

## Short report

## 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.**

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The tumour suppressor gene p53 has an established role in human malignancies and its mutation has been linked with a poor prognosis in various diseases, including haematological malignancies.<sup>1</sup> Consequently, there has been considerable interest in screening the p53 gene for mutations, and several techniques have been applied to this end. Most p53 mutations are found within exons 5–8 of the gene; however, mutations do occur elsewhere. A study of high grade non-Hodgkin's lymphoma found three of 10 mutations outside of this region.<sup>2</sup> Ideally, a method for screening this gene should identify mutations throughout the entire coding region (exons 2–11). In the past, this has been difficult to achieve without sequencing, which has proved both too time consuming and expensive for most laboratories to consider. Therefore, the region of the gene screened in most studies has been limited. The non-isotopic RNase cleavage assay (NIRCA), based on the fact that RNase cleaves both strands of duplex RNA targets at mismatches,<sup>3</sup> has been applied to p53 mutation screening. However, this has again been limited to exons 4–11.<sup>4</sup> We report primer sequences that enable screening of the complete coding sequence of p53, and have applied this simple method to the screening of 130 patients with low grade lymphoproliferative disorders 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.<sup>5</sup>

**RNA EXTRACTION**

Peripheral blood mononuclear cells were isolated on Lymphoprep (Nycomed, Oslo, Norway). RNA, extracted from  $5 \times 10^6$  cells using Trizol following the manufacturer's instructions (Life Technologies, Paisley, UK), was redissolved in 30  $\mu$ l water.

**cDNA SYNTHESIS**

Reverse transcription was performed on 6  $\mu$ l RNA using Superscript II (Life Technologies) and random hexamers (Pharmacia Biotech, Little Chalfont, UK), in a total reaction volume of 10  $\mu$ l, 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'-TAATACGACTCACTATAGG GATGGAGGAGCCGCGAGTCAGA-3' (bp 1–20); and P53-AS: 5'-GTCTGAGT CAGGCCCTTCTG-3' (bp 1179–1160)  
 (II) P53-S2: 5'-GAGCCGCGAGTCAGATCC TAG-3' (bp 7–26); and P53-CT: 5'-TAAT ACGACTCACTATAGGGTCTGAGTC AGGCCCTTCTG-3' (bp 1179–1160).

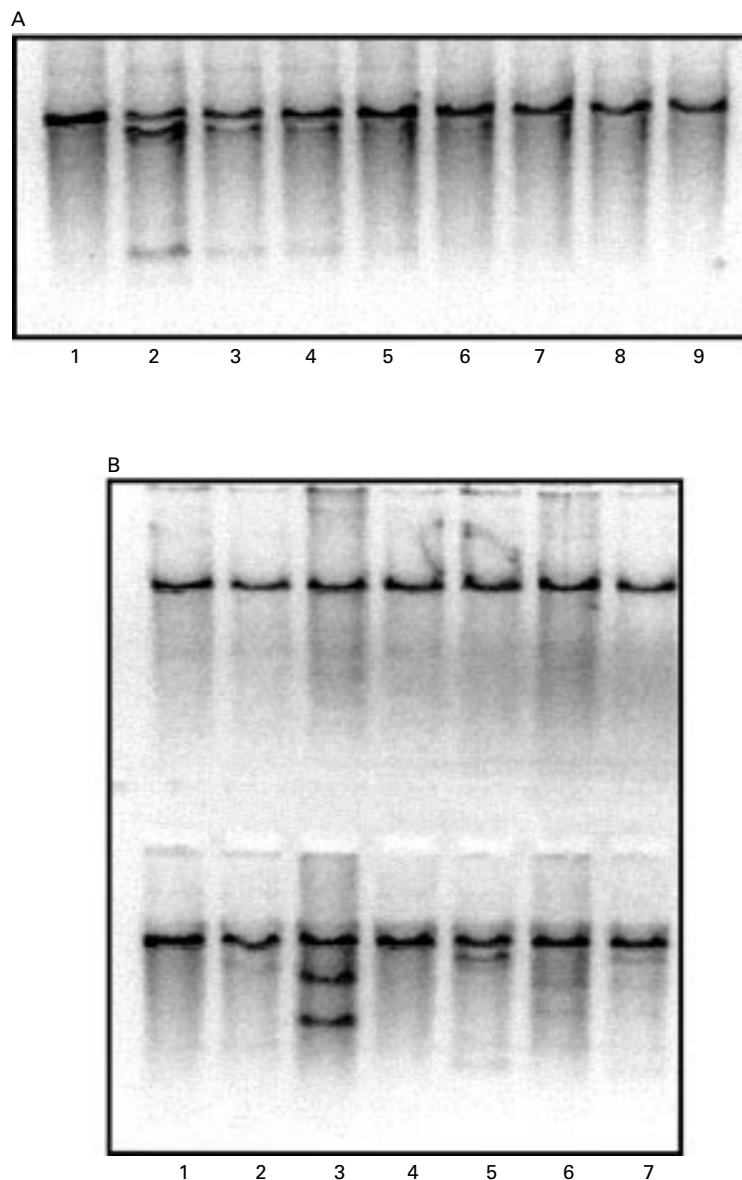
Both primers P53-A and P53-CT have the T7 promoter attached at their 5' end. Nucleotide numbers apply to Genbank accession number X60020.

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**Figure 1** (A) Sensitivity comparison of the adapted (lanes 1–5) and original methods (lanes 6–9). Digestion was performed with RNase T1. Lane 1, no mismatch; lanes 2 and 6, 100% mismatch; lanes 3 and 7, 50% mismatch; lanes 4 and 8, 20% mismatch; lanes 5 and 9, 10% mismatch. (B) Mutation detection in patients with lymphoproliferative disease. The upper panel was digested with RNase I and the lower panel with RNase T1. Lane 1, clone a3 (no mismatch); lane 2, patient 1 PCRII plus clone a3 PCRI; lane 3, patient 1 PCRI plus clone a3 PCRII; lane 4, patient 1 PCRI plus patient 1 PCRII. Lanes 5–7 are as 2–4, respectively, but samples from patient 1 are replaced with samples from patient 4. Note that patient 1 is hemizygous and patient 4 is heterozygous.

The PCR was performed in a reaction volume of 25  $\mu$ l, on 2  $\mu$ l of cDNA, using 0.4  $\mu$ l Platinum Taq (Life Technologies) and the following conditions: 1 $\times$  PCR buffer, 2 mM MgCl<sub>2</sub>, 200  $\mu$ M 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 corre-

sponding 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  $\mu$ l wild-type PCR and 0.4  $\mu$ l 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  $\mu$ l) was added to each hybrid and transcription was carried out at 37°C for one hour. Hybridisation buffer (1  $\mu$ l) 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 I and RNase T1, both diluted at 1/150). RNase digestion buffer (6  $\mu$ l) was added and the samples were incubated at 37°C for 30 minutes before the addition of 1.5  $\mu$ l 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  $\mu$ l of a3 as the wild-type sample and 0.5  $\mu$ l 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 (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

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

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.

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%); three had typical (three of 72; 4%) and three atypical (three of 25; 12%) CLL (table 1).

### 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.<sup>1</sup> 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 a mutation. In our study, the initial screening was against the most common allele in a white population (CGC; 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%,<sup>6</sup> 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%,<sup>1</sup> and we have found a slightly lower p53 mRNA abnormality rate of 6%. p53 abnormalities are more common in CLL/PL,<sup>7</sup> 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.<sup>5</sup> 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.<sup>7</sup> 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.

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