Tanaffos (2011) 10(2), 25-31

Clonality of the Immunoglobulin Heavy Chain Genes in B Cell Non-Hodgkin Lymphoma Using Semi-Nested PCR

Zohreh Mohammad Taheri [1,2], Leila Mohammadi Ziazi [2], Atosa Dorudinia [2], Seyed Alireza Nadji [1], Forozan Mohammadi [2,3]

[1] Virology Research Center, [2] Department of Pathology, [3] Chronic Respiratory Disease Research Center, NRITLD, Shahid Beheshti University of Medical Sciences, Tehran-Iran.

ABSTRACT

Background: *Identification of gene rearrangements and clonality analysis are important techniques for the diagnosis of malignant lymphoproliferative diseases. These methods have various sensitivities based on the type of primer used and method of determination of polymerase chain reaction (PCR) products. This study aimed at determining the clonality of B cell non-Hodgkin lymphoma in Iranian patients using PCR method and 2 primers of FR2 and FR3.*

Materials and Methods: *Paraffin embedded blocks of 67 patients with B cell lymphoma and 19 cases with lymphoid hyperplasia of the lymph nodes who presented to NRITLD, Masih Daneshvari Hospital were retrospectively reviewed. After extracting the genomic DNA using phenol and chloroform, clonal analysis was performed using semi-nested PCR by using two primers: FR2 and FR3. PCR products were determined using 2 techniques of heteroduplex analysis, polyacrylamide gel and silver staining and the conventional method of agarose gel and ethidium bromide staining. Appearance of 1 or 2 bands in the desired location were considered as a sign of clonality.*

Results: *Monoclonal gene rearrangement was observed in 62 out of 67 patients (92.5%) as one or two discrete bands appeared within 60-120 base pairs (bp) and 200-300 bp range. Of the mentioned patients, 53 cases (79.1%) had FR2 and 51 (76.1%) had FR3 rearrangement. Heteroduplex analysis along with silver nitrate staining detected 3 out of the remaining 5 cases of lymphoma to be monoclonal. These cases had been reported negative by the conventional technique. In total, 65 out of 67 patients (97%) showed monoclonal gene rearrangement using both the abovementioned techniques. All hyperplasia cases were polyclonal by this method.*

Conclusion: *Our study showed that evaluation and detection of clonality using PCR, FR2 and FR3 primers along with heteroduplex analysis is a rapid sensitive technique for the diagnosis of malignant lymphomas.* ***(Tanaffos2011; 10(2): 25-31)***

Key words: *Clonality, B cell non-Hodgkin lymphoma, Polymerase Chain Reaction (PCR)*

INTRODUCTION

In the majority of patients suffering from lymphoproliferative disorders, histology along with immunohistochemistry or flow cytometry can distinguish between benign and malignant lymphocytic proliferations. However, in 5-10% of cases establishing a diagnosis is difficult and given the fact that malignant cells are derived from the same clone, clonality analysis can assist the diagnosis (1).

Correspondence to: Mohammad Taheri Z
Address: NRITLD, Shaheed Bahonar Ave, Darabad, TEHRAN 19569, P.O:19575/154, IRAN
Email address: mtaheri@nritld.ac.ir
Received: 9 September 2010
Accepted: 17 January 2011

In many cases, B lymphomas have clonal gene rearrangement. Immunoglobulin (Ig) gene locus includes various segments of V (variable), D (diversity) and J (joining) regions. Rearrangement event joins a single D segment with a J segment; this partially rearranged D-J gene is then joined to a V segment in the heavy chain locus (IgH). In the light chain locus, V segment directly joins the J segment (2). During lymphocyte development, gene rearrangement occurs in Ig and T cell receptor (TCR) genes. During B cell development, gene recombination occurs first in heavy chain genes (IgH), and then in IgK and Igλ and provides a wide range of combinations. Mature B cells in the germinal centers undergo somatic hypermutation in the process of antigen recognition which is a common finding in malignancies of the germinal center or post germinal center origin. Various techniques are available for determination of clonality such as Southern blotting and antigen-antibody based techniques (immunohistochemistry and flow-cytometry). However, at present these techniques have been replaced by PCR methods. PCR is the preferred method especially for extracting DNA from paraffin embedded blocks because it is fast, requires very small amounts of DNA, has fairly good sensitivity and does not require high quality DNA. Sensitivity of PCR for determining clonality is variable from 85%-99% based on the type of primer used and method of determination of PCR products.

