DETECTION OF BCL-2 GENE REARRANGEMENT IN FOLLICULAR LYMPHOMA BY POLYMERASE CHAIN REACTION AND CHEMILUMINESCENCE TECHNIQUE

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The incidence of follicular lymphoma in Saudi Arabia is very low compared to that in Western countries. We analyzed 22 diagnosed cases, based on conventional morphology examination and immunohistochemistry, to detect the Bcl-2 gene rearrangement by polymerase chain reaction (PCR). The DNA was extracted from formalin-fixed paraffin-embedded lymph node tissues by the standard xylene treatment and proteinase K digestion method. Rearrangement of the major breakpoint region was evident in 8 of the 22 cases (36%), determined by visualization of a discrete band hybridized with a chemiluminescence-labeled specific probe. Although the number of cases is small, we believe it denotes a normal detection rate for PCR analysis, using DNA isolated from fixed tissue. With the exception of follicular lymphoma, non-Hodgkin's lymphoma (NHL) analyzed included diffuse large cell lymphoma, lymphoblastic lymphoma, chronic lymphocytic leukemia, mucosa-associated lymphoid tissue and mantle zone lymphomas. No Bcl-2 gene rearrangement was detected in any of these cases. No evidence of Bcl-2 minor cluster sequence gene rearrangement was detected in any of the 38 NHL cases analyzed. *Ann Saudi Med 1997;17(4):423-426.*

Rearrangement of Bcl-2 gene is the molecular consequence of the t(14;18) chromosomal translocation, which is found in approximately 30%-90% of follicular lymphomas (FL) detected by Southern blot analysis.[1-3] The wide range in the frequency of Bcl-2 gene rearrangement in FL may be attributed to geographic differences in the incidence of follicular lymphoma, the predominance of cell type in morphologic examination (as a higher incidence has been observed in small cleaved cells), the status of DNA utilized (intact versus degraded), the method of DNA isolation, as well as the technical procedure.

PCR amplification of Bcl-2 gene rearrangement is a highly sensitive procedure, capable of detecting one malignant cell in 10^5 to 10^6 non-neoplastic cells. However, several limitations should be kept in mind when interpreting the results of these studies. Negative results do not exclude the presence of disease, as only 50%-60% of FL contains Bcl-2 gene detectable by PCR.[4-6] The number of positive cases is further decreased by approximately 20% when only fixed tissue is available.[7,8] Furthermore, follicular lymphomas with t(14;18) breakpoints that occur outside the major breakpoint region (MBR) and the minor cluster sequence (MCS), representing approximately 20%-30% of cases, will be missed with PCR.[5,9]

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Materials and Methods

Genomic DNA was obtained from paraffin-embedded lymph node specimens by standard xylene treatment and proteinase K digestion. Tissue was scraped from the paraffin block using a clean razor blade. Paraffin was removed with two washes of xylene. The tissue was washed twice with 100% ethanol, then dried in an AS160 Automatic SpeedVac (Savant Instruments, Farmingdale, N.Y., USA). The tissue was incubated at 56 °C in 500 µL of digestion buffer containing Tris, EDTA and SDS [10] and 100 µg/mL proteinase K until the tissue appeared dissolved, usually 24 to 72 hours. A 1:5 dilution of this digest was made in water and 1 µL of the dilution was used to confirm the presence of amplifiable DNA by PCR amplification of the erbB-2 gene.

Enzymatic amplification of the Bcl-2 major breakpoint region rearrangement was performed in a Perkin Elmer GeneAmp PCR System 9600 using an oligonucleotide primer of a region 5' to the Bcl-2 MBR and a single consensus J_H primer. The 5' Bcl-2 MBR primer was synthesized on an Applied Biosystem DNA Synthesizer and the J_H primer was synthesized by Oligos, Etc. (Portland, Oregon, USA). The sequences were Bcl-2 MBR

