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Infrequent alteration of the DPC4 tumor suppressor gene in renal cell carcinoma

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Abstract The aim of this study was to investigate the alterations in the DPC4 tumor suppressor gene in renal cell carcinoma (RCC). The study included 32 tumor specimens from Croatian patients with a diagnosis of RCC. Loss of heterozygosity (LOH) was investigated using three specific oligonucleotide primers for the three DPC4 polymorphic markers. Our investigation of mutations in the DPC4 gene was focused on exons 2, 8, 10 and 11. These exons belong to the mad homology domains 1 (exon 2) and 2 (exons 8–11). The presence of previously documented mutation in exons 2 (codon 100), 8 (codon 358), 10 (codon 412), and 11 (codon 493) was investigated by restriction fragment length polymorphism (RFLP) analysis, as a first screening method. Finally, the study was extended to search for any other type of mutation in the four selected exons by single strand conformation polymorphism (SSCP) assay. To increase heterozygosity, all 32 tumor specimens were tested with primers for three polymorphic markers. A total of 30 (94%) were heterozygous (informative). LOH at any of these markers was only revealed in four (13%) of the 30 informative samples. No tumor samples were positive for mutation in the four investigated exons analyzed by RFLP. In addition, no samples showed other types of mutation in denaturing conditions. Genetic alterations were shown only in a minority of patients, probably because mutation analysis of the DPC4 gene has only been partially covered by our work. It seems that exon 2 (belonging to the MH1 domain) and exons 8, 10, 11 (belonging to the MH2 domain) are not altered in RCC. This investigation must be extended on other exons of DPC4 for a better understanding a role of this gene in renal cell carcinoma.

Keywords Tumor suppressor gene · DPC4 · Renal cell carcinoma · Loss of heterozygosity

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Introduction

Renal cell carcinoma (RCC) represents 3% of human cancers. It is the most common malignancy of the kidney in adults, occurring in both sporadic and hereditary forms. Prognosis is mainly related to stage, with a 5-year survival rate of over 90% in stage I disease, falling to 16–32% for stage IV [1]. The most common deletion found in RCC is loss of heterozygosity (LOH) on the short arm of chromosome 3. This leads to the conclusion that the short arm of this chromosome contains several tumor suppressor genes. The best known is the von Hippel-Lindau (VHL) gene. The VHL gene is mapped to the 3p25 region [2] and has the characteristics of a tumor suppressor gene. The inactivation of the VHL gene, in the form of either mutation, loss of heterozygosity or hypermethylation, has frequently been found in sporadic clear cell renal cell carcinoma [3, 4, 5].

The second breakpoint gene, localized at the most common fragile site at chromosome 3p14.2, is the fragile histidine triad gene [6]. This gene has been found altered in a number of cancers, but surprisingly not in sporadic RCC [7].

Other chromosomes potentially associated with renal carcinoma are 6q, 8p, 9p, 13q and 17p [8, 9]. The genes which belong these chromosomes are p53, c-myc, HER2, EGF-R, H-, K-, and N-ras, p14 ^{ARF}, p16 ^{INK4a} [10, 11, 12, 13, 14].

During the development of the kidney, a process termed branching morphogenesis forms the network of collecting ducts. This process arises in the kidney through interaction between the mesenchymal metanephric blastoma and the epithelial ureteric bud [15]. These interactions are mediated by growth factors belonging to the bone morphogenetic protein (BMP) family, a subset of the TGF-β superfamily. The common member of the BMP and TGF-β pathways is the DPC4 (SMAD4) gene, which was originally determined as a tumor suppressor gene in pancreatic carcinoma tissue [16, 17]. In BMP responses, phosphorylated SMAD1 (or SMAD5 and SMAD8) forms a heteromeric complex with the DPC4 (SMAD4) gene, and moves to the nucleus, where DPC4 contributes to DNA binding, which is critical for transcriptional activation [18].

