The detection of specific gene rearrangements in non-Hodgkin's lymphoma using the polymerase chain reaction

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Summary  Characteristic gene rearrangements are present in most non-Hodgkin's lymphomas (NHL). These are usually detected by Southern blotting techniques. In this study, the ability of the polymerase chain reaction (PCR) to detect the t(14;18) chromosomal translocation and immunoglobulin heavy chain (Igh) gene rearrangement was evaluated. DNA from 14 follicular and 42 diffuse B-cell lymphomas was examined using oligonucleotide primers specific for opposing sides of the Igh gene rearrangement on chromosome 14 (towards conserved VH and JH sequences) and opposing sides of the t(14;18) chromosomal translocation (towards the major breakpoint region of the bcl-2 gene on chromosome 18 and conserved Jκ sequence on chromosome 14). The t(14;18) translocation was detected in 57% of follicular lymphomas and 21% of diffuse B-cell lymphomas. Clonal Igh gene rearrangements using PCR were detected in 50% follicular and 52% of the diffuse lymphomas. Either or both of these rearrangements were detected in 93% follicular and in 59% of diffuse lymphomas. PCR is a rapid and easy technique that can detect the abnormal rearrangement of the bcl-2 gene and clonal Igh rearrangement, indicating the presence of lymphoma. This may be of benefit in monitoring response to therapy and in predicting prognosis in this disease.

Malignant lymphomas are a heterogeneous group of tumours with varied clinicopathological features, response to therapy and overall survival. The success of chemotherapy in curing over 50% of patients with intermediate and high grade lymphomas poses new challenges in the management of these tumours (Fisher et al., 1991; Jagannath et al., 1985). Identifying tumour specific prognostic factors that can accurately monitor or predict the behaviour of individual lymphomas may select patients who can benefit from more or less intensive therapy, reduce the morbidity of therapy for some and increase the potential for cure in others. Accurate monitoring of disease may detect residual disease in patients apparently in remission and thus detect recurrence at an earlier stage of tumour progression. Our present understanding of the molecular biology of lymphomas has identified normal and abnormal molecular events which may be used to monitor the disease, understand the pathogenesis and predict prognosis.

During maturation of normal and malignant B-lymphocytes somatic rearrangement of DNA occurs in the immunoglobulin heavy chain (Igh) gene (Tonegawa, 1983). B-cell lymphomas are clonal tumours with genetically identical cells and therefore all the cells have identical immunoglobulin (Ig) gene rearrangements thus providing a unique marker for monitoring disease (Waldmann, 1987).

A number of chromosomal translocations that occur in B-cell Non-Hodgkin's lymphoma (NHL) are associated with the juxtaposition of oncogenes from their original chromosomes, to sites adjacent to antigen receptor genes e.g. the immunoglobulin gene (Yunis et al., 1982; Dalla-Favera et al., 1982; Taub et al., 1982; Croce & Nowell, 1985).

The t(14;18) chromosomal translocation, occurring in over 80% of follicular lymphomas, and in approximately 10-30% of diffuse lymphomas, is the single most common chromosomal abnormality occurring in NHL (Yunis et al., 1987). In this translocation the bcl-2 oncogene is translocated from chromosome 18 to a site juxtaposing the joining (Jκ) region of the Igh gene on chromosome 14 (Tsujimoto et al., 1985a; Cleary & Sklar, 1985; Bakhshi et al., 1985; Cleary et al., 1986a).

These chromosomal changes and abnormalities are detectable using cytogenetics, Southern blotting and more recently the polymerase chain reaction (PCR). While cytogenetics and Southern blotting provide useful information, they have limitations. Karyotype analysis, which provided the original information on chromosomal abnormalities (Yunis, 1981; Rowley & Fukuhara, 1980) is a lengthy procedure and requires highly specialised interpretation. At the molecular DNA level, Southern blotting is also used to identify specific chromosomal changes and to detect clonal Ig rearrangements, however this technique required the use of radioisotopes and is also time consuming.

The PCR is a rapid and sensitive method in which identified and targeted sequences of DNA are exponentially amplified and thereby become detectable. The efficient amplification of a known DNA sequence requires the use of DNA primer sequences on either side of the targeted DNA sequence (Saiki et al., 1988). Using PCR primers which anneal to opposing sides of targeted areas of the Ig gene rearrangements and chromosomal translocations, it is possible to amplify a sequence of DNA that is unique to the lymphoma cells.

