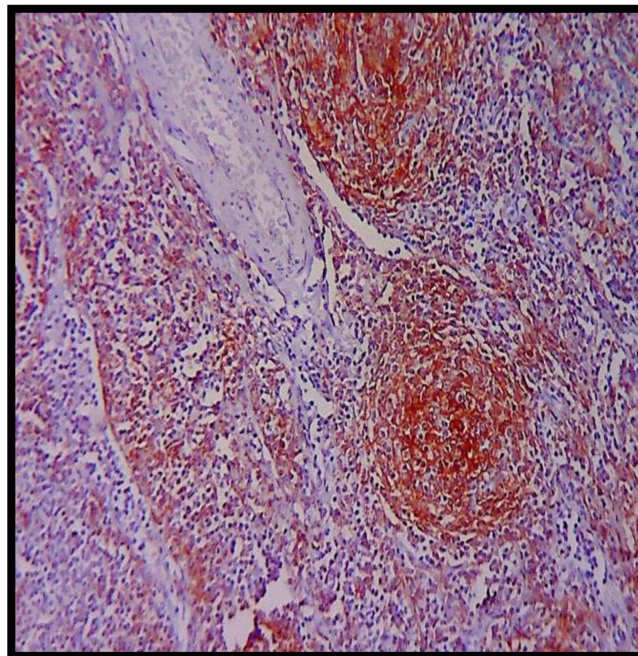


Fig.IIIb. A case of mantle cell lymphoma showing focal follicular expression for CD5 (IHC, x100).

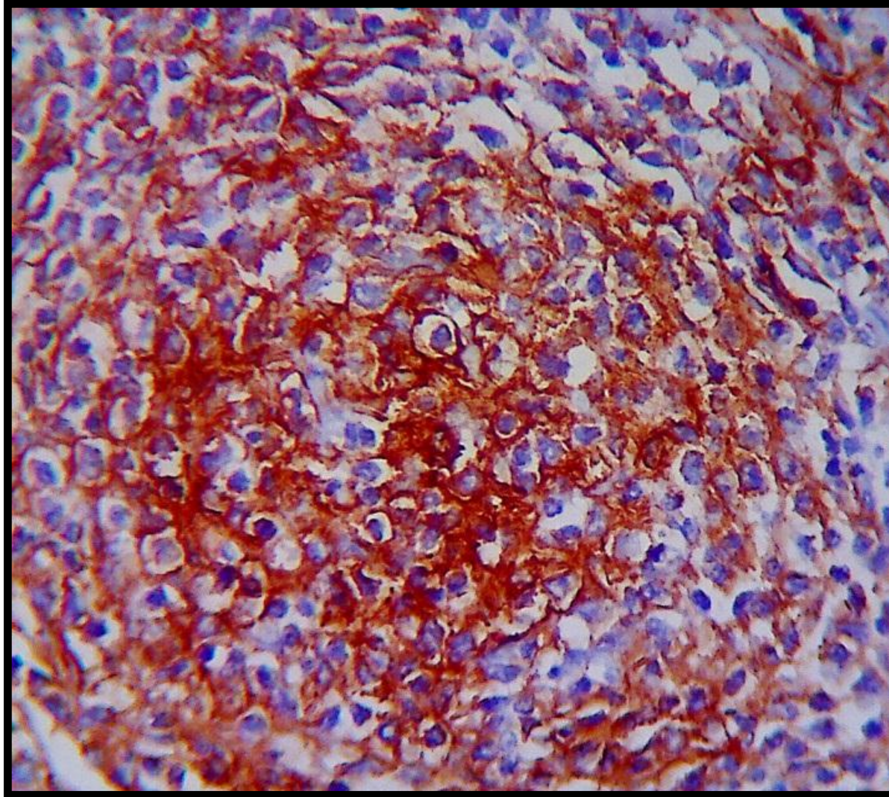


Fig.IIIC. Positive membrane expression for CD5 in the follicles in a case of mantle cell lymphoma (IHC, x400).

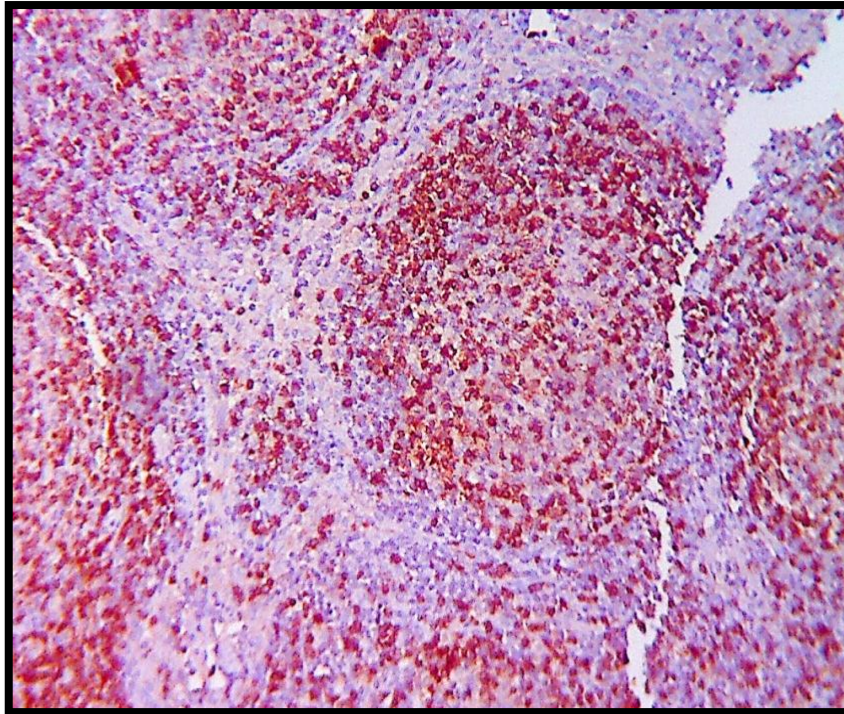


Fig.IVa. A case of T-cell lymphoma showing positive expression for CD3 (IHC, x100).

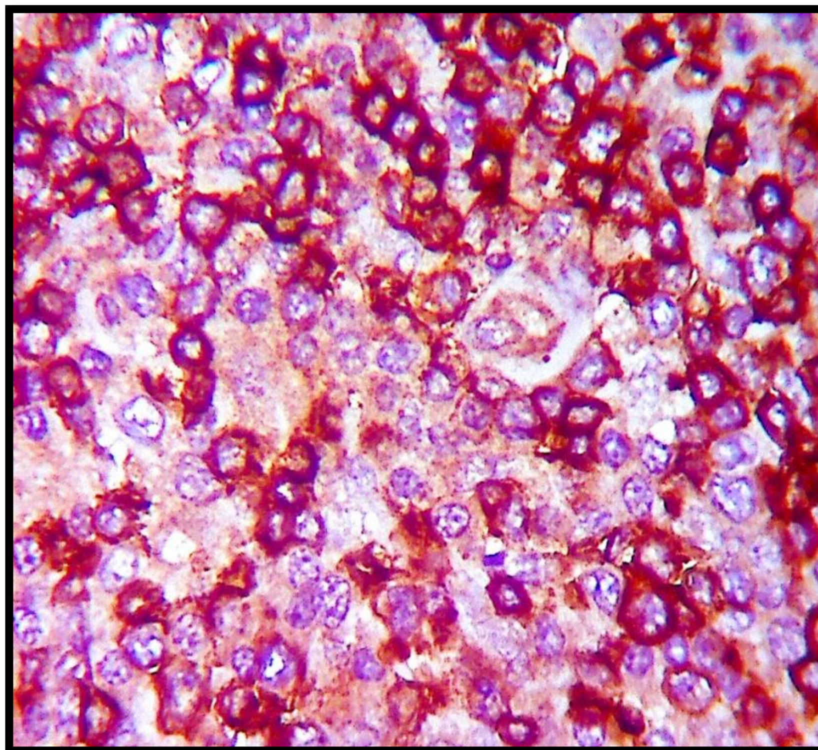
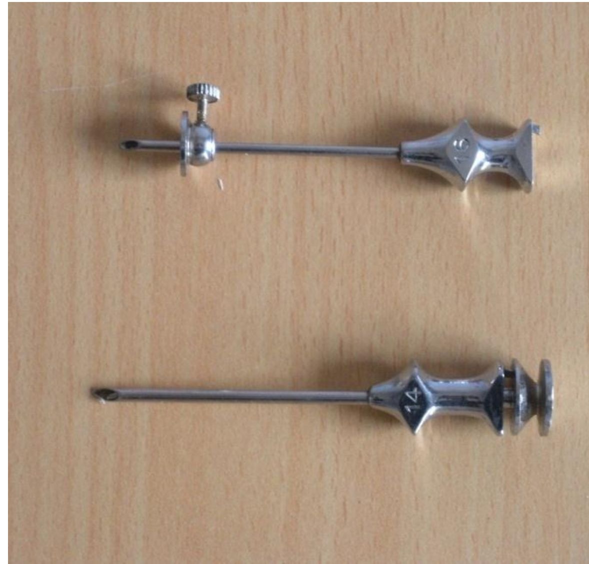
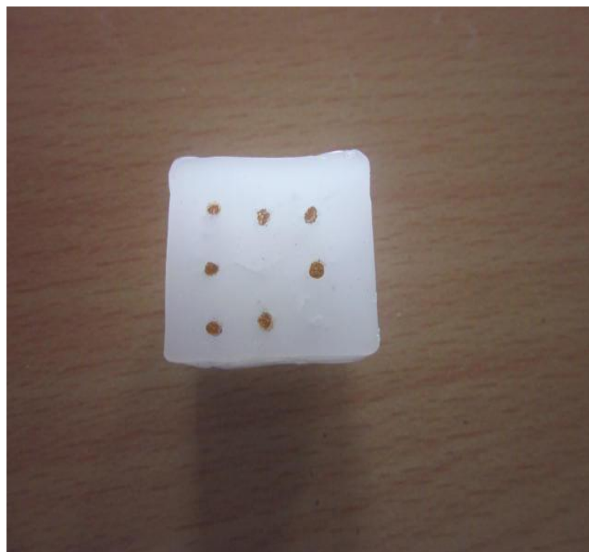