The role of DPC4 in kidney malignancy has not been fully characterized. In this work, we analyzed 32 tumor specimens from Croatian patients with a diagnosis of RCC. To investigate the alterations which affected the DPC4 gene, RCC samples were surveyed for evidence of LOH and also for mutations by restriction fragment length polymorphism (RFLP) and PCR-single strand conformation polymorphism (PCR-SSCP) methods. Our analysis focused on specific parts of the gene; exon 2 (belonging to the MH1 domain) and exons 8, 10, 11 (which belong to the MH2 domain). The MH1 and MH2 domains present highly conserved regions of the DPC4 gene.

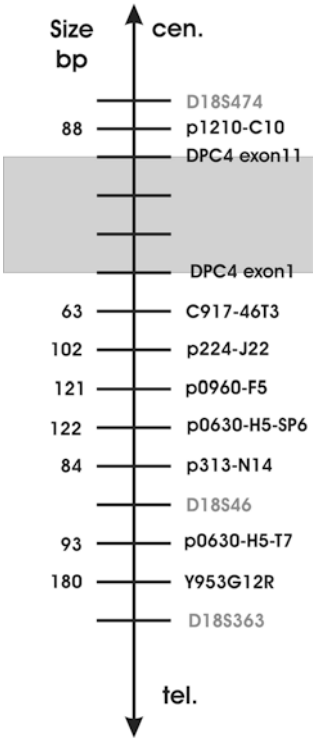

Fig. 1 Physical map of the DPC4 region at chromosome 18q21.1. The *shaded area* is the DPC4 gene region. *Horizontal bars* show the corresponding sequence tagged site (STS). Primer sequences for STS, size in base pairs (*bp*) and the order of markers were reported previously [16]

Materials and methods

Samples

A total of 32 primary tumors from Croatian patients (21 men and 11 women) with a pathological diagnosis of RCC were included in this study. After surgery, the tumor specimens were immediately stored at –80°C until used. The corresponding normal tissue was peripheral blood, and DNA was isolated immediately after transport to the laboratory. All tumor samples were staged according to the TNM-International Union against Cancer classification systems [19]. Thirty one of the tumors were nonpapillary clear cells tumors and only one was classified as a nonpapillary granular cell tumor. Only one tumor was metastatic (T30; tumor stage IV) at the time of diagnosis. DNA isolation was performed using proteinase K digestion and phenol-chloroform extraction [20].

Loss of heterozygosity analysis

The both normal and tumor genomic DNAs were analyzed for allelic loss with primers for three microsatellite markers (D18S474, D18S363, D18S46) lying in the 18q21 region surrounding the DPC4 gene [16, http://www.gdb.org]. Hahn and co-workers [16] reported the order of the markers used (Fig. 1). Table 1 shows the sequences of the primers used.

Genomic DNA (200 ng) was used as the template for the PCR. The PCR mixture (25 µl) contained 5 pM of each primer, 50 µM of each dNTP, and 1 U Taq polymerase (Applied Biosystems). The samples were processed through 35 cycles consisting of 30 s at 96°C, 30 s at 55°C or 58°C (depending on the primer), 30 s at 74°C, and 10 min of final extension. The annealing temperature for each pair of primers was optimized in pilot studies before processing experimental samples. The

Table 1 The sequence of three pairs of primers used in the PCR reaction, followed by loss of heterozygosity (LOH) analysis of the DPC4 gene

Chromosomal location	Amplimer size (bp)	Primer sequence
D18S474	119-139	5′ TGGGGTGTTTACCAGCATC 3′ 5′ TGGCTTTCAATGTCAGAAGG 3′
D18S363	177–247	5′ TTGGGAACTGCTCTACATTC 3′ 5′ GCTTCATTCTCTCACTGGAT 3′
D18S46	129–153	5′ GAATAGCAGGACCTATCAAAGAGC 3′ 5′ CAGATTAAGTGAAAACAGCATATGTG 3′

quality of the PCR products was checked by electrophoresis in an agarose gel.

