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Polymerase chain reaction-single strand conformation polymorphism analysis of the *p53* gene in paraffin-embedded surgical material from human renal cell carcinomas

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Abstract *p53* tumour suppressor gene mutations were studied in 118 renal cell carcinomas using paraffin-embedded surgical material. Optimal results were obtained with analysis of exon lengths between 150 and 200 base pairs for polymerase chain reaction. Single strand conformation polymorphism and sequencing analysis revealed only two point mutations (2/118, 2%): one involving codon 135; TGC→TTC (cysteine→phenylalanine) and the other codon 175; CGC→CAC (arginine→histidine). Both of these cases were classified as granular cell subtype on microscopic observation. The data suggest that the *p53* tumour suppressor gene is not related to tumour initiation, promotion, or progression of renal cell carcinomas. However, there is the possibility that granular cell type carcinomas may have a different genetic background from clear cell type renal neoplasms.

Key words Renal cell carcinoma
p53 tumour suppressor gene
Polymerase chain reaction
Single strand conformation polymorphism

Introduction

The vast amount of paraffin-embedded surgical material accumulated in departments of pathology contains an important fund of potential information, particularly since the advent of methods for gene extraction from nuclei in paraffin-embedded sections made possible by advances in molecular biology. The quantity of extracted genetic material is too tiny and the genes are cut too short to permit analysis by routine approaches such as Southern blotting. The polymerase chain reaction (PCR) method eliminates this problem, although the question of the optimal length of exon base pairs to use for the best result is still debated. The single strand conformation polymorphism (SSCP) method developed by Orita et al. (1989a, b) is capable of detecting point mutations and/or short deletions reliably. Sequencing analysis of all short PCR products is a cumbersome approach and this can be alleviated by SSCP screening. Using this method on frozen material, Suzuki et al. (1992) previously demonstrated only 1 case with a point mutation in the *p53* tumour suppressor gene among 23 renal cell carcinomas (1/23, 4.3%; Lamb and Crowford 1986; Buchman et al. 1988; Rotter and Prokocimer 1991). However, the number of samples was relatively small since renal cell carcinomas usually demonstrate wide histological heterogeneity. It has been reported that the *p53* tumour suppressor gene plays an important role in tumour initiation, promotion, and/or progression in many kinds of malignant tumours, for example hepatocellular carcinomas, colorectal carcinomas, breast cancers and gastric cancers. Although loss of heterozygosity (LOH) for the short arm of chromosome 3 in renal cell carcinomas is well known, it is not known which genes are related to tumour progression, prognosis or histological features. Therefore we analysed a large number of paraffin-embedded renal cell carcinoma samples retrospectively for mutations of the *p53* gene using PCR combined with the SSCP method, taking into account histopathological characteristics and prognosis and investigated the possibility that the gene is altered during tumour progression.

Materials and methods

One hundred and eighteen tumour specimens obtained from patients who underwent nephrectomy at the Jikei University Hospital between 1983 and 1991 were used for the analysis. They consisted of various histological types and nuclear grades (Table 1), including 4 pure papillary carcinomas. The tissues were all routinely fixed with phosphate-buffered 10% formalin and embedded in paraffin. Serial sections were prepared from each tissue block. The first and the third sections were cut at 3 μm in thickness and

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Table 1 Classification of the 118 renal cell carcinomas

Cell type: number		Nuclear grade: number		Stage: number (pT)	
Clear	:95	1	:46	1	: 1
Granular	: 9	2	:60	2	:48
Mixed	:11	3	:12	3a	:30
Sarcomatous	: 3			3b	:33
Papillary	: 4			4	: 6

stained with haematoxylin and eosin for confirmative diagnostic histopathology. The second section was cut at 60 μm in thickness for preparation of DNA.

Extraction of DNA was performed as described by Goelz et al. (1985) with slight modifications. Briefly, the second section was deparaffinized with xylene and hydrated with ethanol in a 2-ml microcentrifuge tube. The tissue was suspended in 600 μl of 100 μg/ml proteinase K buffer [1 × Standard saline citrate (SSC), 1% Sodium dodecyl sulfate (SDS)] and incubated for 120 h at 48° C. Additional proteinase K aliquots of 100 μl of 1 mg proteinase K/1 ml distilled water were added at three different times during the incubation. After extraction by phenol and chloroform, DNA was precipitated in ethanol and resuspended in sterile TE buffer [10 mM Tris, 1 mM ethylenediaminetetracetic acid (EDTA), pH 8.0] for storage.

