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DNA Ploidy Analysis on Lymphoma Using Archival Paraffin Tissue Section

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ABSTRAK

Dalam kajian retrospektif limfoma ini, kandungan DNA dan indeks proliferasi daripada 37 kes limfoma yang disahkan secara histopatologi yang terdiri daripada 29 lelaki dan 8 perempuan diukur menggunakan flowsitometer. Ini dilakukan dengan menggunakan 'embedded paraffin tissue' daripada kes yang diambil dari Bahagian Patologi Hospital Kuala Lumpur dalam jangka masa 1990-1995. Di antara mereka, 24 kes didiagnos sebagai limfoma bukan Hodgkin (NHL) manakala 13 kes lagi sebagai penyakit Hodgkin (HD). Nodus limfa yang menunjukkan perubahan reaktif serta pembuangan tonsil bagi inflamasi kronik digunakan sebagai kawalan. Daripada semua kes yang dikaji bagi NHL (Sel B = 70.8%, Sel T = 8.3% dan kes yang tidak dapat dikenalpasti=20.9%), 12.5% adalah diploid (Indeks DNA, DI = 1.0 - 1.10), 66.7% adalah hiperdiploid (DI = 1.10 - 1.85) dan 20.8% adalah hipodiploid (DI < 1.0). Majoriti kes bagi hiperdiploid adalah sel B jenis NHL. Dalam HD (keberselan bercampur = 1, nodul sklerosis = 5, limfosit predominan = 1 dan tidak diklasifikasikan = 6), 30.8% adalah diploid, 46.1% adalah hiperdiploid dan 23.1% adalah hipodiploid. Penemuan signifikan dalam kajian ini adalah majoriti NHL dan HD menunjukkan indeks proliferasi yang tinggi.

Kata kunci: Analisis DNA, flowsitometri, limfoma Hodgkin, indeks DNA, indeks proliferasi.

ABSTRACT

In this retrospective study of lymphomas, DNA content and proliferative index of 37 histopathologically confirmed cases of lymphoma consisting of 29 male and 8 female were measured using a flowcytometer. This was carried out using paraffin embedded tissues of cases diagnosed in the Department of Pathology, Hospital Kuala Lumpur, over a period of 1990 to 1995. Among them, 24 were diagnosed to be non-Hodgkin's lymphomas (NHL) while 13 were diagnosed as Hodgkin's disease (HD). Lymph nodes showing reactive changes and tonsils removed for chronic inflammation were used as control.

Of all the cases studied for NHL (B cell = 70.8%, T cell = 8.3% and unclassified cases = 20.9%), 12.5% were near diploid (DNA index, DI = 1.0 - 1.10), 66.7% were hyperdiploid (DI = 1.10 - 1.85) and 20.8% were hypodiploid (DI < 1.0). Majority of the hyperdiploid cases were B cell type of NHL. In HD (mixed cellularity = 1, nodular sclerosing = 5, lymphocyte predominant = 1 and unclassified = 6) 30.8% were near diploid, 46.1% were hyperdiploid and 23.1% were hypodiploid. The significant finding of this study was that the majority of the NHL and HD showed a high proliferative index.

Key words: DNA analysis, flowcytometry, Hodgkin's lymphoma, DNA index, proliferative index.

INTRODUCTION

Lymphomas are broad, heterogeneous group of malignancies of the lymph system. On the basis of histological appearance, lymphomas are classified into Hodgkin's and non-Hodgkin's lymphomas (NHL). Hodgkin's disease (HD), which was first discovered by Thomas Hodgkin in 1832, is characterised by the presence of large, binucleated cells called Hodgkin and Reed-Sternberg cells surrounded by a variety of innocent bystander cells such as lymphocytes, macrophages, neutrophils and plasma cells (Jaffe et al. 2002). NHL are a heterogeneous group of malignant lymphoid neoplasm of either a B-cell or T-cell neoplasm. Mature B-cell neoplasm comprises over 85% of all worldwide.

In Malaysia, lymphoma ranked seventh amongst male cancers and eleventh amongst female cancers. Lymphomas constituted 3.7% of all cancers. There is a male preponderance of 3:2. Age standardized incidence rate is highest in the Malay males and Chinese females. Ratio of HD to NHL is 1:9 (National Cancer Registry, 2003). In the UK the incidence of HD is about 2/100 000 with an overall male preponderance of 3:2 male to female ratio (Child et al. 1998). The incidence increases with age continuing into the 6th decade but there is also a distinct peak in the 15-34 year age group. The incidence of NHL is approximately 10/100 000 females per year and 14/100 000 males per year. The two most common types, large B cell lymphoma and follicular lymphoma comprise 50% of all NHL (Child et al. 1998).