Fig.IVb. CD3, showing positive membrane expression in a case of T-cell lymphoma (IHC, x400).

Fig.V. Materials used in the construction of tissue microarray





Discussion

DISCUSSION

The application of tissue microarray for the use of immunohistochemical diagnosis and classification of lymphoma has gained importance in the field of medical research for evaluation of prognosis and treatment modalities.

Lynette K Tumwine[116], in his study "Immunohistochemical and other prognostic factors in B cell NHL", at Kampala, Uganda used TMA for IHC analysis of NHL and studied prognostic factors in B cell lymphoma. According to Christian Hans et al[119] in his study immunostaining of DLBCL using TMA correlated with 71% cases of the GCB group and 88% cases the non-GCB group when compared with gene profiling using cDNA.

The present study included 21 cases of Histomorphologically diagnosed Non- Hodgkin lymphomas. All of the cases were subjected to immunohistochemistry with the help of Tissue microarray, to confirm the histopathological diagnosis and to classify NHL into B-cell type and T-cell type. Subtyping of B-cell Lymphoma has also been done with the help of available immunohistochemical markers. After immunohistochemical study, out of 21 cases 1 case was confirmed as progressive transformation of germinal centre, and in one case opinion was not possible due to tissue loss. Hence out of 21 cases studied 19 cases were taken into the analysis.

Among 19 cases analysed 18 were Non-Hodgkin lymphoma of B-cell type which constituted for 94.73%. One case of T-cell lymphoma was observed which accounted for 5.27%.According to Naresh KN 2000[178], B cell lymphoma formed 79.1% of NHL, 72% according to Kalyan K et al, 2006[179], 86% according to Mushtaq S et al[180], 2008, 96% according to Padhi S et al, 2012[181] and 54% according to Rao et al, 2013[1]. This correlates with the results of the current study. According to K.E.Hunt and Reichard, 2008 [182], diffuse B cell lymphomas are the most frequently occurring B cell Non Hodgkin lymphoma, worldwide. In two studies reported from Mumbai (India) in the year 2000[178] and 2011[183], DLBCL was found to be 34% and 42% respectively. Roy et al, Kalyan et al and Padhi et al found that DLBCL formed 29.3%, 26% and 69% of B cell lymphomas, respectively. In the current study diffuse large B-cell lymphoma formed the bulk. There were 9cases of DLBCL which accounted for 50%. This result was similar to the above mentioned studies.

According to Jaffe ES[184], mantle cell comprises about 2-10% of all Non-Hodgkin lymphoma. Roy et al[185], through his study documented that mantle cell lymphoma ,marginal zone lymphoma and CLL/SLL constituted for 4%, 2.7% and 13% respectively. Naresh et al in his study found that 3.4% of the cases were of mantle cell type, 8.2% of the cases were marginal zone lymphomas and 5.7% of the cases were CLL/SLL. In the current study, cases which showed small cell morphology along with CD20 and CD5

positivity were categorised as CLL/SLL / Mantle cell lymphoma but further classification was not done due to non availability of cyclinD1. This constituted 33.33%. Two cases of mantle cell lymphoma were diagnosed, correlating with cell morphology, immunohistochemistry and one case was associated with lymphomatous polyposis in the ileum. Mantle cell lymphoma accounted for 11.11% and nodal marginal zone lymphoma accounted for 5.5% of cases.

According to Picker LJ[122], all the cases of diffuse large B-cell lymphoma expresses CD20 and Fang JM[124] et al through his study found that 20-30% of the cases of DLBCL are positive for CD10. Vasef MA [130] found that CD5 is nearly negative in all the cases of DLBCL and according to Dogan A[186] CD10 expression is absent in half the cases of diffuse large B-cell lymphomas. In this study, cases with predominantly large cell and mixed cell histomorphology with variable positive membranous expression for CD20 and CD10 were diagnosed to be diffuse large B cell lymphoma. 9 such cases diagnosed, 5 of them showed grade 4+ diffuse positivity with CD20 and 6 of them showed grade1+ diffuse positivity with CD10. The CD20 expression in this study was similar to the above studies but they differed in CD10 expression.

Sumeet Gujaral[187] in his study of 93 cases of mantle cell lymphoma found that 68 cases showed positive expression for CD20 and 61 cases showed positivity for CD5.Arun Roy[188] in his study documented that all

the 13 cases he included in his study showed positive expression for CD5 and CD20. Cheson BD[189] and Matutes E[190] through their study documented that the cells of CLL/SLL shows positive expression for CD20 and CD5 with negative expression for CD10. The cases with predominantly small cell histomorphology with variable positive membranous expression for CD20 and CD5 were categorised as CLL/SLL / Mantle cell lymphoma. 6 such cases were diagnosed.

One case with small cell morphology with 3+ membrane expression for CD 20, 2+ membrane expression for CD5 and 1+ membrane expression for CD3 along with lymphomatous polyposis of ileum was diagnosed as mantle cell lymphoma. Another case with diffuse grade1+ CD20 positivity with follicular grade 3+ positivity for CD5 was diagnosed as mantle cell lymphoma. The results obtained in this study was similar to the above mentioned studies.

Watson et al[191], Kurin PJ[192] and Campo E[193] found that most cases of nodal marginal zone lymphomas show positive expression for CD20 with negative expression of CD3, CD5, CD10. In the current study, the case which showed monotonous lymphoid cell proliferation with marginal zone expansion histomorphologically with positive membrane expression for CD20, CD5 and CD3 was diagnosed as nodal marginal zone lymphoma. The result obtained differed from the above studies.

NHL affects all the age group, commonly involving the elderly individual. The median age of incidence of NHL in Asian countries is significantly lower compared to the population based registration in Western countries. On the basis of data collected by Smith et al [194], the median age of the patients was reported as 68 years by the Hematological Malignancy Research Network. However, the median age of the patient is 54 years in Asia, 54 years in Taiwan (Lee,Tan, Feng, 2005) [195], 52 years in Korea (Y-H Ko et al, 1998)[196] 54.5 years in Japan (Aozasa, et al, 1985) [197], 55 years in Iran (Mozaheb, Farzad, Aledavod, 2011)[198]. In the present study, maximum cases of NHL were over 40 years of age which constitutes for about 47.36% of total NHL cases and 50% of B-cell lymphoma. There were about 15 cases of NHL over 49 years among 19 cases of total NHL and 14cases of NHL over 49 years among 19 cases of B-cell NHL which accounted for 78.94% and 77.77%, respectively.

According to Manzoor Ahmed, Amir Hussain Kahn, Sami Saeed [199], 78.78% of cases of NHL presented with lymph node enlargement. Among these, 40.5% had generalised lymphadenopathy whereas 24% of patients had cervical lymph node enlargement. Sudiptachakravarthi, SupriyaSarkar[200] in their study documented that peripheral lymphadenopathy was noted in 94.7% of cases of NHL with associated symptoms of anemia in 60.5% of the cases. The size of the lymph node also plays an important role in the diagnosis of lymphoma. A diameter larger

than 3cm, firm consistency, white colored nodular cut surface are the features suggestive of neoplastic transformation of the node.

