Evaluation of *IGK* and *IGL* molecular gene rearrangements according to the BIOMED-2 protocols for clinical diagnosis of Hodgkin lymphoma

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Background: Although the analysis of molecular clonality rearrangements of the immunoglobulin light chains (*IGK* and *IGL*) is an alternative approach for diagnosis of B cell non-Hodgkin lymphomas (NHLs) using BIOMED-2 protocols, NHLs have not been extensively confirmed for Hodgkin lymphoma (HL) cases. We evaluated BIOMED-2 protocols in HL cases, which have been suggested previously as gold standard method for molecular clonality analysis on formalin fixed, paraffin-embedded (FFPE) tissue in NHL patients.
Methods: We recruited 50 consecutive FFPE tissues of HL samples to evaluate *IGK* and *IGL* clonality gene rearrangements using BIOMED-2 and Heteroduplex methods.
Results: Our findings revealed a total of 94% (47/50) positive clonality, which consisted of 70% (35/50) for *IGK* and 44% (22/50) for *IGL*. In three cases, clonality was not detected in any of the immunoglobulin gene segments.
Conclusions: Analysis of clonality gene rearrangements in *IGK* and *IGL* genes using BIOMED-2 protocols could be implemented as a valuable method for improving clonality detection rate in HL cases and sensitivity (94%) and accuracy of HL diagnosis similar to that of the NHL samples will be increased.

Keywords: Hodgkin's lymphoma, BIOMED-2, *IGK*, Rearrangements, Clonality

Introduction

Lymphoid neoplasm accounts for approximately 3–4% of all malignancies, which occur worldwide.[1] Hodgkin's lymphoma (HL) is one type of lymphoma disorder, originates from B-cells, and represented 11.8% of all types of lymphomas, which was estimated in 2013.[2] Based on immunophenotypical features and cellular typing, HL was also divided into two forms including, classical Hodgkin's lymphoma (cHL) and nodular lymphocyte-predominant Hodgkin's lymphoma (NLPHL). Cancerous cells in cHL and NLPHL types were termed as Hodgkin and Reed–Sternberg (HRS) and lymphocyte-predominant (LP) cells, respectively.[3,4] Although, HL is considered as an uncommon subtype of lymphoma malignancies in developing countries, whereas it is one of the most common features of hemato-malignancies in the west countries.[4] According to the previous classification, cHL was sub-divided into several forms, such as nodular sclerosis (NS), mixed cellularity (MC), lymphocyte depletion (LD), and lymphocyte-rich (LR) HL, all of which accounts for approximately 95% of cases.[5] HL mainly results from either an infectious agent or activation of NF-κB pathway. In 40% of HRS cells in cHL reconstructed by Epstein–Barr virus (EBV), apoptosis is repressed in GC B cells.[6,7]

Based on feature morphological assessments, HRS cells were exhibited in the background of several cell types, which were originated from the germinal center (GC) or post-GC, during B cell differentiation procedures. Notwithstanding, previous investigations have revealed that the HRS cells could carry high loads of somatic mutations in immunoglobulin (Ig) V gene families,[8] which is confirmed that HRS cells have resulted from GC stage.[5,9] According to the immunophenotyping investigations, HRS cells may be expressed in several surface antigen receptors, such as co-presented in the T cells (CD3, NOTCH1, GATA3), cytotoxic cells (granzyme B, perforin), B

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cells (Pax5, CD20), dendritic cells (Fascin, CCL17), granulocytes (CD15), and activation marker (CD30).[5] Importantly, in the several case investigations, HRS cells could be manifested T cells specific markers and lymphoid neoplasms with T cell origin were identified wrongly as cHL.

In the diagnosis of HL procedures, due to the scarcity of HRS cell population, defect of the surface antigen manifestation and somatic mutation achieved in Ig gene segments, tumor cells' lineage detection, were extremely difficult using immunophenotyping and molecular methods.[10,11]

Polymerase chain reaction (PCR) appraisal based on the amplification of Ig gene rearrangements has been suggested as a practical approach for investigation of lymphoma malignancies.[12–14] Although molecular analysis of clonality Ig gene rearrangements using BIOMED-2 protocols has been approved as gold standard method for clonality detection in NHL patients, there are several limited HL studies have been carried out.[11,15–18] In the present investigation, we performed BIOMED-2 multiplex PCR kit, to assess gene rearrangement clonality in *IGK* and *IGL* for improvement of diagnosis and increasing sensitivity of pathological characteristics of HL.