LOH was determined by microsatellite analysis. About 5 µl of each PCR product were mixed with 3 µl of loading buffer (Eppendorf, Germany) and loaded onto a 1 mm thick, 35×30 cm, 10 or 12% non-denaturing polyacrylamide gel (PAA; acrylamide, N, N methylene-bis-acrylamide, Sigma). Electrophoresis was performed in 1×TBE (Tris/borate acid/EDTA) buffer for 18 h, at 300 V, at ambient temperature. The gel was silver stained. LOH was considered to occur when the tumor DNA did not show or showed a reduced allele which was present in normal DNA.

RFLP analysis

All tumor specimens were tested for intragenic mutations by RFLP analysis. We looked at previously described mutations in exons 2 (codon 100), 8 (codon 358), 10 (codon 412), and 11 (codon 493). Four pairs of primers, whose sequences are listed in Table 2, were used. PCR was carried out under the same conditions as above, except that the annealing temperature was 57°C or 60°C depending on the primer. Ten microliters of amplified product was incubated overnight with 2 U of the appropriate enzyme (*Mae*II, *Mnl*I, *Mae*I, or *Bsp*HI) in a volume of 25 µl, under a drop of paraffin oil. Digestion occurred at the manufacturer's recommended temperature (Table 2).

The products of digestion were analyzed on 2% agarose gels (only in the analysis of exon 2) or on Spreadex EL 300 gels (Elchrom Scientific, Switzerland), in a thermostated circulating bath (Ministat, Huber).

Agarose gels were prepared with ethidium bromide and visualized under UVC light. Spreadex gels were stained with SYBR Green (Molecular Probes, USA) for 30 min, destained in distilled water and analyzed on a UV transilluminator at 254 nm.

PCR-SSCP analysis

All tumor specimens were screened for other mutations in the DPC4 coding region (exons 2, 8, 10, and 11) by PCR-SSCP analysis. Each exon was amplified as above and then 5 µl of the PCR product was mixed with 10 µl freshly prepared denaturing solution (1 ml formamide, Sigma, 10 µl 1 M NaOH). The samples were heated at 95°C for 10 min, cooled on ice and applied to a polyacrylamide gel (6, 9 or 10%) at 4°C under 260 V, for 4 h. For staining single strand DNA, SYBR Gold dye (Molecular Probes, USA) was used.

Results

LOH analysis

The 32 DNAs isolated from matched normal and tumor tissues were analyzed for possible LOH at the DPC4 gene. Three pairs of specific oligonucleotide primers for the three polymorphic markers (D18S474, D18S363, and D18S46), which surround the DPC4 region, were used in the analysis. Normal DNA showed one band (homozygous patient) or two bands (heterozygous patient) at the flanking markers. To increase the frequency of informative (heterozygous) tumors, all samples were investigated with the three primers. Thus, 30 of 32 (94%) investigated samples were informative (Table 3). The heterozygosity at the individual markers was 91% (D18S363), 63% (D18S46), and 59% (D18S474). Only two samples were homozygous at the three DPC4 loci (T8, T24), and four showed homozygosity at two

Table 2 The sequence of four pairs of primers used in the mutation analysis of the DPC4 gene

Exon of the DPC4 (codon)	Amplimer size (bp)	Enzyme/incubation temperature (°C)	Type of changes	Digestion products normal/ mutation	Primer sequence
Exon 2 (100)	526	*Mae*II (50°C)	AGG → ACG	526/319 + 207	5′ TGTATGACATGGCCAAGTTAG 3’ 5′ CAATACTCGTTTTAGCAGTC 3′
Exon 8 (358)	184	*Mnl*I (37°C)	GGA → TGA	117 + 67/184	5′ CTCCTGAGTATTGGTGTTCC 3′ 5′ CTTGCTCTCTCAATGGCTTC 3′
Exon 10 (412)	139	*Mae*I (45°C)	TAC → TAG	139/93 + 46	5′ TTGCGTCAGTGTCATCGACAA 3′ 5′ GATAGCTGGAGCTATTCCAC 3′
Exon 11 (493)	212	*Bsp*HI (37°C)	GAT → CAT	212/183 + 29	5′ TCTGTCAGCTGCTGCTGGAA 3′ 5′ GGTTGTGGGTCTGCAATCGG 3′

