Exon Concatenation to Increase the Efficiency of Mutation Screening by DGGE

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ABSTRACT

For genes that have a substantial number of exons and long intronic sequences, mutation screening by denaturing gradient gel electrophoresis (DGGE) requires the amplification of each exon from genomic DNA by PCR. This results in a high number of fragments to be analyzed by DGGE so that the analysis of large sample sets becomes labor intensive and time consuming. To address this problem, we have developed a new strategy for mutation analysis, lexon-DGGE, which combines the joining of different exons by PCR (also known as lexons) with a highly sensitive technique such as DGGE to screen for mutations. The lexon technique is based on the concatenation of several exons, adjacent or not, from genomic DNA into a single DNA fragment so that this approach could simultaneously be used to check the mutational status of several small genes. To show the feasibility of the approach, we have used the lexon-DGGE technique to analyze all coding exons, intron-exon junctions, noncoding exon 1, and part of the noncoding region of exon 11 of the TP53 gene. The validity and performance of the technique were confirmed by using negative and positive controls for each of the DNA fragments analyzed.

INTRODUCTION

Mutation detection methods usually require the analysis of individual exons amplified by PCR. Direct sequencing requires 35%–40% of mutant DNA in the sample for a mutation to be assigned convincingly (3,16), so it is usually reserved for the detection of germline mutations. For the analysis of tumor samples in which mutant DNA is usually mixed with variable amounts of wild-type DNA from non-tumor cells, mutation scanning methods such as single-stranded conformational polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE) are preferred. It is generally accepted that SSCP has a sensitivity (in terms of potential changes detected) of 70%–90% for PCR products smaller than 200 bp (9), whereas the sensitivity of DGGE reaches 100% with products of 500–600 bp (13). Furthermore, SSCP requires at least 10% mutant molecules in the sample for the mutation to be detected compared to 6%–10% in the case of DGGE (3,16).

To detect all possible mutations including those that involve splicing signals, the analysis of all the genomic fragments by a mutation scanning method may be very laborious and time consuming. Although one of the advantages of DGGE is the possibility of analyzing molecules of 500–600 bp (8,14), this advantage is lost when the analysis involves genes made up of many small exons separated by long intronic sequences. This limitation can be circumvented by linking together two exons from the same or different genes into “lexons” in a single PCR fragment (17).

Therefore, we have combined exon concatenation with DGGE to reduce the number of fragments required to analyze this type of gene. We have tested this approach by analyzing the complete coding region, intron-exon junctions, noncoding exon 1, and part of the noncoding region of exon 11 of *TP53* in patients with acute lymphoblastic leukemia.

TP53 is a tumor suppressor gene frequently mutated in human neoplasia. It is made up of 11 exons of 22–279 bp, except for exon 11, which is 1278 bp. Most *TP53* mutations found in human cancers are located between exons 5 and 8, which encode the conserved DNA binding domain (1,15). In fact, most mutational studies of this gene only analyze exons 5–8. However, some authors have estimated that 10%–25% of *TP53* mutations can take place outside this region (2,5). Therefore, we have developed a combined lexon-DGGE method to scan all the coding sequence and intron-exon junctions of *TP53* in as few fragments as possible.

MATERIALS AND METHODS

Samples

We studied 37 samples from acute lymphoblastic leukemia patients, 29 showing a B-cell phenotype and eight showing a T-cell phenotype. Genomic DNA was extracted from peripheral blood or bone marrow with the QIAamp® DNA Mini kit (Qiagen, Hilden, Germany). The optimization of the technique was carried out by analyzing samples from healthy subjects and mutant controls. The latter were kindly provided

by other laboratories (exons 5–8 and 10) or prepared by PCR using primers with a single nucleotide change in the wild-type sequence (exons 1–4, 9, and 11).