All primers were synthesized using a DNA synthesizer 391 (Applied Biosystems, Foster City, Calif., USA), the primers for exons 4–9 being similar to those used by Nigro et al. (1989). Nucleic acid sequences for each exon were as follows. Exons 4–9 (product length 2.9 kb) no. 1 GTCCCAAGCAATGGATGAT, no. 2 TGGAAACTTTCCACTTGAT; the first half of exon 5 (product length 134 bp) no. 3 GATGAATTCTACTCCCCTGCCTCAACAA (contains *Eco* RI site), no. 71 AAAGCTTGCTTGTAGATGGCCATGGCG (contains *Hind* III site); The last half of exon 5 (product length 147 bp) no. 67 GAATTCGGGTTGATTCCACACCCCCG (contains *Eco* RI site), no. 73 AAAGCTTAGCTGCTCACCATCGCTATC (contains *Hind* III site); exon 6 (product length 144 bp) no. 5 ATAGAATTCGTCTGGCCCCTCCTCAGCA (contains *Eco* RI site), no. 76 AAAGCTTAGTTGCAAACCAGACCTCAG (contains *Hind* III site); exon 7 (product length 127 bp) no. 7 AGAATTCGTTGGCTCTGACTGTACCAC (contains *Eco* RI site), no. 8 GAGAAGCTTTGGAGTCTTCCAGTGTGATG (contains *Hind* III site); exon 8 (product length 153 bp) no. 9 GAGGAATTCTGGTAATCTACTGGGACGGA (contains *Eco* RI site), no. 10 AAAAAGCTTGCTTAGTGCTCCCTGGGGGC (contains *Hind* III site); exon 9 (product length 88 bp) no. 11 GAGGAATTCTGCCCAACAACACCAGCTCC (contains *Eco* RI site), no. 12 GGGAAGCTTTGAAGGGTGAAATATTCTCC (contains *Hind* III site).

In the present experiment the second PCR reaction for exon 5 was tried with one set of primers (nos. 3 and 4) in 40 cases, but successful polymerization only occurred in 12 cases (30%). Therefore, polymerization of exon 5 was performed after preparing two sets of primers (nos. 3 and 71, nos. 67 and 73) with shorter base pairs, and in all 118 cases polymerization succeeded. Exon 6 was successfully polymerized with another set of primers (nos. 5 and 6), but in the SSCP analysis two single strand bands appeared too close to each other to be distinguished. A new set of primers for exon 6 (nos. 5 and 76) was therefore prepared to overcome this problem.

For PCR, DNA was amplified in two stages. The first reaction mixture contained 1 μg genomic DNA in 100 μl of solution with 50 mM potassium chloride, 10 mM Tris-hydrochloric acid at pH 8.3, 1.25 mM magnesium chloride, 200 μM each of dATP, dCTP, dGTP, and dTTP, 1 μl of each primer (10 Optical density (OD), 49.7 μM, final 0.497 μM) for exons 4–9 (nos. 1 and 2), and 0.5 μl DNA polymerase (Wako, Osaka, Japan). Thirty cycles at 95° C for 1 min, 58° C for 1 min, and 72° C for 4 min were applied, followed by terminal conditions at 72° C for 7 min in a DNA thermal cycler (Perkin Elmer Cetus, Norfolk, Conn., USA). The second reaction mixture contained 5 μl of the 2.9 kb PCR product (exons 4–9) in 20 μl PCR reaction buffer with the same composition as described above, 200 μM each of dATP, dGTP, and dTTP, 0.4 μl alpha-32-P-dCTP (3000 mCi/mmol), 0.05 μl of each primer set (10 OD, 34.4 μM, final 0.086 μM), and 0.05 μl DNA polymerase. Thirty-five cycles at 94° C for 40 s, 55° C for 40 s, and 72° C for 90 s were applied, followed by terminal conditions at 72° C for 7 min.

In order to carry out SSCP and sequencing, the amplification products were diluted ten-fold in a buffer consisting of 20 mM EDTA, 95% formamide, 0.05% bromphenol blue, and 0.05% xylene cyanol, heated to 99° C for 1 min, and cooled immediately to 0° C. One microlitre of this sample was loaded onto a 6% neutral polyacrylamide gel with 50% glycerol. Electrophoresis was performed at 60 W for 2.5 h at 18° C. The gel was dried on filter paper and exposed to X-ray film at room temperature with an intensifying screen. The PCR products were subcloned into the pUC119 vector and sequenced using the Sequenase kit (United State Biochemical; version 2.0).