Material and methods

This investigation was carried out on 50 formalin fixed paraffin-embedded (FFPE) samples diagnosed with HL by immunophenotyping and morphological features. Both cHL and NLPHL cases were included in this survey. All of the FFPE blocks were enlisted from the archives of surgical pathology department at Imam Khomeini Hospital of Tehran, between March 2010 and September 2013, under a protocol confirmed by the pathological board. The specimens investigated in our work had no history of hematological disorders, as well as all of the samples were re-evaluated by the pathological board according to the histological features and immunohistochemical (IHC) staining. Clinical demographic parameters revealed that 68% (34/50) of cases were males with the mean age of 34.82 ± 2.72 years (ranging between 12 and 78) and 32% (16/50) were females with the mean age of 38 ± 4.24 years (ranging between 17 and 72).

DNA extraction

To assess molecular gene rearrangement clonality, four slices of 10 μm FFPE tissues from HL cases were used for extraction of genomic DNA according to the protocol previously described by Santos *et al.*[19] In order to ascertain quality and quantity extracted by DNA, PCR amplification was done using BIOMED-2 control genes and UV spectrophotometry (260/280 nm using the NanoDropTM ND-1000, NanoDrop Technology, Wilmington, DE, USA), respectively.[20] The mean DNA concentration was 200 ng/μl and OD260/280 ratio was 1.80 for each of samples. Analysis of clonal gene rearrangements in *IGK* and *IGL* was performed according to the Euro/Clonality BIOMED-2 guideline.[20]

Clonality analysis

For clonality detection, PCR products were denatured for 5 min at 94°C, and then incubated for 60 min at 4°C, and then 10 μl of PCR products was loaded on non-denaturing polyacrylamide gel (PAGE; 8%) and stained with silver nitrate. In each run of electrophoresis, lymphatic tissue (Tonsil) was used 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. Amplification reactions were carried out in a final volume of 25 μl, including 200 ng DNA, 1 pmol of each primer (BIOMED-2 primers, Tables 1 and

Table 1 BIOMED-2 multiplex primer mixes for *IGK* and *IGL* clonality detection

Primer mix		Immunoglobulin gene	Primer sequence (5′–3′)
IGK	*Tube A*: V_K-J_K	$V_{k1f/6}$	TCAAGGTTCAGCGGCAGTGGATCTG
		V_{k2f}	GGCCTCCATCTCCTGCAGGTCTAGTC
		V_{k3f}	CCCAGGCTCCTCATCTATGATGCATCC
		V_{k4}	CAACTGCAAGTCCAGCCAGAGTGTTTT
		V_{k5}	CCTGCAAAGCCAGCCAAGACATTGAT
		V_{k7}	GACCGATTTCACCCTCACAATTAATCC
		$J_{k1–4}$	CTTACGTTTGATCTCCACCTTGGTCCC
		J_{k5}	CTTACGTTTAATCTCCAGTCGTGTCCC
	Tube B: V_K-K_{de} $Intron_{RSS}$-K_{de}	$V_{k1f/6}$	TCAAGGTTCAGCGGCAGTGGATCTG
		V_{k2f}	GGCCTCCATCTCCTGCAGGTCTAGTC
		V_{k3f}	CCCAGGCTCCTCATCTATGATGCATCC
		V_{k4}	CAACTGCAAGTCCAGCCAGAGTGTTTT
		V_{k5}	CCTGCAAAGCCAGCCAAGACATTGAT
		V_{k7}	GACCGATTTCACCCTCACAATTAATCC
		K_{de}	CCTCAGAGGTCAGAGCAGGTTGTCCTA
		$Intron_{RSS}$	CGTGGCACCGCGAGCTGTAGAC
	IGL Tube A: V_λ-J_λ	$V_{L1/2}$	ATTCTCTGGCTCCAAGTCTGGC
		V_{L3}	GGATCCCTGAGCGATTCTCTGG
		$J_{L1/2/3}$	CCCTGGTTCGAGTGGCAGGATC

Table 2 BIOMED-2 multiplex PCR kit for analysis of gene rearrangements in *IGK* and *IGL* genes and expected size band