Table 3 Results of LOH analysis at the three DPC4 loci

Microsatellite markers	Total	Informative (%)	LOH	LOH/informative (%)
D18S363	32	29 (91)	0	0
D18S474	32	19 (59)	1	5
D18S46	32	20 (63)	3	15
TOTAL	32	30 (94)	4	13

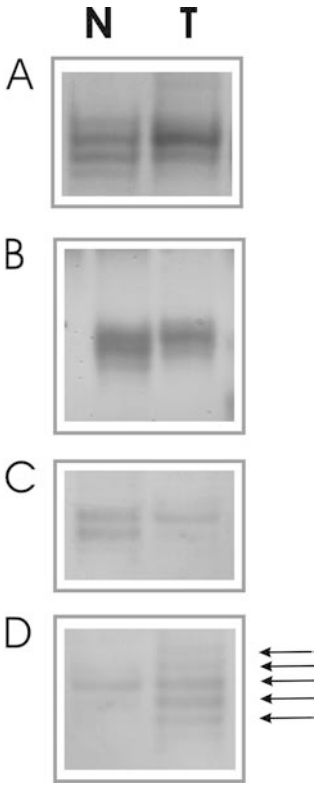

Fig. 2 Three samples with loss of heterozygosity (LOH) (*A*, *B*, *C*) and one sample with microsatellite instability (*D*) at locus D18S46. *N* normal; *T* tumor

investigated loci (T6, T7, T29, and T31). Only one tumor (T31) showed microsatellite instability at the microsatellite marker D18S46 (Fig. 2D).

In our study, LOH occurred in only four of 30 informative tumors (13%); three samples (T10, T22, and T32) at the D18S46 locus (Fig. 2A–C) and one sample (T20) at D18S474 (Fig. 3). The stages of the tumors with LOH were II or III.

Detection of DPC4 mutations by RFLP analysis

In this work, we tested for the presence of previously documented mutations in exons 2, 8, 10, and 11. For this purpose we used the *Mae*II, *Mnl*I, *Mae*I, and *Bsp*HI RFLP sites.

The PCR product of exon 2 has a length of 526 bp. In codon 100, there is normally no site for the action of the *Mae* II enzyme. However, if AGG is changed to ACG, the enzyme found a restriction location, and the sequence of 526 bp was cut into fragments of 207 bp and 319 bp (Table 2). We did not find this type of mutation in renal cell carcinomas collected from Croatian patients. Figure 4A shows some samples of RFLP analysis (exon 2) tested on an agarose gel, prepared with ethidium bromide.

A restriction site for the enzyme *Mnl*I occurs in exon 8, codon 358. When the mutation is present, triplet GGA is changed into TGA, and the PCR product

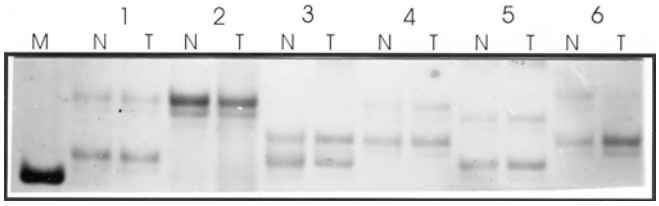

Fig. 3 Loss of heterozygosity at the DPC4 microsatellite locus D18S474. *M*, molecular weight DNA marker pBR322 *Msp*I digest (BioLabs, New England); *N* normal; *T* tumor; *1*, *3*, *4*, *5* heterozygous (informative) without LOH, *2* homozygous (not informative), *6* heterozygous (informative) with LOH

(184 bp) is uncut. All investigated tumor samples contained the *Mnl*I restriction site, which indicates that the mutation is not present. Figure 4B shows some non-mutated representative samples digested with the *Mnl*I enzyme into two fragments (117 bp + 67 bp), separated on a Spreadex EL 300 gel.

The amplified product of exon 10 has a length of 139 bp. The change of normal triplet TAC to mutant TAG in codon 412 allowed its digestion with the *Mae*I enzyme. In the case of a mutation, two fragments occurred of 46 bp and 93 bp. We did not observe such a mutation in any sample of RCC (Fig. 4C).

