Prevalence of epstein barr virus in malignant lymphomas in Iraq

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الخلاصة

الأورام اللمفاوية الخبيثة تعد من الأورام الشائعة التي تنتج عن الإصابة بفيروس ابشتاين بار. رغم العلاقة السببية الوثيقة بين الفيروس والورم اللمفاوي الخبيث المتوطن نوع بركت، فان التوجه العام للعلاقة السببية مع الأورام اللمفاوية المتفرقة الحدوث تشير إلى ازدياد مضطرد في نسبة انتشار مرض هوجكن المصحوب بالإصابة بفيروس ابشتاين بارفي السنوات الأخيرة بالمقارنة بالورم اللمفاوي نوع اللاهوجكن، الأمر الذي من المحتمل أن يدل على وجود دور لهذا الفيروس التسبب بالإصابة بهذا المرض وأحداث الآلية الامراضية له.

الغرض من الدراسة: تقييم العلاقة السببية بين فيروس ابشتاين بار والأورام اللمفاوية بنوعيها- الهوجكن واللاهوجكن وعلاقة ذلك بالعمر عند حدوث الإصابة والجنس والأنواع الفرعية الأربعة لمرض هوجكن.

طرق البحث: تم التقصي عن وجود فيروس ابشتاين بار في المقاطع النسيجية المحضرة من عينات مرضية ماخوذة من العقداللمفاوية لمرضى الأورام اللمفاوية الخبيثة باستخدام تقنية التهجين الموقعي.

النتائج: (ا) تم تقصي الفيروس في 25 حلة من اصل 40 حالة هوجكن، 9 حالات من اصل 30 حالة لاهوجكن، 4 حالات من اصل 10 حالات بركت لمفوما، وفي 5 حالات من اصل 20 حالة لاهوجكن غير البركت لمفوما. (ب) من بين حالات الهوجكن الموجبة لفايروس الـ EB ، كان هناك 19 (76%) حالة من نوع MC وحالة واحدة (%) من كل من نوعي NS و LP و 4(16%) من نوع LD.(ج) أظهرت الدراسة إن سبعة وعشرون حالة هوجكن من اصل 40 حالة أي بنسبة (67.5%) هي الاربعين سنة مقابل 13 من اصل 40 اي (32.5%) فوق سن الاربعين سنة. كما إن التوزيع العمري للحالات اظهر خطا بيانيا ثنائي القمم. أما ضمن الـ 30 حالة لاهوجكن، كانت النتائج المقابلة: 17 (56%) و 13 (43.3%)، على التوالي مظهرة توزيع عمري احادي القمة. (د) لم يلاحظ اختلاف كبير في الإصابة العمرية للإناث والذكور في كلا الحالتين، الهوجكن واللاهوجكن [كان معدل العمر 39.15 (19.15_/+) للذكور المصابين بمرض هوجكن، مقابل 40.80 (9.54_/+) للاناث المصابة بهوجكن، و 30.33 (4.74 _+) للذكور المصابين بمرض اللاهوجكن مقابل 35.26 (6.01_/+) للإناث المصابة باللاهوجكن كانت نسب الذكور للإناث 1:1 لهوجكن و 2:1 للاهوجكن، على التوالي.

نستنتج من هذه الدراسة مايلي: (ا) إن معدل انتشار فيروس ابشتاين بار عالي بين حالات الهوجكن وتتفق هذه النتيجة مع معدلات انتشار الفيروس في هذا المرض المعلنة في دراسات مماثلة سابقة والتي تتراوح بين 20% الى90%. (ب) ان معدل الانتشارالعالي لفيروس ابشتاين بار بين حالات هوجكن بالمقارنة بحالات اللاهوجكن في ؤهذه الدراسة تشير الى الدور الهام لهذا الفيروس في احداث الامراضية لحالات هوجكن الامر الذي يتفق مع ما معروف عن اهمية العوامل البيئية بالمقارنة مع العوامل الوراثية في التسبب بهذا المرض. (ج) النسبة العالية غير المتوقعة لانتشار فيروس ابشتاين بار نوع LD من مرض هوجكن ربما تشير الى التطور المضطرد لبعض حالات هوجكن MC-HD الاقل شراسة الى الحالات المتقدمة الاكثر شراسة من نوع LD-HD .

ABSTRACT

Malignant lymphomas are among the common tumors that are associated with and may complicate Epstein Barr Virus (EBV) infection. Although the strongest association is with the endemic Burkitt lymphomas (BL), the trend of association with sporadic lymhpomas reveals a consistently increasing prevalence of the virus in Hodgkin's disease (HD) in recent years compared to the non-Hodgkin type (NHL) which may point to a possible role for the virus in the predisposition and etio-pathogenesis of the disease.

evaluate the association of EBV with Hodgkin's and non-Hodgkin lymphomas in relation to age, sex and HD subtype retrospectively using archival tissue biopsy sections.

Method: EBV was detected by In Situ Hybridization (ISH) using EBERs RNA probes in paraffin-embedded tissue sections prepared from archival tissue biopsy blocks.

