ABSTRACT

Objective: Proper Characterization of lymphoma cases attending the medical lab by using immunohistochemistry

Material and Methods: Forty cases already diagnosed as lymphoma in the period Jan 2007 to Dec 2008 at Medical laboratory University of Gezira, Sudan. The cases were selected from the records and accordingly the paraffin embedded blocks were collected. Re-embedding of the blocks were done. From these blocks the haematoxylin and eosin stain was performed to assess the preservation of tissue as well as to select the suitable blocks to perform the immunohistochemistry on it. Results: show similar male/female ratio for Hodgkin's and non-Hodgkin's lymphoma. In Hodgkin's lymphoma the cells showed positivity for CD3, CD20, CD15 and CD30 with percentages of 87.6%, 85.7%, 78.6% and 57.1% respectively. The atypical cells in case of non-Hodgkin's lymphoma showed positivity for CD45 with a percentage of 100%. CD3 & CD20 in 23 cases representing 88.5% for each. Twelve cases were found to be CD15 positive with a percentage of 46.2 %, while the remainder 5 cases representing 19.2 % were found to be CD30 positive. Conclusion: The study concluded that the use of immunohistochemistry is very important for diagnosis of lymphoma beside H and E to avoid diagnostic pitfalls. Interpretation of marker studies must be based on a panel and knowledge of a particular antigen's expression in normal, reactive, and neoplastic conditions.

KEYWORDS: Hodgkin’s, Non-Hodgkin’s, Immunohistochemistry.

INTRODUCTION

Malignant lymphoma is the generic term given to tumors of the lymphoid system and specifically of lymphocytes and their precursor cells, whether of T, B, or null phenotypes.[1] Although traditionally tumors presumed to be composed of histiocytes and "reticulum cells" have also been included in the category of malignant lymphoma, it would seem more appropriate to regard them separately for both conceptual and practical reasons.

Although some overlapping exists, the term malignant lymphoma is reserved for those neoplastic processes that initially present as localized lesions and are characterized by the formation of gross tumor nodules. Conversely, neoplastic lymphoid proliferations that are systemic and diffuse from their inception are included among the leukaemias.

Because there are so many different subtypes of lymphoma, the classification of lymphomas is complicated and includes both the microscopic appearance and well-defined immunohistochemical, genetic and molecular rearrangements.

The malignant lymphomas can be divided into two major categories: Hodgkin's disease and all the others which for lack of a better term are known collectively as non-Hodgkin's lymphomas.[2] Both groups are further subdivided into several more or less distinct subcategories.

Hodgkin's disease

The term Hodgkin's disease has been traditionally used for a type of malignant lymphoma in which Reed-Sternberg cells are present in a "characteristic background" of reactive inflammatory cells of various types, accompanied by fibrosis of a variable degree. Thus identification of typical Reed Sternberg cells is necessary for the initial diagnosis of Hodgkin's disease.
As far as the "characteristic background" or "appropriate milieu" is concerned, it is highly variable, but it always lacks the monomorphic appearance of most other malignant lymphomas. Mature lymphocytes, eosinophils, plasma cells, and histiocytes may all be present in greater or lesser amount, depending on the microscopic type.

The etiology of Hodgkin's disease remains unknown, but there is considerable evidence to suggest that the EBV plays an important role. Individuals with a history of infectious mononucleosis have an increased incidence of Hodgkin's disease[3] and patients with Hodgkin's disease have an altered antibody pattern to EBV prior to diagnosis (with marked phenotypic similarities exist between infectious mononucleosis and Hodgkin's disease.[2]

The EBV genomes have been identified in Reed-Sternberg cells in up to half of the cases (particularly in the mixed cellularity subtype, in young patients, and/or in developing countries).[24] There is also evidence for a genetic susceptibility factor.[5]

**Gross features of Hodgkin's lymphoma**

Except for the very early stages, lymph nodes involved by Hodgkin's disease are enlarged, with the consistency varies from soft to hard depending on the amount of fibrosis. Some degree of nodularity is often appreciated, particularly in the nodular sclerosis form and foci of necrosis may be present. Except for lymphocyte-predominance Hodgkin's disease, the cut surface of the node has a more heterogeneous appearance than most non-Hodgkin's lymphomas.

**Reed-Sternberg cell**
The classic Reed-Sternberg cell is a large cell (20 to 50µm in diameter or more) with abundant weakly acidophilic or amphophilic cytoplasm, which may appear homogeneous or granular and lacks a pale zone in the Golgi area. The nucleus is bilobed or polylobed so that the cell appears binucleated or multinucleated.