5' GAG AGT TGC TTT ACG TGG CCT G 3'[11] and J_H 5' AAC TGC AGA GGA GAC GGT GAC C 3'. The reaction mixture contained 10 mM of TrisHC1, pH 8.3; 50 mM KC1; 1.5 mM $MgC1_2$; 0.001% w/v gelatin; 200 uM of each dNTP; 20 pmol/L of each primer; 1 U Taq polymerase (Ampli-Taq, Perkin Elmer-Cetus, Norwalk, CT, USA) and 1 µL of DNA digest in a final volume of 25 µL. The mixture was subject to 30 cycles of PCR following an initial five-minute denaturation step at 94 °C. Each cycle consisted of 30-second denaturation step at 94 °C, a 30-second annealing step at 60 °C and a one-minute elongation step at 72 °C. The last cycle was followed by a nine-minute elongation step at 72 °C. Ten microliters of PCR-amplified product was resolved by electrophoresis on a 1.5% agarose gel and transferred to a nylon membrane. A discrete band of approximately 200 bp is expected for positive results. The membrane was hybridized at 55 °C with the probe 5' CAA CAC AGA CCC ACC CAG AGC 3'.[11] This probe was labelled for chemiluminescent detection using the ECL3' Labelling and Detection Kit (Amersham International, Buckinghamshire, England). In this labelling method,[13] a fluorescein-11-dUTP tail is added to the probe's 3' end using terminal transferase. Following hybridization and stringency washes, the blots are incubated with anti-fluorescein-horseradish peroxidase conjugate. Excess antibody conjugate is removed by washing. The bound peroxidase labelled probe is detected with chemiluminescence detection reagents. When these two reagents are mixed together, the enzymatic reduction of peroxide is coupled to the oxidation of luminol. As the luminol breaks down, 428 nm wavelength light is emitted and detected on X-OMAT AR film (Eastman Kodak Company, Rochester, New York, USA). Amplification of the Bcl-2 minor cluster sequence rearrangement was performed in a Perkin Elmer GeneAmp PCR System 9600, using an oligonucleotide primer of a region 5' to the Bcl-2 MCS and a single consensus J_H primer. The primers were synthesized by Oligos, Etc. (Portland, Oregon, USA). The sequences were Bcl-2 MCS 5' GAC TCC TTT ACG TGC TGG TAC C 3'[12] and J_H5' AAC TGC AGA GGA GAC GGT GAC C 3'. The reaction mixture contained 10 mM of TrisHC1, pH 8.3; 50 mM KC1; 1.5 mM $MgCl_2$; 0.001% w/v gelatin; 200 µM of each dNTP; 100 ng of each primer; 1 U Taq polymerase (Ampli-Taq, Perkin Elmer-Cetus, Norwalk, CT, USA) and 1 µL of DNA digest in a final volume of 25 µL. The mixture was subject to 35 cycles of PCR following an initial five-minute denaturation step at 94°C, a 30-second annealing step at 55 °C and a one-minute elongation step at 72 °C. The last cycle was followed by a nine-minute elongation step at 72 °C. Ten microliters of PCR-amplified product was resolved by electrophoresis on a 1.5% agarose gel and transferred to a nylon membrane. This membrane was hybridized at 50 °C with the chemiluminescent-labelled probe 5' GAT GGC TTT GCT GAG AGG TAT TG 3'.

Results

A total of 38 cases of non-Hodgkin's lymphoma (NHL) were submitted to the Diagnostic Molecular Pathology Section of the Department of Pathology and Laboratory Medicine at King Faisal Specialist Hospital and Research Centre, for the detection of Bcl-2 gene rearrangement. Twenty-two cases with morphologic and immunophenotypic diagnosis of nodal follicular lymphoma (FL) were included. All lymph nodes were formalin-fixed and paraffin-embedded tissues. The DNA was extracted with standard xylene treatment and proteinase K digestion method. Rearrangements of the major breakpoint region (MBR) were evident in 8 out of 22 cases (36%) of FL determined by visualization of a discrete band hybridized by a specific probe and detected by the chemiluminescence technique.

Two cases were repeatedly positive in lymph nodes, bone marrow and peripheral blood. The first case was a 44-year-old female who presented in March 1993 with abdominal mass and generalized lymphadenopathy. Lymph node from the right axilla was examined and the diagnosis of follicular lymphoma, predominantly small cleaved cell, was established. Molecular studies were performed which showed positive Bcl-2 gene rearrangement by PCR. At this time, the bone marrow was involved and the patient received treatment with chemotherapy. In December 1995, the patient presented with recurrence. The biopsy of axillary lymph node and liver showed recurrent lymphoma with progression to large cell type. The bone marrow was again involved and the sample submitted to the molecular diagnostics laboratory demonstrated the same Bcl-2 gene rearrangement.

