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ORIGINAL ARTICLE

Molecular Analysis of *IGH* and Incomplete *IGH D-J* Clonality Gene Rearrangements in Hodgkin Lymphoma Malignancies

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SUMMARY

Background: **We evaluated molecular clonality in immunoglobulin heavy chain (*IGH*) and incomplete *IGH D-J* genes for improvement of clinical diagnosis of Hodgkin's lymphoma (HL). We applied BIOMED-2 protocols in HL cases, which were previously approved by clonality detection in non-Hodgkin lymphoma (NHL) cases.**
Methods: **We investigated 50 consecutive FFPE samples of classical HL (cHL) patients to assess *IGH* and *IGH D-J* clonal gene rearrangements by multiplex PCR protocols, which were provided by the European Biomedicine and Health (BIOMED-2) Concerted Action Project BMH4-CT98-3936.**
Results: **In the present study, there was a monoclonality of 86% (43/50) including a clonality of 74% (37/50) for *IGH* and a clonality of 42% (21/50) in *IGH D-J.* In addition, a lack of clonality was detected in 14% (7/50) of cases. Frequent gene rearrangements were detected in framework (FR) III (54%) and FRII (20%), whereas no clonality was seen in FRI. Furthermore, a monoclonality of 28% and 14% was detected in the $DH_{1\text{-}6}$-*JH* and DH_{7}-*JH* gene rearrangements, respectively.**
Conclusions: **The present study suggests that the complete *IGH* and incomplete *IGH D-J* clonality gene rearrangement assays using BIOMED-2 protocols could be considered a valuable method for detection of clonal gene rearrangements, especially in HL cases.**
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INTRODUCTION

Hodgkin's lymphoma (HL) is one type of lymphoproliferative malignancy, mostly originating from of B-cells [1]. It is of great importance to differentiate Hodgkin's lymphoma (cHL) from of non-Hodgkin lymphoma (NHL) by applying immunopathological features and adopting histomorphology approaches. HL is also categorized into the classical Hodgkin's lymphoma (cHL) and nodular lymphocyte-predominant Hodgkin's

lymphoma (NLPHL) [2-4]. Classical HL is subcategorized into several sub-types such as nodular sclerosis (NS), mixed cellularity (MC), lymphocyte depletion (LD), and lymphocyte-rich (LR) Hodgkin's lymphoma [5]. The neoplastic cells in cHL are known as Hodgkin and Reed-Sternberg (HRS) and are derived from germinal center (GC) or post-GC B cells and undergo somatic hypermutations in variable genes of *IGH* [6].

However, immunophenotyping analyses have shown that HRS cells posses surface antigen receptors, co-presented in the T cells, cytotoxic cells, B cells, dendritic cells, granulocytes, and activation marker [7]. Due to the scarcity of HRS cell population, failure of the surface antigen expression, and acquired somatic mutation, determination of cancerous cell lineage by immunophenotyping and molecular approaches is very difficult [8, 9].

Routinely, histomorphological features and immunohistochemistery assessments are the principle of diagnosis in HL, whereas the availability of BIOMED-2 protocols may increase our ability to improve diagnosis of such cases. Moreover, clonality assays of Ig gene rearrangement (*IGH* and *IGH D-J*) are valuable method for an accurate diagnosis of HL [10].

Molecular clonality analysis of Ig rearrangements have been suggested as an appropriate method for diagnosis of hemato-malignancies [11,12]. To date, few investigations have attempted to reveal monoclonality rearrangements in HL cases [13-16]. In the present study, we used the BIOMED-2 multiplex PCR protocol to evaluate the rate of positive gene rearrangement clonality in *IGH* and *IGH D-J* to improve diagnosis and increase sensitivity of pathological features of HL. In the PCR assessment, monoclonality was confirmed when one band in PCR product background was detected. Observation of more than four clonal rearrangements was considered as oligoclonality and multiple clonal rearrangements were considered as polyclonality.

MATERIALS AND METHODS

Sample preparation

In the present study, we analyzed 50 formalin-fixed, paraffin-embedded (FFPE) tissues, which had been reported as HL malignancies by using immunophenotyping and morphological assessments. All of the FFPE blocks were provided between 2010 and 2013. Definite diagnosis of HL was made as the inclusion criteria. The subjects whose specimens had no history of the other hematologic malignancies were studied, and all samples were re-evaluated by the pathological board on the bases of histological features and immunohistochemical (IHC) staining. All of the participants have given informed written consent and the study protocol was approved by the Ethics Committee of Tabriz University of Medial Sciences (TUMS) which was in compliance with the Helsinki Declaration.

