Multiple PCR analyses on trace amounts of DNA extracted from fresh and paraffin wax embedded tissues after random hexamer primer PCR amplification

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Abstract

Aim—To establish a simple and reliable polymerase chain reaction (PCR) methodology for random amplification of whole genomic DNA from limited histopathological samples.

Methods—Trace amounts of genomic DNA extracted from fresh tissue and individual lymphoid follicles microdissected from archival paraffin wax tissue sections were amplified using a two-phase PCR protocol with random hexamers as primers (RP-PCR). The randomly amplified DNA samples were used as templates for specific PCR amplifications. To check the fidelity of the RP-PCR, products of the specific PCR amplifications were further analysed by single stranded conformation polymorphism (SSCP) or sequencing.

Results—Using a minute fraction of RP-PCR template pool, multiple PCR analyses, including those for β globin gene, p53 gene (exon 5-6, exon 7, exon 8-9 and exon 7-9), and rearranged immunoglobulin heavy chain gene fragments (VH framework 3 to JH and VH framework 2 to JH) were successfully performed. No artefactual mutations were identified in the products of these specific PCR reactions by SSCP or sequencing when compared with the products from the original DNA.

Conclusion—This method is simple and reliable, and permits multiple genetic analyses when only a limited amount of tissue is available.

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The high sensitivity of the polymerase chain reaction (PCR) has facilitated the performance of genetic analysis on scanty pathological samples, such as fine needle aspirates, single tissue sections, or cell populations microdissected from archival tissue sections. In most of these cases there is insufficient DNA for more than a few amplifications. To characterise the genotype of a cell population in a pathological lesion it is often desirable to perform repeated or multiple PCR analyses. Tiny DNA samples are therefore inadequate for this purpose. Random amplification of total DNA in minute samples into expanded template pools for subsequent specific PCR may overcome this limitation.

Amplification of total genomic DNA has been achieved using several methods, such as those using random 15mer primers,¹ degenerate primers,² and linker adaptor ligation.³ It has also been used in the cloning of microdissected chromosomes, single cell genotyping, and generating ancient DNA libraries. In this study we used a mixture of random hexamers that is readily available because it is commonly used for DNA probe labelling, as primers for PCR amplification of whole genomic DNA. Our aim was to establish a simple and reliable methodology for random PCR amplification of minute amounts of DNA from histological samples.

Methods

DNA SAMPLE PREPARATION

High molecular weight DNA was extracted from snap-frozen tissue of a low grade mucosa associated lymphoid tissue (MALT) B cell lymphoma using a standard method. Four individual B cell follicles which had been colonised by the tumour cells⁴ were microdissected from a paraffin wax processed haematoxylin and eosin stained section of the same case, as described before.⁵ Each microdissected follicle was digested in 10 μ l of a solution containing 200 μ g/ml proteinase K, 10 mM TRIS-HCl (pH8.3), 50 mM KCl, 0.1% Triton-X100 for three days at 37°C. DNA samples from cell lines containing mutated p53 exon 5, 6 (HUT78), 7 (BL37), 8 and 9 (BL113)⁶ were used as positive controls for single strand conformation polymorphism analysis (SSCP).

RANDOM HEXAMER PRIMER PCR AMPLIFICATION (RP-PCR)

RP-PCR was performed in two phases. Phase I reactions were run in a 10 μ l solution containing 0.025 units of AmpliTaq polymerase (Perkin Elmer Cetus UK), 10 mM TRIS (pH8.3), 50 mM KCl, 1 mM MgCl₂, 0.001% gelatin, 0.02 mM of each dNTP, 10 μ M of random hexamers (Boehringer, Germany) and 4 μ l of the 10 μ l digest of each microdissected follicle or appropriate amounts (including 40 ng, 4 ng, 400 pg, 40 pg and 4 pg) of high molecular weight DNA. After initial denaturation of DNA at 95°C for five minutes, 10 cycles of one minute at 95°C, one minute at 37°C, and five minutes at 50°C were carried out on a thermal cycler (Hybaid UK). At the end of cycle 10, a 40 μ l solution

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containing 0.5 units Amplitaq, 1.5 mM MgCl₂, 0.2 mM of each dNTP, and the same concentrations of other reagents (without additional template DNA) as in phase I was added. Forty phase II cycles of one minute at 95°C, one minute at 55°C, and two minutes at 72°C were performed. At the end of phase II, a 50 µl product pool was generated. To optimise the random primer concentration, PCR amplification for p53 exon 7–9 (840 base pairs) was performed on the RP-PCR pools generated using serial dilutions (1–100 µM) of the random primers and 400 pg high molecular weight DNA. The products of the specific PCR were analysed using agarose gel electrophoresis.