There is a very large, variously shaped, but usually rounded, highly acidophilic central nucleolus surrounded by a clear halo. In the most typical example of the Reed-Sternberg cell, the two nuclear lobes face each other ("mirror image"), resulting in the "owl eye" appearance. When multilobation occurs, the appearance has been likened to that of an "egg basket." and cells with this set of features but lacking nuclear lobation have been referred to as mononuclear variants of Reed-Sternberg cells or Hodgkin's cells.

At the other end of this spectrum is the Reed-Sternberg cell of giant size and highly pleomorphic hyperchromatic nuclei, having an appearance such as to simulate the cells of anaplastic carcinoma or one of the pleomorphic sarcomas. Another type of Reed-Sterberg cell, characterized by a darkly staining and retracted quality, is referred to as the mummified or necrobiotic variant and appears to be the morphologic expression of apoptosis.

Cells morphologically very similar to Reed-Sternberg cells, representing pleomorphic immunoblasts, can be seen in infectious mononucleosis and other viral diseases, 24.

Finally, some malignant lymphomas of non-Hodgkin's type may be accompanied by cells with the appearance of Reed-Sternberg cells, a fact that raises some questions about the very definition of Hodgkin's disease and the requirement for the presence of classic Reed-Sternberg cells, which is absolute for the initial diagnosis of Hodgkin's disease, can be lessened somewhat in subsequent biopsies from patients with documented Hodgkin's disease. Under these circumstances, the presence of a polymorphic infiltrate with atypical mononuclear cells but not classic Reed-Sternberg cells in a biopsy of bone marrow, liver, or some other organ can be taken as evidence of involvement by Hodgkin's disease.

The immunocytochemical profile of the Reed-Sternberg cell is yet to be totally agreed upon because of the discrepancies among various laboratories. The most important findings in paraffin-embedded material have been the following

- CD15 (Leu-M1): This is expressed in over 80% of the cases; the pattern may be paranuclear (corresponding to the Golgi region), diffuse cytoplasmic, and/or corresponding to the cell membrane.
- CD30 (Ki-1): As recognized by the monoclonal antibody Be-Hz, this is found in about 90% of the cases.
- CD45 RB (LCA): This is expressed in less than 10% of the cases.
- CD20 (L26, B lineage antigen): This is expressed in 10% to 20% of the cases.
- CD40 (a protein present in B cells and nerve growth factor receptor) is expressed in approximately 70%.
- CD74: This is expressed in over 75%.

Restin (an intermediate filament-associated protein) is present in about 80% and the same is true for anaplastic large cell lymphoma but not for other types of non-Hodgkin's lymphoma.

In frozen sections, a large percentage of Reed-Sternberg cells have been found to exhibit reactivity for one or more panT-cell or panB-cell antigens, including the framework antigen of the T-cell receptor beta chain. They also express polyclonal IgG (probably representing passive uptake via the Fc receptor), HLA-DR, CD25 (the interleukin-2 receptor), and CD71 (the transferrin receptor).

Molecular studies have also given rise to controversial results. Most cases of Hodgkin's disease yield a germ
line configuration for immunoglobulin heavy and light chain genes and the beta T-cell receptor genes, but this may simply result from a dilution factor by the non-neoplastic cells; indeed, some studies suggest that an increased number of Reed-Sternberg cells and their variants is associated with a detectable increase in clonal rearrangements of either genes.

Reed-Sternberg cells of B-cell immunophenotype were isolated from 12 cases of "classic" Hodgkin's disease, and found to have rearranged immunoglobulin variable-region heavy-chain (VH) genes, indicating their origin from B cells. In half of the cases the population of Reed-Sternberg cells was polyclonal, and in the other half it was monoclonal or mixed.

The karyotype of these cells is generally hyperdiploid and with structural abnormalities, but no recurring chromosomal abnormalities have yet been detected. Another unresolved issue is the prevalence of t(14;18) in Hodgkin's disease, the reported figures ranging from zero to over 30%; perhaps of significance in this regard is the fact that the bcl-2 protein (a hallmark of the 14:18 translocation) is never overexpressed, except in those exceptional instances of Hodgkin's disease that arise in the setting of follicular lymphoma.

**Microscopic types**

For many years, Jackson and Parker's classification of Hodgkin's disease into granuloma, paragranuloma, and sarcoma variants was widely used because of its reproducibility and clear-cut prognostic implications, the major objection being that too many of the cases (~80%) fell into one of the categories—i.e., Hodgkin's granuloma.