DNA extraction

To analyse the molecular clonality, three sections of 10 µm FFPE samples were used for extraction of DNA according to the protocol previously described by Isola et al. [17]. For assessment of quality and quantity of extracted DNA, amplified BIOMED-2 control genes and UV spectrophotometry (260/280 nm using the NanoDropTM ND-1000, NanoDrop Technology, Wilmington, DE, USA) were used [10]. The mean DNA concentration was 400 ng/µL and OD260/280 ratio was 1.85. All the samples were confirmed according to the Euro/Clonality BIOMED-2 guideline [18].

Immunophenotyping assays

Immunohistochemical staining was carried out on 4 µm thick tissue sections using monoclonal antibodies against CD30 (clone Ber-H2; Dako) and CD20 (clone L26; Dako) for isolation of HRS cells and recognition of B cells. For isolation of monocytes and granulocytes, anti-CD15 (clone LeuM1; Becton-Dickinson) was utilized. Anti-CD45/LCA (leukocyte common antigen) (clone 2B11; Dako) was used for screening of hematopoietic origin cells and anti-facsin (clone 55K-2; Dako) for isolation of the HRS cells. Immunophenotyping was performed as described previously [15].

Clonality analysis

We utilized a multiplex PCR protocol provided by the European Biomedicine and Health (BIOMED-2) Concerted Action Project BMH4-CT98-3936 for accurate diagnosis and analysis of clonal gene rearrangements in lymphoproliferative disorders.

Subsequent to the *IGH* and *IGH D-J* gene amplification, PCR products were denatured for 5 minutes at 94°C and incubated for 60 minutes at 4°C. Afterwards, 10 µL of PCR products was loaded on non-denaturing polyacrylamide gel (8%) and stained with silver nitrate. In each electrophoresis run, lymphatic tissue (Tonsil) was utilized as a polyclonal DNA sample, sterile water as negative and the sensitivity panel IVS-0010 (5%) control clonal DNA (InvivoScribe; catalog No.: 4-088- 0590) as a positive sample. Clonality analysis was performed using amplification on three frameworks (FRs) of *IGH* (V_H *FR1-*J_H, V_H *FR2-*J_H, and V_H *FR3-*J_H) and two segments of *IGH D-J* ($DH_{1\text{-}6}$*-JH* and DH_7*-JH)* gene rearrangements using the multiplex PCR protocols suggested by BIOMED-2 (Table 1) [10]. To avoid pseudoclonality detection, all of the samples were analyzed in duplicate.

Statistical analysis

To determine significant levels between CD30, CD20, CD15, CD45, and Facsin marker expressed in cancerous cells and clonality rates, Pearson's chi-square (χ^2 test) and Fisher's exact tests (SPSS statistical software, version 19.0 (SPSS Inc., Chicago, IL, USA) were performed. P-values < 0.05 were considered statistically significant.

Table 1. BIOMED-2 multiplex primer mixes used for *IGH* (FRI, II, III) and *IGH D-J* clonality detection.