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(a) EBV was detected in 25 of 40 HD cases (62.5%), 9 of 30 (30%) NHL cases, 4 of 10 (40%) BL cases, and in 5 of 20 (25%) other (non-BL) NHL cases. (b) Among the EBV-positive HD cases, 19 (76%) were of the mixed cellularity (MC) subtype, 1 (4%) of the Nodular Sclerosis (NS) subtype, 1 (4%) of the Lymphocyte Predominance (LP) subtype and 4 (16%) cases were of the Lymphocyte Depletion (LD) subtype. (c) Age distribution of HD cases revealed a bi-modal pattern characterized by an early major peak (67.5% of cases) below 35 years and a minor peak (32.5% of cases) above the age of 40. On the contrary, NHL cases revealed a nearly even age distribution (43.3% versus 56.6%) below and above the age of 40, respectively. (d) No difference was observed in the incidence of HD between males and females where the ratio was close to 1:1. On the other hand, a slight male predominance was seen among NHL cases with a male to female ratio of 2:1.
(a) the prevalence rate of EBV infection was high among HD cases and fell within the prevalence rates found in previous similar studies revealing a range of values from 20 to 90%. (b) the higher prevalence of EBV positivity in HD compared to NHL found in this study points to a more substantial role for the virus in the pathogenesis of former compared to the latter disease which also comes in agreement with the greater environmental element compared to the genetic element in the etiology of HD. (c) The unexpected high EBV positivity in the LD subtype of HD may be interpreted as result of the progression of some of the early less aggressive MC-HD cases to the advanced more aggressive LD-HD subtype. (d) the bi-modal age distribution of EBV-positive HD cases follows the same pattern of distribution of the disease in general and testifies for the influence of environmental factors in the incidence of the disease.

INTRODUCTION :

Malignant lymphomas (The Hodgkin and the non-Hodgkin lymphomas) are a diverse group of tumors of the immune system, of B and T cell origin. Hodgkin's disease (HD), which was specifically targeted in this study, is unique among other forms of cancer due to the fact that the malignant H/RS cells constitute a very minor fraction (1-2%) of the tumor mass, surrounded by reactive cellular milieu of various hematopoitic lineages, and a variable degree of fibrotic element[1].
The epidemiologic trend of the disease in developing countries is characterized by bi-modal age-specific incidence with early childhood first peak and a second peak in older age group, where high population density, low socio-economic status, material deficiency and low living standards prevail[2]. This is in contrast to the situation in western countries where the age incidence shows a shift of first peak towards young adult age group and a second putative peak in older age groups, conforming to the late age at exposure to EBV, and the greater incidence of Hodgkin's disease in AIDS-associated lymphoproliferative diseases[3]. The sex-specific incidence in Hodgkin's disease showed a slight preponderance in males, while in NHL twice as many males were affected as females.
EBV is the prototype member of Gamaherpesvirus subfamily, a double-stranded circular DNA virus with B cell lymphotropism. It is the causative agent of acute and chronic Infectious Mononucleosis that typically results in latent type of infection with life-long persistence in infected human host[4].
The causal relationship between EBV and HD was based on several observations including; (a) the presence of EBV-specific antigens and antibodies in large proportion of HD cases[5,6], (b) the rise in antibody titers that precedes development of HD, (c) the increased risk of HD occurrence following Infectious Mononucleosis[7], and (d) the clonal presence in Hodgkin/Reed-Sternberg (H/RS) cells of EBV, indicating that infection with the virus preceded the clonal expansion of the malignant cells[8]. Furthermore, EBV was detected in more than 40% of tumor biopsy specimens from HD cases[9,10]. The viral protein expression profile in HD revealed a latency type II similar to that seen in Undifferentiated NasoPharyngeal Carcinoma (UNPC)[4].

In this study, using the In Situ techniques (In Situ Hybridization and Immunohistochemistry) for the first time in Iraq successfully established and employed in our previous studies (in process for publication), we attempted to evaluate the prevalence rate of EBV infection as reflected by the rate of association of EB viral or its components presence in malignant lymphomas targeting specifically Hodgkin's disease because of the accumulating evidence in support of a causal role for this virus in the etiopathogenesis of the disease.

MATERIALS AND METHODS:

Tissue Samples

A total of 70 archival lymph node and tumor tissue biopsy blocks of malignant lymphoma cases obtained from the Medical City Department of Teaching Laboratories, Division of Histopathology, and from two private laboratories. Of the total, 40 samples were Hodgkin's lymphoma cases, 30 samples from NHL cases divided into 10 Burkitt Lymphoma (BL) samples and 20 samples representing other non-Burkitt NHL cases selected for the years from 1994 to 2004.

Histopathology

All Hematoxilin & Eiosin (H&E) stained tissue slides were previously examined by histopathologist as certified by case signed reports, and subsequently reviewed by a second consultant pathologist to confirm clinicopathologic diagnosis.

Positive and Negative Control samples

(a) Tonsilar biopsy tissues obtained from two patients with acute Infectious Mononucleosis who gave positive results with the Paul-Bunnell test for IgM Heterophil antibodies; (b) EBV positive Lymphoblastoid Cell Lines (LCL); (c) five NPC tissue samples mentioned above, were used as positive controls; (d) Four postmortem lymph node biopsy tissues were obtained from the Institute of Forensic Medicine after signed written consent. Tissue sections from these biopsies were used as negative controls.