The PCR product of the last exon of the DPC4 gene, exon 11, has 212 bp. Normal DNA lacks a *BspH*I restriction site. This is in contrast to the mutation at codon 493 (GAT → CAT) which constitutes a cleavage site. In this case, the products of digestion are two fragments of 183 bp and 29 bp. No tumor sample from our study was positive for this mutation (Fig. 4D).

The restriction products of exons 8, 10, and 11 were checked on commercial Spreadex EL 300 gels (Elchrom Scientific). This gel is very suitable for such analysis, because of its high-resolution power of bands over a small run distance (Fig. 4 B, C, D).

PCR-SSCP analysis

The analysis of mutations was further extended in order to search for any other mutation in the four exons of interest. For this purpose we used a PAA gel, denaturing conditions and SYBR Gold dye.

None of the samples had patterns showing aberrant SSCP variants (Fig. 5A–D).

Discussion

The predominant malignant kidney tumor, RCC or Grawitz tumor, originates from the proximal tubules. To date, there is little data on a connection between the DPC4 gene and the initiation and/or progression of RCC. Cardillo and co-workers [21] investigated the role of the TGF-β pathway in the development of RCC. They analyzed whether alterations in the expression of the TGF-β1 pathway were associated with the grade of

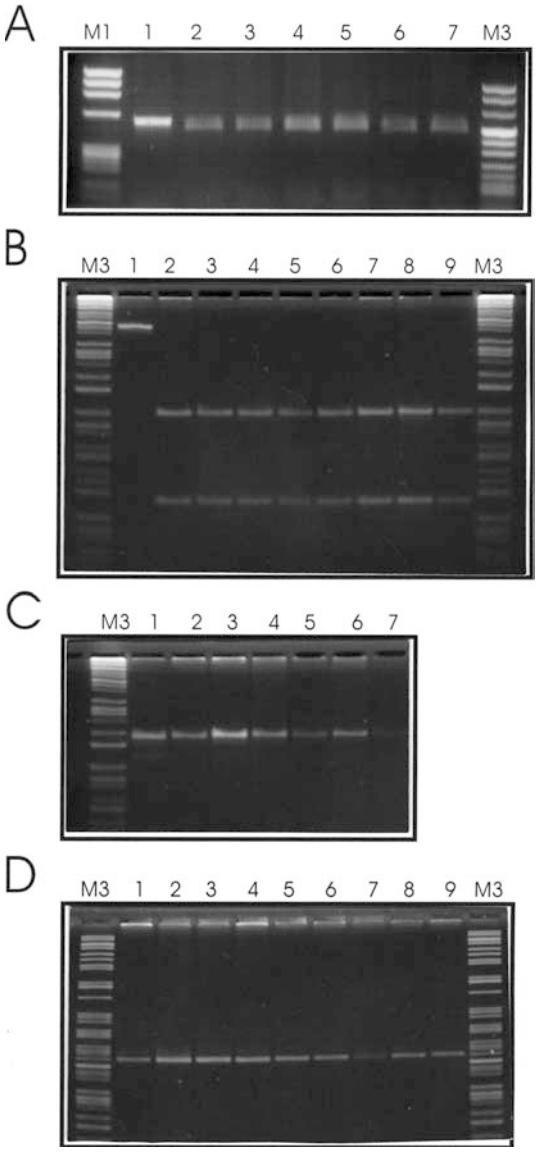

Fig. 4 Examination of mutations in four exons in the DPC4 gene with RFLP analysis. *M1*, molecular weight marker IX (Roche), *M3*, molecular weight DNA marker (Elchrom Scientific, Switzerland). **A** *1* a PCR product of DPC4, exon 2/codon 100 (526 bp); *2*, *3*, *4*, *5*, *6*, *7* samples without a site for *Mae*II