This study aimed at determining the sensitivity of PCR by using 2 regions of FR2 and FR3 for clonality analysis of paraffin embedded blocks of patients with confirmed diagnosis of B cell non-Hodgkin lymphoma.

MATERIALS AND METHODS

Tissue samples: A total of 87 formalin fixed paraffin embedded blocks of tissue samples collected during 2001-2008 were retrieved from the pathology archive of Masih Daneshvari Hospital. Out of which 67 biopsy blocks belonged to patients with B cell non-Hodgkin lymphoma were collected along with 19 control samples taken from lymph nodes with reactive hyperplasia. All the related slides were re-evaluated by 2 pathologists and diagnosis of lymphoma was established based on morphology and immunohistochemistry staining. All the specimens were routinely fixed in 10% neutral buffered formalin for 24-48 hours, processed and embedded in paraffin blocks. Paraffin embedded blocks were a few months to 5 years old. Samples were coded and then based on the amount of tissue embedded in each block, 10-15 micron thick tissue sections were sliced off and transferred into the micro-tubes. The mean age of under study patients was 46 years (range 14-82 yrs). There were 41 males and 26 females.

DNA preparation: DNA was extracted from the tissue sections using phenol chloroform technique. Obtained slides were prepared for extraction in 1.5 ml tubes. First, samples were deparaffinated in 3 phases using xylene: 1000 microliter xylene was added to each tube and tissue sections were processed with alcohol in 2 phases. After each phase, the solution was centrifuged (12,000 cycles) for 5 minutes. The alcohol was discarded and the tubes were dried. Then 200-400 microliter lysis buffer (50 mM Tris, 1 mM EDTA, 20 microliter proteinase K, 5% Tween 20) was added and the tubes were incubated for 24 hours at 60°C and then subjected to extraction with phenol chloroform. At the end, the extracted DNA was preserved in a solution using Tris-EDTA buffer and prepared for the PCR.

PCR: DNA Polymerase Chain Reaction: First, for each specimen 2 separate tubes were prepared as parallel. PCR of the beta-globin gene was performed in order to ensure the method of extraction and quality of DNA and then polymerization reaction was performed using FR2 and FR3 primers. The employed technique was semi-nested PCR.

Adequate master mix conditions for FR2 and FR3 PCR: Total volume of the reaction was 50 microliter that included of 25 pmol each of primer , 200 µM each of dNTP and 4mM MgCl2 in 1X buffer 20mM(NH4)2SO4 ,75mMTris-HCl ,Tween20 1%),100ng of template DNA and 1.5 unit of Taq polymerase.(Fermantase, EU) were used.

FR2/JH primers were applied in the first phase and FR2/VLJH primers were used in the second phase of PCR. In semi-nested PCR, FR3 primers were used instead of FR2. The amount of each of these primers in the master mix was 40 picomole.

The upstream FR2 or FR3 specific primer sequence is as follows

Fr2:5- TGG {A/G} TCCCG {C/A} CAG {G/C}C{T/C}{T/C}CNGG-3 and

Fr3: 5- acacggc{c/t}{g/c}tgtattactgt-3)

The downstream primer was derived from the JH region with the sequence of:

5-TGAGGAGACGGTGACC-3. for nested PCR, the downstream VLJH primer was used:

5-gtgaccagggtnccttggccccag-3 (3-5)

The PCR technique: For primary denaturation: 30 cycles for 3 minutes at 94°C, 40 seconds at 94°C, 40 seconds at 55°C and 40 seconds at 72°C.

For FR3 reaction: Denaturation for 3 minutes at 94°C and 28 cycles for 45 seconds at 94°C, 45 seconds at 60°C and 45 seconds at 72°C.

Analysis of the PCR products: In order to analyze the PCR products 3 methods including; conventional technique using 3% agarose gel with ethidium bromide staining; 6% acrylamide gel with silver staining with or without performing heteroduplex analysis were used.

Three microliter of PCR products were transferred onto the agarose gel and stained with ethidium bromide. If the band was in 60-120 bp range, sample was considered FR3 monoclonal and if the band was in the 200-300 bp range it was considered FR2 monoclonal. If a smear pattern was seen in both cases, sample would be considered as polyclonal.

Another technique employed was heteroduplex analysis and electrophoresis with polyacrylamide gel and then silver staining. In this technique, PCR products were first stored at 94°C for 10 minutes and then underwent an ice shock for an hour. They were then transferred on acrylamide gel 6% and silver stained. In the end, electrophoresis of the samples was performed on acrylamide gel along with silver staining without performing heteroduplex analysis.