Exon Concatenation by PCR

Four different primers are needed for the concatenation of two exons because each exon must first be amplified separately. Figure 1 shows that the antisense primer of exon A must contain a 5'-tail made up of 10–15 bp that is complementary to the sequence of the sense primer for exon C. In turn, the latter primer must contain a 5'-tail of 10–15 bp that is complementary to the sequence of the antisense primer for exon A. In this way, a sequence of 20–30 bp is created that is identical in both primers (17). The concatenation of both exons is accomplished by a second reaction using 1 μ L of the first reaction as a template and the external primers (sense primer of exon A and antisense primer of exon C); as shown,

the PCR products from the first amplifications anneal to each other. This linked fragment is extended in the first cycle of the second reaction, and the following cycles proceed in the usual way by the action of the external primers. All the primers used in this study (Table 1) were designed using OLIGO[®] 4.0 (NBI, Plymouth, MN, USA), and PCR was carried out in a Perkin Elmer 480 thermal cycler in a total volume of 50 μ L, with 2.5 U AmpliTaq Gold[®] polymerase and the buffer supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA), 1.5 mM MgCl₂, 0.4 mM dNTPs, and 0.1 μ M each primer.

The DGGE technique usually requires the presence of a GC-rich clamp at one end of the fragment to be analyzed (14). To determine the most favorable position of the clamp and to analyze the melting profile of each theoretical fragment, we used the program MacMelt[®] 1.0 (Bio-Rad Laboratories, Hercules, CA, USA).

DGGE

Analysis with MacMelt 1.0 provided the theoretical melting profile for each of the fragments that were subjected to DGGE analysis. This is an important parameter to estimate the best denaturing gradient for each fragment because the wider the gradient is, the smaller the difference in migration between the mutant and wild-type molecules. Other parameters such as the duration of electrophoresis and the probability of detecting mutations along the fragment sequence were calculated using the program SQHTX. These parameters provided the initial electrophoresis conditions that were experimentally verified and refined for each fragment.

Electrophoresis was performed on 12% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) using a PROTEAN[®] II xi cell (Bio-Rad Laboratories) at a constant temperature of 55°C in 1 $\times$ TAE buffer in a denaturing gradient. The denaturing solution consisted of a mixture of formamide and urea (100% denaturing = 5.6 M urea + 32% v/v formamide).

To compare the differences between mutant and wild-type sequences, all amplified fragments were subjected to heteroduplex formation (94°C for 12 min, followed by 56°C for 60 min). To maximize the probability of detecting homozygous mutations, heteroduplex formation was performed in two separate reactions for each fragment with or without wild-type DNA.

After electrophoresis, gels were stained with 10 mg/mL ethidium bromide for 12 min. Fragments showing an altered migration pattern compared to the wild-type control were subjected again to the complete lexon-DGGE procedure to confirm the results. Those fragments with at least two different aberrant patterns were purified and sequenced in an ABI PRISM[®] 377 DNA Sequencer (Applied Biosystems).

RESULTS AND DISCUSSION

The aim of this study was to develop a new strategy for mutation detection, combining PCR-mediated exon concatenation (or lexon) (17) with a mutation scanning procedure. Since single-

Figure 1. Concatenation of two exons. (a) Primers needed for the concatenation of two exons; boxes with horizontal and vertical lines indicate homologous regions between primers that are amplifying different exons. Exons are represented as white boxes and introns as solid rectangles. (b) Separate amplification of exons A and C yields fragments containing one homologous end; (c) joining of exons A and C that leads to lexon AC; and (d) the amplification of lexon AC. An example is shown in the photographs. Photograph 1 and 2 show the amplification of exons 10 (152 bp) and 1 (218 bp), respectively. Photograph 3 shows the amplification of lexon 10 + 1 (370 bp). The MW marker is a 1 Kb Plus DNA Ladder[™] (Invitrogen, Carlsbad, CA, USA).

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exon DGGE is relatively laborious and time consuming, lexon-DGGE should result in a reduced number of fragments to be analyzed and a better processivity for genes with many small exons separated by long intronic sequences. We chose the *TP53* gene to optimize this approach because it is frequently mutated in human cancers but is not usually studied in its entirety. The fragments analyzed in this study encompass the complete coding region of *TP53*, all intron-exon junctions, and noncoding regions of exons 1 and 11.

Table 2 shows the PCR conditions required for the first amplification reactions of each separate exon or exon fragment. Melting, annealing, and extension steps were 1 min for these reactions. In contrast, PCR conditions were different for the second reactions, in which two exons or exon fragments are linked into a lexon. A first cycle is performed to achieve the annealing of the fragments obtained in the first reactions (5 min at temperature T1), followed by a 10-min extension step at 72°C, and then the standard PCR conditions (1-min annealing and extension steps) for the number of cycles and annealing temperature T2.

