Mutations of the p53 Gene and p53 Protein Overexpression Are Associated with Sarcomatoid Transformation in Renal Cell Carcinomas

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ABSTRACT

Renal cell carcinomas sometimes show sarcomatoid transformation, thus comprising both carcinomatous and sarcomatous components. Such sarcomatoid renal cell carcinomas are highly malignant with pronounced proliferative activity. The present investigation was conducted to assess the mutational status of the p53 and H-ras genes independently in carcinomatous and sarcomatous portions of individual tumors, applying PCR, subcloning, and sequencing to 14 cases. Sarcomatoid portions showed an extremely high mutation rate for the p53 gene (11 of 14, 78.6%) with two mutational hot spots at codons 278 (6 of 14, 42.9%) and 244 (6 of 14, 42.9%). Five cases showed double mutations, four cases had mutations at codons 278 and 244, and one case had mutations at codons 278 and 248. In contrast, the carcinomatous portions demonstrated a low mutation rate for the p53 gene (2 of 14, 14.3%) and no double mutations were detected. Ten cases showed genetic heterogeneity in the p53 gene between the two tumor components. Furthermore, p53 overexpression was immunohistochemically observed only in those components with p53 mutations, mainly in the sarcomatoid portions. No H-ras mutations were observed. The findings strongly suggest that p53 mutations leading to overexpression of p53 protein are closely associated with sarcomatoid transformation in renal cell carcinomas.

INTRODUCTION

Recent cytogenetic and molecular studies of RCC2 have demonstrated a close association between loss of chromosome 3p and tumor development (1–4). At least three candidate suppressor genes are suspected of being present within this locus (5, 6). Recently, one of the candidates was cloned to be a von Hippel-Lindau disease-related gene (7). The high proportion of 3p deletions observed in both early and late stage tumors suggests that inactivation of suppressor genes may play a causal role in RCC development (1–5). Whether such genes are involved in the mechanisms of progression in RCCs, however, is still undetermined.

The sequence of stages involved in tumor progression are often histologically associated with cellular dedifferentiation, with subpopulations of less differentiated cells growing within lesions, to generate a heterogeneous appearance (8–10). An analysis of genetic alterations among the tumor heterogeneity of differentiation, therefore, should be appropriate to study the roles in tumor progression. RCCs are typical in progressing with such dedifferentiation and, interestingly, sometimes show sarcomatoid transformation (11–13). These sarcomatoid RCCs that consist of carcinomatous and sarcomatous components are characterized by highly malignant biological behavior (13, 14). Recently, we showed this tumor to have the highest proliferative activity of RCC types (15). Thus, the sarcomatoid RCC provides us with a suitable model for studies of cellular heterogeneity and progression within single tumors.

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3 The abbreviation used is: RCC, renal cell carcinoma.
Table 1  p53 mutation and immunohistochemical results for sarcomatoid renal cell carcinomas

<table>
<thead>
<tr>
<th>Case</th>
<th>Components within each tumor</th>
<th>Codon 1</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>Immunohistochemical results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2, Cl</td>
<td>Wild type</td>
<td>CCT to CTT</td>
<td>Pro to Leu</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
<td>278</td>
<td>GGC to TGC/GGC</td>
<td>Gly to Cys</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>2, Mix</td>
<td>Wild type</td>
<td>CCT to CTT</td>
<td>Pro to Leu</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>S</td>
<td>244</td>
<td>GGC to TGC/GGC</td>
<td>Gly to Cys</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>1, Cl</td>
<td>Wild type</td>
<td>CCT to CTT/CCT</td>
<td>Pro to Leu</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>2, S</td>
<td>278</td>
<td>GGC to TGC/GGC</td>
<td>Gly to Cys</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>2, Cl</td>
<td>Wild type</td>
<td>CCT to CTT</td>
<td>Pro to Leu</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>3, Mix</td>
<td>244</td>
<td>GGC to TGC/GGC</td>
<td>Gly to Cys</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>2, Mix</td>
<td>Wild type</td>
<td>CCT to CTT</td>
<td>Pro to Leu</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>2, Mix</td>
<td>Wild type</td>
<td>GTC to TTC/GTC</td>
<td>Val to Phe</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>2, Cl</td>
<td>Wild type</td>
<td>GGC to TGC/GGC</td>
<td>Gly to Cys</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>2, Cl</td>
<td>Wild type</td>
<td>CCT to CTT</td>
<td>Pro to Leu</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>2, Gr</td>
<td>Wild type</td>
<td>GGC to TGC/GGC</td>
<td>Gly to Cys</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>3, Gr</td>
<td>Wild type</td>
<td>CCT to CTT</td>
<td>Pro to Leu</td>
<td>–</td>
</tr>
</tbody>
</table>