Flowcytometry is an emerging technique that involves the separation, classification and quantitation of cell types by cell surface antigen (phenotype), DNA content (ploidy) and cell proliferative activity (S-phase fraction) or % S-phase. The basic technique involves analysing a mononuclear stream of cells through a laser beam and complex computerized system that will then sort the cells into normal and abnormal population of cells. The data is often collected as a bar-histogram, which is displayed

visually as a densitometer tracing of the bar graph; the concentration of each cells in each bar appears as a separate peak for each cell category. Using flourescent dyes, which stain nucleic acids, flowcytometry has been used to measure DNA or ploidy content of solid tumours. DNA diploid tumours are those in which a single peak containing an amount of DNA similar to normal control cells. DNA aneuploid tumours have additional peaks on DNA histogram, presumably representing cells containing more or less nucleic acid than 46 chromosomes. DNA index (DI) is a quantitative method of expression that is the ratio of G0/G1 DNA content of normal diploid reference cells. The greater the deviation of D1 from 1, the greater the aneuploid is the tumour. This serves as a prognostic indicator of solid tumours based on the fact that malignant cells sometimes show abnormalities in total number of chromosomes and the frequency of these abnormalities generally increases with progression to higher-grade tumours (Martinez et al. 1990). A number of studies have demonstrated direct relationship between histological grade, patient's survival and percentage of cells in DNA (Raul 1993).

One of the techniques used to study cell kinetics is the measurement of the percentage of S- phase cells by DNA flowcytometry. % S-phase or the S-phase fraction (SPF) is the percentage of cells preparing for mitosis by their active doubling of DNA. Since tumour cells tend to replicate more readily then normal cells, increased SPF activity correlates with poor differentiation of tumours, increasing tumour size and degree of aggressiveness in tumour spread, which are all bad prognostic significance. Cellular DNA content in human lymphoma cells has been analysed extensively using Flowcytometry performed both from fresh and formalin fixed, paraffin embedded tissues (Hedley 1989).

This is a preliminary study to determine the DNA ploidy status of lymphomas in Malaysian population using archival paraffin embedded tissues.

MATERIAL AND METHODS

PREPARATION OF BLOCKED SPECIMENS INTO SINGLE CELL SUSPENSION

Paraffin embedded tissues from 37 histopathologically confirmed cases of lymphomas sent to Histopathology Unit, Pathology Department, Hospital Kuala Lumpur were selected for this study.

The tissues were deparaffinized and dissociated into a cell suspension according to the modified Hedley's method (Hedley et al. 1983). The nucleus was stained using Cycle Test Plus Kit. A total of 10, 000 cells were studied with the flowcytometer and a DNA histogram was generated.

Measurements were performed on a FAC Scan flowcytometer (Becton-Dickinson, San Jose, USA) for at least 10,000 events/sample, using the CELLFIT software program (Becton Dickinson) and the data were recorded as DNA histogram. This histogram demonstrates presynthetic phase (G1), synthetic phase (S) and post synthetic phase (G2+M). The following parameters were taken note: ploidy, proliferative index (percentage of cells in each phases) and DNA index. Cell suspension from normal and tumour population were analysed separately. The CEN (chicken erythrocyte nuclei) provided in DNA QC particles kit (BD Cat. Num. 95-0023) served as a calibrator for the FAC Scan machine whereas the CTN served as a diploid standard. Normal cells were analysed serving as a diploid standard. Aneuploidy was expressed as DNA index (DI):

$$DI = \frac{\text{modal channel of G0/G1 peak of studied population}}{\text{modal channel of G0/G1 peak of normal cells (CTN)}}$$

Where ratio = 1 indicates diploid or 2c,
ratio = 2 indicates tetraploid or 4c

RESULTS AND DISCUSSION

The result of DNA ploidy analysis for both the non-Hodgkin's lymphoma (NHL) and the Hodgkin's disease (HD) is shown in Table 1. Of all the cases studied for NHL (B cell = 70.8%, T cell = 8.3% and unclassified cases = 20.9%), 12.5 % were near diploid (DI = 1.0 - 1.10), 66.7 % were hyperdiploid (DI = 1.10 - 1.85) and 20.8% were hypodiploid (DI < 1.0). Majority of the hyperdiploid cases were B cell type of NHL. In HD (mixed cellularity = 1, nodular sclerosing = 5, lymphocyte predominant = 1, and

TABLE 1. DNA ploidy analysis on 37 histopathologically confirmed cases of lymphoma

