Journal of Korean Medical Science
Vol. 11, No. 5, 402-408 Oct. 1996

Interphase Cytogenetics of Non-Hodgkin's Lymphoma using Non-Fluorescent in Situ Hybridization in Paraffin Embedded Tissue

Yeong Jin Choi, M.D., Kyungja Han, M.D., Wonbae Lee, M.D.*,
Chang Suk Kang, M.D., Byung Kee Kim, M.D., Sun Moo Kim, M.D.,
and Sang In Shim, M.D.

Department of Clinical Pathology and Pediatrics, Catholic University Medical College, Seoul, Korea.*

Paraffin-embedded tissue samples from 30 cases of non-Hodgkin's lymphoma(NHL) and 10 of reactive hyperplasia, were processed for interphase cytogenetic chromosomal study. We performed non-fluorescent in situ hybridization(NFISH) using the enzymatic method with digoxigenin-labeled DNA centromeric probes for chromosome 7,12,18 and X, and a painting probe for chromosome 18. Chromosomal aberrations were observed in 27(90%) out of 30 cases of NHL. The most commonly observed numerical aberration was extracopy of X chromosome. There were some characteristic aberrations corresponding to each grade and group of NHL by International Working Formulation: In low grade NHL(9 cases), a third were associated with extracopy of chromosome 12, and disomy X was frequently found in small lymphocytic lymphoma(75%). With intermediate grade(16 cases), tetraploidy(25%), translocation of chromosome 18(25%), and extracopy of chromosome 18(19%) were characteristically associated. These results suggest that interphase NFISH is an easily performable method in retrogade cytogenetic study of archival materials. Some specifically correlated chromosomal aberrations corresponding to the histopathologic grades and groups could provide us more valuable information for determining pathologic diagnosis and assessing the clinical outcome of NHL.

Key Words : *Non-Hodgkin's lymphoma, Interphase, DNA probes, Non-fluorescent in situ hybridization*

INTRODUCTION

Non-Hodgkin's lymphoma(NHL) consists of a heterogeneous group of malignant lymphoma(ML) and this accounts for its various histology, immunology and clinical findings(Lukes and Collins,1974 ; The non-Hodgkin's lymphoma pathologic classification project,1982). With the development of molecular biologic analysis of DNA content and ploidy, it is generally accepted that aneuploidy is closely related to malignancy(Juneja et al., 1986 ; Morgan et al.,1986). The conventional cytogenetic investigations have demonstrated that the majority of NHL have various clonal karyotypic abnormalities (Bloomfield et al.,1983 ; Levine et al.,1985 ; Cabanillas et al.,1988). But these techniques require metaphase spread by in vitro culture of tumor cells and have critical limitations in determining the major clone of tumor cells.

With recent development of fluorescent in situ hy-

Address for correspondence : *Yeong Jin Choi, M.D., Department of Clinical Pathology, Catholic University Medical College, Kangnam St. Mary's Hospital, 505 Banpo-Dong, Seocho-Ku, Seoul 135-040, Korea.*
Tel : (02) 590-1603, Fax : (02) 592-4190

bridization(FISH) with chromosome-specific DNA probes for the cytogenetic analysis, rapid identification of chromosomal aberrations, not only on metaphase spread but also on interphase nuclei, has been available(Pinkel et al.,1988 ; Van Dekken and Bauman,1988 ; Lee et al., 1993 a,b,c). Interphase analysis is a startling method of obtaining cytogenetic information from paraffin-embedded solid tumors. Some investigators have performed FISH in the free nuclei obtained from paraffin-embedded tissue sections by modifying Hedley's method (Hedly et al.,1983). It could avoid the disadvantages of performing on tissue sections, such as fragmentation or overlapping of the nuclei(Lee et al., 1993 a,b,c).

Although FISH has been more commonly used in this field, there are several limitations, such as an immediate interpretation and poor preservation of the stained slide. So we performed non-fluorescent in situ hybridization(NFISH) using an enzymatic method to overcome the limitations of FISH.