**Nodular Sclerosis**

Hodgkin's disease is characterized in its fully developed stage by broad collagen bands separating the lymphoid tissue in well-defined nodules. These fibrous bands, which have a birefringent quality when examined under polarized light. In addition to the classic Reed-Sternberg cell, nodular sclerosis Hodgkin's disease also displays a variant known as lacunar or cytoplasmic variant.

In some cases, there is clustering of these lacunar cells, particularly around areas of necrosis. They form sheets and cohesive nests, to the point that a mistaken diagnosis of large cell non-Hodgkin's lymphoma, carcinoma, germ cell tumor, or thymoma can be made. Cases of nodular sclerosis Hodgkin's disease showing prominence of this feature have been referred to as the syncytial, sarcomatoid, or sarcomatous variant.

**lymphocyte predominance**

Hodgkin's disease, the pre-dominant cell is a small B lymphocyte, with or without an accompanying population of benign-appearing histiocytes. Post capillary venules with high endothelium may be prominent. The lymph node architecture is partially or totally effaced, and the infiltrate may have a diffuse or nodular pattern of growth. The latter may be more pronounced as to simulate on low power the appearance of follicular lymphoma; however, the nodules of Hodgkin's disease are more irregular in size and staining quality, and the admixture of lymphocytes and epithelioid cells gives them a mottled appearance.

**lymphocyte depleted**

Which comprises less than 5% of all cases of Hodgkin's disease, includes two morphologically different subtypes, designated as "diffuse fibrosis" and "reticular" in the original Lukes classification. In the diffuse fibrosis subtype, the number of lymphocytes and other cells progressively decreases as the result of heavy deposition of collagen fibers. The reticular subtype is characterized by a very large number of diagnostic Reed-Sternberg cells (many of them of bizarre configuration) among atypical mononuclear cells and other elements.

**Mixed cellularity**

Hodgkin's disease, a large number of eosinophils, plasma cells, and atypical mononuclear cells are admixed with classic Reed-Sternberg cells, which tend to be numerous. Focal necrosis may be present, but fibrosis should be minimal or absent. It is somewhat ironic that mixed cellularity Hodgkin's disease, which fits more closely the histopathologic picture of the disease as depicted in the classical textbooks, has now almost become a diagnosis of exclusion. An alternative approach has been tried by Copplelson et al. and consists of evaluating individually the frequencies of the different cell types. They found that a large number of lymphocytes was associated with a good prognosis, whereas malignant and mononuclear cells and benign-appearing histiocytes independently influenced the prognosis adversely. Reed-Sternberg cells had no prognostic effect independent of the malignant mononuclear cells, and eosinophils and plasma cells had no prognostic value. However, these authors concluded that the Rye classification of Hodgkin's disease furnished more prognostic information than any estimates of individual cell frequencies.

**General and clinical features**

Hodgkin's disease comprises about 20% to 30% of all malignant lymphomas in the United States and Western Europe but a much lower percentage in Japan and other Oriental countries. There is a wide range in age incidence, which varies according to geographic location. In the United States, there is a bimodal distribution, with a peak at 15 to 40 years and a second, smaller peak in the seventh decade. In Japan, the peak in young adulthood is absent. In poorly developed countries, there is a high incidence in children, and the high in incidence in the 15- to 40-year age group, and a third peak later in life. There is a male preponderance (about 1.5 to 1) in all microscopic types except nodular sclerosis. The disease may present in a variety of ways, the most common (about 90% of the cases) being painless enlargement of superficial (usually...
cervical) lymph nodes. Fever, night sweats, and loss of weight (so-called B symptoms) occur in approximately 25% of the cases; their presence influences the clinical staging. Pruritus is also frequent, immunosuppression, or other immune diseases. Although indubitable cases of this association exist (particularly in patients with ataxia-telangiectasia and with AIDS.

**Staging**

The current staging classification for Hodgkin's disease was established by the Ann Arbor Workshop in 1971 and modified at Cotswolds in 1989. Clinical staging refers to all procedures which include physical examination, bone marrow aspiration and biopsy, clinical laboratory evaluation, and numerous imaging studies. Chest x-ray, thoracic and abdominal CT studies have become the norm, and these are supplemented in some centers by bipedal lymphangiogram and gallium scans.

Pathologic staging used to refer to the findings at staging laparotomy during which liver, splenectomy, and biopsies of retroperitoneal lymph nodes, liver, and bone marrow were performed. Although much important information has been obtained from the performance of routine staging laparotomy in patients with Hodgkin's disease, the procedure is now used only sparingly, the reasons being the increased diagnostic power of radiographic techniques, the high efficiency of current therapies, and the occurrence of postsurgical complications, particularly in the pediatric population.