Ploidy Status	Non Hodgkins Lymphoma	Hodgkins Disease
Near Diploid D.I (1.0 - 1.10)	3(12.5%)	4(30.8%)
Hyperdiploid D.I (1.1 - 1.85)	16(66.7%)	6(46.1%)
Hypodiploid D.I < 1.0	5(20.8%)	3(23.1%)

unclassified cases = 6) 30.8% were near diploid, 46.1% were hyperdiploid and 23.1% were hypodiploid. Examples of DNA histogram generated from the various subtype of lymphomas are shown in Figure 1, 2, 3 and 4. It is thus noted from the study that most of the lymphoma cases studied showed aneuploidy. The overall percentage of hyperdiploid in the HD was much lower than the NHL. This was possibly due to the DNA aneuploid cell population in HD being diluted by the presence of a large reactive non-neoplastic diploid cell population (John et al. 1987).

The mean proliferative index and the mean coefficient of variation are shown in Table 2. In this study, a high proliferative index, S-phase fraction (SPF) values of more than 12% were seen in both NHL and HD. High SPF values have been shown to be a useful parameter as a prognostic value in some of the cases studied (Raul 1993). Due to small number of cases, there was no statistically significant correlation between the ploidy status and proliferative index of HD and NHL.

A few of the cases had an unsatisfactory histogram probably due to our early inexperience in the study and abundant cell fragments as a result of the disaggregation procedure (Birger et al. 1989). DNA analysis using archival paraffin tissues section was possible. However, despite the excellent attributes of the flowcytometry technology, interpretive errors in DNA

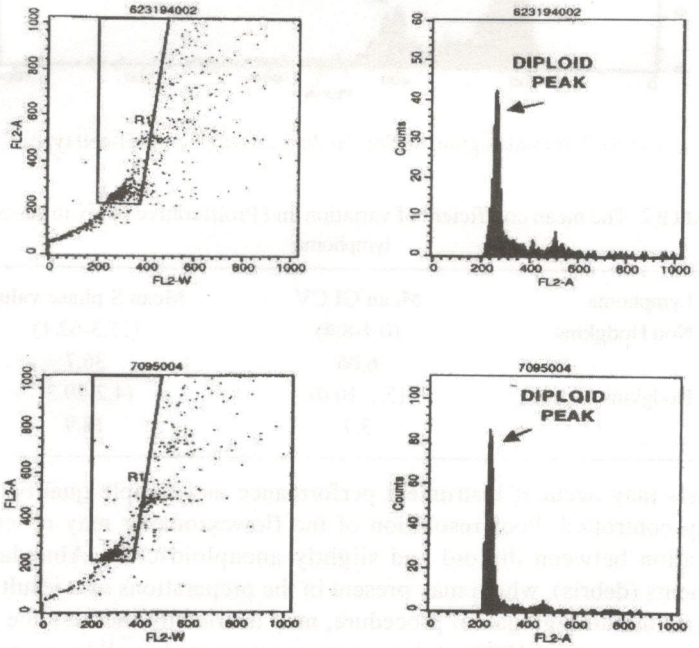

FIGURE 1. Diploid Tumour

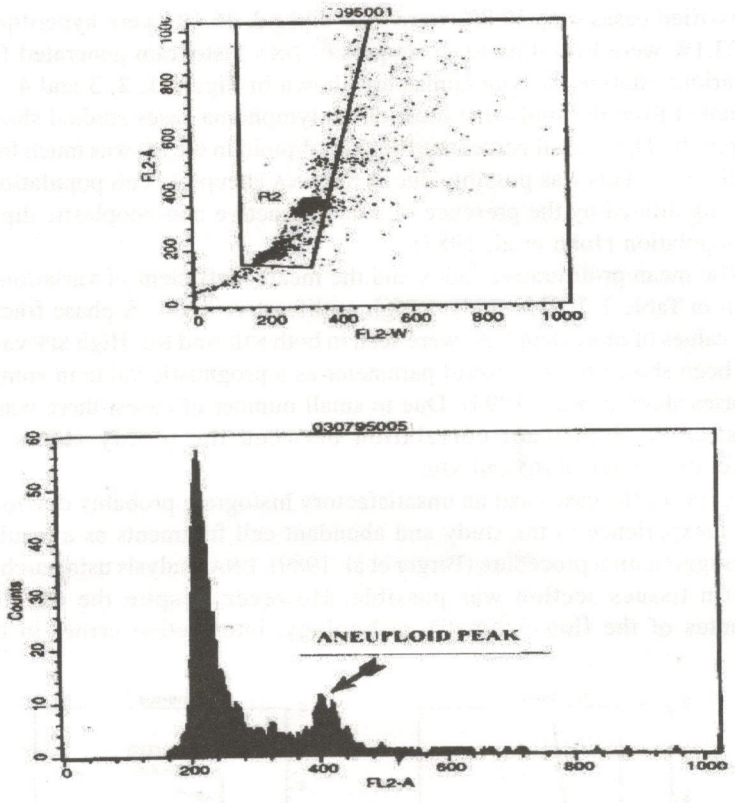

FIGURE 2. DNA histogram of Non-Hodgkin's lymphoma (B-cell type)