enzyme (normal 526 bp; mutation 319 + 207). **B** *1* a PCR product of DPC4, exon 8/codon 358 (184 bp); *2*, *3*, *4*, *5*, *6*, *7*, *8*, *9* *Mnl*I RFLP within exon 8 (normal 117 + 67 bp; mutation 184 bp). **C** *1* a PCR product of DPC4, exon 10/codon 412 (139 bp); *2*, *3*, *4*, *5*, *6*, *7* samples without site for *Mae*I digestion (normal 139 bp; mutation 46 + 93 bp). **D** *1* a PCR product of DPC4, exon 11/codon 493 (212 bp); *2*, *3*, *4*, *5*, *6*, *7*, *8*, *9* uncut samples with *Bsp*HI enzyme (normal 212 bp; mutation 183 + 29 bp)

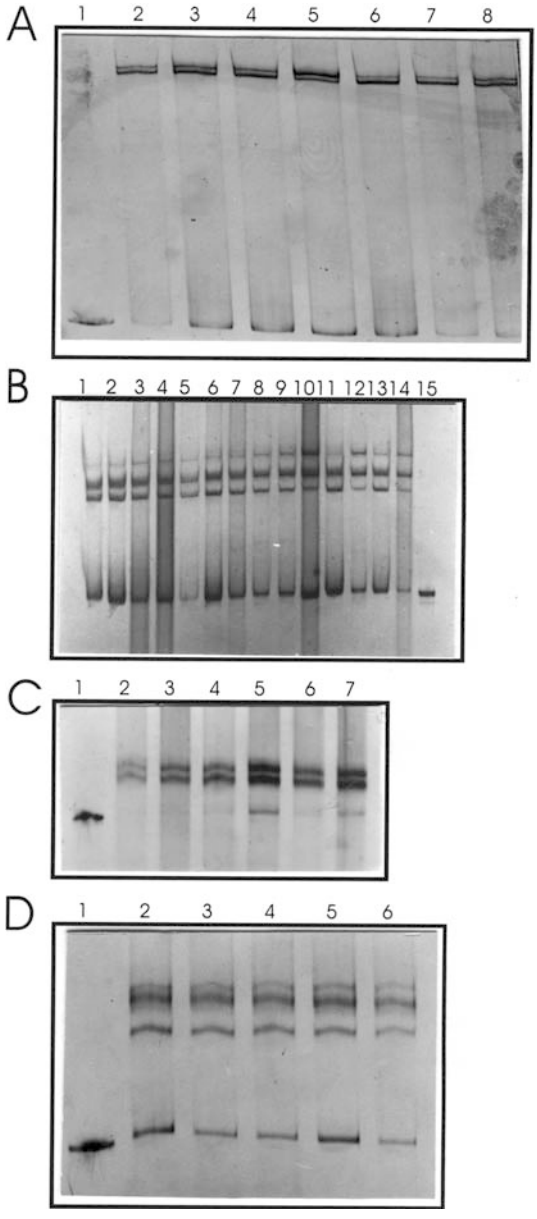

Fig. 5 PCR-SSCP analysis of tumor suppressor gene DPC4 in renal cell carcinoma samples. **A** *1* a PCR product of exon 2; *2*, *3*, *4*, *5*, *6*, *7*, *8* denatured tumor samples, **B** *1–14* denatured tumor samples; *15* PCR product of exon 8, **C** *1* a PCR product of exon 10; *2*, *3*, *4*, *5*, *6*, *7* denatured tumor samples, **D** *1* a PCR product of exon 11; *2*, *3*, *4*, *5*, *6* denatured tumor samples

tumor differentiation and pathological stage. Receptors TGF-β RI and TGF-β RII were more frequently expressed in the tumor than in normal renal tissue. This is the opposite to Smad 2 and Smad 4 genes, for which expression was more frequent in normal than in tumor tissue. The authors, however, did not observe any significant correlation between the histological scores of TGF-β RI, RII and DPC4 with either the histological grade of the malignancy or with TNM clinical stage. The exception was Smad 2, the protein levels of which were significantly lower in grade 3 than in grade 1 tumors. These results indicate that an altered TGF-β/Smad signaling pathway is involved in kidney neoplasm.