It was previously reported with metaphase spread that NHL commonly had the numerical changes of chromosome 3,7,8,10,12,13,18,21 and X, and/or structural changes of chromosome 1,6,14,17 and 18(Bloomfield et al.,1983 ; Levine et al.,1985 ; Hammond et al., 1992). According to these data, we used arbitrarily selected centromeric DNA probes for chromosome 7, 12,18,X and painting probe for chromosome 18 in this study.

MATERIALS AND METHODS

Patients and Control. Paraffin-embedded tumor tissue from 30 patients with ML who were diagnosed at Kangnam St. Mary's Hospital between 1986 and 1993 were evaluated. All patients in this study were not treated before biopsy. Histological classification was done using the International Working Formulation. The number of cases studied in each histologic grades and groups was as follows : Low grade ML(9 cases) included 4 cases of ML, small lymphocytic ; 3 of ML,follicular small cleaved ; 2 of ML,follicular mixed small and large.

Intermediate grade ML(16 cases) included 4 of ML, diffuse small cleaved ; 4 of ML, diffuse mixed small and large ; 8 of ML,diffuse large. High grade ML(5 cases) included 1 of ML,diffuse large immunoblastic ; 2 of ML, small noncleaved ; and 2 of ML, lymphoblastic. Immunophenotypically, there were 23 cases of B cell phenotype, 3 cases of T cell phenotype and 4 cases of non-B, non-T null cell type. Reactive hyperplasia of lymph nodes from 10 patients served as control.

DNA Probes. Digoxigenin-labeled alpha satellite centromeric DNA probes(Oncor, Gaithersburg, USA) for chromosome 7(D7Z1), 12(D12Z3), 18(D18Z1) and X (DXZ1) were used to determine the numerical chromosomal changes in interphase nuclei. In addition, a painting probe for chromosome 18(Oncor) was also used to detect the structural chromosomal changes.

Non-Fluorescent In Situ Hybridization and Detection. We isolated free nuclei by the method of Lee et al. (1993 b,c) which was a modification of Hedley's original method(Hedly et al., 1983). Prepared slides were fixed in a 3:1 solution of methanol : glacial acetic acid for 45 seconds and then air dried. Each of the centromeric probes, prepared by mixing 2 μl of the probes with 10 μl of Hybrisol VIII(Oncor), or 10 μl of painting probe was applied to the prepared slide and coverslipped. After denaturation at 90℃ for 5 minutes, they underwent overnight incubation in a CO_2 incubator at 37℃ . All slides were washed according to the manufacturer's instructions. The signal was detected by digoxigenin-detection kit(Boehringer Mannheim Biochemica, Mannheim, Germany) including color reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. Fast-red was used for counterstaining.

Evaluation of NFISH Results. All NFISH results were evaluated by two experienced pathologists. We counted the signals of more than 100 intact nuclei which were not overlapped or truncated on a oil emmersion power of light microscope. Split or adjacent dots were counted as one. Interpretation of aneuploidy was based on the reference standards for paraffin-embedded tissue(Han et al.,1994 b).

We defined monosomy as more than 15% of nuclei showing only one signal with a centromeric probe or more than 20% with a painting probe. Trisomy or polysomy was defined as nuclei with three or more signals were over 5% with a centromeric and/or a painting probe. Translocation of chromosome 18 was defined as nuclei which revealed two signals with the 18 centromeric probe and revealed more than three signals with the 18 painting probe.