a, sarcomatoid portion; 1, 2, and 3, carcinomatous portions with grades of 1, 2, and 3, respectively; Cl, clear cell type; Gr, granular cell type; Mix, mixed cell type.

RESULTS

p53 Gene Mutations and p53 Protein Overexpression

DNA Sequence Analysis. Eleven of the 14 sarcomatoid RCC cases were found to have mutations in exons 5, 7, or 8 of the p53 gene as shown in Table 1. All the cases with alterations in the p53 gene had point mutations, causing amino acid substitutions by missense. All but...
one (case 8) of the cases showed genetic heterogeneity of p53 within the tumor. In sarcomatoid portions, 8 of 11 cases with alterations of p53 gene showed a point mutation at the second base position of codon 278 (exon 8) (Fig. 1a). The nucleotide change was a C to T transition, which would result in an amino acid substitution of leucine for proline. Six of 11 cases had a point mutation at the first letter of codon 244 (exon 7) with a G to T transversion nucleotide change (Fig. 1b) and an amino acid substitution of cystine for glycine. As for other mutations, sarcomatoid portions of cases 10 and 14 had point mutations at the first base position of codon 157 and the second letter of codon 248, respectively. Five cases showed double missense mutations in the sarcomatoid portion. Four cases (cases 2, 6, 11, and 13) had mutations at codons 278 as well as 244, and in case 14 codons 278 and 248 were involved. In contrast, in carcinomatous portions, only 2 tumors showed mutations in the p53 gene, cases 6 and 8 having point mutations at codons 278 and 244, respectively. As described above and shown in Table 1, 10 cases showed genetic heterogeneity within the p53 gene. Nine cases (cases 1, 2, 3, 5, 7, 10, 11, 13, and 14) had the wild-type p53 gene in carcinomatous portions along with single or double mutations in the sarcomatoid portions. Case 8 had the same mutation at codon 244 in both carcinomatous and sarcomatous portions. No mutations in exons 5-8 of the p53 gene were evident in samples obtained from noncancerous parts of the affected kidneys. In cases 4, 9, and 12, no p53 gene mutations were detected.

Restriction Enzyme Analysis. PCR products of exons 7 and 8 from all of the sarcomatoid portions were digested with restriction enzymes NsiI and EcoRII, respectively. The results were compatible with the sequencing results as shown in Table 1. PCR products including exon 7 from the six cases (cases 2, 3, 6, 8, 11, and 13) were cleaved by NsiI into 98-, 80-, 38-, and 18-base pair subfragments. As shown in Fig. 2a, both of the normal and mutant digestion patterns were observed in the cases showing both of the wild-type and mutant bands in the sequence analysis. EcoRII-digested PCR products, including exon 8 from eight cases (cases 1, 2, 5, 6, 7, 11, 13, and 14), yielded 132-base pair subfragments, which were observed only in mutant PCR products as shown in Fig. 2b. Since the lower subfragments were similar fragment sizes such as 71, 69, and 63 base pairs, it was difficult to distinguish whether both of the normal and mutant PCR products were present or only mutant products.