In current study the patients of NHL presented with lymph node enlargement with one of the case showing additional lesion in the ileum and other case in the skin. Maximum number of patients showed cervical group of enlarged lymph nodes which constituted for 57.89% (11cases). The maximum size of enlarged node was found to be 6cm with minimum size of 0.3cm.

According to Mucci NR et al [201], Schrami et al[202] and Richter J et al[203] tissue loss may be significant using tissue micro array with rate of tissue damage ranging from 15-33%. Hager M et al [34] in his study on renal cell carcinoma using tissue micro array observed tissue loss due to technical problems which accounted for 23%. In the present study tissue loss was found among two cases during the procedure which accounts for 10%. This may be attributed to small core depth, improper embedding, faulty section cutting, and improper antigen retrieval technique. These can be overcome by following standard protocols and obtaining more number of tissue cores especially triplicate cores from the donor block.

The use of chemicals for performing IHC is critical using tissue micro array. We used insulin syringe for dispersal of primary antibody, secondary antibody and DAB chromogen. To analyse single conventional tissue section immunohistochemically, minimum of two drops (0.8IU) of chemical

reagents which includes primary antibody, secondary antibody and DAB chromogen are required. 1 ml insulin syringe can hold 40IU of the reagent which equals 100 drops. Hence, a single tissue section when used conventionally consumes 0.8IU of the reagent whereas in TMA, the same quantity has been used to analyze 6 cores taken from three different cases. So TMA, apart from having the advantage of parallel analysis of multiple sections also decreases the time taken for the IHC procedure and the amount of chemical reagents used. Furthermore, the use of immunohistochemical analysis in NHL by tissue microarray is helpful in comparative analysis of the tumor, sub-classification which is essential for determining treatment, prognosis and standardisation of the chemical reagents.

Summary and Conclusion

SUMMARY AND CONCLUSION

This study was conducted in Department of pathology, Tirunelveli medical college in which tissue micro array was prepared from lymph nodes of 21 cases among which 20 were diagnosed as Non-Hodgkin lymphoma by histomorphology. All the cases were subjected for immunohistochemical analysis using CD3, CD5, CD10 and CD20 from which 19 cases were taken for analysis because of tissue loss in one case. Among 19, 18 cases were Non-Hodgkin lymphoma of B-cell type but for a case of T-cell lymphoma. There were nine cases of diffuse large B-cell lymphoma and six cases of small cell lymphoma/mantle cell lymphoma. Mantle cell lymphoma constituted for two cases and nodal marginal zone lymphoma for a single case.

Most of the Non-Hodgkin lymphomas have aggressive clinical course and hence need to be diagnosed and categorised earlier using immunophenotyping. WHO classification of lymphoid neoplasms (2008), classifies lymphoma based on immunophenotypic features apart from histomorphological and molecular genetics. Hence, basic panel of markers were used on the tissue microarray for the classification of Non Hodgkin lymphoma cases diagnosed based on histomorphological features.

The process of immunohistochemistry using conventional tissue section consumes more reagents, also require control and standardization for

each batch when compared to tissue microarray. By taking representative cores from different cases and performing IHC on them on a single slide not only proved to be economical but also aided in the standardisation of the reagents and procedure. Immunohistochemical analysis with a panel of markers using tissue microarray greatly reduces time and quantity of reagents. Application of a basic required panel of markers for immunophenotyping of the Non Hodgkin Lymphoma aid in the sub-classification as per WHO guidelines and could modify the therapeutic modalities, thereby prognosis. In this scenario, using a basic panel of markers for immunohistochemistry on tissue microarray saves time, cost and aids in precise tissue diagnosis.

Bibliography

BIBLIOGRAPHY

1. Rao SI. Role of immunohistochemistry in lymphoma, Indian J Med PaediatrOncol. 2010;31(4):145–147.
2. Rhodin JAG. Lymph nodes. In: Histology. New York: Oxford University Press, 1974:378–394.
3. Olah I, Röhlich P, Törö I. Lymph node. In: Ultrastructure of lymphoid organs. Philadelphia: JB Lippincott Co, 1975:216–255.
4. Cottier H, Turk J, Sobin L. A proposal for a standardized system of reporting human lymph node morphology in relation to immunological function. Bull World Health Organ 1972;47:375–408.
5. Roitt I. The anatomy of the immune response. In: Essential immunology. London: Blackwell Science, 1997:152–167.
6. Stein H. Lymphocyte differentiation. In: Mason DY, Harris NL, eds. Human lymphoma: clinical implications of the REAL classification. New York: Springer Verlag,1999.
7. van der Walk P, Meijer CJL. Reactive lymph nodes. In: Sternberg SS, ed. Histology for pathologists. Philadelphia: Lippincott-Raven, 1997:651–673.
8. Harris NL. Mature B-cell neoplasms. Introduction, Pathology and Genetics. WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press, 2001;121–126.
9. Gaulard P, d'Agay M-F, Peuchmaur M, et al. Expression of the bcl-2 gene product in follicular lymphoma. Am J Pathol 1992;140:1089–1095.
10. Sheu L-F, Chen A, Meng C-L, et al. Analysis of bcl-2 expression in normal, inflamed, dysplastic nasopharyngeal epithelia and nasopharyngeal carcinoma: association with p53 expression. Hum Pathol 1997;28:556–562.

11. Torlakovic E, Cherwitz DL, Jessurun J, et al. B-cell gene rearrangement in benign and malignant lymphoid proliferations of mucosa-associated lymphoid tissue and lymph nodes. Hum Pathol 1997;28:166–173.
12. van der Valk P, van der Loor EM, Jansen J, et al. Analysis of lymphoid and dendritic cells in human lymph node, tonsil and spleen. A study using monoclonal and heterologous antibodies. Virchows Arch B Cell Pathol Mol Pathol 1984;45:169–185.
13. Papadimitriu CS, Stein H, Papacharalampoulos NS. Presence of a1-antichymotrypsin and a1-antitrypsin in hematopoietic and lymphoid tissue cells as revealed by the immunoperoxidase method. Pathol Res Pract 1980;169:287–297.
14. Crocker J, Williams M. An enzyme histochemical study of the sinuses of reactive lymph nodes. J Pathol 1984;142:31–38.
15. Hodgkin T. On some morbid appearances of the absorbent glands and spleen. Trans R Med Chir Soc Glasgow 1832;XVII:68–114.
16. Robb-Smith AHT, Taylor CR. Lymph node biopsy. New York: Oxford University Press, 1981.
17. Sternberg C. Uber eine Eigenartige unter dem Bilde der Pseudoleukamic verlaufende Tuberculose des lymphateschen Apparates. Ztschr f Heilkd 1898;xix:21–90.
18. Reed DM. On the pathological changes in Hodgkin’s disease, with especial reference to its relation to tuberculosis. Johns Hopkins Hosp Red 1902;10:133–196.
19. Brill NF, Baehr G, Rosenthal N. Generalized giant lymph node hyperplasia of lymph nodes and spleen. A hitherto undescribed type. JAMA 1925;84:668–671.

20. Symmers D. Follicular lymphadenopathy with splenomegaly. A newly recognized disease of the lymphatic system. Arch Pathol Lab Med 1927;3:816–820.
21. Hicks EB, Rappaport H, Winter WJ. Follicular lymphoma: a re-evaluationtion of its position in the scheme of malignant lymphoma, based on a survey of 253 cases. Cancer 1956;9:792–821.
22. Epstein MA, Achong BG. Discovery and general biology of the virus. In: Epstein MA, Achong BG, eds. The Epstein-Barr virus. Berlin: Springer-Verlag, 1979:1–22.
23. Aisenberg AC, Bloch KJ. Immunoglobulins on the surface of neoplastic lymphocytes. N Engl J Med 1972;287:272–276.
24. Barcos MP, Lukes RJ. Malignant lymphomas of convoluted lymphocytes: a new entity of possible T-cell type. In: Sinks LF, Godden JO, eds. Conflicts in childhood cancer: an evaluation of current management. New York: Alan R. Liss,1975.
25. Lukes RJ, Collins RD. Immunologic characterization of human malignant lymphomas. Cancer 1974;34(suppl):1488–1503.
26. Lester JN, Fuller LM, Conrad FG, et al. The roles of staging laparotomy, chemotherapy, and radiotherapy in the management of localized diffuse large cell lymphoma: a study of 75 patients. Cancer 1982;49:1746–1753.
27. Korsmeyer SJ, Hieter PA, Ravetch JV, et al. Developmental hierarchy of immunoglobulin gene rearrangements in human leukemic pre-B-cells. Proc Natl Acad Sci U S A 1981;78:7096–7100.
28. Waldmann TA, Davis MM, Bongiovanni KF, et al. Rearrangements of genes for the antigen receptor on T cells as markers of lineage and clonality in human lymphoid neoplasms. N Engl J Med 1985;313:776–783.