The second case was an axillary lymph node of a 67-year-old male submitted for Bcl-2 gene rearrangement with a peripheral blood sample. Both showed the same size band of Bcl-2 gene rearrangement. Repeated peripheral blood sample was done late in 1995 and again showed the same band size. The remaining 14 cases (64%) of follicular lymphoma (FL) showed no evidence of Bcl-2 (MBR) gene rearrangement.

Rearrangement of the minor cluster sequence (MCS) of the Bcl-2 gene was examined in all 22 cases of FL. However, none demonstrated amplifiable PCR products.

The remaining 16 negative cases for MBR and MCS Bcl-2 gene rearrangement included diffuse large cell lymphoma, lymphoblastic lymphoma, chronic lymphocytic leukemia, mucosa-associated lymphoid tissue and mantle zone lymphomas, and lymphoid hyperplasia.

Discussion

Rearrangement of Bcl-2 gene is the molecular consequence of the t(14;18) chromosomal translocation which is found in approximately 30%-90% of follicular lymphomas.[1-3] The highest detection rate has been reported by Southern blot analysis. However, it is recognized that this technique has several limitations, including length of the procedure, problems of radiation safety and disposal (since the best results are obtained when radio-labeled probes are used), the requirement of large amounts of intact high molecular weight DNA, the high numbers of malignant cells containing the rearrangement which are needed in order to be detected by auto-radiography, and the need for highly skilled laboratory staff to perform this complex procedure. PCR of the Bcl-2 gene rearrangement is a highly sensitive procedure, capable of detecting one malignant cell in 10^5 to 10^6 non-neoplastic cellular elements,[11,14,15] but several limitations should be kept in mind when interpreting the results of these studies. A negative analysis does not always exclude the presence of disease, as only 50%-60% of follicular lymphoma contains the translocation t(14;18) detectable by PCR.[4-6] Liu et al. have shown that the number of positive cases is further decreased by approximately 20% when only fixed tissue is available.[7,8] Furthermore, follicular lymphomas with t(14;18) breakpoints that appear outside the MBR and MCS, representing approximately 20%-30% of cases, will be missed with PCR technique.[5,9] In this retrospective study of archived material, we used DNA derived from formalin-fixed paraffin-embedded lymph node tissue samples, and detected Bcl-2 gene rearrangement in 8 out of 22 cases (36%) of follicular lymphomas diagnosed by conventional morphologic examination and immunohistochemistry. Geographical or racial differences in the incidence of non-Hodgkin's lymphoma and, in particular, follicular lymphoma, as well as the detection rate of Bcl-2 gene rearrangement have been reported in the literature.[16-21] The prevalence of follicular lymphoma in Saudi Arabia is less than 10% of all non-Hodgkin's lymphoma, with diffuse large cell lymphoma being the most common histologic type at the time of presentation.[22] Similar low incidence of follicular lymphoma is also reported from Japan[17] and Hong Kong.[21] However, the low incidence of Bcl-2 gene rearrangement detection in the Japanese patients[20] analyzed by Southern blot technique and cytogenetics is not confirmed in the Chinese group,[21] which had a rate of detection up to 57% of follicular lymphomas, supporting the notion that Bcl-2 translocation is a consistent marker for follicular lymphoma, irrespective of ethnic differences. Furthermore, the study from Japan included a small number of cases, and with large-scale combination studies of cytogenetics, Southern blot analysis and PCR amplification techniques are required. Nasrin et al.[23] have reported a low detection rate (15%) of Bcl-2 gene recombination among 13 cases of follicular lymphomas, suggesting a low incidence of Bcl-2 translocation in follicular lymphomas in Saudi Arabia compared to the Western patients. However, this low detection rate could be due to the small number of cases, the type of tissue (as the majority of lymph nodes analyzed were of formalin-fixed paraffin-embedded material), and the quality of DNA extracted.