**Immunohistochemical Analysis.** The relationship between mutation and protein expression of the p53 gene was assessed by immunohistochemical demonstration of p53 protein with two antibodies, DO-7 and CM-1. Both antibodies showed the identical results as summarized in Table 1, although CM-1 demonstrated higher background staining than did DO-7. As shown in Table 1 and Fig. 3, 12 of 13 portions with a missense mutation (including 11 sarcomatoid and 2 carcinomatous portions) exhibited intense staining for p53 protein in most of the nuclei of the cancer cells. Although the sarcomatoid portion of case 7 had a point mutation of the p53 gene, no positive reaction was observed immunohistochemically. Control immunohistochemical analysis for p53 showed intensely positive reactions in the nuclei of colon and lung carcinomas. Negative controls were not stained.

**H-ras Gene Mutations**

Analysis of exons 1 and 2, including codons 12, 13, and 61, revealed no mutations in sarcomatoid, carcinomatous, or noncancerous portions.

**DISCUSSION**

The present report, to our knowledge, is the first to describe a strong association between p53 gene alteration and sarcomatoid transformation in RCC. Thus, the 11 of 14 cases (79%) of sarcomatoid portions of sarcomatoid RCC showing point mutations of the p53 gene showed a clear contrast to the 2 cases (14%) for carcinomatous portions. Including 1 case with a single mutation of p53 gene in the ordinary carcinomatous portion, which had double mutations in the sarcomatous portion, 10 cases showed genetic heterogeneity of the p53 gene within a single tumor. These findings strongly suggest that p53 may play an important role in a unique type of tumor progression in RCC, sarcomatoid transformation.

The fact that eight cases had mutations at the second base position of codon 278 (CCT to CTT) and six cases showed alterations of the first letter of codon 244 (GGC to TGC) indicates the existence of a “hot spot.” While it cannot be completely excluded that artifacts could occur with the presently applied method, the relevant PCR controls were always negative, and sequencing was performed on DNA extracted from mix cultures with at least 50 recombinant colonies. DNA samples isolated from noncancerous parts of the kidneys all demonstrated the wild type. In addition, for the tissues with mutations at codons 278 and 244, new DNA samples were extracted from paraffin blocks and PCR to sequencing procedures were repeated with the results being identical to the original ones in all cases. Furthermore, the PCR products on exons 7 and 8, including the two hot spots, were cut directly with restriction enzymes, and the results were compatible with the sequencing results. Therefore, we conclude that the high mutational frequency at codons 278 and 244 of the p53 gene in sarcomatoid portions is unlikely to be an artifact.

There have been a few previous investigations of alterations of the p53 gene and chromosome 17 in RCCs. Cytogenetic studies revealed...
p53 MUTATIONS IN SARCOMATOID RCC

Fig. 2. Restriction enzyme analysis. (a) PCR products containing p53 exon 7 are digested with NspI. NspI cleaves the normal PCR products into subfragments of 98 and 38 base pairs (bp). The mutation at codon 244 results in creation of another NspI site; therefore, the mutant PCR product is cleaved into 80-, 38-, and 18-base pair fragments. The DNA samples are derived from normal (N) and sarcomatoid portions of case 2 (2) and case 3 (3). Both of the normal and mutation digestion patterns are observed in cases 2 and 3. (b) EcoRII-digested normal PCR products of exon 8 yields 71-, 69- and 63-base pair fragments (N). In the sarcomatoid portions, the mutation at codon 278 leads to loss of one of the two EcoRII sites and a 132-base pair fragment is detected. 1, case 1; 6, case 6.

frequent abnormalities of chromosome 3p but not 17p. More recently, loss of heterozygosity of 17p using restriction enzyme fragment length polymorphism was found at varying frequencies, with 21% (5 of 24 cases) reported by Morita et al. (5), 16% (5 of 31 cases) by Presti et al. (29), and 6% (2 of 22 cases) by Anglard et al. (4). It was suggested by the authors that allelic losses at 17p, together with those at other chromosomes such as 11p and 13q, might contribute to the progression of RCC. This is consistent with our present results, although greater numbers of lesions need to be studied for confirmation.

