Sequence analysis of polymerase chain reaction amplified t(14;18) chromosomal breakpoints in formalin fixed, paraffin wax embedded follicular lymphoma

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Abstract

Aims: To determine whether junctional sequences of rearranged chromosomes can be amplified by use of the polymerase chain reaction (PCR) and whether direct sequence analysis of the PCR products is possible, using DNA from formalin fixed, paraffin wax embedded biopsy specimens.

Methods: DNA was extracted from paraffin wax embedded, formalin fixed lymphoma specimens, and junctional sequences of rearranged chromosomes were amplified by the PCR. The products were used as templates for asymmetrical PCR. Subsequently, direct sequence analysis was performed using the chain termination method.

Results: Formalin fixed, paraffin wax embedded biopsy specimens and PCR amplification could be used to determine the nucleotide sequences of junctional regions of rearranged chromosomes t(14;18) from patients with follicular lymphoma.

Conclusion: The identification of junctional sequences of the translocation in follicular lymphoma provides a molecular "fingerprint" of t(14;18) of the lymphoma of an individual patient and can be used for the detection of clone specific DNA in any biopsy tissue obtained from the patient. The strategy used for rapid sequence analysis of PCR amplified DNA sequences will be useful in many areas of molecular pathology.

A specific interchromosomal translocation t(14;18) (q32;q21) occurs in 70-85% of human follicular lymphomas and in about 20% of diffuse large cell lymphomas.¹⁻⁴ This rearrangement results in the transposition of a putative oncogene bcl-2 on chromosome 18 to a joining region (J_H) of immunoglobulin heavy chain genes on chromosome 14. The translocation results in a bcl-2/immunoglobulin fusion gene and a hybrid transcript, translated into a normal but much increased bcl-2 protein.⁵ The putative oncogenic potential of an activated bcl-2 gene has been shown in in vivo studies in mice, where gene transfer of bcl-2 resulted in a lymphoproliferative disease with substantial expansion of follicular centre cells.⁶ The trans-

location of the bcl-2 gene in lymphatic tissue can be detected by Southern blot analysis of endonuclease digested DNA. This technique, however, is associated with severe limitations. The translocation can only be identified if present in at least 1% of the cells of the analysed tissue. Furthermore, relatively large amounts (several micrograms) of high quality DNA are required, thus frequently limiting the analysis of DNA from preserved tissue.

In contrast, detection of specific DNA sequences by use of the polymerase chain reaction (PCR)⁷ is several orders of magnitude more sensitive than any conventional method and sequences can be detected even if present in only one cell among a background of about 10⁵-10⁶ cells.^{8,9} Small amounts (nanograms or less) of degraded DNA can also be used as a template for the PCR, thus facilitating the analysis of DNA from paraffin wax embedded tissue.

In most lymphomas with the t(14;18) translocation breakage on chromosome 18 occurs in a focused 150 base pair area within a major breakpoint region (mbr) 3' of the translated region of the bcl-2 gene.¹⁰ Breakage on chromosome 14 occurs at the immunoglobulin heavy chain locus within or 5' of one of the six J_H genes.⁵ The clustering of most breakpoints allowed for the design of oligonucleotide primers for PCR amplification of the junctional region produced by the translocation.^{9,11} This permitted the rapid and sensitive detection of t(14;18) chromosomal translocations in fixed, paraffin wax embedded tissues, when PCR products were subsequently analysed by oligonucleotide hybridisation techniques.^{12,13}

Although clustering in certain regions, the breakpoints on chromosome 14 and 18 occur at different sites in lymphomas of different clones. Furthermore, at the junction between rearranging chromosomes, short sequences of random nucleotides are inserted (N-region). This junctional sequence represents a specific molecular marker that is unique for an individual lymphoid clone. Accurate nucleotide sequence analysis of the junctional region of rearranged chromosomes leads to the identification of a molecular fingerprint, specific for the lymphoid malignant clone of an individual patient. Direct DNA sequence analysis of PCR amplified chromosomal breakpoints has been performed using DNA from fresh or snap-frozen