**Prognosis**

The International Prognostic Index for HL, includes the following 7 risk factors:

1. Male sex
2. Age 45 years or older
3. Stage IV disease
4. Albumin less than 4.0 g/dL
5. Hemoglobin less than 10.5 g/dL
6. Elevated White blood cell (WBC) count of 15,000/mL.
7. Low lymphocyte count less than 600/mL or less than 8% of total WBC

The absence of any of the above risk factors is associated with an 84% rate of control of Hodgkin disease, whereas the presence of a risk factor is associated with a 77% rate of disease control. The presence of 5 or more risk factors was associated with a disease control rate of only 42%. The current overall 5-year survival rate of Hodgkin's disease is approximately 75%.

**Non-Hodgkin's lymphoma**

The classification of non-Hodgkin's lymphoma that was most widely used until the early 1980s in the United States and many other countries was that proposed by Rappaport in 1966. This represented a slight modification of the classification that Gall and Rappaport had presented at a Seminar of the American Society of Clinical Pathologists held in New Orleans, Louisiana, in 1963. This, in turn, was based on the classification proposed by Gall and Mallory as part of their comprehensive critical study of 618 lymphomas. Rappaport's classification was, of necessity, based entirely on morphologic grounds. Numerous independent clinicopathologic studies have shown its reproducibility, usefulness, and clinical relevance.

Analysis of the data showed that all six classifications were successful in predicting the prognosis in a large number of lymphoma patients and that no classification appeared superior to any other in this respect. It also confirmed that lymphomas with a follicular pattern of growth (a feature consistently identified by all reviewers) had a more favorable prognosis than those with diffuse patterns within the same cytologic subtypes. This was true whether the nodularity was extensive or only partial.

Finally, it confirmed the suspicion that within the "histiocytic lymphoma" category of Rappaport there was a variety of morphologically recognizable neoplasms with a somewhat different natural history.

**Classification of non-Hodgkin's lymphoma**

The classification of non-Hodgkin's lymphomas (NHL's) has been a mess for years and the most common types of NHL's in the REAL (revised European-American lymphoma) classification include.

**Small Lymphocytic Lymphoma:** Small and well-differentiated B lymphocytes, with diffuse effacement of nodal architecture and no follicles. Positive expression for CD19, CD5; Bcl-2 and Bcl-6. Seen in older adults, it is essentially the solid tissue (lymph nodal) component of chronic lymphocytic leukemia. The disease tends to be generalized with indolent course and prolonged survival but some may transform to more aggressive lymphomas.

**Follicle Center Lymphoma (predominantly small cell):**

Nodal architecture is effaced by monotonous and crowded follicles composed of monomorphic small cleaved B-lymphocytes. Shows positivity for CD19, CD20, CD79a with t(14:18) and Bcl-2 expression. Most common type, seen in adults, often involves multiple lymph nodes with indolent course, showing prolonged survival, though some may transform to a large cell lymphoma.

**Diffuse Large B-cell Lymphoma**

Cells are large, with prominent nucleoli and abundant cytoplasm and many mitoses. Most are B-cell, but 20% are T-cell phenotype with CD19, CD20, CD79a positive. Some have t(14:18); some have Bcl-2 and Bcl-6 expression; with linked to EBV infection and negative TdT. Though often localized, they tend to be aggressive extranodal masses and seen in adults and children, specially with HIV infection.
Burkitt's Lymphoma: Intermediate sized B-cell lymphocytes (small-cleaved cells), show CD10, CD19, CD20, CD79a positivity and t(8:14) is characteristic. African form linked to EBV infection with negative TdT. Endemic in Africa with mandibular and abdominal involvement. Sporadic elsewhere with abdominal involvement and affects mainly children and young adults.

High-grade B-cell Lymphoma (small non-cleaved)
Burkitt-like: Intermediate sized B-lymphocytes (small non-cleaved, with CD19, CD20 positive. Sporadic; but may be seen with HIV infection. [28]

Staging of Lymphoma
Staging of lymphomas, as in all cancers, is based on the microscopic examination and on the results of imaging studies and related tests that reveal the extent of the cancer involvement.

Stage I (early disease):- Lymphoma located in a single lymph node region or in one area or organ outside the lymph node.

Stage II:-(locally advanced disease) - Lymphoma located in two or more lymph node regions all located on the same side of the lymphoma or in one lymph node region and a nearby tissue or organ.

Stage III:- (advanced disease) - Lymphoma affecting two or more lymph node regions, or one lymph node region and one organ, on opposite sides of the diaphragm

Stage IV:- (widespread or disseminated disease) - Lymphoma outside the lymph nodes and spleen that has spread to another area or organ such as the bone marrow, bone, or central nervous system.