Suzuki et al. (30) investigated mutations of the p53 gene in 23 cases of surgically resected low grade RCCs by PCR-single strand conformational polymorphism and direct sequencing analysis and reported that only one tumor carried a mutated p53 gene. They thus concluded that p53 mutations could not play an important role. However, Reiter et al. (31) found a 48% loss of heterozygosity on chromosome 17p and a 33% rate of p53 mutations in RCC cell lines. As a possible explanation they stressed that all cell lines evaluated were derived exclusively from patients with advanced disease, making the point that cell lines rarely are contaminated with normal cells such as lymphocytes and stromal cells. However, possibility of mutations occurring in genes during establishment of a cell line cannot be completely ruled out. Consequently, the present description of a relation between primary RCCs and p53 gene mutational status is the first to provide unequivocal evidence.

Several authors have proposed that p53 mutations are associated with the progression of various cancers such as those in the colon, brain, breast, and liver (19-21). However, there have been no reports of mutational clustering at specific codons related to tumor progression. Mutated codons, codons 244 and 278, observed as hot spot in the present study, were reported previously; however, there has been no such clustering as our findings (17-19). Two possible explanations for our results can be put forward. First, some particular chemical carcinogen may be associated with RCC development, especially in the progression stage. In lung cancer related to smoking, a predominant G to T transversion has been observed. This may reflect the action of benzopyrene, which is found in tobacco smoke (32). In hepatocellular carcinomas occurring in aflatoxin B1-contaminated areas such as China and southern Africa, a G to T transversion at codon 249 has been found at high frequency (25, 33). Furthermore, skin cancers associated with UV irradiation often contain the unique double change base, CC to TT (34, 35). These previous reports have suggested that individual chemical carcinogens may cause specific base changes, sometimes at specific codons. For RCCs, environmental factors or chemical carcinogens have never been determined, but in this context, our results do suggest involvement of some specific agent in progression. A second possibility, however, is a link between a specific base change at a specific codon which can occur with dedifferentiation. A previous examination of the p53 gene in carcinomas of the thyroid.

Fig. 3. p53 immunostaining exhibiting nuclear overexpression in the sarcomatoid portion of case 11 (a), but not in the adjacent carcinomatous portion (b) (×180).
gland revealed a unique association between mutations and undifferentiated but not differentiated lesions (36). Furthermore, one-half of their cases (3 of 6 cases) showed p53 mutations at the same codon 248. This and our results may imply that particular mutations might occur with presentation of specific histological features.

The present study demonstrated five cases with double missense mutations limited to the sarcomatoid portions. Such accumulation of p53 mutations may either cause or be due to clonal expansion. Thus, an additional mutation might increase the potential for progression. On the other hand, tumor cells are known to be less stable genetically than normal cells and this instability leads in itself to the occurrence of more mutations. There are only a few reports describing double mutations in the literature (21), and no definite conclusion is possible at present.

It has been described that overexpression of p53 protein correlates with the existence of missense mutations (37). This is directly supported by our findings that most RCC samples with single or double missense mutations were immunohistochemically positive for binding of antibodies against p53 protein. Staining was observed limited to the nuclei of tumor cells. Recently, Moll et al. (38) investigated the relation between localization of p53 protein and p53 mutation and demonstrated that cytoplasmic accumulation in breast carcinomas is not linked with any mutation. However, this was not a feature of our study. In the present series, was an exception in case 7 showing no positive staining with p53 antibodies, despite having a missense mutation at codon 278 of the p53 gene and demonstrating a positive reaction for other nuclear proteins such as proliferating nuclear antigen (15). We therefore speculated that this negative result might be due to other genetic alterations leading to abnormality of mRNA transcription and resulting in a truncated p53 protein.

Our results for the -ras gene, with no mutation observed in exons 1 and 2, including codons 12, 13, and 61, in any carcinomatous or sarcomatoid RCC tissue, are in line with the report of Naus et al. (16), who found only 1 positive RCC of 47 RCCs and 3 renal oncocytomas examined for mutations of H-, K-, and N-ras genes. They speculated that only advanced stage RCC lesions might demonstrate ras mutations in RCC, since their case was metastatic. However, Nagata et al. (39) revealed 16 cases of RCC to be negative and, considering these previous reports together with our results, we conclude that ras gene mutations are very rare in RCCs.

REFERENCES

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