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Figure 1 Direct DNA sequence analysis of PCR amplified junctional region of rearranged chromosomes 14 and 18 of case 1 (left) and case 2 (right). In DNA from case 1, the *bcl-2* gene is transposed to a *J_{H4}* gene on chromosome 14 and five nucleotides are inserted. In DNA from case 2 the *bcl-2* gene translocates to a *J_{H6}* gene and an intervening sequence of 16 nucleotides is identified.

biopsy samples.¹⁴⁻¹⁶ We report the rapid determination of the nucleotide sequence of the junctional region using DNA from formalin fixed, paraffin wax embedded lymphoma tissue.

Methods

Two formalin fixed, paraffin wax embedded specimens of follicular lymphoma from two different patients, positive for the t(14;18) translocation as determined by the polymerase chain reaction and dot blot analysis, were chosen for this study. Specimen 1 was a biopsy specimen of an inguinal lymph node from a 42 year old man, who presented in 1983 with lymphadenopathy. Specimen 2 was a biopsy specimen of a submandibular lymph node, obtained in 1988 from a 40 year old man. Three adjacent 25 μ m thick sections were cut from each paraffin wax block and placed into two 1.5 ml microfuge tubes. The blade of the microtome was changed between samples to avoid cross-contamination. After 1 ml of xylene had been added the slices were vortexed vigorously for 10 seconds. The tissue was centrifuged and the pellet washed with ethanol and air dried. The pellet was resuspended in 100 μ l of 0.2 M TRIS buffer, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulphate, and proteinase K was added to a final concentration of 0.5 mg/ml. The reaction was incubated at 37°C overnight on an orbital rotator and the DNA was extracted as described.¹⁷

As primers for PCR amplification, oligo-1, annealing immediately 5' to the 150 base pair area within the major breakpoint region on

chromosome 18, and oligo-4, complementary to a universal *J_H* sequence on chromosome 14, were used.⁹ As sequencing primer, the internal hybridisation probe⁹ annealing to the major breakpoint region internal to primer oligo-1 was used. For PCR amplification, 50 μ l reactions were performed with 3U Taq polymerase (Perkin Elmer Cetus), a 1 $\times$ reaction buffer as supplied by the manufacturer, 1 μ l of the DNA solution, and 300 ng of primer oligo-1 and oligo-4, overlaid with 80 μ l mineral oil. Reaction conditions using an automated thermal cycler (Perkin Elmer Cetus) were 45 cycles of 94°C for one minute, 55°C for one minute, and 72°C for one minute, except that denaturation in the first cycle lasted for three minutes. The entire reaction products were loaded on an agarose gel and electrophoresed. The bands representing the amplified DNA were excised from the gel and DNA was eluted overnight in 100 μ l of water; 35 μ l of the eluate were used as template for asymmetrical PCR to generate mostly single stranded DNA.¹⁸ Amplification conditions were identical with those of symmetrical PCR except that primers were used with a ratio of 1:50 (6 ng of primer oligo-1 and 300 ng of oligo-4). The asymmetric PCR products were washed using a Centricon 100 (Amicon, Danvers, Massachusetts, USA) and the entire eluate was used as template for sequencing reactions according to the chain termination method¹⁹ using a modified T7 DNA polymerase (Sequenase 2.0; United States Biochemicals). As sequencing primer 300 ng of the "internal hybridisation probe"⁹ was used.

Results

The PCR amplified junctional sequences of rearranged chromosomes of both patients showed distinct bands when analysed by agarose gel electrophoresis and ethidium bromide staining (data not shown). Direct sequence analysis of the products led to the identification of the chromosomal breakpoints as well as inserted nucleotides between rearranged fragments in DNA from lymphoma of both patients (fig 1). In DNA from case 1 the *bcl-2* gene is transposed to a *J_{H4}* gene on chromosome 14, with five nucleotides inserted in between, while in DNA from case 2 the *bcl-2* gene translocates to a *J_{H6}* gene on chromosome 14, and 16 nucleotides are inserted (figs 1 and 2).