The more common rearrangements identified in B-cell NHL include clonal IgH rearrangement and the t(14;18) translocation. In this study the ability of PCR assays to identify these specific molecular events in patients with B-cell NHL was evaluated. The detection of these and other molecular abnormalities in NHL may help to understand its pathogenesis, be of prognostic significance and be of benefit in monitoring the disease.

Materials and methods

Patient materials

DNA was extracted using standard techniques (Sambrook et al., 1989) from snap frozen, lymph node tumour biopsy specimens, obtained from 56 patients. All tumours were confirmed histopathologically to be non-Hodgkin's lymphomas and the B-cell phenotype was confirmed by immunohistochemistry (Picker et al., 1987).

Primers

Clonal rearrangement of the IgH gene was detected, by using two primers to amplify the DNA sequence across the Ig gene.
V_{H}D_{0}J_{H} rearrangement (McCarthy et al., 1990) (Figure 1). An oligonucleotide primer complementary to the majority of variable segments (V_{H} consensus sequence) was used in conjunction with a consensus joining region (J_{H}) oligomer. In the non-rearranged immunoglobulin heavy chain gene, the V_{H} and J_{H} regions are separated by a distance of 100 kb and are thus, not amenable to amplification (Tonegawa, 1983).

Approximately 60% of breakpoints on chromosome 18 occur at the major breakpoint region (MBR) over a 150 bp region in the bcl-2 gene (Cleary et al., 1986b; Lipford et al., 1987). The presence of t(14;18) was detected using two oligonucleotide primers, one complementary to the bcl-2 gene close to the MBR and the J_{H} consensus sequence, to amplify the DNA sequence across the t(14;18) breakpoint fragment, i.e. bcl-2-J_{H} rearrangement (Lee et al., 1987; Cresczenzi et al., 1988) (Figure 1). In normal genomic DNA the bcl-2 gene and J_{H} region would be on different chromosomes and thus not exponentially amplifiable by PCR. The DNA sequences of the primers used are shown in Table I.

**PCR conditions**

One μg purified DNA was subjected to 45 cycle PCR amplification using the Perkin-Elmer DNA thermal cycler. The reaction mix (100 μl) contained 10 mM Tris-Cl, 1.5 mM MgCl$_2$, 50 mM KCl, 0.3 μM (primers 1 and 2, Table I) or 0.2 μM (primers 2 and 3, Table I), 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2.5 U Taq polymerase (Amplitaq, Cetus) and 1 μg DNA template. The reaction mixture was overlaid with 60 μl of light mineral oil (Sigma). The PCR cycle consisted of denaturation at 94°C for 2 min, annealing at 61°C for 3 min and synthesis at 72°C for 3 min.

| Primers | Sequences |
|---------|-----------|
| 1. V_{H} | 5' CTG-TCG-ACA-CCG-CCG-TGT-ATT-AGT-G 3' |
| 2. J_{H} | 5' ACC-TGA-GGA-GAC-GTG-GAC-GGT 3' |
| 3. bcl-2 (MBR) | 5' TTA-GAG-AGT-TGC-TTC-TA-CG-TG 3' |
| 4. bcl-2 (internal hybridisation probe, alkaline phosphatase labelled) | 5' GCC-TGT-TTC-AAC-ACA-GAC-CC 3' |

**Results**

DNA from 56 biopsy specimens obtained from patients with confirmed NHL, including 42 diffuse lymphomas and 14 follicular lymphomas was subjected to PCR amplification using the bcl-2/J_{H} and V_{H}/J_{H} primers.

The t(14;18) translocation was detected in 8/14 (57%) of follicular lymphomas and 9/42 (21%) of the diffuse B-cell lymphomas. In Figure 2 examples of the PCR amplified breakpoint fragments across the t(14;18) translocation junction are shown for a number of lymphoma samples (lanes 1–7). From other studies, it is known that the bcl-2/J_{H} breakpoint fragments vary in size between 100–300 bp in length (Shibata et al., 1990). Under the PCR conditions, used with an annealing temperature of 61°C, background amplification products consistently appear at approximately 600 bp and greater in most samples (Figure 2; lanes 3–11).