29. Harris NL, Jaffe ES, Stein H, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood 1994;84:1361–1392.
30. Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov). SEER stat database: mortality—all COD, public-use with state, total U.S. for expanded races/Hispanics (1990–2003), National Cancer Institute, DCCPS, Surveillance Research Program, Cancer Statistics Branch, released April 2006. Underlying mortality data provided by NCHS (www.cdc.gov/nchs), 2006.
31. Paltiel O, Schmit T, Adler B, et al. The incidence of lymphoma in first-degree relatives of patients with Hodgkin disease and non-Hodgkin lymphoma: results and limitations of a registry-linked study. Cancer 2000;88:2357–2366.
32. Chatterjee N, Hartge P, Cerhan JR, et al. Risk of non-Hodgkin's lymphoma and family history of lymphatic, hematologic, and other cancers. Cancer Epidemiol Biomarkers Prev 2004;13:1415–1421.
33. Wang SS, Slager SL, Brennan P, et al. Family history of hematopoietic malignancies and risk of non-Hodgkin lymphoma (NHL): a pooled analysis of 10 211 cases and 11 905 controls from the International Lymphoma Epidemiology Consortium (InterLymph). Blood 2007;109:3479–3488.
34. Wiernik PH, Wang SQ, Hu XP, et al. Age of onset evidence for anticipation in familial non-Hodgkin's lymphoma. Br J Haematol 2000;108:72–79.
35. Straus SE, Jaffe ES, Puck JM, et al. The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. Blood 2001;98:194–200.

36. Linet MS, Pottern LM. Familial aggregation of hematopoietic malignancies and risk of non-Hodgkin's lymphoma. Cancer Res 1992;52:5468s–5473s.
37. Sandlund JT, Downing JR, Crist WM. Non-Hodgkin's lymphoma in childhood. N Engl J Med 1996;334:1238–1248.
38. Murphy SB, Fairclough DL, Hutchison RE, et al. Non-Hodgkin's lymphomas of childhood: an analysis of the histology, staging, and response to treatment of 338 cases at a single institution. J Clin Oncol 1989;7:186–193.
39. Jaffe ES, Harris NL, Stein H, et al. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon, France: IARC Press, 2001.
40. Suarez F, Lortholary O, Hermine O, et al. Infection-associated lymphomas derived from marginal zone B cells: a model of antigen-driven lymphoproliferation. Blood 2006;107:3034–3044.
41. Pozzato G, Mazzaro C, Crovatto M, et al. Low-grade malignant lymphoma, hepatitis C virus infection, and mixed cryoglobulinemia. Blood 1994;84:3047–3053.
42. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984;1:1311–1315.
43. Parsonnet J, Friedman GD, Vandersteen DP, et al. Helicobacter pylori infection and the risk of gastric carcinoma. N Engl J Med 1991;325:1127–1131.
44. Parsonnet J, Hansen S, Rodriguez L, et al. Helicobacter pylori infection and gastric lymphoma. N Engl J Med 1994;330:1267–1271.
45. Wotherspoon AC, Doglioni C, Diss TC, et al. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid

tissue type after eradication of Helicobacter pylori. Lancet 1993;342:575–577.

46. Ioachim HL. Immune deficiency: opportunistic tumors. In Bertino JR, ed., Encyclopedia of Cancer, 2nd ed. San Diego: Academic Press, 2002;2:469–487.

47. Lim ST, Levine AM. Recent advances in acquired immunodeficiency syndrome (AIDS)-related lymphoma. CA Cancer J Clin 2005;55:229–241.

48. Centers for Disease Control. HIV/AIDS surveillance report, 1-22. Atlanta, GA: CDC, 1990.

49. Ioachim HL, Cooper MC. Lymphomas of AIDS [letter]. Lancet 1986;1:96–97.

50. Ioachim HL, Dorsett B, Cronin W, et al. Acquired immunodeficiency syndrome-associated lymphomas: clinical, pathologic, immunologic, and viral characteristics of 111 cases. Hum Pathol 1991;22:659–673.

51. Gaidano G, Carbone F, Dalla-Favera R. Pathogenesis of AIDS-related lymphomas. Molecular and histogenetic heterogeneity. Am J Pathol 1998;152:623–630.

52. Pelicci PG, Knowles DM, Arlin Z, et al. Multiple monoclonal B-cell expansions and c-myc oncogene rearrangements in AIDS-related lymphoproliferative disorders: implications for lymphomagenesis. J Exp Med 1986; 164:2049–2060.

53. Capello D, Rossi D, Gaidano G. Post-transplant lymphoproliferative disorders: molecular basis of disease histogenesis and pathogenesis. Hematol Oncol 2005;23:61–67.

54. Isaacson P, Wright DH. Malignant lymphoma of mucosa-associated lymphoid tissue. A distinctive type of B-cell lymphoma. Cancer 1983;52:1410–1416.

55. Bunim JJ, Talal N. The association of malignant lymphoma with Sjogren's syndrome. Trans Assoc Am Physicians 1963;76:45.
56. Lindsay S, Dailey ME. Malignant lymphoma of the thyroid gland and its relation to Hashimoto disease: a clinical and pathologic study of 8 patients. J Clin Endocrinol 1955;15:1332.
57. Holm LE, Blomgren H, Lowhagen T. Cancer risks in patients with chronic lymphocytic thyroiditis. N Engl J Med 1985;312:601–604.
58. Krikorian JG, Burke JS, Rosenberg SA, et al. Occurrence of non-Hodgkin's lymphoma after therapy for Hodgkin's disease. N Engl J Med 1979;300:452–458.
59. Doll DC, List AF. Burkitt's lymphoma in a homosexual. Lancet 1982;1:1026–1027.
60. Delecluse HJ, Anagnostopoulos I, Dallenbach F, et al. Plasmablastic lymphomas of the oral cavity: a new entity associated with the human immunodeficiency virus infection. Blood 1997;89:1413–1420.
61. Cesarman E, Chang Y, Moore PS, et al. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med 1995;332:1186–1191.
62. Rosenberg SA, et al. Lymphosarcoma. Medicine 1961;40:31.
63. Bilsel Y, Balik E, Yamaner S, et al. Clinical and therapeutic considerations of rectal lymphoma: a case report and literature review. World J Gastroenterol 2005;11:460–461.
64. Batchelor T, Loeffler JS. Primary CNS lymphoma. J Clin Oncol 2006;24:1281–1288.
65. Shenkier TN, Connors JM. Primary extranodal non-Hodgkin's lymphomas. In: Canellos GP, Lister TA, Young BD, eds. The lymphomas, 2nd ed. Philadelphia: Saunders, Elsevier, 2006:325–347.

66. Hugh JC, Jackson FI, Hanson J, et al. Primary breast lymphoma. An immunohistologic study of 20 new cases. Cancer 1990;66:2602–2611.
67. Zaharia L, Gill PS. Primary cardiac lymphoma. Am J Clin Oncol 1991;14: 142–145.
68. Calvo KR, Traverse-Glehen A, Pittaluga S, et al. Molecular profiling provides evidence of primary mediastinal B-cell lymphoma as a distinct entity related to classic Hodgkin lymphoma: implications for mediastinal grey zone lymphomas as an intermediate form of B-cell lymphoma. Adv Anat Pathol 2004;11:227–238.
69. Stein H, Johrens K, Anagnostopoulos I. Non-mediastinal grey zone lymphomas and report from the workshop. Eur J Haematol 2005;75(Suppl 66):42–44.
70. Dogan A. Grey zone lymphomas. Hematology 2005;10(Suppl 1):190–192.
71. Traverse-Glehen A, Pittaluga S, Gaulard P, et al. Mediastinal grey zone lymphoma: the missing link between classic Hodgkin's lymphoma and primary mediastinal large B-cell lymphoma. Am J Surg Pathol 2005;29:1411–1421.
72. Jaffe ES, Harris NL, Stein H, Vardiman JW. Tumours of haematopoietic and lymphoid tissues. Lyon, France: World Health Organization-IARC Press, 2001.
73. Siebert JD, Harvey LA, Fishkin PA, et al. Comparison of lymphoid neoplasm classification. A blinded study between a community and an academic setting. Am J Clin Pathol 2001;115:650–655.
74. Glaser SL, Dorfman RF, Clarke CA. Expert review of the diagnosis and histologic classification of Hodgkin disease in a population-based cancer registry: interobserver reliability and impact on incidence and survival rates. Cancer 2001;92:218–224.

