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Short reports

Nested PCR-SSCP assay for the detection of p53 mutations in paraffin wax embedded bone tumours: improvement of sensitivity and fidelity

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Abstract

DNA extraction and PCR amplification from paraffin wax embedded bone tumour specimens present several difficulties, firstly, because of the abundant matrix they contain and, secondly, because decalcification often causes degradation of DNA. In this report, comparative studies were carried out to determine the most efficient method for DNA extraction and PCR amplification from such specimens. The results indicated that nested PCR produced appropriate strong reaction products with minimal background contamination. A method for DNA extraction from paraffin wax embedded bone tissue and a nested PCR-SSCP technique have been developed for use in such diagnostic specimens.

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Keywords: DNA extraction, PCR amplification, SSCP, paraffin wax embedded bone tumour specimens.

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Although tumours of bone are infrequently encountered in clinical practice, they are none the less of great importance because of their diverse origins from the many cell types found in bone. Malignant bone tumours account for 40%[1] of bone neoplasms and are amongst the most lethal, widely metastasising human malignancies. The extremely variable biological behaviour of malignant bone tumours is a diagnostic and prognostic challenge for clinicians and histopathologists alike. Recent studies have shown that alterations of the tumour suppressor gene p53 are a crucial step in the development of human malignancies.[2] Screening of p53 mutations with a rapid PCR-SSCP method has been widely used by many investigators for a variety of tumours.[3] It has been shown—for example, that p53 mutations may be an important prognostic indicator for carcinomas of the breast and stomach and many others.[3] We reasoned that detection of p53 mutations in primary bone cancers may provide a possible prognostic marker. Therefore, we used the PCR-SSCP method to screen retrospectively 94 specimens of paraffin wax embedded bone tumours. During these studies we found a number of difficulties in performing the PCR-SSCP procedure from bone tumour samples. Firstly, we were unable to obtain enough suitable DNA from the paraffin wax embedded tissues because they often contained abundant bone matrix. Secondly, DNA samples, even in a good yield, often could not be amplified by normal PCR as the decalcification process caused degradation of DNA. In the present study we describe optimisation of the conditions for DNA extraction and PCR reaction and development of a nested PCR-SSCP method for detecting p53 mutations.

Methods

Formalin fixed and paraffin wax embedded surgical specimens of various bone tumours, including 74 giant cell tumours and 20 osteosarcomas, were obtained from the Department of Pathology, Sun Yat-sen University of Medical Sciences, Guangzhou, P.R. China. Specimens had been stored for two to 12 years.

EXTRACTION OF DNA FROM PARAFFIN WAX EMBEDDED TISSUE

Histopathological examination of haematoxylin and eosin stained sections of the tumours enabled the selection of representative areas consisting predominantly of tumour, from which DNA could be extracted. Ten, 5 μm thick sections were cut and placed into an Eppendorf tube using sterile toothpicks. After trimming of each block, the blade was wiped clean with xylene to prevent carry over from one paraffin wax block to another. Tumour samples were digested with proteinase K (20 mg/ml; Promega) in 200 μl digestion buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.5% Tween 20) at 55°C for 48 hours on a rotating wheel apparatus, according to a method described previously with only slight modifications.[4] The following procedures, however, were used in an attempt to enhance the quantity and quality of DNA extracted from paraffin wax embedded tissue: (1) tissue sections were dewaxed by washing three times in xylene after proteinase K digestion[5]; alternatively (2) sections were heat treated at 90°C for 40 minutes to remove paraffin wax after digestion with proteinase K[5]; and (3) phenol-chloroform extraction and sodium acetate precipitation were also carried out to purify the DNA.[5]