Establishment of Lymphoblastoid Cell Line (LCL)

The protocol used by Neitzel (1986)[11] and modified in a previous study (submitted for publication)[12] was used to provide positive control samples to validate the ISH test. In summary; Ten ml of whole blood was drawn with heparinized (200ul of 1000U/ml Heparin) 10ml syringe from a patient with acute Infectious Mononucleosis, and placed in sterile 30ml universal tube containing equal volume of RPMI 1640 medium without serum. The blood-RPMI 1640 mixture was placed over 10ml Ficoll-Hypaque gradient (lymphoprep) in a 50ml sterile centrifuge tube, and centrifuged for 40 minutes at 1200rpm (400g). The lymphocyte rich zone was carefully drawn with sterile pasture pipette into 15ml centrifuge tube, washed 3x10 minutes by centrifugation at 750 (200g) rpm with RPMI 1640 to remove platelets, and cells were counted using Neuber chamber and then adjusted from $3x10^6$ to $1x10^6$/ml in RPMI 1640 containing 20% fetal calf serum and antibiotics and incubated at 36C° for 24 hours to check for contamination. Fifty ml of human cord blood were collected in heparinized sterile container, and lymphocytes were separated by the same method as above, counted, and placed in a $40cm^2$ tissue culture flask containing RPMI 1640 [constituted with 20% fetal calf serum, 2mM L-glutamine, penicillin-streptomycin (200ug/ml) and Fungizone (50ug/ml)], at a concentration of 1x106 cells/ml in a total volume of 10ml. The culture was incubated at 36C° until inoculation. Lymphocytes separated from patient blood were enriched for B cell by T-cell depletion using cyclosporine A at a concentration of 2ug/ml. A concentrated culture $1x10^7$/ml (in complete RPMI 1640 medium) of HUCL was co-cultured with equal volume of patient lymphocytes adjusted to a concentration of $3x10^5$/ml, and incubated at 36C°.

After 24 hours, cells were centrifuged and suspended in fresh complete RPMI 1640 medium containing 2ug/ml cyclosporine A. The medium was changed once a week by replacing half of the volume with fresh medium containing 1ug/ml cyclosporine A. The initial culture was sub-cultured after 2 weeks, then after 3 weeks. The medium was changed at each sub-culture with fresh medium containing 10% fetal calf serum without cyclosporine A. Successful transformation was determined by microscopic detection of (a) Blast formation of lymphocytes and (b) the development of cell aggregates of proliferating lymphoblasts. The yield of B cell enrichment was evaluated by staining a sample of the culture for the B cell-specific marker CD19 using FITC-labeled anti-CD19 by direct immunofluorescence test as previously shown12. A drop of LCL cell suspension was placed on a positive-charged slide, spread over slide surface, dried and fixed with Acetone: Methanol mixture. Fixed cyto-preparations were then tested for the presence EBV by In situ Hybridization with EBERs-specific probes. Stained slides served as positive control in the detection of EBV in lymphoma cases by the same technique.

Detection of EBV by In Situ Hybridization (ISH)

ISH was carried out on all test and control samples using EBERs probe. This detects the presence of EBERs (EBV-encoded, differentially spliced, non-polyadenylated, non-coding small mRNAs known as EBER1and 2 RNAs) transcripts that is characteristic of the latent phase of infection. The probe is an Oligonucleotide Probe containing a cocktail of three oligonucleotides of 19-21 bases. The probe was purchased as 1ml Ready-To-Use flurousceinlabeled from Innogenex (San Ramon CA, USA. Product number: PR-1010-01). ISH Detection Kit. InnoGenex^{TM} ISH Kit: In Situ Hybridization Detection Kit-BCIP/NBT. (Product Number: SH-2008-01, from InnoGenex, San Ramon CA. USA.). The test was carried out as in previous studies (in press)[(12)], in summary: Four to 5 micron-sections were cut by standard microtom and placed onto positive charged slides (Fischerbrand type). Positive and negative control sections were also prepared and used within 24-48 hours, plus one H&E stained section for histopathologic review. Overnight dewaxing was carried out at 60-70C° in dry oven from 1-16 hours followed next morning by baking for one hour at 70-80C°. After baking, slides were placed sequentially in xylene and alcohol containing jars as follows: Exylene 1x for 5 minutes; Fresh Exylene 1x for 5 minutes; 100% Ethanol 1x for 5 minutes; 95% Ethanol 1x for 5 minutes; 70% Ethanol 1x for 5 minutes; De-ionized H_2O containing 0.2% RNase block 2 x 5 minutes. Slides were subsequently subjected for Protease digestion by proteinase K at a concentration of 20 microgram (ug)/ml in proteinase K buffer (supplied with the kit) for 15 minutes at 37C°, then slides were placed in bath containing 0.1M Triethanolamine (TEA), ph 8.0, and acetic anhydride was then added fresh twice to a final concentration of 0.5% during an incubation time of 5 minutes. Slides were then washed once with 2X PBS or 2X SSC buffer. After proteinase K treatment and acetylation, slides were washed twice with de-ionized RNase free water, and post-fixed by placing them in 1% formaldehyde in PBS for 10 minutes at RT, then washed twice in the same washing solution for 5minutes each time. For Pre-Hybridization, slides were drained and carefully whipped with paper towel, then a volume of 50-100 microliter (according to section area) of Hybridization solution without probe (provided in the kit) was added and the slides were covered with coverslips or parafim strips carefully avoiding air bubble entrapment and then placed in a humid chamber and denatured in the Hybridization oven preset at 85C° for 5 minutes. Before Hybridization, the probe was diluted in the Hybridization solution to the optimal concentration that gave the best signal (150ng/ml). Having determined the optimal probe concentration, the slides were drained and whipped around sections carefully, and Hybridization mixture solution (50-100uL per section) with probe was added to sections, covered with coverslips or parafilm pieces and placed in humid chamber. Next, slides were placed in Hybridization oven set at 85C°for 10 minutes to denature the probe and the target at the same time in a one-step denaturation.