75. The Non-Hodgkin's Lymphoma Classification Project. A clinical evaluation of the International Lymphoma Study Group. Classification of non-Hodgkin lymphoma. Blood 1997;89:3909–3918.
76. Lennert K, Mohri N, Stein H, Kaiserling E, Muller-Hermelink HK. Malignant Lymphomas other than Hodgkin's disease. Berlin, Heidelberg and New York, 1977 [in press].
77. Non-Hodgkin's Lymphoma Pathologic Classification Project. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: summary and description of a working formulation for clinical usage. Cancer 1982;49:2112–2135.
78. Serdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW (eds) WHO classification of tumors of hematopoeitic and lymphoid tissues. IARC: Lyon 2008.
79. Aster JC. Diseases of white blood cells, lymph nodes, spleen and thymus. In Robbins and Cotran Pathologic Basis of Disease. Kumar V, Abbas AK, Fausto N (eds). Robbins and Cotran Pathologic Basis of Disease. 7th ed. Elsevier Saunders:Philadelphia, PA;2005. Figure 14-4, Origin of lymphoid neoplasms; p.669.
80. Greer JP, Williams ME. Non-Hodgkin Lymphoma in Adults. In Greer JP , Foerster J , Rodgers GM, Paraskevas F, Glader B, Arber DA, Means Jr RT (eds). Wintrobe's Clinical Hematology. 12th Edition. Lippincott Williams & Wilkins: Philadelphia, PA;2009.Figure 91.1, Cellular origins of non-Hodgkin lymphoma by B- and T-cell differentiation pathways; p.2145.
81. Coons AH, Creech HJ, Jones RN. Immunological properties of an antibody containing a fluorescent group. Proc Soc Exp Biol Med. 1941;47:200.

82. Avrameas S. Enzyme markers: Their linkage with proteins and use in immunohistochemistry. Histochem J. 1972;4:321.
83. Nakane PK, Pierce GBJ. Enzyme-labeled antibodies for the light and electron microscopic localization of tissue antigens. J Cell Biol. 1967;33:307.
84. Mann G, ed. Physiologic histology. Oxford: Oxford University Press; 1902.
85. Taylor CR, Burns J. The demonstration of plasma cells and other immunoglobulin containing cells in formalin-fixed,paraffin-embedded tissues using peroxidase labeled antibody. J Clin Pathol. 1974;27:14.
86. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975;256:495.
87. Huang SN. Immunohistochemical demonstration of hepatitis B core and surface antigens in paraffin sections. Lab Invest.1975;33:88.
88. Shi S-R, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem. 1991;39:741.
89. Shi S-R, Tandon AK, Cote C, et al. S-100 protein in human inner ear: Use of a novel immunohistochemical technique on routinely processed, celloidin-embedded human temporal bone sections. Laryngoscope. 1992;102:734.
90. Shi S-R, Tandon AK, Haussmann RR, et al. Immunohistochemical study of intermediate filament proteins on routinely processed, celloidin-embedded human temporal bone sections by using a new technique for antigen retrieval. Acta Otolaryngol (Stockh). 1993;113:48.

91. Shi S-R, Cote C, Kalra KL, et al. A technique for retrieving antigens in formalin-fixed, routinely acid-decalcified, celloidin-embedded human temporal bone sections for immunohistochemistry. J Histochem Cytochem. 1992;40:787.
92. Taylor CR, Shi S-R, Chen C, et al. Comparative study of antigen retrieval heating methods: Microwave, microwave and pressure cooker, autoclave, and steamer. Biotech Histochem. 1996;71:263.
93. Taylor CR, Shi S-R, Cote RJ. Antigen retrieval for immunohistochemistry: Status and need for greater standardization. Appl Immunohistochem. 1996;4:144.
94. Fraenkel-Conrat H, Brandon BA, Olcott HS. The reaction of formaldehyde with proteins. IV: Participation of indole groups. J Biol Chem. 1947;168:99.
95. Fraenkel-Conrat H, Olcott HS. The reaction of formaldehyde with proteins. V: Cross-linking between amino and primary amide or guanidyl groups. J Am Chem Soc. 1948;70:2673.
96. Fraenkel-Conrat H, Olcott HS. Reaction of formaldehyde with proteins. VI: Cross-linking of amino groups with phenol, imidazole, or indole groups. J Biol Chem. 1948;174:827.
97. Gown AM, de Wever N, Battifora H. Microwave-based antigenic unmasking: A revolutionary new technique for routine immunohistochemistry. Appl Immunohistochem. 1993;1:256.
98. Cattoretti G, Pileri S, Parravicini C, et al. Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. J Pathol. 1993;171:83.
99. Van Regenmortel MHV. The recognition of proteins and peptides by antibodies. In: van Oss CJ, van Regenmortel MHV, eds. Immunochemistry. New York: Marcel Dekker; 1994:277-300.

100. Shi Y, Li G-D, Liu W-P. Recent advances of the antigen retrieval technique. Linchuang yu Shiyan Binglixue Zazhi (J Clin Exp Pathol). 1997;13:265.
101. Evers P, Uylings HB. Microwave-stimulated antigen retrieval is pH and temperature dependent. J Histochem Cytochem. 1994;42:1555.
102. Straus W. Phenylhydrazine as inhibitor of horseradish peroxidise for use in immunoperoxidase procedures. J Histochem Cytochem.1972;20:949.
103. Straus W. Use of peroxidase inhibitors for immunoperoxidase procedures. In: Feldman R, ed. Proceedings of the first international symposium on immunoenzymatic techniques. Amsterdam: Elsevier; 1976:117.
104. Li C-Y, Zeismer SC, Lacano-Villareal O. Use of azide and hydrogen peroxide as an inhibitor for endogenous peroxidise method. J Histochem Cytochem. 1987;35:1457.
105. Schmid KW, Hittmair A, Schmidhammer H, et al. Non-deleterious inhibition of endogenous peroxidase activity (EPA) by cyclopropanone hydrate: A definitive approach. J Histochem Cytochem. 1989;37:473.
106. Colvin RB, Bhan AK, McCluskey RT, eds. Diagnostic Immunopathology. 2nd ed. New York: Raven Press; 1995.
107. DeLellis RA, Sternberger LA, Mann RB, et al. Immunoperoxidase techniques in diagnostic pathology: Report of a workshop sponsored by the National Cancer Institute. Am J Clin Pathol. 1979;71:483.
108. Heras A, Roach CM, Key ME. Enhanced polymer detection system for immunohistochemistry.Mod Pathol. 1995;8:165A.
109. Taylor CR. A history of the Reed-Sternberg cell.Biomedicine. 1978;28:196.

110. Zola H, Swart B, Nicholson I, et al. CD molecules 2005: human cell differentiation molecules. Blood.2005;106:3123–3126.
111. Chen CC, Raikow RB, Sonmez-Alpan E, Swerdlow SH. Classification of small B-cell lymphoid neoplasms using a paraffin section immunohistochemical panel. Appl Immunohistochem Mol Morphol.2000;8:1- 11.
112. Barberis A, Widenhorn K, Vitelli L, Busslinger M. A novel B-cell lineagespecific transcription factor present at early but not late stages of differentiation.Genes Dev. 1990;4:849–859.
113. Bonzheim I, Geissinger E, Roth S, et al. Anaplastic large cell lymphomas lack the expression of T-cell receptor molecules or molecules of proximal T-cell receptor signaling. Blood.2004;104:3358–3360.
114. Wang T, Lasota J, Hanau CA, Miettinen M. Bcl-2 oncoprotein is widespread in lymphoid tissue and lymphomas but its differential expression in benign versus malignant follicles and monocytoid B-cell proliferations is of diagnostic value. APMIS.1995;103:655–662.
115. Zutter M, Hockenbery D, Silverman GA, Korsmeyer SJ. Immunolocalization of the Bcl-2 protein within hematopoietic neoplasms.Blood.1991;78:1062– 1068.
116. Tumwine LK, Agostinelli C, Campidelli C, et al. Immunohistochemical and other prognostic factors in B cell non Hodgkin lymphoma patients, Kampala, Uganda. BMC Clin Pathol. 2009;9(11)
117. Jambhekar NA, Chaturvedi AC, Madur BP. Immunohistochemistry in surgical pathology practice: A current perspective of a simple, powerful, yet complex, tool. Ind J Pathol Microbiol 2008;51:2-11.

