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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 *DH1-6-JH* and *DH7-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.


KEY WORDS

Hodgkin’s lymphoma, monoclonality, BIOMED-2, *IGH D-J*, rearrangement

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 subcategori-
ized 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 germin-
al 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 possess 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 sur-
face antigen expression, and acquired somatic mutation,
dermination of cancerous cell lineage by immunophe-
notyping and molecular approaches is very difficult [8,
9].

Routinely, histomorphological features and immunohis-
tochemistry 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 rearrange-
ment (IGH and IGH D-J) are valuable method for an ac-
curate 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 investiga-
tions have attempted to reveal monoclonality rearrange-
ments in HL cases [13-16]. In the present study, we
used the BIOMED-2 multiplex PCR protocol to evalu-
ate 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. Obser-
vation of more than four clonal rearrangements was
considered as oligoclonality and multiple clonal rearr-
rangements 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 re-
ported as HL malignancies by using immunophenotyp-
ing and morphological approaches. 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 bas-
es of histological features and immunohistochemical
(IHC) staining. All of the participants have given in-
formed written consent and the study protocol was ap-
proved by the Ethics Committee of Tabriz University
of Medical 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 ex-
tracted DNA, amplified BIOMED-2 control genes and
UV spectrophotometry (260/280 nm using the Nano-
DropTM ND-1000, NanoDrop Technology, Wilming-
ton, DE, USA) were used [10]. The mean DNA concen-
tration 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 utili-
zed. Anti-CD45/LCA (leukocyte common antigen)
(clone 2B11; Dako) was used for screening of hemato-
poietic origin cells and anti-fascin (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) Con-
certed 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 polyacryl-
amide gel (8%) and stained with silver nitrate. In each
electrophoresis run, lymphatic tissue (Tonsil) was uti-
lized as a polyclonal DNA sample, sterile water as neg-
ative 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
(VH FR1-JH, VH FR2-JH, and VH FR3-JH) and two seg-
ments of IGH D-J (DH 1,2-JH and DH 3,4-JH) gene rear-
rangements using the multiplex PCR protocols suggest-
ed by BIOMED-2 (Table 1) [10]. To avoid pseudoclo-
inality detection, all of the samples were analyzed in du-
plicate.

Statistical analysis
To determine significant levels between CD30, CD20,
CD15, CD45, and Facsin marker expressed in cancer-
ous cells and clonality rates, Pearson’s chi-square (χ²
test) and Fisher’s exact tests (SPSS statistical software,
version 19.0 (SPSS Inc., Chicago, IL, USA) were per-
formed. 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.