Immunohistological investigation of the *p53* tumour suppressor gene was not carried out.

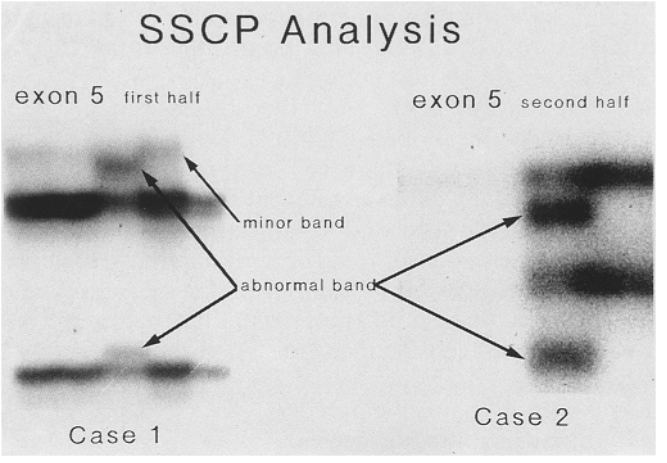

Fig. 1 So-called minor bands and abnormal bands in single strand conformation polymorphism (SSCP) analysis

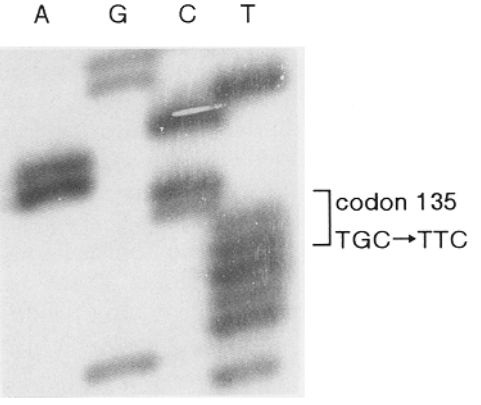

Fig. 2 Sequencing analysis revealed an alteration: codon 135, TGC → TTC (cysteine → phenylalanine) in case 1

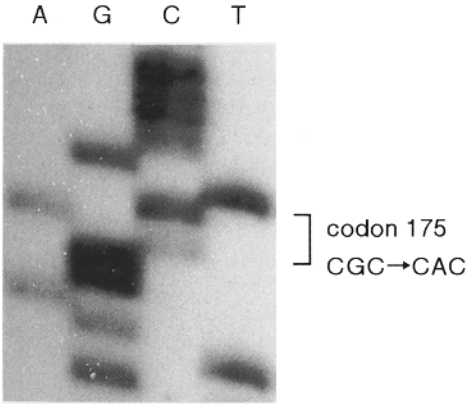

Fig. 3 Sequencing analysis revealed an alteration: codon 175, CGC → CAC (arginine → histidine) in case 2

Results

In the SSCP analysis, so-called minor bands often appeared, which necessitated their distinction from abnormal bands (Fig. 1). The latter could, however, be distinguished because the so-called minor bands always appeared in the same position in most of the lanes. The minor bands did not appear using labelled primer and non-labelled dNTPs; therefore they might be non-specific products in the prior PCR. On sequencing analysis of the cases that expressed minor bands, there were no point mutations, rearrangements, or insertions. Therefore direct sequencing of the minor bands was not carried out.

Only 2 cases of point mutations among 118 renal cell carcinomas were identified after SSCP analysis, se-