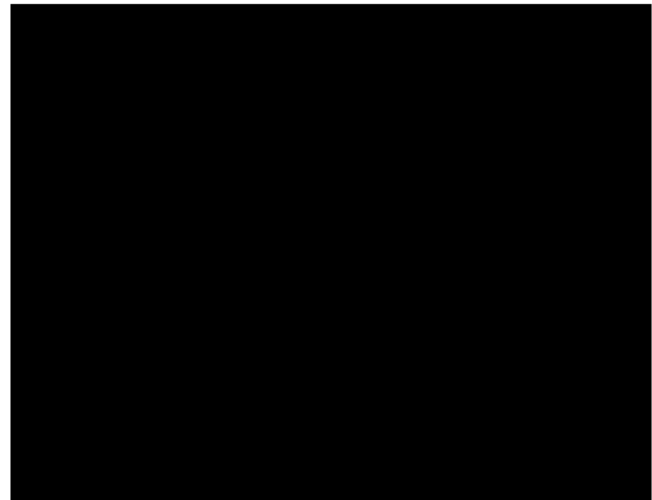

FIGURE 1. Ethidium bromide-stained 6% polyacrylamide gel of PCR products amplified with the Bcl-2 MBR and single-consensus J_H primers. M) 100 base pair ladder marker, 1) follicular lymphoma, 2) follicular lymphoma, 3) follicular lymphoma, 4) follicular lymphoma, 5) follicular lymphoma (negative), 6) positive control, 7) chronic lymphocytic leukemia, 8) normal DNA, and 9) water.

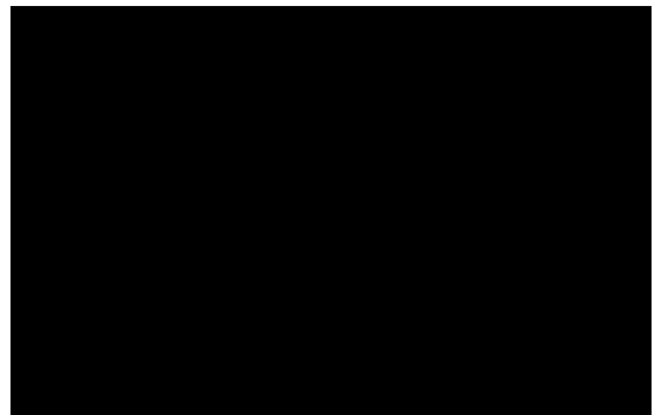

FIGURE 2. Detection by chemiluminescence technique of Bcl-2 translocation amplified by PCR. Lane 1) Follicular lymphoma, 2) follicular lymphoma (faint band), 3) follicular lymphoma, 4) follicular lymphoma, 5) follicular lymphoma, 6) positive control, 7) chronic lymphocytic leukemia, 8) normal DNA, and 9) water.

Using primers for the Bcl-2 gene, we were able to detect rearrangements in 8 out of 22 cases (36%) of follicular lymphoma in formalin-fixed paraffin-embedded

lymph node tissues hybridized with specific probe and detected by chemiluminescence technique. Although the number of cases is small compared to Western studies, the rate of detection in our study falls within the range reported by others for PCR analysis using DNA from fixed material.[7,8,24]

The PCR is highly sensitive and false-positive results due to contamination are possible. In this study, contamination was unlikely because the t(14;18) PCR products were, as expected, of varying sizes (Figure 1), except for those cases with reproducible bands on different materials examined, such as bone marrow aspirates and peripheral blood. Furthermore, all cases were hybridized with specific probe and detected by the chemiluminescence technique (Figure 2). All positive samples were from follicular lymphomas (8/22) and none of the other 16 non-Hodgkin's lymphoma cases were positive, including cases of lymphoblastic lymphoma, diffuse large cell lymphoma, chronic lymphocytic leukemia, mucosa-associated lymphoid tissue and mantle zone lymphomas. The presence of amplifiable DNA was confirmed by amplification of the erbB-2 gene in all specimens, denoting no false-negative results due to DNA quantity. The minor breakpoint cluster sequence (MCS) for the Bcl-2 gene was examined in all 38 specimens, however, no single case was detected.

In conclusion, the present study serves to further define the utility of PCR analysis for detection of the Bcl-2 gene rearrangement in formalin-fixed samples and the high specificity when hybridized with specific probe and detected by a quick method such as chemiluminescence. The presence of PCR products can be used to test for minimal residual disease in peripheral blood and/or bone marrow samples following chemotherapy or bone marrow transplantation. The incidence of Bcl-2 gene rearrangement (36%) in formalin-fixed paraffin-embedded tissue in the present study is within the range reported by others.[7,8,24] We believe that the detection rate of t(14;18) depends on several factors, including the availability of fresh tissue compared to fixed material, the molecular technique, Southern blot versus PCR, the method of DNA extraction from fresh and fixed tissues, and the hybridization with specific DNA probes.

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