Primer Mix		Immunoglobulin Gene	Primer Sequence (5' - 3')
IGH D-J	Tube A: D_{H1-6} - J_H	D_{H1}	GGCGGAATGTGTGCAGGC
		D_{H2}	GCACTGGGCTCAGAGTCCTCT
		D_{H3}	GTGGCCCTGGGAATATAAAA
		D_{H4}	AGATCCCCAGGACGCAGCA
		D_{H5}	CAGGGGGACACTGTGCATGT
		D_{H6}	TGACCCCAGCAAGGGAAGG
		J_H	CTTACCTGAGGAGACGGTGACC
	Tube B: D_{H7} - J_H	D_{H7}	CACAGGCCCCCTACCAGC
		J_H	CTTACCTGAGGAGACGGTGACC
IGH	Tube A: *VH-FRI*	V_{H1}	GGCCTCAGTGAAGGTCTCCTGCAAG
		V_{H2}	GTCTGGTCCTACGCTGGTGAAACCC
		V_{H3}	CTGGGGGGTCCCTGAGACTCTCCTG
		V_{H4}	CTTCGGAGACCCTGTCCCTCACCTG
		V_{H5}	CGGGGAGTCTCTGAAGATCTCCTGT
		V_{H6}	TCGCAGACCCTCTCACTCACCTGTG
		JH-FR	CTTACCTGAGGAGACGGTGACC
	Tube B: *VH-FRII*	V_{H1}	CTGGGTGCGACAGGCCCCTGGACAA
		V_{H2}	TGGATCCGTCAGCCCCCAGGGAAGG
		V_{H3}	GGTCCGCCAGGCTCCAGGGAA
		V_{H4}	TGGATCCGCCAGCCCCCAGGGAAGG
		V_{H5}	GGGTGCGCCAGATGCCCGGGAAAG
		V_{H6}	TTGGGTGCGACAGGCCCCTGGACAA
		V_{H7}	TGGATCAGGCAGTCCCCATCGAGAG
		JH-FR	CTTACCTGAGGAGACGGTGACC
	Tube C: *VH-FRIII*	V_{H1}	TGGAGCTGAGCAGCCTGAGATCTGA
		V_{H2}	CAATGACCAACATGGACCCTGTGGA
		V_{H3}	TCTGCAAATGAACAGCCTGAGAGCC
		V_{H4}	GAGCTCTGTGACCGCCGCGGACACG
		V_{H5}	CAGCACCGCCTACCTGCAGTGGAGC
		V_{H6}	GTTCTCCCTGCAGCTGAACTCTGTG
		V_{H7}	CAGCACGGCATATCTGCAGATCAG
		JH-FR	CTTACCTGAGGAGACGGTGACC

RESULTS

Clonal gene rearrangement assays

CHL and NLPHL constituted 86% (43) and 14% (7) of the 50 evaluated FFPE tissue blocks. CHL consisted of 8 (19%) cases with nodular sclerosis (NS), 10 (23%) cases with mixed cellularity (MC), 1 (2%) case with lymphocyte depletion (LD), and 24 (56%) cases with cHL with sub-types which were not determined. Following Ig genes amplification, a monoclonality of 86% (43/50) was detected, in which 74% (37/50) of positive monoclonality rearrangements were related to the *IGH* gene and 42% (21/50) were for *IGH D-J*. Thirteen cases (13/50, 26%) did not show any monoclonality. The frequent clonal gene rearrangements were detected in FRIII (54%) and FRII (20%), whereas no clonality was identified in FRI. A 28% rate of positive monoclonality was detected in the DH_{1-6}-*JH*, while a 14% of monoclonality was seen in the DH_7-*JH* gene rearrangements (Table 2). There was a statistically significant difference between positive CD30, CD20, CD45, CD15, and Facsin markers expressed in cancerous cells and monoclo-

Table 2. Frequencies of clonality gene rearrangements assessed in the three frameworks (FRI, FRII, FRIII) for *IGH* and two gene segments (*DH_{1-6}-JH* and *DH_{7}-JH*) for *IGH D-J* according to BIOMED-2 protocols.

Diagnosis		*IGH*			*IGH D-J*	
		FR_{I}-JH	*FR_{II}-JH*	*FR_{III}-JH*	*DH_{1-6}-JH*	*DH_{7}-JH*
cHL	*NS* n = 8	0 (0%)	0 (0%)	5/8 (62.5%)	3/8 (37.5%)	2/8 (25%)
	MC n = 10	0 (0%)	2/10 (20%)	6/10 (60%)	2/10 (20%)	3/10 (30%)
	LD n = 1	0 (0%)	1/1 (100%)	0 (0%)	0 (0%)	0 (0%)
	HL (Not-typed) n = 24	0 (0%)	6/24 (25%)	11/24 (46%)	8/24 (33.5%)	2/24 (8.5%)
NLPHL	*LR* n = 7	0 (0%)	1/7 (14.3%)	5/7 (71.5%)	1/7 (14.3%)	0 (0%)
Total n = 50		0 (0%)	10/50 (20%)	27/50 (54%)	14/50 (28%)	7/50 (14%)

NS - nodular sclerosis, MC - mixed cellularity, LR - lymphocyte-rich, LD - lymphocytic depletion.

nal gene rearrangements in immunoglobulin genes ($p < 0.005$). A statistically significant difference was found between the number of positive CD30 cells and rate of positive *IGH* monoclonality ($p < 0.005$). However, no significant difference was demonstrated in *IGH D-J* gene rearrangement assays ($p = 0.171$). In addition, in CD20 positive cases, we found a significant difference for *IGH D-J* ($p = 0.004$) rearrangements, whereas none was found for *IGH* clonality ($p = 0.078$). In the CD15/CD3 positive cells, a statistically significant difference was found between *IGH* ($p < 0.005$ in CD15 cells) and *IGH D-J* ($p < 0.005$ in CD3 cells) clonality.