After denaturation, slides were rapidly cooled on cold platform for about 2-5 minutes, and then hybridized at 37C° overnight in humid chamber.After hybridization, slides were dipped in 2X PBS-0.1% Tween-20 to let coverslip fall off the slides, then slides were placed in the same buffer jar and post-hybridization wash was carried out by placing in water bath at 55C° for 10 minutes. Then slides were rinsed with 1X PBS-0.1% Tween-20 from washing bottle. One ml of the same buffer was then placed onto sections and incubated for 3 minutes at RT, followed by draining and blotting. To minimize non-specific binding of antibodies to highly charged sites, a proteinacious blocking reagent was used. Enough quantity was added to cover the section, incubated at RT for 5 minutes, then drained and blotted. The Detection System: Is based on the streptavidin-Alkaline phosphatase conjugate enzyme detection with BCIP/NBT substrate that gives dark-blue or purple color at sites of linker antibody binding. The detection system includes in addition: Anti-flurouscein primary antibody; Biotinylated secondary antibody; Counterstain (Nuclear Fast Red) which gives a red nuclear background. Following color development, the slides were dehydrated by sequential dipping of in the following series of xylene and alcohol: 70% Ethanol Alcohol 3minutes; 95% Ethanol 3minutes; 100% Ethanol 5 minutes; xylene 5 minutes; Fresh Xylene 5 minutes. Permanent Mounting Medium (DPX).

RESULTS:

Age-specific incidence

Age-specific incidence of HD showed that 27 cases out of 40 (67.5%) occurred below the age of 40 years where the highest incidence occurred. Of the 27 cases, 19 (70%) cases fell within the age interval 15 to 25 which represented an early major peak incidence around the age of 20. Thirteen out of the 40 cases (32.5%) occurred above the age of 40 years. Of these 13 cases 6 (46%) fell within the age interval 50 to 70 years which represented a second minor peak around the age of 60. In the 40 to 50 age group only one case was observed as shown in figure 1.A. These results reflected a bimodal age-specific incidence of the disease.

In NHL, cases were scattered more or less evenly within different age groups, with 13 out of 30 cases (43.3%) above, and 17 out of 30 cases (56.6%) below the age of 40. Unlike HD, No peaks of incidence were noted among the different age groups and bimodality of age distribution was not observed.

The Mean age-specific incidence in Hodgkin's disease was 32.67 (+ 23.77) and 39.70 (+ 23.19) in non-Hodgkin Lymphoma.

Sex-related incidence

As shown in figure 1.B, no difference was observed in the incidence of HD between males (21 of 40) and females (19 of 40). And the sex ratio was nearly equal, i.e., 1:1 ratio. A slight male preponderance was observed among cases of NHL, with 20 male and 10 female cases out of a total of 30 cases, giving a male to female ratio of 2:1, as shown in figure 1.D.

Evaluation of the sex effect within the HD group, revealed no significant difference in the occurrence of HD between the two sex groups ($p>0.05$). Among the NHL cases, similar results were obtained, but within the male group the observed value was higher then the expected value, which might reflect some degree of male association with the disease.

Combined Age and Sex Effect on Disease Incidence

No statistically significant difference was found between the mean age of males and females within HD as well as NHL cases (39.15 and 40.80 versus 30.33 and 35.26, respectively), as shown in figure 1.C.