TABLE 2. The mean coefficient of variation and Proliferative index in cases of lymphoma

Lymphoma	Mean GI CV	Mean S phase value
Non Hodgkins	(0.4-8.4)	(11.3-62.1)
	6.06	36.7
Hodgkins	(5.2-10.0)	(4.2-29.3)
	3.7	18.9

analysis may occur if instrument performance and sample quality are not rigidly controlled. Poor resolution of the flowcytometer may obscure the separation between diploid and slightly aneuploid cells. Abundant cell fragments (debris), which may present in the preparations as a result of cell death or the disaggregation procedure, may artificially increase the size of the S-fraction (Raul 1993). A large retrospective study will be necessary to determine the clinical impact of this technique in the analysis of lymphomas.

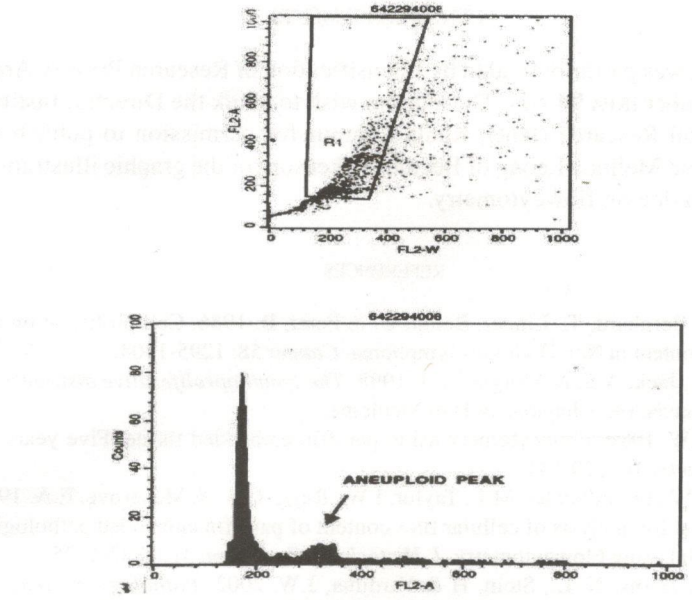

FIGURE 3. DNA histogram of Non-Hodgkin's lymphoma (T-Cell type)

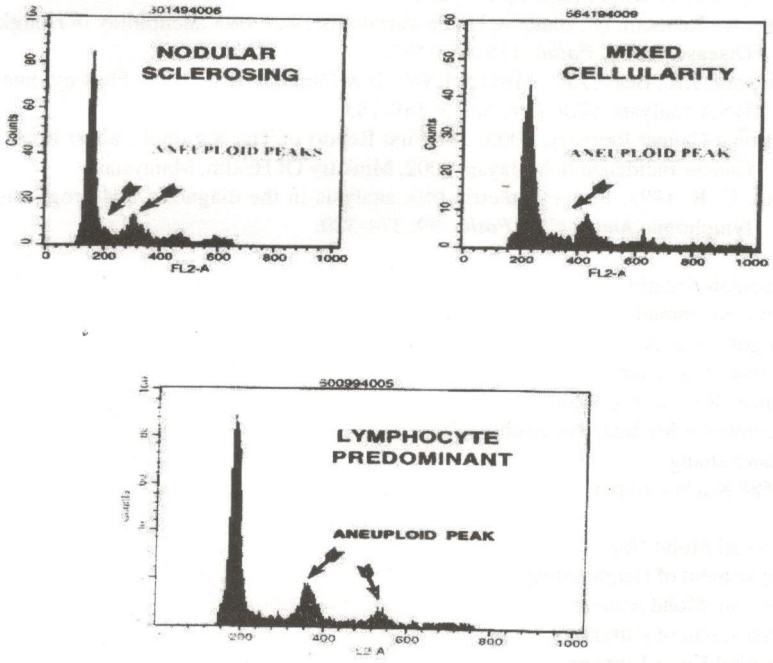

FIGURE 4. Multiple Aneuploid in Hodgkin's disease

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