Target genes		Size band
IGK	*Tube A*: V_K-J_K	120–160 bp ($V_{k1f/6}$/ V_{k7}-J_k)
		190–210 bp (V_{k3f}-J_k)
	Tube B: V_K-K_{de}	260–300 bp (V_{k2f}/ V_{k4}/ V_{k5}-J_k)
	$Intron_{RSS}$-K_{de}	210–250 bp ($V_{k1f/6}$/ V_{k7}-K_{de})
		350–390 bp (V_{k2f}/ V_{k4}/ V_{k5}-K_{de})
		270–300 bp (V_{k3f}/$Intron_{RSS}$-K_{de})
IGL	*Tube A*: V_λ-J_λ	140–165 bp (V_λ-J_λ)

2), and 2x master mix red with 0.2 units/µl Ampliqon Taq DNA polymerase (Ampliqon A/S, Stenhuggervej 22, Denmark). Clonality analysis was performed using amplification on two tubes for *IGK* (V_K-J_K) and (V_K-$Intron_{RSS}$/K_{de}), and one tube for *IGL* (V_L-J_L) using multiplex PCR protocols, recommended by BIOMED-2 concerted action BMH4-CT98-3936.[20] In order to increase fidelity of detected clonality, all of the samples were evaluated in duplicate. For clonality detection, individual reproducible clonal gene rearrangements of identical size can be reliably elucidated as monoclonal. The presence of two different PCR products could indicate biclonality, whereas it can often more easily be certified to the occurrence of biallelic rearrangements in a single clone. Therefore, IGK/IGL PCR analysis was performed on multiple aliquots of each DNA sample. For standard reporting, the findings were interpreted according to the EuroClonality Ig/TCR guidelines.[21]

Statistical analysis

To determine the relationship between CD30 markers expressed in cancerous cells and clonality rates, the Pearson's chi-square (χ^2) test and Fisher's exact test were performed. The findings obtained were assessed using the SPSS statistical software, version 20.0 (SPSS Inc., Chicago, IL, USA). The *P*-values <0.05 were considered significant statistically.

Results

Molecular gene rearrangement analysis

Of the 50 FFPE blocks evaluated, 43 cases (86%) were diagnosed as cHL, and 7 cases (14%) as NLPHL. Generally, cHL consisted of 8 (19%) cases with NS, 10 (23%) cases with MC, 1 (2%) case with LD, and 24 (56%) cHL cases did not have determined subtypes. Following IHC staining, the percentage of Ki67 expression in HRS cells was scored and placed in three groups: <10%, between 10 and 50%, and >50% in cases. In 25.5% of cHL cases, the Ki67 expression was in ranges of more than 50%. Surprisingly, approximately 70% of cases showed positive expression in 10–50% and only one case (2.5%) revealed less than 1%. In NLPHL cases, HRS cells showed positive expression ranging between 43 (10–50%) and 57% (>50%) in all of the LP cases.

The density of CD30-positive cancerous cells in cHL cases was ranging between 30 and 70%. In addition, the number of background CD20-positive B cells was ranging between 15 and 23%.

A total of NLPHL cases showed considerable lymphocytic and histiocytic CD20-positive cells. In all of the samples, the frequencies of CD20-positive cancerous cells were scored as low density, and the number of background CD20-positive B cells was considered to be high. Remarkably, all NLPHL cases revealed high-density B cell nodules and low lymphocytic and histiocytic density. In reality, lymphocytic and histiocytic cells accounted for <2–11% of cellularity.

In our investigation, we used Heteroduplex (HD) gel electrophoresis for clonality detection in HL cases. Following gene targets amplification, 47 of the 50 (94%) HL samples indicated monoclonality rearrangements in *IGK* and *IGL* together. In *IGK* clonality detection, 35 out of 50 cases (70%) demonstrated clear gene rearrangements. Importantly, the most rate of positive monoclonality was detected in $Intron_{RSS}$-K_{de} rearrangements. In order to increase the sensitivity of monoclonality detection, we assessed an additional clonality gene rearrangement in *IGL*. Our findings indicated 22 out of 50 (44%) HL samples; monoclonality was detectable in *IGL* analysis. The results obtained from the clonality gene rearrangements assessed on FFPE tissue of HL are shown in Table 3.

In addition, we assessed whether, between rates of monoclonality detected and positive CD30 marker cells, there is any statistically significant difference or not. In our findings, we observed statistically significant difference between the CD30 marker expressed in cancerous cells and the rate of monoclonality gene rearrangements. Regarding the number of CD30 cells positive and *IGL* monoclonality detected, statistically significant difference was found ($P = 0.030$), whereas no statistically significant difference in clonality *IGK* rearrangements was detected ($P = 0.305$).