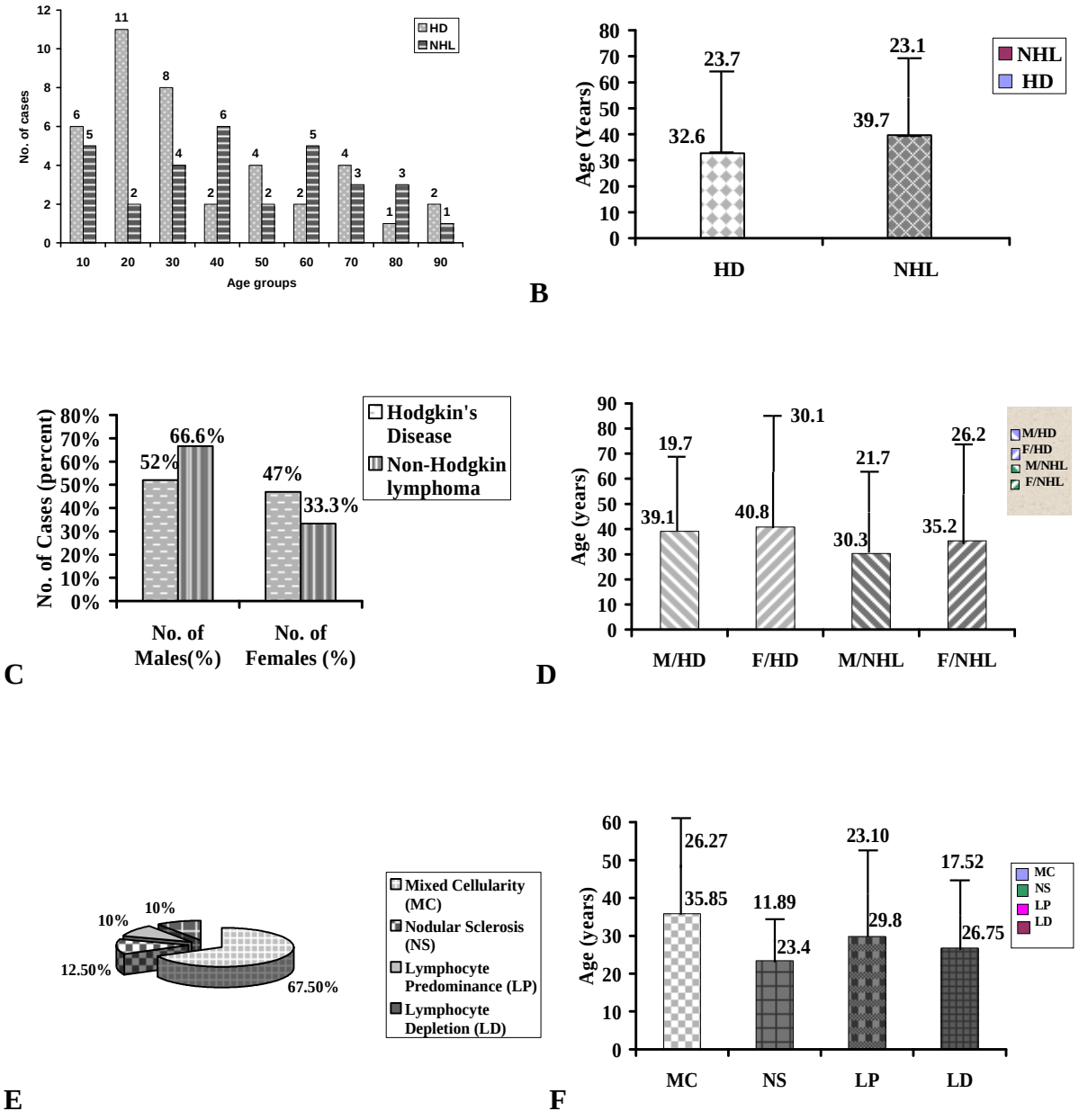

FIGURE 1 . A: AGE DISTRIBUTION OF HODGKIN AND NON-HODGKIN LYMPHOMAS. B: MEAN AGE-SPECIFIC INCIDENCE IN HODGKIN AND NON-HODGKIN LYMPHOMAS. C: SEX DIFFERENCE FOR AGE AMONG CASES OF HODGKIN AND NON-HODGKIN LYMPHOMAS. D: SEX DIFFERENCES FOR AGE IN HODGKIN AND NON-HODGKIN LYMPHOMAS. E: PREVALENCE OF THE FOUR HD SUBTYPES. F: MEAN AGE-SPECIFIC INCIDENCE OF THE DIFFERENT HODGKIN'S DISEASE SUBTYPES.

Prevalence of HD Subtypes and Relation to Age

Figure 1.E illustrates the prevalence of HD subtypes. Of the 40 cases of HD cases included in this study, 27 (67.5%) were of the Mixed Cellularity (MC) subtype, 5 (12%) cases were of the Nodular Sclerosis (NS) subtype, and 4 (10%) cases of each of the Lymphocyte Predominance (LP) and Lymphocyte Depletion (LD) subtypes, which was based on histopathologic-morphologic diagnosis. Highly significant difference was observed in the frequency of occurrence of the different HD sub-types ($P>0.001$) as shown in comparison between the observed value and expected value for the subtypes showed an excess of MC subtype compared to other subtypes which showed lower observed values than the expected ones, indicating rarity of the other subtypes and greater prevalence of MC sub-type, the calculated values of which contributed to the significant difference obtained among the four subtypes of the disease. Comparison between age-specific incidence and each of the MC, NS, LP, and LD subtypes revealed no significant difference in age-specific incidence among the four subtypes (35.85, 24.40, 29.75, and 26.75, respectively), as shown in figure 1.F.

Although the mean age-specific incidence was higher in MC subtype than the other subtypes, the difference did not reach significance level due to the obvious difference in the prevalence of the four subtypes and the limited number of cases in the study.

When the age distribution among MC cases was taken alone, it clearly reflected the same bi-modal pattern of distribution as that observed when all HD cases were taken together. Hence, the MC subtype alone can be representative of the disease age-wise.

Detection of EBV in Malignant Lymphomas

In Situ Hybridization. This technique was used for the detection of EBV in Hodgkin's and non-Hodgkin lymphomas using oligonucleotide probes (Oligo-probes) specific for the EBV encoded small RNAs, EBER1 and EBER2, the most abundant viral RNA species expressed in infected cells. The results depicted in table (1) which shows that 25 out of 40 (62.5%) HD cases, and 9 out of 30 (30%) NHL cases tested were EBV positive. Among the 10 Burkitt lymphoma cases tested, 4 cases (40%) were EBV positive, while only 5 cases of 20 (25%) non-Burkitt-NHL showed positive results for EBV.

Table 1 . Results of testing for EBV presence in different tumors, inflammatory and normal tissues.