RESULTS

Analysis of Chromosomal Aberrations of Reactive Hyperplasia and NHL. In 10 cases of reactive hyperplasia, all were diploid for chromosome 7, 12, 18 and X. Ninety to ninety-three percent of all nuclei showed

Table 1. Chromosomal aberrations in 30 cases of non-Hodgkin,s lymphoma using non-fluorescent in situ hybridization

Centromeric DNA probe	No. of cases				
	Monosomy	Disomy	Trisomy	Polysomy	Total(%)
Xc	2	8	4	2	12 (40)
7c	9	-	4	3	13 (43)
12c	6	-	5	2	11 (37)
18c	8	-	4	2	12 (40)

Table 2. Chromosomal aberrations associated with specific histologic grades of non-Hodgkin's lymphoma

Grade	No.of cases	Chromosomal aberrations	
		Type	No. of cases(%)
Low	9	Tri & polysomy 12	3 (33)
Intermediate	16	Tetraploidy	4 (25)
		Translocation of chromosome 18	4 (25)
		Tri & polysomy 7	3 (19)
		Tri & polysomy 18	3 (19)
High	5	Tri & polysomy X	2 (40)

the expected two distinct signals in all four centromeric probes(Fig. 1a). The percentage of nuclei with one signal ranged from 6 to 9%, and that with more than three signals was less than 1%. However,the detection rate with the 18 painting probe was only 85% of all nuclei for two signals(Fig. 1b) and 14% for one signal. The size of two signals for chromosome 18 tended to be equal.

In contrast to reactive hyperplasia, aberrant chromosomes were observed in 27(90%) out of 30 cases of NHL(Table 1). The cases having more than two types of chromosomal aberrations (17 cases, 66%), outnumbered the cases having only a single chromosomal aberration (10 cases, 23%). The numerical chromosomal changes were most frequently found in chromosome 7(13 cases, 43%), followed by X and 18(12 cases, 40%, respectively) and 12(11 cases, 37%). The extracopy of chromosome X was most commonly observed (14 cases, 47%) and the translocation of chromosome 18 was found in 5 cases(16.7%).

Specific Chromosomal Aberrations corresponding to Histologic Grades and Groups of NHL.

Some characteristic chromosomal aberrations were found in each grade(Table 2) and group(Table 3) of NHL.

In low grade ML(9 cases), the extracopy of chromosome 12 was found in 3 cases(33%), but extracopy of

Table 3. Chromosomal aberrations associated with specific histologic groups of non-Hodgkin's lymphoma

Pathologic diagnosis	No.of cases	Chromosomal aberrations	
		Type	No. of cases
ML, SL	4	Disomy X	3
		Monosomy 7,12&18	2
ML, FSC	3	Trisomy 12	2
		Monosomy 7	2
ML, FM	2	-	-
ML, DSC	4	Tetraploidy	1
		Translocation of chromosome 18	1
ML, DM	4	Tetraploidy	2
ML, DL	8	Disomy X	3
		Monosomy 12	3
		Trisomy 18	3
		Translocation of chromosome 18	3
		Tetraploidy	1
ML, DLIb	1	Tri & polysomy 18	1
ML, SNC	2	-	-
ML, Lb	2	Trisomy X	2
		Monosomy 18	2
		Translocation of chromosome 18	1

Abbreviations : ML, malignant lymphoma ; SL, small lymphocytic ; FSC, follicular small cleaved ; FM, follicular mixed small & large ; DSC, diffuse small cleaved ; DM, diffuse mixed small & large ; DL, diffuse large ; DLIb, diffuse large immunoblastic ; SNC, small noncleaved ; Lb, lymphoblastic

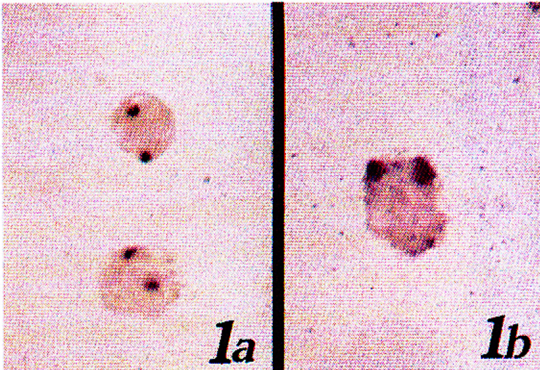

Fig. 1a. Reactive hyperplasia reveals two signals with X centromeric probe. **1b.** Two equal sized signals with chromosome 18 painting probe.