Amplification of bcl-2/J_{H} products was confirmed by
Southern blotting using an internal bcl-2 probe (Figure 3; positive samples lanes 1–7). No hybridisation was observed in the negative samples (lanes 8–11). In the positive samples (lanes 3, 4, 5, 7) there is hybridisation of larger PCR products (~500 bp or greater). This is probably due to the use of the consensus Jα primer which may anneal to other adjacent Jα regions, if present, as a consequence of the IgH rearrangement (see Figure 1). This would result in the appearance of larger PCR products, however, sequencing information would be necessary to confirm this. The slight variation in size of the breakpoint fragments between individual samples is due to firstly, the variation in the exact location of the breakpoint in the bcl-2 gene and secondly, the introduction of extranucleotide regions (N-segments) during the rearrangement process of the IgH gene Dα-Jα regions, during which the t(14;18) translocation is thought to occur (Desiderio et al., 1984).

Using the consensus Vα and Jα primers, amplification of the Vα-Dα-Jα junction was carried out. In 50% (n = 14) of follicular and 52% (n = 42) of diffuse lymphomas amplified breakpoint fragments, indicative of clonal B-cell populations, were observed using the Vα/Jα primers. In clonal B-cell populations this results in the amplification of a single or double band (Figure 4). Again, due to the insertion of the extranucleotide N-regions during the rearrangement process there is variation in the size of the breakpoint fragments (75–200 bp) obtained between individual lymphoma samples. Larger bands are also observed in some samples with the Vα/Jα primers, which as described above could be due to priming of adjacent Jα regions or due to background amplification products (although, they do not appear in the control tonsil tissue (T)). Two Vα/Jα breakpoint fragments are apparent in lane 4 (Figure 4) and may represent IgH rearrangement of both alleles or the presence of two B-cell clones in the tumour. In both diffuse and follicular lymphoma samples, 14% had breakpoint amplification products with both sets of primers.

The ability of PCR to detect the presence of a malignant clone of lymphocytes in tissue samples may be potentially the most valuable application of this technique. Combining the results from using either or both sets of primers to detect the t(14;18) translocation and clonal B-cell populations, for individual tissue samples, it was possible to detect the malignant clone in 93% follicular lymphomas and 59% of diffuse B-cell lymphomas (Table II).

Discussion

Our results demonstrate the ability of the PCR, to detect the presence of the t(14;18) translocation, and clonal IgH rearrangements in tissue from diffuse and follicular lymphomas. The presence of the t(14;18) translocation was demonstrated using a primer specific for the major breakpoint region. The majority of t(14;18) translocations on chromosome 18 occur within this well defined region which has been shown by sequence analysis to encode a portion of the 3′ untranslated region of the bcl-2 gene (Cleary et al., 1986b; Lipford et al., 1987). On chromosome 14 the breakpoints have been shown to occur with or near an IgH joining segment, and a single consensus Jα primer was used on this side of the breakpoint for PCR amplification. Using this set of primers for the MBR, the t(14;18) translocation was
Table II Summary of results of Follicular and Diffuse NHL patients with t(14;18) translocations and clonal B-cell populations as determined by specific PCR assays

| NHL subtype | No. of t(14;18) translocation (MBR) | PCR Amplification Clonal B-cell | PCR Amplification concurrently bcl-2/JH and VH/JH | Identification of malignant clone |
|-------------|------------------------------------|---------------------------------|-----------------------------------------------|----------------------------------|
| Follicular  | 14                                 | 8/14 (57%)                      | 7/14 (50%)                                    | 13/14 (93%)                     |
| Diffuse     | 42                                 | 9/42 (21%)                      | 22/42 (52%)                                   | 25/42 (59%)                     |

Our results for detection of MBR using the specific primers are similar to those found in other studies (Crescenzi et al., 1988; Shibata et al., 1990). We have also used two rounds of PCR amplification and single or nested sets of V<sub>H</sub>-J<sub>H</sub> primers, as suggested by Wan et al. (1990), but did not find that it significantly increased the detection rate of IgH rearrangements in B-cell NHL (data not shown). Due to the inclusion of variable N-regions and J<sub>H</sub> segments, bcl-2 breakpoints are highly variable, as shown by direct sequencing studies (Eick et al., 1990; Cotter et al., 1991). Clearly, all IgH gene rearrangements cannot be detected by a single set of primers, because of the variability in the targeted sequences, and the frequent occurrence of specific translocations at this locus. In order to reliably identify the malignant clone in all lymphoma samples, groups and nested sets of appropriate primers would be required.