MULTIPLE PCR ANALYSES

Seven different specific PCR analyses (β globin gene, p53 gene (exon 5–6, exon 7, exon 8–9 and exon 7–9) and rearranged immunoglobulin (Ig) heavy chain gene fragments (VH framework 3 to JH and VH framework 2 to JH)) were performed on individual RP-PCR product pools generated from the microdissected follicles, and 40 ng, 4 ng, 400 pg, 40 pg, 4 pg high molecular weight DNA using the primers and conditions, as described before.^{7–9} Each of these specific PCR analyses required 2 µl of the 50 µl RP-PCR product pool as template. For comparison, the same PCR analyses were also performed on 2 µl of 50 µl solutions containing 40 ng, 4 ng, 400 pg, 40 pg and 4 pg of the same high molecular weight DNA without RP-PCR amplification. Ten microlitres of PCR products were separated by electrophoresis on 2% or 3% agarose gels with ethidium bromide added and viewed under ultraviolet light.

PCR-SSCP ANALYSIS

To examine any introduced sequence changes, the products of the multiple specific PCR reactions generated from one of the RP-PCR template pools of the microdissected follicles were analysed by SSCP. The products of the same specific PCR reactions from high molecular weight DNA without RP-PCR amplification were run in parallel.

A minute portion of the specific PCR products (1 µl of 1 in 100 dilution) was used as a template for a second PCR amplification. This was performed in a 15 µl volume under the same conditions described above in the presence of 1 µCi ³²P-dCTP (Amersham UK). The product of the second PCR was diluted with 5 to 25 volumes of a solution

containing 0.1% sodium dodecyl sulphate and 10 mM EDTA. Five to 10 µl of this mixture was further diluted with one volume of a loading solution containing 95% formamide, 20 mM EDTA, 0.05% xylene cyanol and bromophenol blue. The samples were denatured at 90°C for three minutes, chilled on ice, and loaded on to 6% acrylamide gels (2% C). The gels were run at 1 watt for 11 to 21 hours at room temperature, cooled by a water jacket. The acrylamide gels were dried on filter paper and exposed to x ray film at –70°C with an intensifying screen for five to 24 hours.

CLONING AND SEQUENCING ANALYSIS

To analyse further any possible artefactual mutations, products of the Ig gene PCR amplification generated from the above RP-PCR template pool and high molecular weight DNA without RP-PCR amplification were cloned and sequenced. The products were run on a 4% low melting temperature agarose gel. The discrete bands were excised and purified using the Mermaid Kit (Bio 101 Inc, California, USA). The purified fragments were ligated into pBluescript SKII phagemid (Stratagene, La Jolla, California, USA) and the recombinant DNAs were used to transform SURE bacteria (Stratagene). Colonies were screened by PCR amplification for the cloned Ig fragment. Three positive recombinant DNAs from each original PCR reaction with and without RP-PCR amplification were sequenced by the Sanger method using the Sequenase kit (United States Biochemical, Cleveland, Ohio).

Results

All concentrations of random hexamer primers used were able to generate the 840 base pair fragment of p53 exon 7–9, except 1 µM. Ten microlitres was selected as the standard concentration for the primer in all the subsequent RP-PCR reactions.

Among the seven specific PCR reactions performed on RP-PCR template pools generated from the four microdissected follicles, six produced discrete fragments with expected sizes (ranging from 100 to 413 base pairs). The fragment of p53 exon 7–9 (840 base pairs) could not be amplified from any of these pools. The results of the multiple PCR reactions performed on the RP-PCR template pools generated from various amounts of fresh DNA are summarised in the table.

Before RP-PCR amplification the minimum amount of high molecular weight DNA template used for individual PCR reactions ranged from 16 pg (2 µl from 50 µl of the 400 pg dilution) to 1.6 ng (2 µl from 50 µl of the 40 ng dilution). After RP-PCR amplification 0.16 pg (2 µl from 50 µl of the 4 pg pool) to 16 pg (2 µl from 50 µl of the 400 pg pool) of fresh DNA was shown to be sufficient for the same PCR analyses. Thus RP-PCR increased the sensitivity of specific PCR reactions by at least 100 times.