Discussion

The purpose of our investigation was to evaluate an auxiliary approach for the improvement of diagnosis of HL cases by applying BIOMED-2 molecular gene rearrangement protocols on FFPE tissues. We studied clonality gene rearrangements in *IGK* and *IGL* genes in 50 FFPE cases, which were diagnosed as several sub-types of HL consisting of NS, MC, LD, and NLPHL neoplasms. We assessed the rate of positive monoclonality gene rearrangements in *IGK* and *IGL* genes. In fact, *V* gene families are more prone to somatic hypermutations and lead to an increase in negative pseudoclonaity,[10,22] whereas the

Table 3 The clonality rates of gene rearrangements assessed according to BIOME-2 protocols

Diagnosis		*IGK*		*IGL*	
		V_K-J_K	V_K-K_{de}	$Intron_{RSS}$-K_{de}	V_L-J_L
cHL	NS, $n = 8$	1/8 (12.5%)	1/8 (12.5%)	7/8 (87.5%)	6/8 (75%)
	MC, $n = 10$	1/10 (10%)	2/10 (20%)	6/10 (60%)	3/10 (30%)
	LD, $n = 1$	0 (0%)	0 (0%)	0 (0%)	1/1 (100%)
	HL (no-typed), $n = 24$	3/24 (12.5%)	2/24 (8.5%)	5/24 (21%)	7/24 (29.2%)
NLPHL	LR, $n = 7$	0 (0%)	2/7 (28.6%)	5/7 (71.5%)	5/7 (71.5%)
Total, $n = 50$		5/50 (10%) 70%	7/50 (14%)	23/50 (46%)	22/50 (44%) 44%

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

IGK, *LGL* clonal rearrangements analysis, which are less than affected to SHM and will be increased the rate of positive monoclonality detected.[22] In our findings, monoclonality was identified in 47 out of 50 (94%) HL cases.

In addition, we evaluated the usefulness of the BIOMED-2 protocols for *IGK* molecular rearrangement assays on FFPE in HL samples. Of the 68% (34/50) monoclonality detected, 63% (27/43) was detected for cHL, and 100% (7/7) for NLPHL. Although the sensitivities of *IGK* gene rearrangements in previous studies[11,15,17,18] were similar to our investigation, they are significantly lower than that in the present report. Based on the above discussion, BIOMED-2 protocols have been acknowledged as the reliable and valuable method for clonality detection in lymphoproliferative disorders, whereas a number of factors including DNA sample concentration, storage time, fixative type, DNA integrity, density of neoplastic cells, thickness of the samples can be significantly affected by sensitivity and accuracy of gene rearrangement assessment on FFPE samples.[20]

In conformation to the previous investigations, we found no statically significant difference between *IGK* gene rearrangements and density of positive CD30 cells ($P = 0.305$). However, according to these results,[15,17,18] positive clonality detected in *IGK* analysis was low relatively to the number of positive CD30 cells. Our findings showed that in regard to the rate of positive clonality in *IGL* assay and density of positive CD30 cells, there was no statically significant difference ($P = 0.030$). Owing to the low number of cancerous cells present in HL, it has been implicit conventionally that display of clonal populations in HL is remarkably problematical, lacking the use of microdissection. This method is well demonstrated by the WHO 2008 Blue Book, which affirms: 'the clonal gene rearrangements are commonly demonstrable only in the DNA of separated single HRS, and not in entire tissue DNA'.[23]

To determine the increase in sensitivity of clonal gene rearrangement detection, we included *IGL* that is less prone to SHM.[22]

In our study, we revealed that 39.5% (17/43) positive monoclonality in *IGL* and 71.5% (5/7) in cHL and NLPHL cases (total clonality of 44%). Notably, there is no reported evidence for the rate of positive monoclonality gene rearrangements in *IGL* as of now. Although investigators have applied several different methods (Gene Scanning and HD gel electrophoresis) to detect clonality rearrangements, both the approaches have similar sensitivities in clonality PCR assays.[20] However, further studies are needed to clarify these findings.

Conclusion

Overall, clonality detection in HL is less than that investigated in NHL. In addition, the rate of positive monoclonality detection is closely related to the density of cancerous cells (HRS). We conclude that the analysis of clonality gene rearrangements in *IGK* and *IGL* using BIOMED-2 protocols could be implemented as a valuable and reliable method for increase of sensitivity and accuracy of HL diagnosis similar to that of NHL.

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Ethics approval Yes.

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