The great advantage of PCR is its unique sensitivity and specificity in detecting targeted sequences of DNA and RNA in both fresh and paraffin-embedded tissue. It is easier to use and more widely applicable than either Southern blotting or karyotyping. In comparison to these techniques, analysis by PCR is much quicker and less labour intensive with results in less than 24 h as compared to 5–10 days. Apart from contamination, the other major drawbacks with PCR are, the inability to accurately quantify the volume of cells with the targeted abnormality and, because of the specificity of the oligonucleotide primers, any variation in the targeted sequence of DNA can give a false negative result.

The ease and rapidity with which the t(14;18) translocation and IgH rearrangements can be detected by PCR is useful in the detection of lymphoma, in the differential diagnosis of reactive from malignant infiltrations, and in differentiating between lymphoid and non-lymphoid tumours. Our study suggests that combining PCR reactions to detect the t(14;18) translocation and IgH rearrangements will allow detection of almost all follicular lymphomas (93%). Detecting the t(14;18) translocation may be of prognostic significance in patients with diffuse NHL. As a result of the sensitivity of this technique, the positive detection of V<sub>H</sub>-J<sub>H</sub> or bcl-2 rearrangements may be used in individual patients to monitor disease and determine their response to therapy. This may be of particular importance in detecting minimal disease in patients considered for autologous bone marrow transplantation.

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detected in 57% of follicular lymphomas. This correlates with the incidence found using Southern blotting for the MBR region (Weiss et al., 1987). The t(14;18) translocation occurs in approximately 85% of follicular lymphomas. However, a significant proportion of translocations (up to 25%) occur at a second site on chromosome 18, distal to the MBR region by 20 kb. This site is known as the minor cluster region (MCR) and would require a separate MCR specific primer, in conjunction with the J<sub>H</sub> primer, to allow PCR amplification (Cleary et al., 1986a; Ngan et al., 1989).

Each individual lymphocyte has a unique IgH gene rearrangement with a specific V<sub>H</sub>-D<sub>H</sub>-J<sub>H</sub> sequence. Each malignant lymphoma is composed of a clone of cells, all with the same IgH gene rearrangement. Using primers specific for consensu regions within the variable and joining segments, clonal IgH gene rearrangements were detected in the presence of a malignant clone in approximately 50% of both follicular and diffuse lymphomas. Our failure to detect a malignant clone in all cases of B-cell NHL is probably due to the heterogeneity of the targeted V<sub>H</sub>-D<sub>H</sub>-J<sub>H</sub> DNA sequences. Detection of clonal IgH gene rearrangements can thus provide valuable information for individual patients, but is of limited sensitivity as a tumour marker in detecting and monitoring disease in all B-cell NHL.

However, when the results of both PCR assays are combined for each patient, we found that the malignant clone could be detected in 93% of follicular and 59% of diffuse lymphomas. This suggests that using more than one set of primers in follicular lymphomas is of benefit in detecting and monitoring disease. A valuable use of PCR has been demonstrated by Gribben et al. (1991) in the detection of the t(14;18) translocation in marrow samples prior to transplantation. They presented strong evidence that reinfusion of malignant cells contributed to relapse in autologous marrow transplantation. The absence of residual lymphoma after immunologic purging of autologous marrow prior to transplantation was associated with a significant increase in disease-free survival and reduced relapse rate in these patients.

The significance of the t(14;18) translocation in the oncogenesis of NHL is not fully understood. It is thought to occur as an early event during IgH rearrangement. The translocation results in the increased expression of the bcl-2 oncogene, and this increased bcl-2 oncoprotein expression has been shown to promote survival of B-cells (McDonnell et al., 1991). The prognostic value of bcl-2 rearrangement in diffuse NHL remains controversial (Yunis et al., 1989). Detected in between 10–30% diffuse lymphomas, the bcl-2 rearrangement correlates with a poor response to therapy and shorter survival in a number of studies.
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