Of all possible combinations of fragments, only those with similar melting profiles were selected for the potential concatenation into lexons. Computational analysis provided the initial gradient range and DGGE conditions that were further optimized experimentally. Table 3 shows the final experimental DGGE conditions for all the fragments analyzed.

Even though computational analysis with SQHTX had predicted that all possible mutations could be found under these conditions, we verified this experimentally using wild-type and mutant control DNA. Computational predictions were supported by the experimental data because all mutant control samples showed an altered electrophoretic pattern compared to the wild-type controls.

We also carried out dilution experiments to assess the detection limit of the technique. Mixing mutant and wild-type DNA in various ratios, we were able to detect a mutant pattern when the amount of mutant DNA was 5%–10% of the total DNA present in the sample (Figure 2).

Table 1. Sequence of All Primers Used

Fragment	Primer Sequence
Exon 1	E1-R: AGCCCCTTACGCGCTAAAAGTTTTGAGCTTCTCAA E1-I: <i>CLAMP</i> -CCCGTGACTCAGAGAGGACTCATCA
Exon 2	E2-R: GCTGGATCCCCACTTTTCCT E2-I: GACAGGGGCTGGTGGCCTGCCCTTCCAAT
Exon 3	E3-R: <i>CLAMP</i> -CAGCCCCCTAGCAGAGACCT E3-I: AGAGGCCTGGGTCCCAGCCCAACCCCTTGTC
Exon 4-A	E4A-R: GAAGCGGTGGTGCTCTTTTCACCCATCTAC E4A-I: <i>CLAMP</i> -TCTGGGAGCTTCATCTGGAC
Exon 4-B	E4B-R: CGGACGATATTGAACAATGG E4B-I: <i>CLAMP</i> -CTGCCCTGGTAGGTTTTCTG
Exon 4-C	E4C-R: CAGGCCACCAGGCCCTGTCTCTTCTGTC E4C-I: <i>CLAMP</i> GCCAGGCATTGAAGTCTCAT
Exon 5	E5-R: <i>CLAMP</i> -TGCCCTGACTTTCAACTCTGT E5-I: CAACCAGCCCTGTCGTCTCTCC
Exon 6	E6-R: GGGCTGGGACCCAGGCCTCTGATTCCTCAC E6-I: AGACCCAGTTGCAAACCCAG
Exon 7	E7-R: <i>CLAMP</i> -ACTGGCCTCATCTTGGGCCTGTGTT E7-I: GCAAGAGGCAGCAAGCAGAGGCTGGGGCACAGCAG
Exon 8	E8-R: TGCCTCTTGCTTCTCTTTTCCTATC E8-I: GAAAAGAGCACCACCGCTTCTTGCTCTGCTTGCTT
Exon 9	E9-R: ATGCCTCAGATTCACCTTTATCACCTTTCC E9-I: <i>CLAMP</i> -TCCACTTGATAAGAGGTCCCAAGACTTAGT
Exon 10	E10-R: CTCCCCCTCCTCTGTTGCTG E10-I: CTTTTAGCGCGTAAGGGGCTGAGGTCACTC
Exon 11-A	E11A-R: <i>CLAMP</i> -GGCACAGACCCTCTCACTCATGTGATGTCA E11A-I: CATCCTCCTCCCCACAACAAAACACCAGTG
Exon 11-B	E11B-R: AAGTTGGCCTGCACTGGTGTGTTTGTGTTG E11B-I: <i>CLAMP</i> GAGAGGGAGAGGGAACAGGCACCCTCAAGG
The clamp sequence is 5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGGCCCCGCCCCG-3'.	

To check the feasibility of the technique, 37 samples from patients with acute lymphoblastic leukemia were analyzed. We found a total of 15 altered patterns. Three of these alterations were found to correspond to a previously described silent polymorphism in exon 6 caused by a g13399A>G transition (Arg213) (numbered as in sequence X54156). Seven alterations were due to an exon 4 polymorphism (g12139G>C, A72P) that has been previously described. The remaining five alterations were missense mutations. In three of

Figure 2. Sensitivity test. M, mutant control. Lanes 1–6 contain 50%, 25%, 20%, 15%, 10%, and 5% mutant DNA, respectively. WT, wild-type control. The bands corresponding to the heteroduplex between wild-type and mutant DNA (arrow) are shown.