Lymphoma Grading
Low grade:
These are often called "indolent" lymphomas as they usually do not require immediate treatment unless organ function is compromised. They are rarely cured and can transform over time to a combination of indolent and aggressive types.

Intermediate grade
These are rapidly growing (aggressive) lymphomas that usually require immediate treatment, but they are often curable.

High grade
These are very rapidly growing and aggressive lymphomas that require immediate, intensive treatment and are much less often cured.

MATERIALS AND METHODS

Study Type:- This is laboratory based retrospective descriptive study

Study Area:- The study was conducted at the Medical Laboratory, Faculty of Medicine, University of Gezira, Sudan.

Study Population:- All diagnosed lymphoma cases attending the Medical lab, University Of Gezira from Jan 2007- Dec 2008.

MATERIALS
The cases were selected from the records and accordingly the paraffin embedded blocks were collected. Re-embedding of the blocks were done .From these blocks the haematoxylin and eosin stain was performed to assess the preservation of tissue as well as to select the suitable blocks to perform the immunohistochemistry on it.

Equipments
The salanized slides, slide labels, eppendorf pipettes, glass jars, microwave hot oven were used to perform the immunohistochemistry steps. The reagents from Labtech company representative of Dako company were used.

METHODS

The samples were stained with Haematoxylin and eosin stain as follows
- Brought the sections to distilled water
- Nuclei were stained with the alum haematoxylin.
- Rinse in running tap water
- Differentiate with 0.3% acid alcohol.
- Rinse in running tap water
- Rinse in Scott's tap water substitute.
- Rinse in tap water
- Stain with eosin 2 mins
- Dehydrate, clear and mount.

After doing H/E stain the suitable block selected to perform immunohistochemistry as follows.

Immunohistochemistry Steps

Tissue and Slide Processing
- The tissue sections cut at three or four microns in thickness.
- Sections are floated on water and picked up on slides that are coated with some adherent material. Slides prepared with a coating of albumin.
- Wax removed completely, by heating the slides to about 60°C to soften the wax, and then reversing the procedure described in Detailed De-Waxing Protocol, A, below.
- The slide is immersed in Xylene, 100 percent alcohol and then diminishing concentrations of alcohol until the final buffer is fully aqueous. Stained tissue with immunostaining.

RESULTS AND DISCUSSION

Most of Hodgkin's Lymphoma cases found among the age group less than 20 years of age and represent (50%) of cases. Non-Hodgkin's Lymphoma is most common among age group of more than 60 years of age representing (53.8%).
The majority of cases were found among male account for (71%) of cases where as the female r3
50epresent (29%).The below figure showed Non-
Hodgkin's Lymphoma cases with male predominance representing (65%) of cases and the rest of cases (35%) were female.

Table No (I): Hodgkin's Lymphoma, CD markers.

<table>
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<th>Marker</th>
<th>R.S + (ve)</th>
<th>%</th>
<th>R.S –(ve)</th>
<th>%</th>
<th>Total no of cases</th>
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<tr>
<td>CD 45</td>
<td>14</td>
<td>100</td>
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<td>14</td>
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<tr>
<td>CD 3</td>
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<td>3</td>
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<td>2</td>
<td>14.3</td>
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</tr>
<tr>
<td>CD 15</td>
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<td>3</td>
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<td>14</td>
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<tr>
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<td>57.1</td>
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</tr>
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</table>

the total number of R.S CD45 (+/ve were found to be 14 cases (100%), the CD3 marker (+) ve were 11 (78.6 %), CD20 were 12 (85.7%), CD15 were 11 (78.6).

The remainders 8 cases representing (57.1 %) of the 14 Hodgkin's lymphoma were found to be CD30 (+) ve. Table (II) shows that all non- Hodgkin's Lymphoma cases with Atypical cells (26) show positivity for CD45, 23(88.5%) cases were CD30 (+) ve were 23(88.5%), CD230 positivity in 23case (88.5%), positivity for CD15 were 12 (46.2%) and the last 5 cases (19.2%) were found to be CD30 positive.

CONCLUSION
1. The immunohistochemistry is very important for diagnosis of lymphoma beside H and E to avoid diagnostic pitfalls.
2. Interpretation of marker studies must be based on a panel and knowledge of a particular antigen's expression in normal, reactive, and neoplastic conditions.

RECOMMENDATION
1. Increased the sample size with using more markers (Complete Lymphoma panel).
2. Molecular techniques is highly recommended for better assessment of the outcome.

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