Tumors, reactive, and normal tissues tested (total No. of cases)	Number of EBV-Positive Cases (%) by ISH-EBERs & IHC- LMP-1
Hodgkin's Disease (40)	25 (62.5%)
Non-Hodgkin Lymphoma (30)	9 (30%)
Burkitt lymphoma (10)	4 (40%)
Other, Non-Burkitt-NHL (20)	5 (25%)
NasoPharyngeal Carcinoma (5)	5 (100%)
Nornal lymph node (4)	0 (0%)
Tonsilar hyperplasia (2)	2 (100%)

No significant difference was found in the frequency of detection of EBV between Burkitt and non-Burkitt NHL ($P>0.05$), as revealed in table (2).

Table 2 . Significance of differences in the incidence of EBV-positive cases between Burkitt lymphoma and other NHL.

Type of lymphoma	Observed/ Expected value	EBV-positive	EBV-negative	Total	X2 (p value)
Burkitt lymphoma	O value	4	26	30	
	E value	4.50	25.50		
Other NHL	O value	5	25	30	0.131
	E value	4.50	25.50		(p>0.05)
Total		30	30	60	

DISCUSSION:

Age and Sex relation to the incidence of lymphomas

Age-specific incidence

In this study, an obvious difference was noticed in the incidence of Hodgkin's disease among different age groups with the majority of cases (67.5%) occurring in the younger age groups below 40 years versus 32.5% of cases occurring above that age, with a peak around the age of 20 years (table 3). This age-specific incidence found in this study follows the same epidemiologic trend of the disease in developing countries that is characterized by bi-modal age-specific incidence with early childhood first peak and a second peak in older age group (figure 1.A), where high population density, low socio-economic status, material deficiency and low living standards prevail[(2)]. These conditions provide solid grounds for the build-up of predisposing factors to the disease, most important of which are early exposure to viral infection and Immune suppression, and thus are considered significant correlates with EBV infection. In these countries most people acquire EBV infection early during childhood and the significant correlation that was observed between exposure to EBV and the development of Hodgkin's disease, may explain this mode of age related incidence. After the age of 40, disease incidence revealed a slight, barely discernible peak age-wise, a finding which is in agreement with the recently revised issue of bimodality of disease incidence[(13)]. This is in contrast to the situation in western countries where the age incidence shows a shift of first peak towards young adult age group and a second putative peak in older age groups, conforming to the late age at exposure to EBV, and the greater incidence of Hodgkin's disease in AIDS-associated lymphoproliferative diseases[(3)].

In NHL, cases were scattered more or less evenly among different age groups, a finding that is in agreement with the epidemiologic trend of the disease.

Table 3 . Age distribution of Hodgkin and non-Hodgkin lymphomas.

Disease (Total Number of cases)	No. below Age 40 years (%)	No. above Age 40 years (%)
Hodgkin's lymphoma (40 cases)	27 (67.5%)	13 (32.5%)
Non-Hodgkin lymphoma (30 cases)	17 (56.6%)	13 (43.3%)

Sex-specific incidence

The sex-specific incidence in Hodgkin's disease showed a slight preponderance in males, while in NHL twice as many males were affected as females (table 2.). Both of these observations did not differ from sex relation to the disease elsewhere[13]. Absence of sex effect on the incidence of both diseases indicates that the genetic background has no effect on disease incidence with respect to age (table 4.).

Table 4 . Sex-specific incidence among Hodgkin and non-Hodgkin lymphomas.

Type of lymphoma (Total No. of cases)	No. of Males (%)	No. of Females (%)	Male to Female Ratio
Hodgkin's Disease (40)	21 (52%)	19 (47%)	~ 1 : 1
Non-Hodgkin lymphoma (30)	20 (66.6%)	10 (33.3%)	2 : 1

Prevalence of Hodgkin's disease subtypes and relation to age.

The significant predominance of the MC-HD subtype of Hodgkin's disease compared to the other subtypes despite the limited number of the cases tested stands in contrast to the case in western countries where the NS-HD subtype prevails[14,15]. This can be explained on the basis that the morphologic differences among the different subtypes were governed by such factors as the malignant RS cell behavior and the host immune response to the disease, which, in turn, is influenced by a myriad of factors including genetic, ethnic, racial, and environmental factors[16] at manipulate the immune response type and reactivity to the tumor in various ways. The malignant RS cells have recently been found to express different and complex profiles of cytokines and chemokines that modulate the surrounding microenvironment resulting in the different proportions of the three major components of the tumor mass (the malignant, the reactive and the fibrotic components) that characterize each subtype of the disease[17].

With respect to the relation between HD subtypes and age-specific incidence, previous epidemiologic studies have shown that the MC subtype is the most prevalent in the younger age group and the lymphocyte depletion LD subtype in the more advanced age groups[13,15]. In this study, no significant relation between subtype and age was observed (figure 1.F), and, although the greater proportion of MC subtype conformed to the first peak in younger age group, the relation between the two did not reach statistical significance due to the limited number of cases in the study.

Detection of EBV in lymphomas

EBV-associated Hodgkin's disease

The finding that 62.5% of the HD cases tested positively for EBV in this study was in agreement with the results of previous studies which showed an incidence of EBV positive HD cases in the range from 20-40% in western countries[10,14] to more than 90% in the under-developed countries[18,19]. It is evident from this wide variation of incidence that environmental factors play a major role in the development of this tumor.