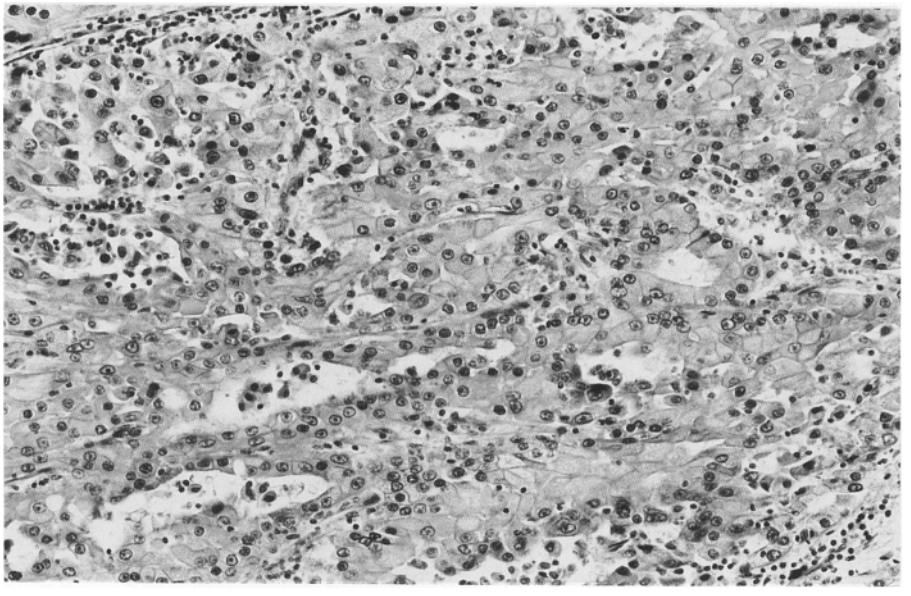

Fig. 4 Renal cell carcinoma, common cell type, granular cell subtype, alveolar and tubular pattern, grade 2 in case 1. Haematoxylin and eosin (H&E) × 50

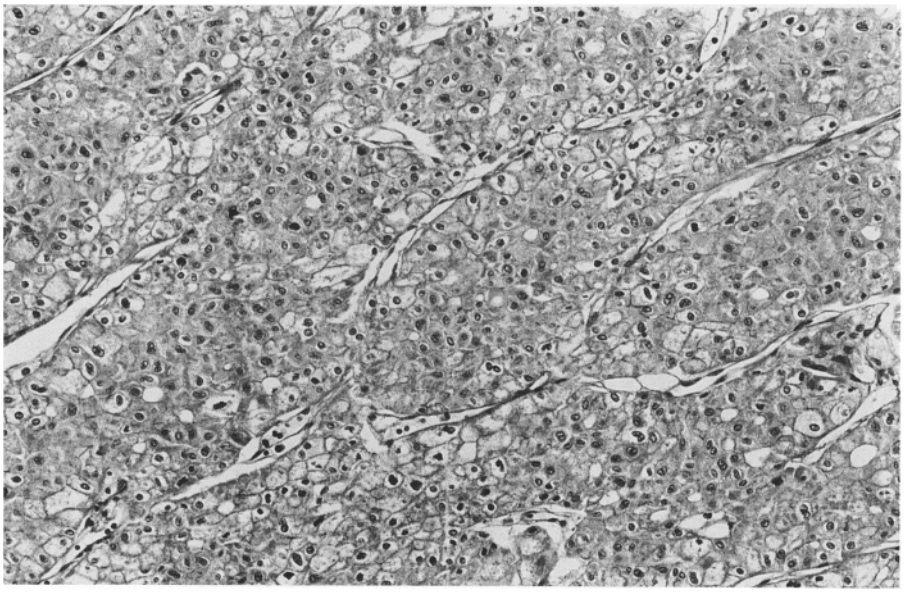

Fig. 5 Renal cell carcinoma, common cell type, granular cell subtype, alveolar pattern, grade 2 in case 2. H&E, × 50

quencing analysis revealing the following alterations: codon 135, TGC → TTC (cystiene → phenylalanine) in case 1 (Fig. 2); and codon 175, CGC → CAC (arginine → histidine) in case 2 (Fig. 3). Case 1 was a 61-year-old male at presentation. His tumour, 20 × 25 mm in maximum dimensions, was histologically diagnosed to be granular cell type with alveolar and tubular patterns, and was classified as grade 2 (Fig. 4). He is still alive and disease free 24 months after the operation. Case 2 was a 36-year-old female at presentation. Her tumour, which measured 30 × 28 × 40 mm, was a granular cell type with an alveolar pattern, and was classified as grade 2 (Fig. 5). She is also still disease free 53 months after her operation. We were unable to analyse the difference between these two positive cases and the other negative cases statistically, because of their limited number.

Discussion

The length of exon 5 that was not successfully polymerized in 70% of the cases was 201 bp, including the extended area of restriction enzyme sites. In contrast, polymerization of 156 bp was successful in all cases. Therefore there might be a limitation of between 150 and 200 bp for PCR using DNA extracted with phenol and chloroform after proteinase K treatment of paraffin-embedded surgical materials as a template. However, Heller et al. (1991) amplified beta-globulin fragments that were from 300 to 500 bp in length using a sonication method. However, it is not possible to find bind shifts when the amplified DNA fragments are very long in SSCP analysis. We conclude that DNA fragments between 100 and 150 bp in length are best for SSCP analysis at the present time.