DISCUSSION

The present study was designed to assess an auxiliary method to improve clinical diagnosis by means of the BIOMED-2 molecular gene rearrangements protocol in FFPE tissue of HL samples. Clonal gene rearrangements in *IGH* and *IGH D-J* genes in 50 FFPE cases, reported as several sub-types of HL involving NS, MC, LD, and NLPHL neoplasms, were investigated. We studied clonal gene rearrangements in FRI, FRII, and FRIII of complete *IGH* and incomplete *IGH D-J* genes. Our results indicated a total monoclonality of 86% (43/50). Monoclonality was identified in 37 out of 50 (74%) HL cases. With respect to the rate of clonal rearrangements in FRI-J_H, no monoclonality was detected in any of the sub-types of HL. These findings are compatible with those of the previous investigations [9,11,13-15]. In the analysis of FRII-J_H and FRIII-J_H gene rearrangements, we demonstrated a positive clonality in 71% (31/43) and 86% (6/7) of cHL and NLPHL types, respectively. A positive clonality of 74% was identified in the present study, which was a significant improvement compared to other studies, which reported a clonality of 15.5%, 44.5%, 50%, 10%, and 24% [9,11,13-15], respectively. We have also observed a monoclonality of 86% in the *IGH* assay within NLPHL cases, whereas other reports failed to show any positive clonality in this regard [15,19]. Several factors such as histological structures, sample types, and specificity assessments have been suggested to affect the rate of the positive monoclonality rearrangements assay by means of BIOMED-2 protocols [10].

In order to enhance of the positive rate of detection of clonal gene rearrangements, we included additional Ig genes such as *IGH D-J*, which are less susceptible to somatic hypermethylation. A positive monoclonality rate of 28% and 14% were seen in *DH_{1-6}-JH* and *DH_{7}-JH* gene rearrangements.

Subsequent to assessment of *IGH D-J* clonal gene rearrangements, a positive clonality was identified in 17/43 (39.5%) of cHL samples. This was higher in NLPHL (57.2%). In contrast, assessment of the relationship between clonal *IGH D-J* rearrangements and variation in density of cell surface antigens showed a significant difference in positive CD2 and CD3 cells as well as LCA ($p < 0.005$). It is worth mentioning that no statically significant difference was seen between positive CD30 cell population and *IGH D-J* rearrangements. A monoclonality of 8 - 22% in *IGH D-J*, which was declared not to be applicable to cHL assessments, was reported previously [9,14]. However, the present study reports a monoclonality, which is significantly higher than that of previous research (57.2% *v* 8% - 22%).

Furthermore, we analyzed a significant difference between positive CD30 cells and the rate of *IGH* monoclonality rearrangements. In agreement with previous research [11,14,15], we found increases as the number of the positive CD30 population cells and the rate of

monoclonality rearrangements were enhanced. Thus, it is conceivable that one of the most important differences of positive clonality is detected in association with the density of cancerous cells. However, further studies are needed to clarify these differences. In spite of the fact that different approaches (Gene Scanning and Heteroduplex gel electrophoresis) have been used to detect such rearrangements, these approaches have similar sensitivities in clonality PCR assays [10]. In addition, the relatively high percentage of clonality in the present study, compared to preceding research, may be a result of the relatively greater amounts of the DNA samples and high density of neoplastic CD30 positive cells.

CONCLUSION

The present study suggests that the rate of clonal gene rearrangements in HL was less than reported in NHL and the rate of positive monoclonality was closely related to the density of HRS. In conclusion, assessments of clonal gene rearrangements in *IGH* and *IGH D-J* using BIOMED-2 protocols could be considered as a valuable method to increase sensitivity and accuracy of determining HL, similarly to NHL.

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Declaration of Interest:
The authors declare that there is no conflict of interest.

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