RESULTS

Sixty-seven patients including 26 female and 41 male and a mean age of 46 years (range 14-82 yrs) were included(Table 1). Pathologic diagnosis of the samples included 57 cases of diffuse large B cell lymphoma (85%), 4 cases of follicular lymphoma (6%), 4 cases of marginal zone lymphoma (6%), 1 case of lymphoblastic lymphoma (1.5%), and 1 case of Burkitt's lymphoma (1.5%).

After amplification with FR3 primers, single or dual band patterns in 51 out of 67 cases (76.1%) within 60-120 bp range appeared on the gel. Amplification with FR2 primers in monoclonal cases showed 1 or 2 bands in 200-300 bp range in 53 out of 67 patients (79.1%). Simultaneous use of FR2 and FR3 caused 1 or 2 bands in 62 cases (92.5%). In both cases, if it was polyclonal, a smear like pattern was observed.

Our control group consisted of 19 samples of lymph nodes with the diagnosis of reactive hyperplasia which all showed polyclonal pattern.

Sixty two samples (92.5%) showed monoclonal pattern on agarose gel with ethidium bromide staining and 51 cases (76.1%) showed monoclonal pattern on acrylamide gel with silver staining. Data are summarized in Table 2.

Electrophoresis with agarose gel: Sixty two out of 67 lymphoma patients (92.5%) showed

monoclonality; among those 53 cases (79.1%) were monoclonal for FR2; and 51 (76.1%) were monoclonal for FR3. Five cases showed polyclonal pattern.

Electrophoresis with acrylamide gel and silver nitrate staining: Clonality analysis was performed on all lymphoma samples using silver staining after performing PCR. Twenty six lymphoma cases showed monoclonal pattern with one of the primers (FR2 showed monoclonal and FR3 showed polyclonal or vice versa) and 26 cases demonstrated monoclonal pattern with both primers. Fifteen cases (14 cases of diffused large B cell lymphoma and 1 case of mucosa associated lymphoid tissue) were polyclonal for both primers. After performing heteroduplex analysis, 3 out of 5 cases which were morphologically diagnosed as lymphoma and had showed polyclonality on agarose gel and ethidium bromide staining showed monoclonality with silver staining. The 2 remaining samples were polyclonal with silver staining as well. Therefore, we can say that 65 out of our 67 cases (97%) were monoclonal.

Table 1: Clinico-pathologic data and clonality results in 67 cases with B cell non-Hodgkin lymphoma with both staining methods.

Sex	Age	Editium Bromid	Silver Stain	Diagnosis	Site of Biopsy
Male	67	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lymph node
Female	16	FR3 and FR2	Polyclonal	Diffuse Large B Cell Lymphoma	Mediastinum
Male	18	FR3 and FR2	Polyclonal	Diffuse Large B Cell Lymphoma	Ileum
Male	20	FR3 and FR2	FR2	Diffuse Large B Cell Lymphoma	Mediastinum
Male	20	FR3 and FR2	FR2	Diffuse Large B Cell Lymphoma	Mediastinum
Male	22	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lymph node
Female	22	Polyclonal	Polyclonal	Diffuse Large B Cell Lymphoma	Mediastinum
Female	23	FR2	Polyclonal	Diffuse Large B Cell Lymphoma	Lymph node
Male	23	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Mediastinum
Male	23	FR3 and FR2	Polyclonal	Diffuse Large B Cell Lymphoma	Mediastinum
Male	23	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lymph node
Male	25	FR3	FR3	Diffuse Large B Cell Lymphoma	Mediastinum
Male	25	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Pleura
Female	27	FR3 and FR2	Polyclonal	Diffuse Large B Cell Lymphoma	Lung
Male	28	FR3 and FR2	FR3	Diffuse Large B Cell Lymphoma	Mediastinum
Male	30	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lymph node
Female	30	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Mediastinum
Male	31	FR3	FR3 and FR2	Diffuse Large B Cell Lymphoma	Bone Marrow
Female	32	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Mediastinum
Male	33	FR3	FR3	Diffuse Large B Cell Lymphoma	Lymph node
Male	34	FR3	FR3	Diffuse Large B Cell Lymphoma	Mediastinum
Female	36	FR2	FR3	Diffuse Large B Cell Lymphoma	Lymph node
Male	39	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lymph node
Male	40	FR3 and FR2	Polyclonal	Diffuse Large B Cell Lymphoma	Lymph node
Male	41	Polyclonal	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lung
Female	41	FR3 and FR2	FR3	Diffuse Large B Cell Lymphoma	Bronchus
Male	42	FR2	FR2	Diffuse Large B Cell Lymphoma	Lymph node
Male	42	FR2	FR2	Diffuse Large B Cell Lymphoma	Thymus
Female	42	FR3 and FR2	Polyclonal	Diffuse Large B Cell Lymphoma	Kidney
Female	43	FR3 and FR2	FR2	Diffuse Large B Cell Lymphoma	Lymph node
Male	43	FR2	FR2	Diffuse Large B Cell Lymphoma	Lymph node
Male	44	FR3 and FR2	FR2	Diffuse Large B Cell Lymphoma	Ileum
Male	45	FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lung
Male	47	FR2	Polyclonal	Diffuse Large B Cell Lymphoma	Stomach

