Screening of the entire coding region of p53 in low grade lymphoproliferative disorders

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Abstract
This report details a rapid method for screening the entire p53 coding region (exons 2–11). This method, based on the non-isotopic RNase cleavage assay, uses novel primer sequences and an adaptation of the MutationScreener™ method. A mutation in 20% of the sample was easily detectable by this method, whereas mutations below 50% were undetectable using the original method. Alterations to the wild-type p53 mRNA sequence were found in nine of the 130 patients with low grade lymphoproliferative disorders screened, and this was confirmed by DNA sequencing in eight of eight samples. The method is a simple and reliable technique for screening for p53 mutations.

Methods

PATIENTS
Peripheral blood was obtained from 130 patients: 28 with unclassified low grade lymphoproliferative disorders (not chronic lymphocytic leukaemia (CLL)), two prolymphocytic leukaemias (PL), three CLL/PL, and 97 B cell CLL (B-CLL). CLL cases were classified as typical (T-CLL) or atypical (A-CLL), according to their surface immunophenotype and morphology. Typical CLL (n = 72) had an immunophenotype of CD5/19 positive, CD23 positive, weak surface immunoglobulin (sIg), FMC7 negative, and morphology typical of CLL. Atypical CLL (n = 25) had either one atypical surface marker or atypical morphology.

RNA EXTRACTION
Peripheral blood mononuclear cells were isolated on Lymphoprep (Nycomed, Oslo, Norway). RNA, extracted from 5 × 10^6 cells using Trizol following the manufacturer’s instructions (Life Technologies, Paisley, UK), was redissolved in 30 µl water.

cDNA SYNTHESIS
Reverse transcription was performed on 6 µl RNA using Superscript II (Life Technologies) and random hexamers (Pharmacia Biotech, Little Chalfont, UK), in a total reaction volume of 10 µl, following the manufacturer’s instructions. RNA complementary to the cDNA was removed by incubation at 37°C for 20 minutes with 1 U of RNase H (Helena Biosciences, Sunderland, UK). Before the polymerase chain reaction (PCR) cDNA was heated at 95°C for five minutes.

POLYMERASE CHAIN REACTION
Two PCR reactions were performed for each sample using the following primer pairs (each amplifying exons 2–11).

(I) P53-A: 5'-TAATACGACTCACTATAGGGATGGAGGAGCCGCAGTCAGA-3' (bp 1–20); and P53-AS: 5'-GTCTGAGTCAGGCCCTTCTG-3' (bp 1179–1160)

(II) P53-S2: 5'-GAGCCGCAGTCAGATCCTAG-3' (bp 7–26); and P53-CT: 5'-TAATAGACTCTATAGGAGTCTGAGTCAGGCCCTTCTG-3' (bp 1179–1160).

Both primers P53-A and P53-CT have the T7 promoter attached at their 5’ end. Nucleotide numbers apply to Genbank accession number X60020.
The PCR was performed in a reaction volume of 25 µl, on 2 µl of cDNA, using 0.4 µl Platinum Taq (Life Technologies) and the following conditions: 1× PCR buffer, 2 mM MgCl₂, 200 µM dNTPs, 100 ng each primer, and 1 M betaine (Sigma, Gillingham, UK). After an initial incubation of two minutes at 97°C the reactions were submitted to 35 cycles of one minute at 95°C, 30 seconds at 67°C, and one minute at 72°C.

WILD-TYPE CONTROL

The wild-type control was obtained by the cloning of the PCR product from a known wild-type patient, heterozygous for the CGC/CCC polymorphism at codon 72. This resulted in two wild-type clones, namely: a3 corresponding to the arginine form (CGC) and d2, corresponding to the proline form (CCC).

NON-ISOTOPIC RNase CLEAVAGE ASSAY

NIRCA was performed by a modified protocol of the MutationScreener² kit (Ambion, Texas, USA) using the reagents provided with the kit. Typically, hybrids consisted of 0.6 µl wild-type PCR and 0.4 µl patient PCR products (volumes were adjusted to take account of variability in PCR efficiency). Two hybrids were set up for each patient, wild-type PCRI plus patient PCRII and vice versa so that each hybrid contained the T7 promoter on opposing strands. Transcription mix (2 µl) was added to each hybrid and transcription was carried out at 37°C for one hour. Hybridisation buffer (1 µl) was added and the hybrids were heated at 98°C for five minutes, followed by five minutes at room temperature. The hybrids were split between two RNase digestions (RNase 1 and RNase T1, both diluted at 1/150). RNase digestion buffer (6 µl) was added and the samples were incubated at 37°C for 30 minutes before the addition of 1.5 µl of loading dye. Digested hybrids were run on a 2% agarose gel at 90 V for approximately one hour.

SEQUENCING

Sequencing of the PCR product from amplification with primers P53-S2 and P53-AS was performed by Cambridge Bioscience (Cambridge, UK) using primers P53-S2 and P53AS.

SENSITIVITY DETERMINATION

Comparison of sensitivity between the original MutationScreener protocol and the modified method was performed using clones a3 and d2. The PCR was performed as above for the modified method and with primers P53-A and P53-CT, together in a single reaction, for the original method. Mixes of the PCR products representing 100%, 50%, 20%, and 10% mismatch were made as follows: 100%, d2 only; 50%, a3 : d2 (1 : 1); 20%, a3 : d2 (4 : 1); 10%, a3 : d2 (9 : 1). NIRCA was performed using 0.5 µl of a3 as the wild-type sample and 0.5 µl of the above mixes as patient samples.