Table 1 Primers for PCR amplification of exons 5–8 of the p53 gene

Exon	*Primer oligonuncleotide sequences*	*Product size (base pairs)*
5	P1 α 5'-TCTTCCTGCAGTACTCCCCT-3'	
	P1 z 5'-AGCTGCTCACCATCGCTATC-3'	205
	P2 α Exon 5-F CTG TTC ACT TGTGCC CTGAC	
	P2 z Exon 5-R AGT GAG GAATCA GAG GCCTG	304
6	P1 α 5'-GATTGCTCTTAGGTCTGGCC-3'	
	P1 z 5'GCAAACCAGACCTCAGGCGG-3'	136
	P2 α Exon 6-FGCTGCTCAGATA GCGATG GT	
	P2 z Exon 6-R ACT GACAACCACCCTTAA CC	265
7	P1 α 5'-TTGTCTCCTAGGTTGGCTCT-3'	
	P1 z 5'-GCTCCTGACCTGGAGTCTTC-3'	130
	P2 α Exon 7-FCTTGCCACA GGTCTC CCC AA	
	P2 z Exon 7-RGAAATC GGTAAGAGGTGG GC	259
8	P1 α 5'TCCTGAGTAGTGGTAATCTA-3'	
	P1 z 5'-GCTTGCTTACCTCGCTTAGT-3'	157
	P2 α Exon 8-F GGACCTGATTTCCTT ACT GC	
	P2 z Exon 8-R ATA ACT GCA CCC TTG GTCTC	236

PCR ASSAY

Oligomers were designed according to published sequences of exons 5–8 of the p53 gene.[4] Table 1 summarises the sets of primers used for both non-nested PCR and nested PCR.

Non-nested PCR

Reaction mixtures contained 0.4 μM of each of the second set primers as indicated in table 1, 25 mM $MgCl_2$, 200 mM of dNTPs, one unit of Taq DNA polymerase (Biotech Int., Perth, Australia) and 1 μl of the solution containing the extracted DNA. Amplification was carried out in a Perkin Elmer Gene Amp PCR System 2400 with 38 cycles at 94°C for 30 seconds (DNA denaturation), 55°C for 30 seconds (primer annealing) and 62°C for 30 seconds (DNA extension). PCR was completed with a 10 minute extension step at 72°C.

Nested PCR

Reaction mixtures were as above, except that 0.4 μM of first set primers (as indicated in table 1) were used and amplification was for 22 cycles. Two microlitres of the product of this reaction were transferred to a second reaction mixture, made as described above with 0.4 μM of the second set primers. This was amplified with 30 cycles. To increase the specificity and enzyme stability of the PCR, various concentrations of formamide and triton X-100 were also tested.[6 7] To visualise the PCR products, 2 μl of reaction product was run on a 2% agarose gel and viewed under ultraviolet transillumination.

SSCP/SILVER STAINING ASSAY

SSCP/silver staining was used to detect gene mutations, as described previously.[8] In brief, 5 μl of PCR product plus 5 μl of the formamide loading buffer were denatured at 95°C for 10 minutes and loaded onto non-denaturing 15% polyacrylamide (30:1 acrylamide:bisacrylamide)/10% glycerol gels cast on a Miniprotean Apparatus (BioRad). Samples were run in 1 × TBE at 150 volts for 3.75 hours (fixed tissue) and 4.50 hours (fresh tissue). Gels were soaked in 10% ethanol for three minutes, 1% nitric acid for three minutes, stained in 0.1% silver nitrate solution for 10 minutes under subdued lighting, and developed in sodium carbonate solution (60 mg/ml) and formaldehyde (1.2 μl/ml). Development was halted by soaking the gel in 10% acetic acid for five minutes. The presence of mutations was confirmed by separate PCR and SSCP.

Results and Discussion

Extraction of DNA from paraffin wax embedded tissue is always a challenge because many factors, including fixation, the temperature of tissue processing and the age of the paraffin wax block, can affect the integrity of DNA.[5] In the case of bone tumours degradation of DNA often occurs because decalcification of specimens causes depolymerisation of DNA.[9] In the present study several methods of DNA extraction were assessed. The results indicated that the best way to obtain suitable DNA for PCR amplification is to digest the tissue sample with proteinase K (20 mg/ml, Promega) in digestion buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.5% Tween 20) at 55°C for 48 hours, followed by heat treatment at 90°C for 40 minutes. The DNA solution should then be centrifuged at 13 000 rpm for five minutes to remove the paraffin wax. Xylene extraction, phenol-chloroform extraction and sodium acetate precipitation complicated the process of DNA extraction and did not improve the recovery of DNA.