- Continued (Table 1).

Male	49	FR3 and FR2	FR3	Diffuse Large B Cell Lymphoma	Lymph node
Female	52	FR3 and FR2	FR2	Diffuse Large B Cell Lymphoma	Mediastinum
Female	53	Polyclonal	Polyclonal	Diffuse Large B Cell Lymphoma	Lymph node
Male	54	FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lymph node
Male	57	FR3	Polyclonal	Diffuse Large B Cell Lymphoma	Bronchus
Female	58	FR3	FR2	Diffuse Large B Cell Lymphoma	Lymph node
Male	60	FR3 and FR2	Polyclonal	Diffuse Large B Cell Lymphoma	Bone Marrow
Female	60	FR3 and FR2	FR3	Diffuse Large B Cell Lymphoma	Sinus
Male	63	FR3 and FR2	FR2	Diffuse Large B Cell Lymphoma	Mediastinum
Female	63	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Pleura
Female	63	FR3 and FR2	FR2	Diffuse Large B Cell Lymphoma	Lymph node
Female	64	FR2	FR2	Diffuse Large B Cell Lymphoma	Pleura
Male	65	FR3 and FR2	Polyclonal	Diffuse Large B Cell Lymphoma	Lymph node
Male	65	Polyclonal	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lymph node
Male	65	Polyclonal	FR2	Diffuse Large B Cell Lymphoma	Mediastinum
Male	68	FR3	Polyclonal	Diffuse Large B Cell Lymphoma	Lung
Female	70	FR3 and FR2	FR2	Diffuse Large B Cell Lymphoma	Mediastinum
Male	72	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Liver
Female	78	FR3 and FR2	FR2	Diffuse Large B Cell Lymphoma	Lung
Male	79	FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lymph node
Female	82	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Pleura
Male	17	FR3 and FR2	FR3	Diffuse Large B Cell Lymphoma	Lymph node
Female	46	FR3 and FR2	FR3 and FR2	Diffuse Large B Cell Lymphoma	Lymph node
Female	59	FR3 and FR2	FR3 and FR2	Follicular Lymphoma	Lymph node
Female	67	FR2	FR3 and FR2	Follicular Lymphoma	Lymph node
Female	70	FR3 and FR2	FR2	Follicular Lymphoma	Lymph node
Female	79	FR3 and FR2	FR3 AND FR2	Follicular Lymphoma	Lymph node
Male		FR3 and FR2	FR3 AND FR2	Lymphoblastic Lymphoma B	Lymph node
Female	38	FR3 and FR2	FR3 AND FR2	Marginal zone lymphoma	Lung
Male	70	FR3	Polyclonal	Marginal zone lymphoma	Lung
Male	17	FR3 and FR2	FR2	Marginal zone lymphoma	Lymph node
Male	72	FR3	FR3 and FR2	Marginal zone lymphoma	Thyroid
Male	53	FR3 and FR2	FR3 and FR2	Burkitt's	Bone Marrow

Table 2. Comparison of clonality results with both FR2 and FR3 primers with each of the silver and ethidium bromide staining.