Discussion

We have shown that the junctional region of rearranged chromosomes 14 and 18 can be accurately determined by direct sequence analysis of PCR amplified DNA from formalin fixed, paraffin wax embedded lymphoma specimens. Compared with detection of the t(14;18) chromosomal translocation by Southern blot or dot blot analysis of PCR amplified material, the precise molecular determination of the breakpoints as well as intervening sequences offers the advantage of identification of a unique molecular marker sequence for the lymphoid malignant clone of the individual patient. This should facilitate studies of the

Case 1:

bcl-2 *J_{H4}*
TCTCATGGCTGTCTTCAGGGTCTTCCTGAAATGCAGTGGTGC CAAAG ATTGAC
Chromosome 18 N Chromosome 14

Case 2:

bcl-2 *J_{H6}*
AGAGCCCTCCTGCCCTCTCCGCGGGGGGCTTT GGAGGTCGGGGGCCCT CTACTAÇA
Chromosome 18 N Chromosome 14

Figure 2 Schematic representation of the DNA sequence of the *bcl-2/J_H* junctional regions as determined by direct sequence analysis (fig 1). The dots below three nucleotides of the rearranged *J_{H6}* gene of case 2 indicate single base pair differences of the *J_{H6}* sequence compared with its germline equivalent. These differences are frequently found in rearranged t(14;18) sequences of lymphomas.¹⁶

clonal evolution of the lymphoma. For example, with the knowledge of this clonotypic sequence, a primer for PCR amplification specific for the identified clone can be designed. Using this primer, it can be determined whether lesions occurring at different anatomical sites or at different times during the course of the disease belong to the same clone or to a different clone at the molecular level.

In a few cases with the t(14;18) translocation breakage on chromosome 18 does not occur in the major breakpoint area, but within a 500 base pair minor cluster region about 20 kilobases distant from the bcl-2 gene.²⁰ As oligonucleotide primers for PCR amplification of this region can be designed,¹⁵ direct sequence analysis of junctional rearranged segments from paraffin wax embedded tissue should also be possible.

Essential for successful direct sequence analysis of a PCR product is the quality of the amplified material. A single discrete and intense PCR product, as detected by ethidium bromide staining of an agarose gel after electrophoresis, yields the best results. This may require optimisation of PCR amplification conditions, which is often only achieved by performing several reactions with various conditions. In our experience, when analysing DNA from fixed paraffin wax embedded tissue, the use of less DNA as a template for the PCR generally produces better results. This may be due to the inhibiting effects of certain fixatives on the Taq DNA polymerase. In some cases dilution of the DNA solution (1 in 1000) and use of 1 µl of this dilution as a template for the PCR increases the yield and specificity of the PCR reaction. The success of PCR amplification may also depend on the fixative used, with DNA from samples fixed in ethanol giving the best results, followed by DNA from formalin fixed tissue. Mercury based fixatives, such as B-5, and fixatives containing picric acid, such as Bouin's, extensively degrade DNA and frequently only amplification of very short sequences (~100 base pairs) is possible (abstract presented at 1991 Biotechnology Winter Symposium, Miami).²¹

Direct DNA sequence analysis of PCR amplified products offers several advantages compared with sequence analysis of individual clones generated by ligation of PCR products into vector, and transformation into bacteria. Data obtained by direct sequence analysis is representative of the sequence of the entire DNA population. This decreases the probability that a misincorporation event that occurred in the synthesis of an individual molecule during the extension step of PCR will be visible when analysing the sequencing ladder.

Direct sequence analysis of PCR products is also more rapid, and sequence data can be obtained within one to two days after extraction of DNA from the paraffin wax block. This will

facilitate analysis of a larger number of samples. As direct sequence analysis is feasible for almost every gene of which flanking sequences are known for the design of oligonucleotide primers for PCR amplification, the strategy presented will be important in molecular pathology.

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