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

T J Bromidge, D J Howe

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 × 10⁶ 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'-TATACAGACTCACTATTAGGGATGAGGAGCCGCAGTCAGA-3' (bp 1–20); and P53-AS: 5'-GTCTGAGTCAGGCCCTTCTG-3' (bp 1179–1160)
(II) P53-S2: 5'-GAGCCGCAGTCAGATCCAG-3' (bp 7–26); and P53-CT: 5'-TATACAGACTCACTATTAGGGATGAGGAGCCGCAGTCAGA-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 MutationScreener™ 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 PCR 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**

The modified NIRCA method, using the clones of the codon 72 polymorphism, enabled a mismatch to be observed when a mutation was present in 20% of the test sample. When the mutation comprised 10% it was possible to see digestion by RNase T1, but at such a level that would make it difficult to draw a definite conclusion. A lower level of sensitivity was obtained using the original MutationScreener method (with T7 primers on both strands of the PCR products), with no mutation being detected below 50% (fig 1A).

**LYMPHOPROLIFERATIVE DISORDER SCREENING**

In total, nine patients showed a positive result upon NIRCA (table 1). NIRCA results from patient 1 (hemi-zygous) and patient 4 (heterozygous) are illustrated in fig 1B. Alteration from the wild-type sequence was confirmed in eight...
patients by the sequencing of the PCR product. The level of the cleavage signal in the remaining patient (patient 9) was low, suggesting the presence of a small clone; therefore, sequencing was not performed on this sample. Samples from one patient with CLL/PL and one with PLL (negative by NIRCA) were sequenced and showed no mutation. Both of these patients were known to have only one copy of the p53 gene by fluorescence in situ hybridisation (FISH).

Sequencing confirmed missense mutations in five patients (patients 1, 2, 4, 6, and 8), all resulting in amino acid substitutions. Two (patients 3 and 7) had alternative splice products, resulting from an intronic splice site mutation causing disruption of the exon 7–8 boundary in patient 3 (TJ Bromidge et al, unpublished results, 2000) and disruption of correct exon 9–10 splicing in patient 7. The remaining patient (patient 5) had a known polymorphism in exon 6 and is therefore excluded from further analysis (table 1).

One in nine patients with mantle cell lymphoma and one in 19 with low grade unclassified lymphoproliferative disease had a p53 mutation. Six patients with CLL had abnormal p53 mRNA (six of 97; 6%).

Table 1 Details of p53 mRNA alterations

<table>
<thead>
<tr>
<th>Patient</th>
<th>T-CLL</th>
<th>A-CLL</th>
<th>UN</th>
<th>9 T-CLL</th>
<th>Small clone, not sequenced</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>248 CGG-GGG arg/gly</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>135 TGC-TTC cys/phe</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>163 TAC-TGC try/cys</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>213 CGA/CGG arg/arg</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>266 GGA/CGA gly/arg</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>135 TGC-TTC cys/phe</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>248 CGG-GGG arg/gly</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>266 GGA/CGA gly/arg</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></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 MutationScreeener 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 addition, the reaction volumes can be reduced of PCRs performed (nesting is not required); in addition, the reaction volumes can be reduced making the technique more economical. 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 MutationScreeener 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.