	FR3 (%)	FR2 (%)	FR3+FR2 (%)	Polyclonal (%)	Total
Agarose Gel	51 (76.1)	53 (79.1)	62 (92.5)	5 (7.5)	67
Silver Stain	35 (52.2)	43 (64.2)	51 (76.1)	15 (22.4)	67

DISCUSSION

Determination of clonality is an important complementary technique for the diagnosis of malignancy in lymphoproliferative diseases. At present, PCR-based techniques for detection of clonality in lymphoproliferative diseases are a good substitute for Southern blot analysis. However, primers and method of detection of PCR products can affect the results. In this study by using FR2 and FR3 primers and heteroduplex analysis of PCR products, 92% of malignant lymphomas showed clonality. Most studies use consensus primers that complement one of the segments of the framework of variable and joining regions. Sensitivity and specificity of PCR have shown to be 75%-90% in different studies using these primers. However, most of these studies have not used heteroduplex analysis and have employed simple gel technique (1).

When using primers for the VDJ segment of the

immunoglobulin heavy chain (IgH) gene, we can expect 15-30% false negative results based on the type of primer used and because of the somatic hypermutation (3, 6-8).

False negative results are due to the improper annealing of the primer to the rearranged segment which per se might be due to 2 different reasons: first is that precise detection of all gene segments of V, D and J requires the use of large number of primers which is not feasible. Secondly, mismatch primers may be due to somatic hyper-mutation which commonly occurs in cases of mature post germinal center lymphoma and can result in false negativity.

In our study, 2 cases with false negative results were post germinal center large cell lymphoma. This can be due to the lack of annealing of the used primers with the rearranged gene segment in this type of lymphoma.

False positive results can be due to the presence of lymphocytes which include a population of polyclonal B cells. In order to avoid false positive results, heteroduplex analysis can be used. In this technique, PCR products are first denatured and then double stranded molecules are formed by dramatic decrease of the temperature in the form of homoduplex in monoclonal cases and heteroduplex in polyclonal cases. Although no false positive result was detected when using simple agarose gel technique, heteroduplex analysis and use of polyacrylamide gel and silver staining increased sensitivity and resulted in detection of 3 more cases of large cell lymphoma (65 out of 67 cases) (97%)which had been shown negative by the first technique.

Determination of clonality has its own limitations. For example, presence of clonality is not necessarily indicative of malignancy; as we can see in monoclonal gammopathies and primary phase of EBV positive lymphoproliferative diseases in immunodeficient patients. Therefore, molecular clonality results should always be interpreted in conjunction with morphology and immuno-phenotyping studies (1).

CONCLUSION

Using PCR by applying 2 primers of FR2 and FR3 and heteroduplex analysis has a high sensitivity for detection of non-Hodgkin lymphomas and can be employed as a complementary test for the diagnosis of malignant lymphoproliferative disorders.

REFERENCES

1. van Dongen JJ, Langerak AW, Brüggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. ***Leukemia*** 2003; 17 (12): 2257- 317.
2. McPherson RA, Pincus MR, Henry's Clinical Diagnosis and Management by Laboratory Methods. 21st ed. Philadelphia, PA: Saunders; 2007.
3. Aubin J, Davi F, Nguyen-Salomon F, Leboeuf D, Debert C, Taher M, et al. Description of a novel FR1 IgH PCR strategy and its comparison with three other strategies for the detection of clonality in B cell malignancies. ***Leukemia*** 1995; 9 (3): 471- 9.
4. Yamada M, Hudson S, Tournay O, Bittenbender S, Shane SS, Lange B, et al. Detection of minimal disease in hematopoietic malignancies of the B-cell lineage by using third-complementarity-determining region (CDR-III)-specific probes. ***Proc Natl Acad Sci U S A*** 1989; 86 (13): 5123- 7.
5. Chuang SS, Lin CN, Shen FC, Liao PJ, Liao YL, Chang JH, et al. Detecting clonal rearrangement in non-Hodgkin's lymphomas in Taiwan by polymerase chain reaction. ***Leuk Lymphoma*** 2003; 44 (1): 117- 21.

6. Rockman SP. Determination of clonality in patients who present with diagnostic dilemmas: a laboratory experience and review of the literature. ***Leukemia*** 1997; 11 (6): 852- 62.
7. McCarthy KP, Sloane JP, Wiedemann LM. Rapid method for distinguishing clonal from polyclonal B cell populations in surgical biopsy specimens. ***J Clin Pathol*** 1990; 43 (5): 429-32.
8. Segal GH, Wittwer CT, Fishleder AJ, Stoler MH, Tubbs RR, Kjeldsberg CR. Identification of monoclonal B-cell populations by rapid cycle polymerase chain reaction. A practical screening method for the detection of immunoglobulin gene rearrangements. ***Am J Pathol*** 1992; 141 (6): 1291- 7.