118. Kwak LW, Wilson M, Weiss L, et al. Clinical significance of morphologic subdivision in diffuse large cell lymphoma. Cancer 1991;68:1988–1993.
119. Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood.2004; 103: 275-282.
120. Li T, Medeiros LJ, Lin P, et al. Immunohistochemical Profile and Fluorescence In Situ Hybridization Analysis of Diffuse Large B-Cell Lymphoma in Northern China. Arch Pathol Lab Med. 2010;134:759-65.
121. Strauchen JA, Mandeli JP. Immunoglobulin expression in B-cell lymphoma.Immunohistochemical study of 345 cases. Am J Clin Pathol 1991;95:692–695.
122. Picker LJ, Weiss LM, Medeiros LJ, et al. Immunophenotypic criteria for the diagnosis of non-Hodgkin's lymphoma. Am J Pathol 1987;128:181–201.
123. Piris M, Brown DC, Gatter KC, et al. CD30 expression in non-Hodgkin's lymphoma. Histopathology 1990;17:211–218.
124. Fang JM, Finn WG, Hussong JW, et al. CD10 antigen expression correlates with the t(14;18)(q32;q21) major breakpoint region in diffuse large B-cell lymphoma. Mod Pathol 1999;12:295–300.
125. Lai R, Weiss LM, Chang KL, et al. Frequency of CD43 expression in non-Hodgkin lymphoma. A survey of 742 cases and further characterization of rare CD43+ follicular lymphomas. Am J Clin Pathol 1999;111:488–494.
126. Gaulard P, d'Agay M, Peuchmaur M, et al. Expression of the bcl-2 gene product in follicular lymphoma. Am J Pathol 1992;140:1089–1095.

127. Lorsbach RB, Shay-Seymoore D, Moore J, et al. Clinicopathologic analysis of follicular lymphoma occurring in children. Blood. 2002;99:1959-1964.
128. Davies AJ, Rosenwald A, Wright G, et al. Transformation of follicular lymphoma to diffuse large B-cell lymphoma proceeds by distinct oncogenic mechanisms. Br J Haematol 2007;136(2):286-293.
129. Said JW, Pinkus JL, Shintaku IP, et al. Alterations in fascin-expressing germinal center dendritic cells in neoplastic follicles of B-cell lymphomas. Mod Pathol 1998;11:1–5.
130. Vasef MA, Medeiros LJ, Koo C, et al. Cyclin D1 immunohistochemical staining is useful in distinguishing mantle cell lymphoma from other low-grade B-cell neoplasms in bone marrow. Am J Clin Pathol 1997;108:302–307.
131. Cheuk W, Wong KO, Wong CS, et al. Consistent immunostaining for cyclin D1 can be achieved on a routine basis using a newly available rabbit monoclonal antibody. Am J Surg Pathol 2004;28:801–807.
132. Willemze R, Jaffe ES, Burg G, et al. WHO-EORTC classification for cutaneous lymphomas. Blood 2005;105:3768–3785.
133. Picker LJ, Weiss LM, Medeiros LJ, et al. Immunophenotypic criteria for the diagnosis of non-Hodgkin's lymphoma. Am J Pathol 1987;128:181–201.
134. Rudiger T, Weisenburger DD, Anderson JR, et al. Peripheral T-cell lymphoma (excluding anaplastic large cell lymphoma):results from the Non-Hodgkin's Lymphoma Classification Project. Ann Oncol 2002;13:140–149.
135. Abdel-Ghafar, Afaf Abdel-Aziz, et al. Immunophenotyping of chronic B-cell neoplasms: flow cytometry versus immunohistochemistry. Hematology reports 2012;4(1):e3.

136. Mazur MT, Katzenstein AL. Metastatic melanoma: the spectrum of ultrastructural morphology. Ultrastruct Pathol 1980;1:337–356.
137. Llombart-Bosch A, Blache R, Peydro-Olaya A. Ultrastructural study of 28 cases of Ewing's sarcoma: typical and atypical forms. Cancer 1978;41:1362–1373.
138. Gould VE. Neuroendocrinomas and neuroendocrine carcinomas: APUD cell system neoplasms and their aberrant secretory activities. Pathol Annu 1977;12:33–62.
139. Ioachim HL, Finkbeiner JA. Pseudonodular patterns of T-cell lymphoma. Cancer 1980;45:1370–1378.
140. Caulet S, Audouin J, le Tourneau A, et al. Angio-immunoblastic lymphadenopathy (AIL) or T-cell malignant lymphoma of AIL-type. A histopathological, immunohistochemical and ultrastructural study of 8 cases. Pathol Res Pract 1988;183:724–734.
141. Rappaport H. Tumors of the hematopoietic system. In: Atlas of tumor pathology. Series I, Fascicle 8. Washington, DC: Armed Forces Institute of Pathology, 1966.
142. Lukes RJ, Collins RD. New approaches to the classification of the lymphomata. Br J Cancer 1975;31[Suppl 2]:1–28.
143. Greiner TC, Medeiros J, Jaffe ES.Non-Hodgkin's lymphoma. Cancer 1995;75:370–380.
144. Krishnan J, Wallberg K, Frizzera G. T-cell rich large B-cell lymphoma: a study of 30 cases supporting its histologic heterogeneity and lack of clinical distinctiveness. Am J Surg Pathol 1994;18:455–465.
145. Hsi ED. The search for meaningful prognostic markers in diffuse large B-cell lymphoma. Am J Clin Pathol 2001;115:481–483.

146. Fijten GH, Blijham GH. Unexplained lymphadenopathy in family practice.An evaluation of the probability of malignant causes and the effectiveness of physicians' workup. J Fam Pract 1988;27:373:6.
147. Pangalis GA, Vassilakopoulos TP, Boussiotis VA, et al. Clinical approach to lymphadenopathy. Semin Oncol 1993;20:570–582.
148. Johnson JT, Myers EN. Evaluation of cervical masses in the elderly. Geriatrics 1983;38:99–106.
149. Osborne BM, Butler JJ. Clinical implications of nodal reactive follicular hyperplasia in the elderly patient with enlarged lymph nodes. Mod Pathol 1991;4:24–30.
150. Nathwani BN, Winberg CD, Diamond LW, et al. Morphologic criteria for the differentiation of follicular lymphoma from florid reactive follicular hyperplasia: a study of 80 cases. Cancer 1981;48:1794–1806.
151. Abbondanzo SL. Epstein-Barr virus-associated lymphadenitis: the differential diagnosis of diffuse paracortical lymphoid hyperplasia. Pathol Case Rev 2004;9:192–198.
152. Shear NH, Spielberg SP. Anticonvulsant hypersensitivity syndrome: in vitro assessment of risk. J Clin Invest 1988;82:1826–1832.
153. Jeng YM, Tien HF, Su IJ. Phenytoin-induced pseudolymphoma: reevaluation using modern molecular biology techniques. Epilepsia 1996;37:104–107.
154. Braddock SW, Harrington D, Vose J. Generalized nodular cutaneous pseudolymphoma associated with phenytoin therapy. Use of T-cell receptor gene rearrangement in diagnosis and clinical review of cutaneous reactions to phenytoin. J Am Acad Dermatol 1992;27:337–340.
155. Chamulak GA, Brynes RK, Nathwani BN. Kikuchi-Fujimoto disease mimicking malignant lymphoma. Am J Surg Pathol l990;14:514–523.

156. Davies JD, Stansfeld AG. Spontaneous infarction of superficial lymph nodes. J Clin Pathol 1972;25:689–696.
157. Elie H, Joubert B, Mandard JC. Infarctus ischémique spontané massif d'un ganglion lymphatique cervical. Ann Pathol 1982;2:240–242.
158. Casper C. The aetiology and management of Castleman disease at 50 years: translating pathophysiology to patient care. Br J Haematol 2005;129:3–17.
159. Keller AR, Hochholzer L, Castleman B. Hyaline-vascular and plasma-cell types of giant lymph node hyperplasia of the mediastinum and other locations. Cancer 1972;29:670–683.
160. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844–847.
161. Cordon-Cardo C: Mutation of cell cycle regulators. Biological and clinical implications for human neoplasia. Am J Pathol 1997;147:545–560.
162. Yan P, Seelentag W, Bachmann A, Bosman FT. An agarose matrix facilitates sectioning of tissue microarray blocks. J Histochem Cytochem 2007; 55: 21–4.
163. Borel N, Mukhopadhyay S, Kaiser C, et al. Tissue MicroArray (TMA) analysis of normal and persistent Chlamydophila pneumonia infection. BMC Infect Dis 2006;6:152.
164. Chan JK, Wong CS, Ku WT, Kwan MY. Reflections on the use of controls in immunohistochemistry and proposal for application of a multitissue spring-roll control block. Ann Diagn Pathol 2000;4:329–36.
165. Wan WH, Fortuna MB, Furmanski P. A rapid and efficient method for testing immunohistochemical reactivity of monoclonal antibodies

against multiple tissue samples simultaneously. J Immunol Methods 1987; 103: 121–9.