Because amounts of amplifiable DNA obtained from bone tumour specimens were generally very low, we developed a sensitive nested PCR method for DNA amplification. Nested PCR amplifies the target DNA in two steps.[10] In the first step an initial pair of primers for each exon was used to generate a long sequence that contains the target DNA sequence. A small amount of this product was then used in a second round of amplification,

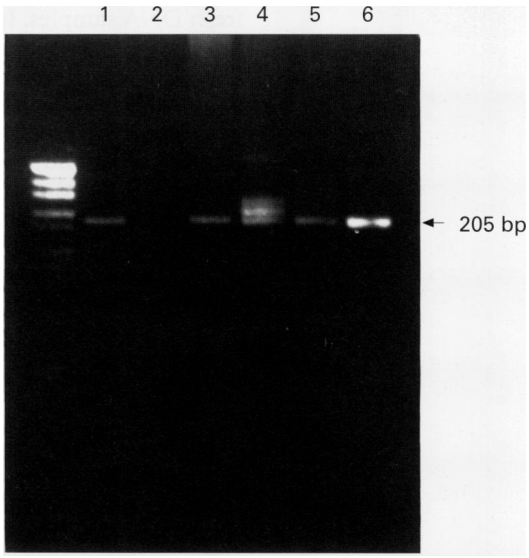

Figure 1 Amplification of a 205 base pair sequence of p53 at exon 5 using nested and non-nested PCR in paraffin wax embedded and fresh tissues. Lane 1, nested PCR of DNA extracted from a paraffin wax embedded section of giant cell tumour (GCT). Lane 2, non-nested PCR of paraffin wax embedded GCT. Note the lack of product. Lane 3, non-nested PCR of DNA extracted from fresh osteosarcoma tissue. Lane 4, nested PCR using 100 ng DNA from fresh osteosarcoma tissue; multiple bands of amplified product are seen. Lane 5, nested PCR using 10 ng DNA from fresh osteosarcoma tissue. Lane 6, nested PCR using 1 ng DNA from fresh osteosarcoma tissue; amplified product is clearly seen.

Table 2 Recommended protocol for nested PCR of paraffin wax embedded bone tumours

	1st run (22 cycles)	*2nd run (30 cycles)*
1% Triton X-100	0.5	1.0
12.5% Formamide	0.5	1.0
H_2O	1.45	2.95
25mM $MgCl_2$	0.5	1.0
5 × Buffer	1.0	2.0
Primer (pair)	0.5	1.0
Taq polymerase	0.05	0.5
Sample DNA	0.5	1.0
Total volume	5.0 µl	10.0 µl

which used primers directed against the final target DNA. The efficiency of the second round of amplification was enhanced because of the more rapid and more complete denaturation of the first reaction product compared with the total genome. Nested PCR can overcome false positives caused by contamination and improves the specificity, efficiency and fidelity of the reaction.[10 11] Using nested PCR-SSCP, we were able to amplify 50 (67%) of 74 cases of giant cell tumours and all 20 cases of osteosacoma. Figure 1 shows the comparision of nested and non-nested PCR for amplification of DNA extracted from paraffin wax embedded and fresh tissues. It should be noted that the non-nested PCR did not amplify the reaction product from the former, even when the number of PCR cycles was increased to 38. In contrast, nested PCR amplified the correct sized product (fig 1). In fig 2 the SSCP gel shows the nested PCR product, and has revealed the presence of various bands, with minimal background contamination. Mutations of p53 at exon 5 in osteosarcomas and giant cell tumours can be seen clearly in lanes 4, 5, 1, and 7, respectively.