Table 2. PCR Conditions for the Amplification of the Fragments Studied

Fragments	Primers	Annealing Temperature (°C)	No. of Cycles
Exon 1	E1-R/E1-I	68	30
Exon 2	E2-R/E2-I	62	25
Exon 3	E3-R/E3-I	68	30
Exon 4-A	E4A-R/EA-I	65	30
Exon 4-B	E4B-R/EB-I	58	30
Exon 4-C	E4C-R/EC-I	69	30
Exon 5	E5-R/E5-I	60	30
Exon 6	E6-R/E6-I	61	30
Exon 7	E7-R/E7-I	64	30
Exon 8	E8-R/E8-I	56	25
Exon 9	E9-R/E9-I	62	35
Exon 10	E10-R/E10-I	65	30
Exon 11-A	E11A-R/E11A-I	68	35
Exon 11-B	E11B-R/E11B-I	68	30
Lexon 10 + 1	E10-R/E1-1	T1, 55; T2, 65	30
Lexon 2 + 4-C	E2-R/E4C-1	T1, 63; T2, 62	30
Lexon 3 + 6	E3-R/E6-1	T1, 62; T2, 61	25
Lexon 8 + 4-A	E8-R/E4A-1	T1, 54; T2, 56	25

T1, temperature required for concatenation of the fragments. T2, annealing temperature used in the second reaction after concatenation.

Table 3. Experimental DGGE Conditions for the Fragments Analyzed

Fragments	Gradient (%)	Run Time (h)
Exon 10 + Exon 1	60–90	5
Exon 2 + Exon 4-C	50–80	8
Exon 3 + Exon 6	30–80	6.5
Exon 8 + Exon 4-A	40–90	7
Exon 4-B	60–100	7
Exon 5	60–90	6
Exon 7	40–90	4.5
Exon 9	40–80	3
Exon 11-A	40–80	5
Exon 11-B	30–80	6

these, a mutation in exon 5 was observed (g13214C>T, H179Y). This mutation is already described in *TP53*-specific mutation databases. The remaining two mutant patterns corresponded to a novel S366A missense mutation, resulting from a g17674T>G transversion in exon 10 of *TP53*. This mutation has not

been previously described (Figure 3).

Our results indicate a frequency of missense mutations (13.5%, 5 out of 37) clearly higher than that reported in other studies for acute lymphoblastic leukemia patients (approximately 2% higher) (7,10,11). Although this could be overestimated in our study because

of sample size, the high number of mutations and polymorphisms detected in this sample set suggests that our strategy is very sensitive and is detecting most, if not all, nucleotide changes along *TP53*. It is interesting to note that in two different samples one missense mutation was found outside the so-called *TP53* mutational hot spot that encompasses exons 5–8. In this regard, other studies suggest that the frequency of *TP53* mutations is underestimated in hematological malignancies (12) and that 10%–37% of *TP53* mutations could affect nucleotides outside the DNA binding domain encoded by exons 5–8 (2,4–6). Our preliminary data support these suggestions and further highlight that using a sensitive mutation detection method such as DGGE could identify novel mutations in exons that are usually considered to be poor in mutational events.

We have developed an approach that should facilitate the analysis of the entire coding region of complex genes or several small genes simultaneously. In this case, the coding region of *TP53*, including intron-exon boundaries and noncoding transcribed regions, was analyzed in 10 different fragments, requiring only six types of denaturing gradients. This approach could accelerate the mutational analysis of genes and help to establish the true frequency of mutations, especially in cancerous samples.

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Figure 3. DGGE patterns and sequencing of mutations found in this study. Upper panels, some of the altered patterns found by DGGE in lexons 10+1 and 3+6 and exons 4-B and 5. M, mutant sample; WT, wild-type; and C, mutant control. Lower panels, sequence analysis of the same samples that demonstrate the sequence change.

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