We evaluated the status of the DPC4 tumor suppressor gene in 32 samples of RCC, with 31 samples of nonpapillary clear cell tumor and only one sample of nonpapillary granular cell tumor. First, we investigated LOH of this tumor suppressor gene, since inactivation of a tumor suppressor gene plays an important role in the development of human malignancies. We chose primers

for the three microsatellite markers D18S474, D18S363, and D18S46 which are usually used in such analysis [16, 22]. In order to increase the number of informative (heterozygous) specimens, all samples were tested using all three markers. Of these, 94% (30/32) were informative. However, LOH at the DPC4 loci was found in only four (13%) of the 30 informative samples; one sample (5%) at the D18S474 locus, and three (15%) at the D18S46 locus. We did not observe LOH in any sample at the D18S363 locus, although we found the highest frequency of heterozygous samples at this locus.

Detection of allelic losses by microsatellite analysis has been a widely accepted method for evaluating the implications of tumor-suppressor genes in tumors. The frequency of LOH observed in this work suggests that the DPC4 gene is active in the genesis of RCC. A correlation between LOH at the 18q21 region and tumor stage was not found, although in our specimens LOH was observed in samples with II and III stage of tumors.

The majority of cancer cells escape TGF-ß mediated growth control. The mechanism of resistance to the growth inhibitory effects by TGF-ß is not clear. TGF-ß signaling is initiated when the type I receptor phosphorylates the SMAD proteins Smad2 and Smad3, which function as transcription factors regulating gene expression. Mutations in Smad2 have been detected in human colon and lung cancers, but there are no precise data on the involvement of Smad2 in kidney tumor. Li and co-workers [23] recently publish work in which they confirm that TGF-ß is a potent mediator in renal fibrosis and that the Smad proteins are critical intracellular mediators in TGF-ß signaling. This work reported that renal fibrogenesis in the tubular epithelial cells is associated with the activation of Smad2. This fibrotic process, however, could be blocked by overexpression of Smad7. This suggests that treatment targeting the inactivation of Smad2 by overexpression of Smad7 may provide a new therapeutic strategy for renal fibrosis. Taken together, it is possible that Smad2 plays important role in RCC.

Tumors show a hypermutable feature, called microsatellite instability, that occurs in many types of cancer [24, 25]. However, in our study we observed only one case with genetic instability (sample T31 at the D18S46 locus, Fig. 2D). Some authors believe that LOH at a distinct locus may be preceded by a mutation in the specific gene. A recent study suggests that LOH of 18q may be independent of a DPC4 mutation [26]. The combined genetic and protein expression data support a model in which allelic loss of DPC4 (and also p53) is the first hit in biallelic inactivation during pancreatic carcinogenesis. In the second step, inactivation of the second allele occurs by mutation.

Numerous genetic aberrations have been documented in the DPC4 gene, of which the best understood are those that occur in pancreatic tumors. About 90% of the reported mutations are located in the C-terminal region [16, 27]. This finding prompted us to investigate whether DPC4 aberrations exist in RCC samples from Croatian patients, especially in those with LOH. It was reasonable to use the unique restriction site and RFLP analysis as a first screening method to detect some mutations at the specific site/codon within DPC4 gene. These mutations were previously documented in the literature, with a precisely defined site of enzyme action, and with known restriction products [16, 28]. For the visualization of restriction products we used Spreadex EL 300 gels, which were shown to be a powerful tool for the RFLP analysis of small and very tight bands.

In the second step, screening for other mutations in the highly conserved domains of the DPC4 gene was performed by SSCP analysis using PAA gels. None of the tested samples was positive for mutation by either of the methods used (Figs. 4, 5).

Alterations in genes which function as regulators of cell growth and differentiation are considered to be crucial in the progression of human cancers. Our results show that the parts of the DPC4 gene tested in our study do not suffer the alterations at high frequency in RCC. The TGF-ß signaling pathway contains many members. It is possible that some other genes in this pathway are inactivated in RCC and contribute to the malignant progression. This should be confirmed in future work.

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