Our results have also indicated that care must be taken if nested PCR is to be used on fresh DNA samples. In fig 1 note that only very low amounts of DNA (less than 1 ng) are needed for nested PCR, otherwise multiple bands of PCR product appear. Moreover, Triton X-100 and formamide enhanced enzyme stability and specificity of the PCR assay (data not shown). Our recommended protocol for nested PCR of paraffin wax embedded bone tumours is summarised in table 2.

In summary, our results have indicated that nested PCR produces strong reaction products with minimal background contamination in samples of DNA extracted from paraffin wax embedded bone tumours.

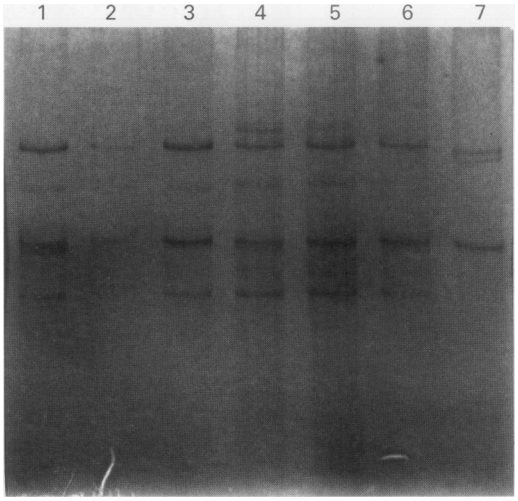

Figure 2 Nested PCR-SSCP of GCT and osteosarcoma. Various alleles with minimum background contamination are seen. Lane 1, GCT 157; lane 2, GCT 160; lane 3, GCT 177; lane 4, osteosarcoma 40; lane 5, osteosarcoma 55; lane 6, GCT 145; lane 7, GCT 129. Mutations of p53 at exon 5 in GCT and osteosarcoma samples are clearly seen in lanes 1, 4, 5, and 7. (The numbers following GCT and osteosarcoma represent patient numbers.)

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1 Liu ZJ, Li RZ, Liu CM, Zhang RY, Han X, Yang SY, *et al.* Pathological survey and analysis of 12404 cases of tumours and tumour-like lesions of bone. *Chinese J Orthopaedics* 1986;**6:**162–9.
2 Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. *Nature* 1991;**351:**453–6.
3 Bosari S, Viale G. The clinical significance of p53 aberrations in human tumours. *Virchows Arch* 1995; **427:**229–41.
4 Sparrow LE, Soong R, Dawkins HJS, Iacopetta BJ, Heenan PJ. p53 gene mutation and expression in naevi and melanomas. *Melanoma Res* 1995;**5:**93–100.
5 Greer CE, Wheeler CM, Manos MM. Sample preparation and PCR amplification from paraffin-embedded tissues. *PCR Methods Appl* 1994;**3(Suppl):**113–22.
6 Cha RS, Zarbl H, Keohavong P, Thilly WG. Mismatch amplification mutation assay (MAMA): Application to the c-H-ras gene. *PCR Methods Appl* 1992;**2:**14–20.
7 Cha RS, Thilly WG. Specificity, efficiency, and fidelity of PCR. *PCR Methods Appl* 1993;**3(Suppl):**18–29.
8 Bassam BJ, Caetano-Anolles G, Gresshoff PM. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* 1991;**196:**80–3.
9 Koshiba M, Ogawa K, Hamazaki S, Suglyama T, Ogana O, Kitajima T. The effect of formalin fixation on DNA and the extraction of high-molecular-weight DNA from fixed and embedded tissues. *Pathol Res Pract* 1993;**189:**66–72.
10 Porter-Jordan K, Rosenberg El, Keiser JF, Gross JD, Ross AM, Nasim S, Garrett CT. Nested polymerase chain reaction assay for the detection of cytomegalovirus overcomes false positives caused by contamination with fragmented DNA. *J Med Virol* 1990;**30:**85–91.
11 Mullis KS, Falloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* 1989;**155:**335–50.