Abnormalities in the *p53* tumour suppressor gene were found in only 2% of a total of 118 renal cell carcinomas in our series. The mutations were found in the highly conservative regions, and were on GC-dinucleotides in which a high frequency of mutations was reported in *p53*. Suzuki et al. (1992) similarly detected only 1 case of point mutation among 23 renal cell carcinomas using DNA templates obtained from snap frozen materials. Although in terms of percentage their value (4.3%) was a little higher than ours, the difference is negligible. In contrast, Reiter et al. (1993) detected *p53* mutations in 11 of 33 cell lines, the differences in findings being presumably due to variation in the materials. There are two possibilities. Cell lines from renal cell carcinomas with *p53* mutations may be established more easily than those without mutations and/or the mutations are formed when culture starts. Murakami et al. (1991) reported detection of *p53* gene aberrations only in advanced hepatocellular carcinomas, and not in early lesions, suggesting their involvement in a late stage of hepatocellular carcinoma development. Kikuchi-Yanoshita et al. (1992) reported that genetic changes in both alleles of the *p53* gene through mutation and LOH may play a role in the conversion of adenoma to early carcinoma of the colorectum. However Davidoff et al. (1991) described mutations within a highly conserved region of the *p53* gene leading to overexpression of the protein product and showed that these could occur in the earliest recognized phase of breast cancer. This alteration was maintained during progression from intraductal to infiltrating carcinoma. Sano et al. (1991) reported that LOH on chromosome 17p, where the *p53* tumour suppressor gene is located, is a common event in gastric carcinomas and is found from an early stage. Our results suggest that the *p53* tumour suppressor gene does not play a major role in the initiation, promotion, and/or progression of renal cell carcinomas. Similar evidence has been presented by Nanus et al. (1990) for abnormalities in *ras*, which were also found in only 2% of renal cell carcinomas. The coincidence of low prevalence of common oncogene and suppressor gene mutations is of clear interest. There may thus be different mechanisms in the development of renal cell carcinoma in relation to a stepwise alteration of oncogene mutations than those thought to exist for other tumours such as colonic and rectal adenocarcinomas and breast carcinomas.

The two cases with *p53* mutations in the present study were both classified as granular subtype lesions. Kovacs et al. (1992) reported that renal cell tumours were characterized by the loss of chromosome 3p and trisomy 5q segments (common, non-papillary renal cell carcinoma), by trisomy of chromosome 7 and 17 and loss of the Y chromosome (papillary renal cell carcinoma), by random karyotype changes and mitochondrial alterations (renal oncocytoma), or by LOH of 3p, 5q, 17p and 17q (chromophobe renal cell carcinoma). Ogawa et al. (1992) reported that allelic losses at 17p in the granular cell type of renal cell carcinoma were significantly more frequent (44%; 4 out of 9) than in clear cell type (6%; 1 out of 18). Hout et al. (1993) reported LOH for 3p was present in 22 out of 36 clear cell tumours, whereas none of 15 non-clear cell tumours were affected. On this basis they discussed whether non-clear cell tumours may have a different genetic background. Our data suggest the same possibility for granular cell type carcinomas. The present approach allowed histological heterogeneity to be taken into account accurately by using DNA extraction techniques on adjacent sections to those used for microscopic observation. This is especially important with renal cell carcinomas, which have a wide variety of forms or present as complex mixtures.

In conclusion, molecular biological analyses of DNA extracted from paraffin-embedded surgical materials is useful for retrospective studies with the proviso that appropriate lengths of DNA for PCR amplification are applied to screening by the SSCP method. In particular, analyses are most effective when the relationship between gene mutations and differences or histological heterogeneity can be discussed. Our data indicate that the *p53* tumour suppressor gene is not generally related to tumour initiation, promotion, and/or progression in renal cell carcinoma, although the granular cell subtype

may be an exception. Thus mutations of the *p53* tumour suppressor gene were found in none of 95 clear cell subtype carcinomas but in two of nine granular cell subtype carcinomas. The possibility that granular cell type carcinomas may have a different genetic background from clear cell type carcinomas requires further elucidation.

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