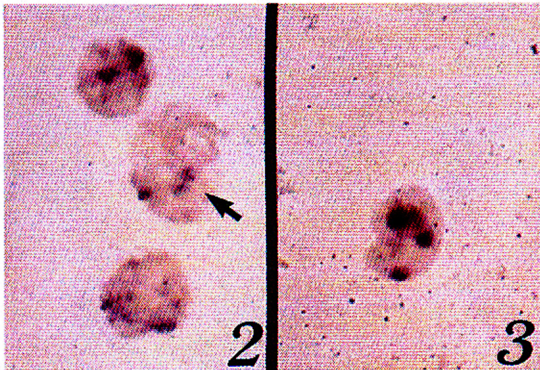

Fig. 2. Small lymphocytic lymphoma(case 2, male) with X centromeric probe reveals two signals. A cell in the center shows two signals with splitting(arrow).

Fig. 3. Diffuse mixed lymphoma(case 15, male) reveals three signals with X centromeric probe.

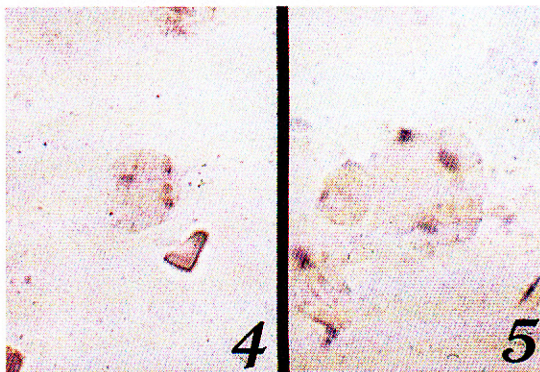

Fig. 4. Diffuse large cell lymphoma (case 19) with trisomy 7 shows three signals.

Fig. 5. Diffuse large cell lymphoma(case 21) with 12 centromeric probe reveals three signals.

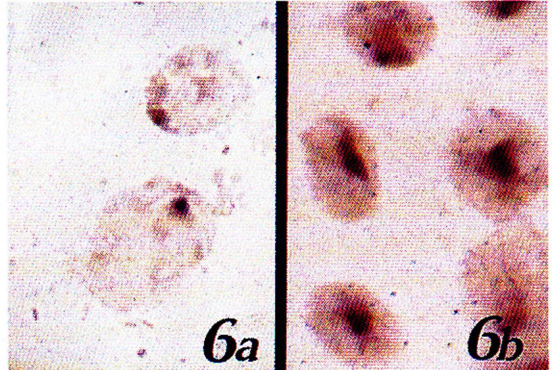

Fig. 6a. Diffuse large cell lymphoma(case 18) with 18 centromeric probe reveals only one signal. **6b.** Only one signal with 18 painting probe.

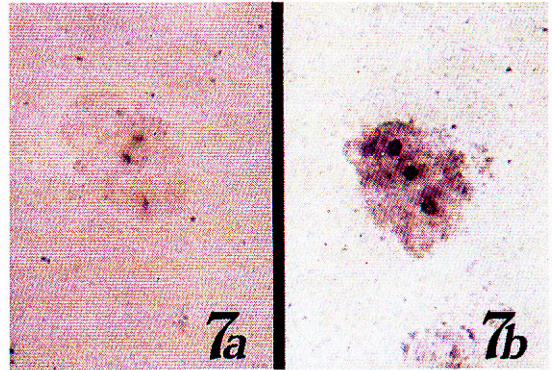

Fig. 7a. Diffuse large cell lymphoma(case 23) with trisomy 18 showing three signals. **7b.** Equal sized three signals with 18 painting probe.