Most evidence in favor of an etiologic association between Hodgkin's disease and EBV available thus far are only circumstantial at best, and no unified theme exists to date that links the fragmented pieces of evidence into a model that convincingly explains the mechanism(s) by which EBV triggers the development of HD.

One evidence is the association between positive IM history and HD development. In our retrospective study, it was not possible to evaluate this association due to lack of clinical information regarding past history of IM, save for the fact that it was not one of the objectives in this study to evaluate this association. Nevertheless, review of data from previous studies by Sleckmen, Hjalgrim and Alexander[20,21,22], confirmed this association, and found an increased risk of both EBV-positive and EBV-negative HD in cases with past history of IM, and that this increased risk was greater for young adult EBV-positive cases compared to other age groups. The clonal presence of EBV in H/RS cells of EBV-positive HD cases and the constant viral copy number per case represented the strongest evidence that link the virus to the disease.

Non-EBV- associated Hodgkin's disease

In contrast to the situation in developing countries, most HD cases in western countries are EBV negative[19], and these cases account for the young adult first peak. However, in an attempt to resolve the dilemma of absence of such a strong candidate etiologic agent like EBV from other HD cases, another likely viral agent was actively sought for on the basis that most epidemiologic studies suggest that delayed exposure to a ubiquitous infectious agent may play a role in such cases. Most likely candidates were members of the Herpesvirus family, among others. However, molecular studies failed to consistently show the presence of any single viral agent, and most studies concluded that any virus directly involved in the causation of EBV-negative HD is currently unknown[23].

While the absence of EBV from a proportion of HD cases may seem to argue against a role for this virus in tumor causation, this finding does not defy the notion of association completely. This is because other possible explanations do exist though most of which still await confirmation. At least three such explanations has been set forth in this respect; one potential explanation suggests that in EBV-negative HD cases, the virus exists in very low copy number in its usual episomal form, that is beyond the detection power of the most sensitive tests thus far available[24]. However, this explanation seems far from being true considering the high capabilities of the molecular amplification techniques in detecting very small viral copy number in various types of cells.

An alternative explanation suggests that EBV is the etiological agent of all HD cases but is using the "Hit and Run" mechanism in EBV-negative cases[25]. By this mechanism the virus triggers the transforming event and induces cell cycle deregulation but then disappears from the malignant clone with the development of effective immune response during tumor progression. In this case, it is likely that young adult HD cases, most of whom are EBV-negative, are more able to mount an effective immune response which selects against H/RS cells expressing viral proteins. In a similar manner, this mechanism may also explain the increasing incidence of HD in immune suppressed patients.

A third explanation was raised which is based on the observation that in some cases of EBV-negative sporadic Burkitt lymphoma, fragments of the EBV genome were detected in biopsy tissue using a combination of southern blot analysis, quantitative PCR and Fluorescence in situ hybridization (FISH) with probes spanning the entire viral genome[26]. It was suggested that these fragments are remnants of previously existing complete virions that contain the reading frame that encodes the viral oncoprotein LMP-1 responsible for cellular transformation. However, Staratschek-Jox and coworkers[27] failed to detect such fragments in a study aimed at a search for similar viral fragments in EBV-negative HD cases.

Therefore, considering the explanations given above and the high sensitivity of the tests used, the possibility that some other cellular defect playing a major role in the pathogenesis of the disease, cannot be excluded. Moreover, this likely defect must operate in the genesis of both EBV-positive and EBV-negative HD cases at the same magnitude because there is no reason to believe the contrary.

Overall, it seems that at least in EBV-negative HD cases the possible presence of EBV as a transient event in the cell life time is a prelude to the emergence of a putative cellular defect that may lead to the development of the tumor.

Burkitt lymphoma

In this study 40% of the sporadic Burkitt lymphoma (BL) tested were EBV-positive, a quite interesting finding because it fits with the rate of EBV presence in the recent epidemiologically defined third type of sporadic BL, the so-called Mediterranean or Middle-eastern BL, in which the frequency of association with EBV was found to be midway between that of the African BL, where the association exceeds 90%, and that of the American BL where less than 10% of the sporadic cases are associated with EBV infection.
The rate of association of EBV with the middle eastern type BL is explained on the basis that the same epidemiologic risk factors (socio-economic level, the living standards, material deficiency..etc) that govern the prevalence of EBV infection in the region differ in type and magnitude from those prevalent in the other geographical types of BL.

Non-Hodgkin lymphomas (other than Burkitt's)

The rate of EBV association with NHL found in this study comes in agreement with those previously reported[3,28] where different studies conducted in different region showed a wide range of variation in the association with the virus, and in comparison, our results approximated the lower end of the range when BL was excluded (table 1.).
The types of NHL most commonly associated with EBV infection are; the Burkitt lymphoma, large B cell lymphomas and NHL arising in the setting of AIDS associated immune suppression. Apart from the BL in which EBV association is consistently high, the association with the other types of NHL is variable.
Many reasons can be put forth to explain the wide variation in the results, most important of which include; (a) the great diversity of diseases included within the entity of NHL, each exhibiting different rate of association with EBV. (b) The prevalence of the various diseases differs in different geographical regions. (c)The extent to which different types of NHL impair the immune response, in particular the defective T-cell regulation (d) Some studies may have investigated a restricted number of diseases or may have been confined to the more common types of diseases. (e) The number of cases in the cohorts studied certainly influences the significance of the results.
Although different types of NHL were included in this study, the limited number of cases tested made it difficult to draw conclusions as to what extent our results reflected the actual association between virus and this group of diseases collectively.