166. Parsons MWG H. How to make tissue microarrays. Diagnostic Histopathology 2009;15:142-50. Figure 3, Low magnification picture of an H&E stained TMA section; p.145.
167. Parsons MWG H. How to make tissue microarrays. Diagnostic Histopathology 2009;15:142-50. Figure 5b, Close up view of the front of the machine with recipient block in block holder which is attached to the rest of the machine via a magnet; p.146.
168. Howat WJ, Warford A, Mitchell JN, Clarke KF, Conquer JS, McCafferty J. Resin tissue microarrays: a universal format for immunohistochemistry. J Histochem Cytochem 2005; 53: 1189–97.
169. Zhou L, Hodeib M, Abad JD, Mendoza L, Kore AR, Hu Z. New tissue microarray technology for analyses of gene expression in frozen pathological samples.Biotechniques 2007; 43: 101–5.
170. Wen CH, Su YC, Wang SL, Yang SF, Chai CY. Application of the microarray technique to cell blocks. Acta Cytol 2007; 51: 42–6.
171. Datta MW, Kahler A, Macias V, Brodzeller T, Kajdacsy-Balla A. A simple inexpensive method for the production of tissue microarrays from needle biopsy specimens: examples with prostate cancer. Appl Immunohistochem Mol Morphol 2005; 13: 96–103.
172. Torhorst J, Bucher C, Kononen J, et al. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol 2001; 159: 2249–56.
173. Sauter G, Mirlacher M. Tissue microarrays for predictive molecular pathology. J Clin Pathol 2002; 55: 575–6.
174. Gulmann C, O'Grady A. Tissue microarrays: an overview. Curr Diagn Pathol 2003; 9: 149–54.

175. Camp RL, Charette LA, Rimm DL.Validation of tissue microarray technology in breast carcinoma.Lab Invest 2000; 80: 1943–9.

176. Hager M, Kolbitsch C, Tiefenthaler W, et al. Tissue microarrays from renal cell tumors: exclusion criteria and rate of exclusion. Scand J Urol Nephrol 2007; 41: 485–9.

177. Hidalgo A, Piña P, Guerrero G, et al. A simple method for the construction of small format tissue arrays. J Clin Pathol 2003;56:144–146.

178. Naresh KN, Srinivas V, Soman CS. Distribution of various subtypes of non-Hodgkin's lymphoma in India: a study of 2773 lymphomas using R.E.A.L. and WHO Classifications. Ann Oncol.2000;11 Suppl 1:63-67.

179. Kalyan K, Basu D, Soundararaghavan J. Immunohistochemical typing of non-Hodgkin's lymphoma-comparing working formulation and WHO classification. Indian J Pathol Microbiol.2006 Apr;49(2):203-7.

180. Mushtaq S, Akhtar N, Jamal S, et al., Malignant lymphomas in Pakistan according to the WHO classification of lymphoid neoplasms. Asian Pac J Cancer Prev.2008 Apr-Jun;9(2):229-232.

181. Padhi S, Paul TR, Challa S, et al., Primary Extra Nodal Non Hodgkin Lymphoma: A 5 Year Retrospective Analysis. Asian Pacific J Cancer Prev.2012: 13 (10), 4889-4895.

182. Hunt KE, Reichard, KK. Diffuse large B-cell lymphoma. Archives of pathology & laboratory medicine.2008;132(1):118-124.

183. Sengar M, Akhade A, Nair R, et al., A retrospective audit of clinicopathological attributes and treatment outcomes of adolescent and young adult non-Hodgkin lymphomas from a tertiary care center. Indian J Med Paediatr Oncol.2011;32:197-203.

184. Jaffe ES. Hematopathology: integration of morphologic features and biologic markers for diagnosis. Modern pathology.1999;12(2):109.

185. Roy A, Kar R, Basu D, et al. Spectrum of histopathologic diagnosis of lymph node biopsies: A descriptive study from a tertiary care center in South India over 5½ years. Indian J Pathol Microbiol.2013;56:103-108.

186. Dogan A, Bagdi E, Munson P et al. CD10 and Bcl-6 expression in paraffin sections of normal lymphoid tissues and B-cell lymphomas. Am J Surg Pathol 2000 ; 24(6) : 846-852.

187. S.Gujaral, A.Agarwal,V.Gota,R.Nair et al. A clinicopathologic study of mantle cell lymphoma in a single center study in india. Indian J Pathol Microbiol. 2008;51:315-322.

188. Roy A, Kar R, Basu D, et al. SpectrumNodal mantle cell lymphoma: A descriptive study from a tertiary care center in South India. Indian J Pathol Microbiol.2013;56(2):94.

189. Cheson BD, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood 1996;87:4990–4997.

190. Matutes E, Polliack A. Morphological and immunophenotypic features of chronic lymphocytic leukemia. Rev Clin Exp Hematol 2000;4:22–47.

191. Watson P, Wood KM, Lodge A, et al. Monoclonal antibodies recognizing CD5, CD10 and CD23 in formalin-fixed, paraffin embedded tissue: production and assessment of their value in the diagnosis of small B-cell lymphoma. Histopathology. 2000;36(2):145-150.

192. Kurtin PJ, Hobday KS, Ziesmer S, et al. Demonstration of distinct antigenic profiles of small B-cell lymphomas by paraffin section immunohistochemistry. Am J Clin Pathol. 1999;112(3):319-329.
193. Campo E, Miquel R, Krenacs L, et al. Primary nodal marginal zone lymphomas of splenic and MALT type. Am J Surg Pathol. 1999;23(1):59-68.
194. Smith A, Roman E, Howell D, et al. The Haematological Malignancy ResearchNetwork (HMRN): a new information strategy for population based epidemiology and health service research. British Journal of Haematology.2010;148(5):739-753.
195. Lee MY, Tan TD, Feng AC, et al. Clinicopathological analysis of malignant lymphoma in Taiwan, defined according to the World Health Organization classification. Haematologica.2005; 90(12):1703.
196. Ko YH, Kim CW, Park CS, et al. REAL classification of malignant lymphomas in the Republic of Korea. Cancer,1998;83(4):806-812.
197. Aozasa K, Tsujimoto M, Sakurai M, et al. Non-Hodgkin's lymphomas in Osaka, Japan. European Journal of Cancer and Clinical Oncology.1985;21(4):487-492.
198. Mozaheb Z, Aledavood A, Farzad F. Distributions of major sub-types of lymphoid malignancies among adults in Mashhad, Iran. Cancer epidemiology,2011;35(1):26-29.
199. Ahmad M, Khan AH, Mansoor A et al. Non-Hodgkin's lymphoma - clinicopalhological pattern. Journal- Pakistan Medical Association 1992; 42:205-7.
200. Chakrabarti S, Sarkar S, Goswami BK et al.Hodgkin’s and Non-Hodgkin’s Lymphomas in an Indian Rural Medical Institution: Comparative Clinicopathologic Analysis. Asian Pacific J Cancer Prevention.2010;11:1605-1608.

201. Mucci NR, Akdas G, Manely S, Rubin MA. Neuroendocrine expression in metastatic prostatic cancer: evaluation of high throughput tissue microarrays to heterogenous protein expression. Hum Pathol. 2000;31:406-414.
202. Schrami P, Kononen J, Bubendorf L, et al. Tissue microarrays for gene amplification surveys in many different tumors types. Clin cancer res. 1999;5:1966-75.
203. Richter J, Wagner U, Kononen J, et al. High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. Am J Pathol. 2000;157:787-794.