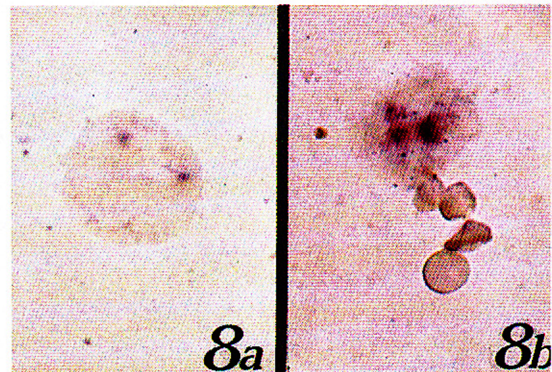

Fig. 8a. Diffuse large cell lymphoma(case 22) with 18 centromeric probe reveals two signals. **8b.** 18 painting probe reveals one big and two smaller signals, indicating translocation of chromosome 18.

Table 4. Correlations between immunophenotypes of non-Hodgkin,s lymphoma and specific chromosomal aberrations

Cell type	No. of cases	Specific chromosomal aberrations	
		Type	No. of cases(%)
B	23	Polysomy X	4 (17)
		Trisomy 18	5 (22)
T	3	-	
Null	4	-	

chromosome 7 or 18 was not observed. Disomy X was frequently found in 3 out of 4 cases of ML, small lymphocytic(Fig. 2). ML, follicular, small cleaved(3 cases) showed trisomy 12(2 cases) and monosomy 7(2 cases). There were no common chromosomal aberrations in ML,follicular,mixed small and large.

In intermediate grade ML(16 cases), tetraploidy and translocation of chromosome 18 were specifically found in 4 cases(25%), and extracopy of chromosome 7 and 18 were found in 3 cases(19%) each. Tetraploidy, in which over 5% of nuclei showed 4 signals for more than two types of chromosomes, indicated the presence of a 4N clone. It was detected in ML, diffuse, small cleaved(1 case), ML, diffuse, mixed small and large(2 cases), and ML, diffuse, large(1 case). ML, diffuse, mixed small and large showed trisomy X in 1 out of 4 cases(Fig. 3). ML, diffuse, large(8 cases) revealed 3 cases of disomy X, 2 cases of trisomy 7(Fig. 4) and 1 case of trisomy 12(Fig. 5). Moreover, monosomy 7, 12, and 18(Fig. 6 a,b) were also observed in 2 cases, 3 cases and 2 cases, respectively. The numerical and structural changes for chromosome 18, including 3 cases of trisomy 18(Fig. 7 a,b) and 3 cases of translocation 18(fig. 8 a,b), were frequently observed in this group.

In high grade ML(5 cases), there were no specific chromosomal aberrations. ML, small noncleaved showed monosomy 18 in 1 out of 2 cases. Even though there were only two cases of ML, lymphoblastic, each of them revealed trisomy X and monosomy 18.

Correlations between Immunophenotypes of NHL and Chromosomal Aberrations.

Immunophenotypical difference of cytogenetic aberrations was not characteristic, but polysomy X and trisomy 18 were detected only in B cell NHL, while aberrations of chromosome 7 were not found in T cell NHL.

DISCUSSION

Chromosomal in situ hybridization of interphase nuclei has been widely accepted as a strong tool used on not only fresh tissue but also paraffin-embedded archival tissue(Drut et al.,1992 ; Lee et al.,1993 b,c ; Han et al.,1994 b). It can be used for slow-growing tumor with little or no mitotic activities, in which it is not feasible to carry out metaphase analysis. Therefore, it is very useful for retrograde chromosomal study on paraffin-embedded archival tissue of solid tumors(Lee et al.,1993 b,c ; Han et al.,1994 b).