Age Distribution of Hodgkin and Non-Hodgkin Lymphomas by EBV-status

In a large cohort retrospective study of archival HD cases23, found that EBV-positive and EBV-negative HD cases have quite distinct age distribution, with the EBV-negative cases showing uni-modal age distribution accounting for the young-adult peak, while the EBV-positive cases exhibiting the more typical bi-modal age distribution.
In this study (table-5.), such a difference in the mode of age distribution was less recognizable, where in both EBV-positive and negative HD cases an obvious first peak in childhood and young adulthood was noted with the age incidence curve becoming flatter afterwards. The reason for the incompatibility is obviously the limited number of cases in this study. Furthermore, the lower age incidence in HD paralleled the higher overall occurrence of HD in the lower age group in developing countries and particularly among the lower socio-economic class, where individuals acquire EBV infection early during childhood. These results are in agreement with the observed significant epidemiologic linkage between EBV infection and HD incidence[29].

In NHL, the situation was obviously different, where EBV-positive and negative cases showed more or less even distribution among age groups with slightly higher incidence in lower age group which was statistically not significant. The difference in age incidence of EBV associated cases between the two diseases may be attributed to the difference in the magnitude of influence of genetic and environmental predisposing factors in the etiology of the two diseases, where genetic imbalances contribute to the etiology of NHL to a greater extent than do the environmental factors and the reverse is true in HD. Taken together with the overall higher incidence of EBV infection in HD, it is not unexpected that the age distribution in EBV associated HD reflected the typical bi-modal age distribution of the disease.

Table 5 . Difference in age distribution between EBV positive and negative HD cases.

EBV status (total No. of cases)	No. of cases below age 40 years (%)	No. of cases above age 40 years (%)
EBV-positive (25)	17 (68%)	8 (32%)
EBV-negative (15)	10 (66.6%)	5 (33.3%)

Relation between Hodgkin's disease Subtypes and EBV status

In this study, the lymphocyte depletion (LD-HD) subtype showed the highest rate of association with EBV infection (table -6.) where all cases (100%) tested, gave positive results for EBV. This finding was quite surprising because most previous studies investigating the epidemiology and pathology of Hodgkin's disease concluded that the proportion of EBV-associated disease was greatest in the MC-HD subtype followed by the NS-HD subtype with the least association seen in the LP-HD subtype[16,30,31]. In these studies the differences in EBV association were statistically significant. This was in agreement with the results obtained in our study when the MC subtype, taken alone, was compared with the other subtypes for the association with EBV wherein most of the EBV-associated HD cases were contributed by the MC subtype. Moreover, the proportion of EBV-associated HD cases was greater in childhood where most of the cases were of the MC subtype, a finding in this and others studies that lend further support to the strong association between the MC subtype and EBV presence in the malignant clone.

Table 6 . Number of EBV- positive and EBV negative cases among different Hodgkin's disease Subtypes.

Hodgkin's disease subtype (Total No. of cases)	No. out of the total of each subtype (%)	No. out of The total of EBV-positive HD cases (%)
Mixed Cellularity (27)	19 (70.3%)	19 (76%)
Nodular Sclerosis (5)	1 (20%)	1 (4%)
Lymphocyte Predominance (4)	1 (25%)	1 (4%)
Lymphocyte Depletion (4)	4 (100)	4 (16%)

In western countries where NS is the most prevalent subtype, EBV was also found to be most prevalent in the MC subtype. Pallesen and colleagues[31] observed that the LP subtype of the tumor was rarely linked with EBV, whereas approximately 30% of NS cases and up to 90 % of MC and LD were positive for the virus. In this study, the association between LD subtype and EBV was unexpected but certainly requires careful consideration because the association was apparently very strong despite the fact that it did not approach statistical significance.

Provision of an explanation for this interesting finding at the moment is difficult before conducting a study on a large-scale including a greater number of LD cases that allows for evaluation of the statistical significance of the results. However, one potential explanation is based on the longstanding pathological observation that many cases of Hodgkin's disease exhibited a morphologic change of subtype from the more benign MC subtype in early stages of the disease to the more aggressive LD subtype in the more advanced stages at a later age. The implication is that the MC subtype known to be strongly associated with EBV presence may eventually give way to the LD subtype as the disease progresses to more the advanced stages. Since the latter subtype, by definition, contains larger proportion of malignant RS cells within the tumor mass, these cells would be EBV positive in those LD-HD cases that evolved from the MC subtype which may, in part, explain the observed high association of LD subtype with EBV in this study.

Even more surprising, was the higher frequency of EBV positivity in the LP sub-type, a finding that was not reported before, since most previous studies indicated that EBV is very rarely detected in LP-HD. The latter finding might be explained on the basis that many of the cases morphologically diagnosed as LP-HD might actually prove to be LRCHD cases.

In conclusion, We found that high proportion of HD cases were positive for EBV with a prevalence rate not oddly deviant from that found in previous studies, hence providing solid grounds to surmise that EB virus and its products are responsible for the initial transformation and the maintenance of the transformed phenotype that leads to the development of the cancer.

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