Results of multiple PCR on fresh DNA samples before (B) and after (A) RP-PCR

Multiple PCR	Size (base pairs)	1.6 ng (B) (A)	0.16 ng (B) (A)	16 pg (B) (A)	1.6 pg (B) (A)	0.16 pg (B) (A)	H ₂ O (B) (A)
Ig FR3	105	+/+	+/+	+/+	–/+	–/+	–/–
Ig FR2	260	+/+	+/+	–/+	–/+	–/–	–/–
β globin	250	+/+	+/+	+/+	–/+	–/+	–/–
p53,5–6	408	+/+	+/+	+/+	–/+	–/+	–/–
p53,7	139	+/+	+/+	+/+	–/+	–/+	–/–
p53,8–9	330	+/+	+/+	–/+	–/+	–/–	–/–
p53,7–9	840	+/+	–/+	–/+	–/–	–/–	–/–

Figure 1 Ethidium bromide stained agarose gel showing products of specific PCR amplifications on RP-PCR template pools. (A) Template pool generated with 400 pg of high molecular weight DNA; (B) template pool generated with DNA prepared from a microdissected lymphoid follicle. Lane M: Φ X/HinfI molecular weight marker (size in base pairs); lanes 1–7: β globin, Ig (Fr2-JH), Ig (Fr3-JH), p53 exon 5–6, p53 exon 7, p53 exon 8–9, p53 exon 7–9.

No difference in mobility was identified on the SSCP gels between the products of the six specific PCR reactions generated from the RP-PCR template pool of the microdissected follicle and the products of the same PCR reactions from the original high molecular weight DNA without RP-PCR amplification (figs 1 and 2).

The sequences of the clones analysed (three from original high molecular weight DNA sample and three from a RP-PCR product pool of a microdissected follicle of the same case) were identical (FR3 primer-ACG TCA TAA TGT AGT AGT AGC ACC CAT TGG AGT AGC AGC TAC CAC CAC CGT AAA CCT CTC GAC-JH region primer).

Discussion

Using the random hexamers from an oligo-labelling kit as primers, we have developed a

simple method, referred to here as RP-PCR, for amplification of total genomic DNA. As little as 4 pg of DNA from fresh tissue or DNA from a single follicle of an archival paraffin wax section can be readily amplified by this method into a template pool. A minute fraction of this pool contains sufficient DNA for a number of specific PCR analyses.

One major concern about PCR random amplification of trace amounts of DNA is the fidelity of the amplified products. To achieve good fidelity it is critical to minimise errors in the DNA copying process during early PCR cycles, as these errors can be further amplified later. For this reason, we divided our RP-PCR into two phases. In phase I we reduced the amount of Taq polymerase and dNTP to restrict the errors caused by the enzyme¹⁰ and used a recommended extension temperature at which a good combination of fidelity and efficiency of the enzyme could be obtained.¹¹ We also performed the phase I reaction in a small volume to ensure accurate temperature control. In phase II standard PCR conditions were applied to increase yield of the products. Sequencing and SSCP analysis of the specific PCR products confirmed the high fidelity of the RP-PCR.

In titration tests of high molecular weight template DNA RP-PCR increased the sensitivity of specific PCR by over 100 times. Amplification of DNA fragments up to 400 base pairs in archival material and 800 base pairs in fresh tissue extracts could be achieved by standard PCR when RP-PCR products were used as templates. The successful amplification of a number of different gene fragments has also indicated that RP-PCR can generate a representative template pool. These features should meet the requirements for most current PCR analyses.

The main advantage of RP-PCR over other methods, such as the random 15mer¹ and linker adaptor methods³ for PCR random amplification of genomic DNA, is that the random hexamer primers are inexpensive and readily available. RP-PCR, together with some newly developed techniques, such as microdissection,⁵ should make it feasible to perform multiple genetic analyses on defined cell populations on tissue sections or scanty amounts of pathological material.

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Figure 2 SSCP analysis of PCR products of p53 gene amplified from DNA templates with and without RP-PCR. (A) p53 exon 5–6; (B) p53 exon 7; (C) p53 exon 8–9. Lanes 1–4: original DNA; DNA after RP-PCR; positive control DNA from cell lines (HUT78 for p53 exon 5–6, BL37 for p53 exon 7, BL113 for p53 exon 8–9); negative control DNA from placenta.

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