To date, most of human chromosome-specific DNA probes have been developed and now widely used for cytogenetic studies in various materials such as cultured cells from fresh specimens(Pinkel et al., 1986, 1988 ; Wullich et al.,1991 ; Zhao et al.,1993), on old slides of bone marrow smears(Lee et al.,1993a) and cells from paraffin-embedded sections. Repetitive satellite centromeric probes are very useful for detection of numerical chromosomal aberrations(Willard,1985 ; Van Dekken and Bauman,1988) and the recent development of a painting probe, unique DNA sequences spanning the length of the target chromosome, enables us to detect even structural chromosomal aberrations(Pinkel et al.,1988 ; Lengauer et al.,1991).

There were several reports that FISH in metaphase nuclei detected the chromosomal abnormalities which could not be revealed by conventional cytogenetic studies(Lee et al.,1993 a,b,c ; Zhao et al.,1993 ; Han et al.,1994 a,b). These results suggest the possibility of overcoming the disadvantage of the conventional cytogenetic method by chromosomal in situ hybridization.

There were many reports in the literature that about 80-90% of NHL had chromosomal aberrations by conventional cytogenetic method(Bloomfield et al.,1983 ; Levine et al.,1985 ; Cabanillas et al.,1988 ; Hammond et al.,1992). However, we could not yet find any comparable report about interphase cytogenetic study of NHL using paraffin-embedded tissue. In our study, we unexpectedly detected aberrant chromosomes in 27 out of 30 cases(90%) by only five kinds of chromosomal DNA probes.

The correlations between chromosomal aberrations and histological grades and groups by International Working Formulation were found in this study. The extracopy of chromosome 12 was associated with low grade ML. This was consistent with the result by Hammond et al.(1992), where chromosome X, 12 and 3 were frequently gained in low grade.

Although frequent association of trisomy 12 with ML, small lymphocytic was reported by metaphase spread (Levine et al.,1985), our study showed frequent association(two out of three cases) of it with ML, follicular, small cleaved. We observed more frequently disomy X (3 cases), monosomy 7,12 and 18(2 cases each) rather than trisomy 12(1 case) in small lymphocytic lymphoma. High frequency of t(14 ;18)(q32 ; q21) was reported in ML, follicular, small cleaved and follicular, mixed small and large, but we had no case with translocation of chromosome 18.

Among 16 cases of intermediate grade ML, tetraploidy and extracopy of chromosome 7 were specifically found. We found increased incidence of tetraploidy and extracopy of chromosome X rather than trisomy 12 and trisomy 18 in ML, diffuse, mixed small and large, consistent with the previous report by Bloomfield et al. (1983). Among 8 cases of ML, diffuse large, trisomy 18 and translocation of chromosome 18 were characteristically frequent(38%), compared with any other groups. The extracopy and translocation of chronosome 18 were found not in low grade but only in intermediate and high grade ML. These results are consistent with that of Cabanillas et al.(1988), in which most common abnormalities of diffuse, large cell lymphoma was t(14 ; 18)(q32 ; q21), followed by trisomy 12, trisomy 3, and trisomy 7.

In 5 cases of high grade ML, we could not find any specific chromosomal abnormalities. Although there was a report that translocation involving chromosome 8 and 14 was frequently found in ML, small noncleaved and lymphoblastic(Levine et al.,1985), we did not put these chromosomal DNA probes in our study.

In conclusion, we demonstrated a number of numerical and structural chromosomal aberrations in the paraffin-embedded tissue of NHL more frequently than expected, and confirmed the principal role of chromosomal aberrations in the pathogenesis of NHL. The association of some specific chromosomal aberrations with histopathologic grades and groups suggest that the cytogenetic analysis by NFISH could give an valuable information for pathologic diagnosis and clinical outcome of NHL. In addition, as we didn't use enough numbers of chromosomal DNA probes, some aberrations may have not been revealed. So we hope in the future there will be more extensive study of NHL with a sufficient battery of DNA probes to identify the full spectrum of chromosomal aberrations in NHL.

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