Annexures

Annexure I – Master Chart

MASTER CHART

SN	IP no	HPE no	AGE	SEX	SPECIMEN	SITE	SIZE (largest dimension in cm)	HPE DIAGNOSIS	No OF CORES	TMA BLOCK NO	TMA CORE POSITION	PATTERN OF STAINING IN IHC				IHC GRADING				FINAL DIAGNOSIS
												CD3	CD20	CD5	CD10	CD3	CD20	CD5	CD10	
1	25758	1567/11	65	F	LYMPH NODE	FEMORAL	6	LARGE CELL ANAPLASTIC LYMPHOMA	3	BLOCK1	1A1,1A2,1A3	NEGATIVE	DIFFUSE	DIFFUSE	DIFFUSE	0	4+	2+	1+	DIFFUSE LARGE B CELL LYMPHOMA
2	29234	1593/11	62	F	LYMPH NODE	AXILLA	4	DIFFUSE NHL	2	BLOCK1	1B1,1B3	NEGATIVE	DIFFUSE	DIFFUSE	NEGATIVE	0	4+	2+	0	CLL/SLL OR MANTLE CELL LYMPHOMA
3	34538	1813/11	65	F	LYMPH NODE	CERVICAL	1	DIFFUSE SMALL & LARGE CELL LYMPHOMA	2	BLOCK1	1C1,1C2	NEGATIVE	DIFFUSE	NEGATIVE	DIFFUSE	0	4+	0	1+	DIFFUSE LARGE B CELL LYMPHOMA
4	55544	2857/11	15	M	LYMPH NODE	CERVICAL	2	FOLLICULAR LYMPHOMA	3	BLOCK2	2D1,2D2,2D3	FOCAL-INTERFOLLICULAR	DIFFUSE	DIFFUSE	NEGATIVE	1+	4+	2+	0	NODAL MARGINAL ZONE LYMPHOMA
5	54433	2860/11	60	M	LYMPH NODE	CERVICAL	2	DIFFUSE SMALL CELL LYMPHOMA	2	BLOCK2	2E1,2E2	FOCAL-FOLLICULAR	DIFFUSE	DIFFUSE	NEGATIVE	2+	4+	3+	0	CLL/SLL OR MANTLE CELL LYMPHOMA
6	43617	2426/11	78	M	LYMPH NODE	CERVICAL	1.5	NHL	2	BLOCK2	2F1,2F2	NEGATIVE	DIFFUSE	DIFFUSE	NEGATIVE	0	4+	2+	0	CLL/SLL OR MANTLE CELL LYMPHOMA
7	51884	2551/12	60	M	LYMPH NODE	CERVICAL	2	DIFFUSE MIXED CELL TYPE OF NHL	3	BLOCK3	3G1,3G2,3G3	FOCAL-INTERFOLLICULAR	DIFFUSE	DIFFUSE	DIFFUSE	2+	3+	3+	1+	DIFFUSE LARGE B CELL LYMPHOMA
8	45082	2072/12	11	M	LYMPH NODE	CERVICAL	1.5	RFH WITH PTGC	2	BLOCK3	3H1,3H3	FOCAL-INTERFOLLICULAR	FOLLICULAR	FOCAL-FOLLICULAR	NEGATIVE	2+	3+	3+	0	PROGRESSIVE TRANSFORMATION OF GERMINAL CENTRE
9	64414	3143/12	11	M	LYMPH NODE	CERVICAL	3	NHL WITH RDD CHANGES	3	BLOCK3	3I2,3I3	NEGATIVE	DIFFUSE	DIFFUSE	NEGATIVE	0	3+	3+	0	CLL/SLL OR MANTLE CELL LYMPHOMA
10	52954	2444/13	65	M	LYMPH NODE	CERVICAL	1	NHL,DIFFUSE TYPE	3	BLOCK4	4J1,4J2,4J3	FOCAL	DIFFUSE	NEGATIVE	DIFFUSE	1+	3+	0	1+	DIFFUSE LARGE B CELL LYMPHOMA
11	45789	1883/13	53	F	LYMPH NODE	MESENTRIC	0.5	NHL, MIXED TYPE	1	BLOCK4	4K2	NEGATIVE	DIFFUSE	NEGATIVE	DIFFUSE	0	3+	0	1+	DIFFUSE LARGE B CELL LYMPHOMA
12	56822	2607/13	45	F	LYMPH NODE	MESENTRIC	0.3	MANTLE CELL LYMPHOMA	2	BLOCK4	4L1,4L3	FOCAL-FOLLICULAR	DIFFUSE	DIFFUSE	NEGATIVE	1+	3+	2+	0	MANTLE CELL LYMPHOMA
13	58077	0026/11	55	M	LYMPH NODE	AXILLA	1.5	LARGE CELL ANAPLASTIC LYMPHOMA	3	BLOCK5	5M1,5M2,5M3	NEGATIVE	DIFFUSE	DIFFUSE	DIFFUSE	0	3+	1+	2+	DIFFUSE LARGE B CELL LYMPHOMA
14	160424	2928/13	60	F	LYMPH NODE	CERVICAL	1.5	NHL	2	BLOCK5	5N1,5N3	NEGATIVE	DIFFUSE	DIFFUSE	NEGATIVE	0	3+	1+	0	CLL/SLL OR MANTLE CELL LYMPHOMA

15	39870	1438/13	65	F	LYMPH NODE	CERVICAL	1	NHL, FOLLICULAR TYPE	3	BLOCK5	5O1,5O2	FOCAL	DIFFUSE	DIFFUSE	DIFFUSE	2+	3+	3+	1+	DIFFUSE LARGE B CELL LYMPHOMA
16	18724	1104/14	65	M	LYMPH NODE	AXILLA, INGUINAL	3	ANGIOIMMUNOBLASTIC LYMPHADENOPATHY	3	BLOCK6	6P1,6P2,6P3	DIFFUSE	NEGATIVE	FOCAL	NEGATIVE	2+	0	2+	0	T CELL LYMPHOMA
17	33143	980/14	53	F	LYMPH NODE	AXILLA	0.7	NHL	2	BLOCK6	6Q1,6Q2	NEGATIVE	DIFFUSE	DIFFUSE	NEGATIVE	0	1+	2+	0	CLL/SLL OR MANTLE CELL LYMPHOMA
18	13933	0095/14	44	M	LYMPH NODE	OMENTUM	1	SMALL CELL CARCINOMA/LYMPHOMA	2	BLOCK6	6R1,6R3	NEGATIVE	DIFFUSE	FOCAL-FOLLICULAR	NEGATIVE	0	1+	3+	0	MANTLE CELL LYMPHOMA
19	35334	1998/14	60	M	LYMPH NODE	INGUINAL	2	NHL	2	BLOCK7	7S2,7S3	NEGATIVE	DIFFUSE	DIFFUSE	DIFFUSE	0	1+	1+	2+	DIFFUSE LARGE B CELL LYMPHOMA
20	22556	1428/14	75	M	LYMPH NODE	AXILLA	3	LYMPHOPROLIFERATIVE DISORDER	2	BLOCK7	7T1,7T3	LOSS	LOSS	DIFFUSE	LOSS	LOSS	LOSS	1+	LOSS	NOT POSSIBLE
21	14458	768/14	60	M	LYMPH NODE	CERVICAL	3	NHL	1	BLOCK7	7U1	NEGATIVE	DIFFUSE	LOSS	LOSS	0	2+	LOSS	LOSS	DIFFUSE LARGE B-CELL LYMPHOMA-NOS

KEY TO THE MASTER CHART

CD	–	Cluster of Differentiation.
F	–	Female.
HPE.NO	–	Histopathological Examination Number.
IHC	–	Immunohistochemistry.
IP.NO	–	In patient number.
M	–	Male.
NHL	–	Non Hodgkin Lymphoma.
PTGC	–	Progressive Transformation of Germinal Centre.
RFH	–	Reactive Follicular Hyperplasia.
RDD	–	Rosai Dorfman Disease.
S.N	–	Serial Number.

Annexure II – TMA layout

ANNEXURE 2

TMA LAYOUT

1567/11
1593/11
1813/11
2857/11
2860/11
2426/11
2551/12
2072/12
3143/12
2444/13
1883/13
2607/13
0026/11
2928/13
1438/13
1104/14
980/14
95/14
1998/14
1428/14
768/14

BLOCK 1	1	2	3
A	1567/11	1567/11	1567/11
B	1593/11	BLANK	1593/11
C	1813/11	1813/11	BLANK
BLOCK2			
D	2857/11	2857/11	2857/11
E	2860/11	2860/11	BLANK
F	2426/11	2426/11	BLANK
BLOCK3			
G	2551/12	2551/12	2551/12
H	2072/12	BLANK	2072/12
I	BLANK	3143/12	3143/12
BLOCK4			
J	2444/13	2444/13	2444/13
K	BLANK	1883/13	BLANK
L	2607/13	BLANK	2607/13
BLOCK5			
M	0026/11	0026/11	0026/11
N	2928/13	BLANK	2928/13
O	1438/13	1438/13	BLANK
BLOCK6			
P	1104/14	1104/14	1104/14
Q	980/14	980/14	BLANK
R	95/14	BLANK	95/14
BLOCK7			
S	BLANK	1998/14	1998/14
T	1428/14	BLANK	1428/14
U	768/14	BLANK	BLANK

BLOCK1	1A1	1A2	1A3
	1B1	BLANK	1B3
	1C1	1C2	BLANK
BLOCK2	2D1	2D2	2D3
	2 E1	2 E2	BLANK
	2F1	2F2	BLANK
BLOCK3	3G1	3G2	3G3
	3H1	BLANK	3H3
	BLANK	1I2	3I3
BLOCK4	4J1	4J2	4J3
	BLANK	4K2	BLANK
	4L1	BLANK	4L3
BLOCK5	5M1	5M2	5M3
	5N1	BLANK	5N3
	5O1	5O2	BLANK
BLOCK6	6P1	6P2	6P3
	6Q1	6Q2	BLANK
	6R1	BLANK	6R3
BLOCK7	BLANK	7S2	7S3
	7T1	BLANK	7T3
	7U1	BLANK	BLANK