Results

SENSITIVITY DETERMINATION

Comparison of sensitivity between the original MutationScreener protocol and the modified method was performed using clones a3 and d2. The PCR was performed as above for the modified method and with primers P53-A and P53-CT, together in a single reaction, for the original method. Mixes of the PCR products representing 100%, 50%, 20%, and 10% mismatch were made as follows: 100%, d2 only; 50%, a3 : d2 (1 : 1); 20%, a3 : d2 (4 : 1); 10%, a3 : d2 (9 : 1). NIRCA was performed using 0.5 µl of a3 as the wild-type sample and 0.5 µl of the above mixes as patient samples.

LYMPHOPROLIFERATIVE DISORDER SCREENING

In total, nine patients showed a positive result upon NIRCA (table 1). NIRCA results from patient 1 (hemizygous) and patient 4 (heterozygous) are illustrated in fig 1B. Alteration from the wild-type sequence was confirmed in eight...
Table 1 Details of p53 mRNA alterations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino acid</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T-CLL</td>
<td>7</td>
<td>248</td>
<td>CGG-GGG</td>
<td>arg/arg</td>
<td>Hemizygous</td>
</tr>
<tr>
<td>2</td>
<td>T-CLL</td>
<td>5</td>
<td>135</td>
<td>TGC-TTC</td>
<td>cys/phe</td>
<td>Hemizygous</td>
</tr>
<tr>
<td>3</td>
<td>A-CLL</td>
<td>AS</td>
<td>5</td>
<td>163</td>
<td>TAC-TGC</td>
<td>try/cys</td>
</tr>
<tr>
<td>4</td>
<td>UN</td>
<td>5</td>
<td>213</td>
<td>CGA-GGG</td>
<td>arg/arg</td>
<td>Polymorphism/heterozygous</td>
</tr>
<tr>
<td>5</td>
<td>A-CLL</td>
<td>6</td>
<td>270</td>
<td>TTT/CTT</td>
<td>phe/leu</td>
<td>Alternative splice product</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>8</td>
<td>266</td>
<td>GGA/CGG</td>
<td>gly/arg</td>
<td>Hemizygous</td>
</tr>
<tr>
<td>7</td>
<td>A-CLL</td>
<td>AS</td>
<td>Multiple aberrant splice products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>A-CLL</td>
<td>8</td>
<td>218</td>
<td>GGA/CGA</td>
<td>gly/arg</td>
<td>Small clone, not sequenced</td>
</tr>
<tr>
<td>9</td>
<td>T-CLL</td>
<td>7</td>
<td>248</td>
<td>CGG/GGG</td>
<td>arg/gly</td>
<td>Hemizygous</td>
</tr>
</tbody>
</table>

A-CLL, atypical B cell chronic lymphocytic leukaemia; AS, alternative splice products; M, mantle cell lymphoma; T-CLL, typical B cell chronic lymphocytic leukaemia; UN, low grade unclassified non-Hodgkin’s lymphoma.

Discussion

This adaptation of the NIRCA method requires two separate PCRs for each patient but the increase in sensitivity obtained justifies the approach. The method is no more cumbersome than the original MutationScreener method because there is no increase in the total number of PCRs performed (nesting is not required); in addition, the reaction volumes can be reduced making the technique more economical. The increased sensitivity (fig 1A) results from the reduction in the number of matched hybrids that can form: limiting transcription to opposing strands of wild-type and test samples ensures that no wild-type–wild-type or patient–patient hybrids are produced. Single strand conformational polymorphism (SSCP), commonly used for screening the p53 gene, results in an equivalent degree of sensitivity (20%), but restricts the region of the gene screened to approximately 400 bp.

Our NIRCA method enables screening of the entire p53 coding region in a single experiment, giving a more complete picture of the status of the gene.

The most common polymorphism of the p53 gene occurs in exon 4 and results in an amino acid substitution at codon 72 (arg/pro). Screening the entire coding sequence of the p53 gene will inevitably pick up this polymorphism, which should not be confused with any mutation. In our study, the initial screening was against the most common allele in a white population (CGG; 71%). The polymorphism results in an easily recognisable cleavage pattern, allowing easy discrimination from mutations. The exon 6 polymorphism discovered in patient 5 is rare in white populations, a French study reported its occurrence as 3%,6 and its incidence in our present study was only one in 130. Cloning of a control for this polymorphism would enable the exclusion of patients showing this cleavage pattern by a second NIRCA reaction.

Previously reported rates for p53 mutations in CLL are approximately 10%,1 and we have found a slightly lower p53 mRNA abnormality rate of 6%. p53 abnormalities are more common in CLL/PL,7 the strict classification applied to our cohort of patients excludes CLL/PL and ensures that only true CLL patients are included. Studies of the incidence of trisomy 12 in CLL have found that the application of strict classification criteria results in a lower rate of abnormality than that reported previously, and have also found this karyotypic abnormality to be more common in atypical than typical CLL.5 In our present study, the p53 mutation rate was higher in atypical CLL (12%) than in typical CLL (4%) and this is in keeping with a higher degree of genomic instability in this subtype.

A strong correlation has been observed between p53 gene deletion and mutation of the remaining copy.1 Therefore, the PCR products from two hemizygous patients, negative for mutation by NIRCA, were sequenced; only the wild-type sequence was present, confirming the NIRCA result.

In summary, the use of novel primer sets with an adaptation of the MutationScreener method enables the rapid screening of the complete coding region of p53. Considering the simplicity, sensitivity, and reliability of this method it seems unnecessary to continue to restrict the exons of the p53 gene studied and risk missing rare mutations.

This work was supported by the Musgrove Leukaemic Group, Somerset.