<table>
<thead>
<tr>
<th>Primer Mix</th>
<th>Immunoglobulin Gene</th>
<th>Primer Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IGH D-J</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube A: $D_{III}$ - $J_{H}$</td>
<td>$D_{H1}$</td>
<td>GGCAGAATGTGGTGCAGGCC</td>
</tr>
<tr>
<td></td>
<td>$D_{H2}$</td>
<td>GCACCTGAGCTCAGTCTCTCT</td>
</tr>
<tr>
<td></td>
<td>$D_{H3}$</td>
<td>GTGGCCCTGGGAATATAAAA</td>
</tr>
<tr>
<td></td>
<td>$D_{H4}$</td>
<td>AGATCCCCAGGACGCAGCA</td>
</tr>
<tr>
<td></td>
<td>$D_{H5}$</td>
<td>CAGGGGGCAGACTGTGCATGT</td>
</tr>
<tr>
<td></td>
<td>$D_{H6}$</td>
<td>TGACCCCCAGCAAGGGAGG</td>
</tr>
<tr>
<td></td>
<td>$J_{H}$</td>
<td>CCTACCTGAGGAGACGGTGACC</td>
</tr>
<tr>
<td>Tube B: $D_{H7}$ - $J_{H}$</td>
<td>$D_{H7}$</td>
<td>CACAGGGCCCTACACGC</td>
</tr>
<tr>
<td></td>
<td>$J_{H}$</td>
<td>CCTACTGAGGACGCGTGACC</td>
</tr>
<tr>
<td><strong>IGH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube A: VH-FRI</td>
<td>$V_{H1}$</td>
<td>GGCCTCAGTGAAGGTCTCTGCAAG</td>
</tr>
<tr>
<td></td>
<td>$V_{H2}$</td>
<td>GTCTGCTTACGGCTGTTAAAA</td>
</tr>
<tr>
<td></td>
<td>$V_{H3}$</td>
<td>CTGGGCGGCTCAGTCTCTCT</td>
</tr>
<tr>
<td></td>
<td>$V_{H4}$</td>
<td>CTTCGGAGCACCTGCCACCT</td>
</tr>
<tr>
<td></td>
<td>$V_{H5}$</td>
<td>CAGGGGATCTCTGAGATCTTCTG</td>
</tr>
<tr>
<td></td>
<td>$V_{H6}$</td>
<td>TCGAGACCTCCTCTACCTACCTG</td>
</tr>
<tr>
<td></td>
<td>JH-FR</td>
<td>CCTACTGAGGACCGTGACC</td>
</tr>
<tr>
<td>Tube B: VH-FRII</td>
<td>$V_{H1}$</td>
<td>CTGGCGTCGCAAGGCCCTTGGACA</td>
</tr>
<tr>
<td></td>
<td>$V_{H2}$</td>
<td>TGGACTCGTGCAGGCCCCAGGAAG</td>
</tr>
<tr>
<td></td>
<td>$V_{H3}$</td>
<td>GTGCCGCAAGCTCAGGG</td>
</tr>
<tr>
<td></td>
<td>$V_{H4}$</td>
<td>TGGCGTCGCAGGCCCCAGGAGG</td>
</tr>
<tr>
<td></td>
<td>$V_{H5}$</td>
<td>GGTGCGCAGATGCCGGGAAAG</td>
</tr>
<tr>
<td></td>
<td>$V_{H6}$</td>
<td>TGGGTGTCGACAGCCAGGAGG</td>
</tr>
<tr>
<td></td>
<td>$V_{H7}$</td>
<td>TGATCCAGGACGACCTACG</td>
</tr>
<tr>
<td></td>
<td>JH-FR</td>
<td>CCTACTGAGGAGCGTGACC</td>
</tr>
<tr>
<td>Tube C: VH-FRIII</td>
<td>$V_{H1}$</td>
<td>TGGAGCTGACAGCCGCTTGAGATG</td>
</tr>
<tr>
<td></td>
<td>$V_{H2}$</td>
<td>CAAAGACCATGAGGAGGTG</td>
</tr>
<tr>
<td></td>
<td>$V_{H3}$</td>
<td>TCGTAACGAAATGGCAAGCTCAG</td>
</tr>
<tr>
<td></td>
<td>$V_{H4}$</td>
<td>GAGCTCTTGACGCGCGCGAG</td>
</tr>
<tr>
<td></td>
<td>$V_{H5}$</td>
<td>CAGCACCGCTAATGAGAGG</td>
</tr>
<tr>
<td></td>
<td>$V_{H6}$</td>
<td>GTCTCCCTGACGATCTGAG</td>
</tr>
<tr>
<td></td>
<td>$V_{H7}$</td>
<td>CAGCACGCGCATATCTGAGATC</td>
</tr>
<tr>
<td></td>
<td>JH-FR</td>
<td>CCTACTGAGGAGCGTGACC</td>
</tr>
</tbody>
</table>

**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 $DH_{I}$-$J_{H}$, while a 14% of monoclonality was seen in the $DH_{I}$-$J_{H}$ gene rearrangements (Table 2). There was a statistically significant difference between positive CD30, CD20, CD45, CD15, and Fas-sin 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 (DH₁,σ-JH and DH-JH) for IGH D-J according to BIOMED-2 protocols.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>IGH</th>
<th>IGH D-J</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FRγ-JH</td>
<td>FRµ-JH</td>
</tr>
<tr>
<td>NS n = 8</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>MC n = 10</td>
<td>0 (0%)</td>
<td>2/10 (20%)</td>
</tr>
<tr>
<td>LD n = 1</td>
<td>0 (0%)</td>
<td>1/1 (100%)</td>
</tr>
<tr>
<td>HL (Not-typed) n = 24</td>
<td>0 (0%)</td>
<td>6/24 (25%)</td>
</tr>
<tr>
<td>LR n = 7</td>
<td>0 (0%)</td>
<td>1/7 (14.3%)</td>
</tr>
<tr>
<td>Total n = 50</td>
<td>0 (0%)</td>
<td>10/50 (20%)</td>
</tr>
</tbody>
</table>

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

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-JH, 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-JH and FRIII-JH 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 significant compared to other studies, which reported a monoclonality 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 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 DH₁,σ-JH and DH-JH gene rearrangements.

Subsequent to assessment of IGH D-J clonal gene rearrangements, a positive monoclonality 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 CD2 and CD3 cells as well as LCA (p < 0.005). It is worth mentioning that no statistically significant difference was seen between positive CD2 and CD3 cells as well as LCA (p < 0.005).
IGH Gene Rearrangement Assays in HL

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.

References: