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PROGNOSTIC FACTORS IN RENAL-CELL CARCINOMA: IMMUNOHISTOCHEMICAL DETECTION OF p53 PROTEIN VERSUS CLINICO-PATHOLOGICAL PARAMETERS

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Immunoreactivity for p53 protein was assessed in 100 cases of primary renal-cell carcinoma (RCC). The results were correlated with clinical survival data (follow-up 24 to 84 months; mean: 39 months) and with clinico-pathological parameters, including nuclear grade, tumour stage, cell type, tumour architecture and tumour diameter. In all, 32% of the tumours were p53-positive; there was no difference in survival between p53-positive and -negative cases. Similarly, p53 expression did not correlate with any of the clinico-pathological parameters mentioned. Nuclear grade (grade 1 + 2 *vs.* grade 3 + 4) had a striking impact on prognosis and so, to a lesser extent, did tumour stage and the occurrence of a spindle-cell component. The immunohistochemical detection of p53 in RCC is not of prognostic value. The estimation of nuclear grade, however is a major predictor of prognosis.

Progress through the cell cycle is controlled by positive and negative regulatory substances. An important negative regulator is the p53 protein. Originally discovered in SV40-transformed tumour cells, the *p53* gene is now regarded a major tumour-suppressor gene (Levine *et al.*, 1991). The *p53* gene is localized on the short arm of chromosome 17 (17p) (McBride *et al.*, 1986). A mutation in the gene may result in an altered protein that has lost its negative regulatory effect. In contrast to wild-type p53 protein, point-mutated p53 protein can usually be detected immunohistochemically in paraffin-embedded material (Porter *et al.*, 1992). Immunohistochemically detectable p53 has, in some studies, been linked to a worse prognosis (Harris and Holstein, 1993).

Renal-cell carcinoma (RCC) is a tumour with several histologically distinct subtypes related to more or less specific cytogenetic defects, *e.g.* a deletion at chromosome 3p in clear-cell RCC (Kovacs *et al.*, 1989; Ogawa *et al.*, 1991; Presti *et al.*, 1991). Limited data are available regarding mutations in the *p53* gene in RCC (Oka *et al.*, 1991). Using polymerase chain reaction and single-strand conformational polymorphism analysis of RNA, Torigoe *et al.*, (1992) concluded that p53 mutations occur in only 10% of RCC. Deletions at 17p are found in a sizeable number of RCCs (Presti *et al.*, 1991; Ogawa *et al.*, 1992). In 2 studies, deletions at 17p in RCC were associated with granular cell type (generally associated with a worse prognosis) (Presti *et al.*, 1991) and higher tumour grade (Ogawa *et al.*, 1992). This would fit the hypothesis that loss of p53 cell-cycle control in RCC may lead to a more malignant phenotype which would show immunoreactivity for p53 protein. We therefore performed an immunohistochemical study on p53 protein using archival paraffin-embedded samples of 100 cases of RCC and examined the relation of p53 protein with established prognostic factors and with survival. In this way, we hoped to determine the validity of immunohistochemical assessment of p53 protein as a prognostic factor.

MATERIAL AND METHODS

Clinical details and tissue preparation

A total of 118 RCC specimens from patients treated in the University Hospital, Dijkzigt, in the 5-year period 1985–1990, were examined. The follow-up period ranged from 24 to 92 months (mean: 39 months; median: 34 months). Eighteen patients were excluded because of loss to follow-up or insufficient material, leaving 100 patients for analysis. In total, 87 patients were treated by complete nephrectomy and 13 were treated by partial nephrectomy or tumour enucleation. In some cases, lymph-node dissection was performed as well. No pre-operative treatment directed at the tumour was given. Clinico-pathological details of patients and tumours are summarized in Table I. Tumour cell type was categorized as either clear-cell, granular-cell, spindle-cell or oncocytic, according to standard pathological criteria. Architecture was categorized as papillary or non-papillary, the latter including solid, trabecular or tubulo-acinar subtypes. When a tumour was of mixed type it was classified according to the appearance of the dominant part.

The Fuhrman system of nuclear grading (Fuhrman *et al.*, 1982) and the UICC TNM system for tumour staging were employed.

The specimens had been routinely processed, including fixation in 10% buffered formalin and embedding in paraffin wax. For each tumour a representative tissue block was selected from the archives. Whenever possible this tissue block contained uninvolved kidney tissue as well, for purposes of comparison. Follow-up data for each patient were drawn from the hospital charts.

Immunohistochemistry

The monoclonal antibody DO-7 (DAKO, Glostrup, Denmark) was used throughout the studies. This antibody was designed for the immunohistochemical detection of p53 in paraffin-embedded human material and is produced by a hybridoma from mice immunized with recombinant human wild-type p53 (Vojtěšek *et al.*, 1992); it detects wild-type as well as mutant p53. DO-7 was tested in dilutions ranging from 1:5 to 1:200. A dilution of 1:25 was found to give the optimal signal to noise (= non-specific staining) ratio. In 10 cases, available frozen tumour material was compared with the formalin-fixed material, yielding similar results.

Briefly, 5-μM sections were cut, mounted on 3-amino propyltrioxy-silane (Sigma)-coated glass slides and heat-fixed in an incubator overnight at 37°C. The slides were then deparaffinized in xylene, rinsed with alcohol and incubated for 10 min in 3% H_2O_2 in methanol to block endogenous peroxidase activity. After a thorough wash in water, the antigen retrieval method described by Shi *et al.* (1991) and modified for p53 detection by one of us (K.S.K.) was employed. To this end, the slides were immersed in deionized water and heated in a microwave oven (700 W power) to 100°C for 2 × 5 min. Between each period of heating, evaporated fluid was replenished. After a 25-min cooling period, the slides were rinsed in PBS and preincubated in 10% rabbit serum in PBS for 30 min at room temperature. Then DO-7 was applied (1:25, in PBS) and the slides were stored overnight at 4°C.

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TABLE I - p53 EXPRESSION IN RCC *VERSUS* CLINICO-PATHOLOGICAL CHARACTERISTICS

Characteristic	Number	p53 positive (%)
Total	100	32 (32)[1]
Sex:		
Male	66	23 (35)
Female	34	9 (26)
Diameter:		
< 5 cm	40	16 (40)
5–10 cm	44	11 (25)
> 10 cm	16	5 (31)
Cell type:		
Clear cell	74	22 (30)
Granular cell	18	7 (39)
Spindle cell	7	3 (43)
Oncocytic	1	0 (0)
Architecture:		
Papillary	14	6 (43)
Non-papillary	86	26 (30)
Nuclear grade:		
G1	22	7 (32)
G2	45	17 (38)
G3	26	6 (23)
G4	7	2 (29)
Tumor stage:		
T1	10	5 (50)
T2	54	16 (30)
T3	36	11 (31)
Treatment		
Total nephrectomy	87	25 (29)
Tumor enucleation/ partial nephrectomy	13	7 (54)

[1]18 of moderate, 7 of strong and 7 of very strong intensity.

After the overnight incubation with DO-7, the slides were washed in PBS and monoclonal rabbit anti-mouse antibody (1:50 in PBS/5% BSA) was applied for 30 min at room temperature. Following washing in PBS, the third-step reagent consisted of monoclonal mouse peroxidase-anti-peroxidase complex (PAP) (1:100 in PBS) for 30 min at room temperature again followed by a PBS wash. The slides were then developed using 3,3′-diaminobenzidinetetrahydrochloride (DAB) as substrate and lightly counterstained with Mayer's haematoxylin. Control slides were prepared, omitting the primary antibody, and consistently found to be negative. The PAP system was preferred to a biotinylated system because of the presence of endogenous biotin activity in kidney (and tumour) tissue.

Analysis

p53 staining was typically nuclear, and classified as either non-existent or weak, of moderate, strong or very strong intensity. In the majority of tumours the staining pattern was heterogeneous. A tumour was considered positive when in a field at 250× magnification at least 50% of the nuclei were at least moderately positive. Cytoplasmic staining was not observed.

Survival curves were made using Kaplan-Meier analysis, and compared with the log-rank test. The differences in p53 scores between subgroups of RCC were tested for significance with a Chi-square test.

RESULTS

p53 protein expression

A typical example of p53 immunostaining of RCC is shown in Figure 1. The results of the immunohistochemical staining of p53 protein in relation to clinicopathological parameters are summarized in Table I.

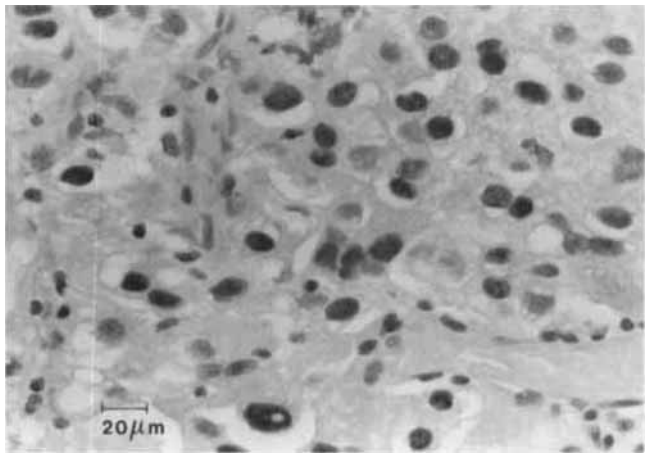

FIGURE 1 – Typical renal-cell carcinoma with a majority of the nuclei staining dark brown/black indicating p53 immunoreactivity. Scale bar, 20 μm.

A total of 32 tumours were p53-positive (32%) (18 of moderate, 7 of strong and 7 of very strong intensity) and 68 were p53-negative. Normal kidney never showed positive reactivity with anti-p53. No significant difference in p53 reactivity was seen between tumours composed of diverse cell types or between tumours with papillary *versus* non-papillary architecture. Among different classes of nuclear grade, no statistically significant difference in p53 reactivity was noted. This was also the case when low-grade (G1 + G2) tumours were compared with high-grade (G3 + G4) tumours ($\alpha > 0.2$). Equally, T-category, sex of patient and tumour diameter did not correlate with p53 expression. In very small tumours (diameter of 2.5 cm or less), which by some are regarded as adenomas, the distribution of p53-positive and -negative cases was analogous to the distribution in larger tumours. When the same analysis was performed with only strongly p53-reactive tumours rated as positive, the results were identical. This eliminates a potential bias due to subjective assessment of staining intensity.

Survival analysis

A Kaplan-Meier survival analysis demonstrated no difference in survival between patients with p53-positive tumours and those with p53-negative tumours (Fig. 2). Equally, no difference in survival could be detected after stratification for grade or T-category.

Among the conventional clinicopathological parameters, nuclear grade had the strongest effect on prognosis. Patients with G3 and G4 tumours had a significantly lower chance of survival than did those with low-grade (G1 and G2) tumours ($p < 0.001$) (Fig. 3*a*).

T-category was another important determinant of prognosis. T1 and T2 tumours (limited to the kidney and differing only in size) shared the same prognosis, T3 tumours (extension into peri-renal fatty tissue and/or the renal vein) clearly had a worse prognosis ($p = 0.02$) (Fig. 3*b*).

Clear-cell type and granular-cell type showed no significant difference in survival curves ($p = 0.2$). Spindle-cell type, however, was related to a definitely worse prognosis (Fig. 3*c*).

No significant differences in survival were found between papillary and non-papillary tumours (Fig. 3*d*) and with increasing tumour diameter (data not shown).

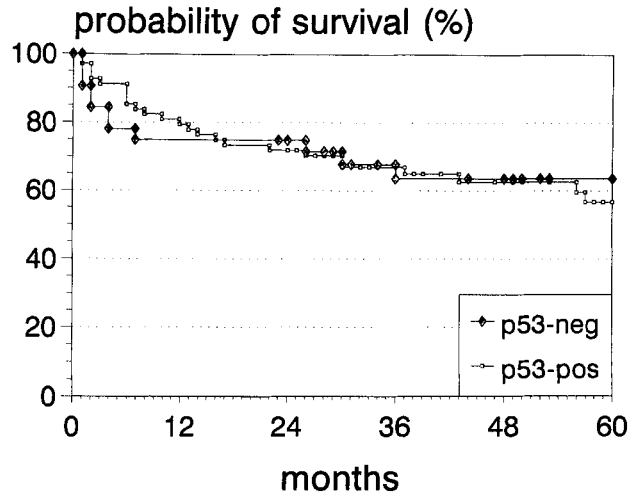

FIGURE 2 – Kaplan-Meier survival analysis comparing cases with p53-negative RCC and cases with p53-positive RCC. The Y-axis shows the probability of survival and the X-axis the length of survival in months. There is no difference in survival between p53-negative and p53-positive cases.

DISCUSSION

This study shows that immunoreactivity for p53 protein in the nuclei of RCC cells is not related to prognosis or to established, prognostically relevant, clinico-pathologic parameters.

In order to obtain a sufficiently strong p53 signal, the use of antigen-retrieval (Shi *et al.*, 1991), adapted for p53 detection, was a necessity in formalin-fixed material, as otherwise hardly any p53 reactivity could be detected. Artifacts due to unequal duration of fixation may play a role in this routinely processed material, and could be an explanation for the heterogeneous staining pattern in many tumours.

In several studies on other types of cancer, p53 expression emerged as a negative prognostic factor (Harris and Holstein, 1993). In 2 studies on breast carcinoma, however, p53 expression did correlate with unfavourable prognostic factors, but not with prognosis (Davidoff *et al.*, 1991; Bosari *et al.*, 1992). Furthermore, p53 expression or immunostaining did not correlate with prognosis in laryngeal carcinoma (Dolcetti *et al.*, 1992), lung carcinoma (McLaren *et al.*, 1992) and breast carcinoma (Scott *et al.*, 1991).

The lack of correlation between p53 staining and prognosis in renal-cell carcinoma observed in this study is therefore not unique. An explanation for this might be that, in RCC, carcinogenesis occurs along a pathway that does not involve p53. The predominant type of RCC, the clear-cell carcinoma, is characterized by a distinct cytogenetic abnormality: a deletion on the short arm of chromosome 3 involving a putative tumour-suppressor gene (Ogawa *et al.*, 1991; Yamakawa *et al.*, 1991). This pathway may not require a p53 mutation to confer malignant behaviour upon a cell. Presumably, a mutation in the *p53* gene may occur at the same time, but it is not clear as yet what its role might be in the development of malignant behaviour.

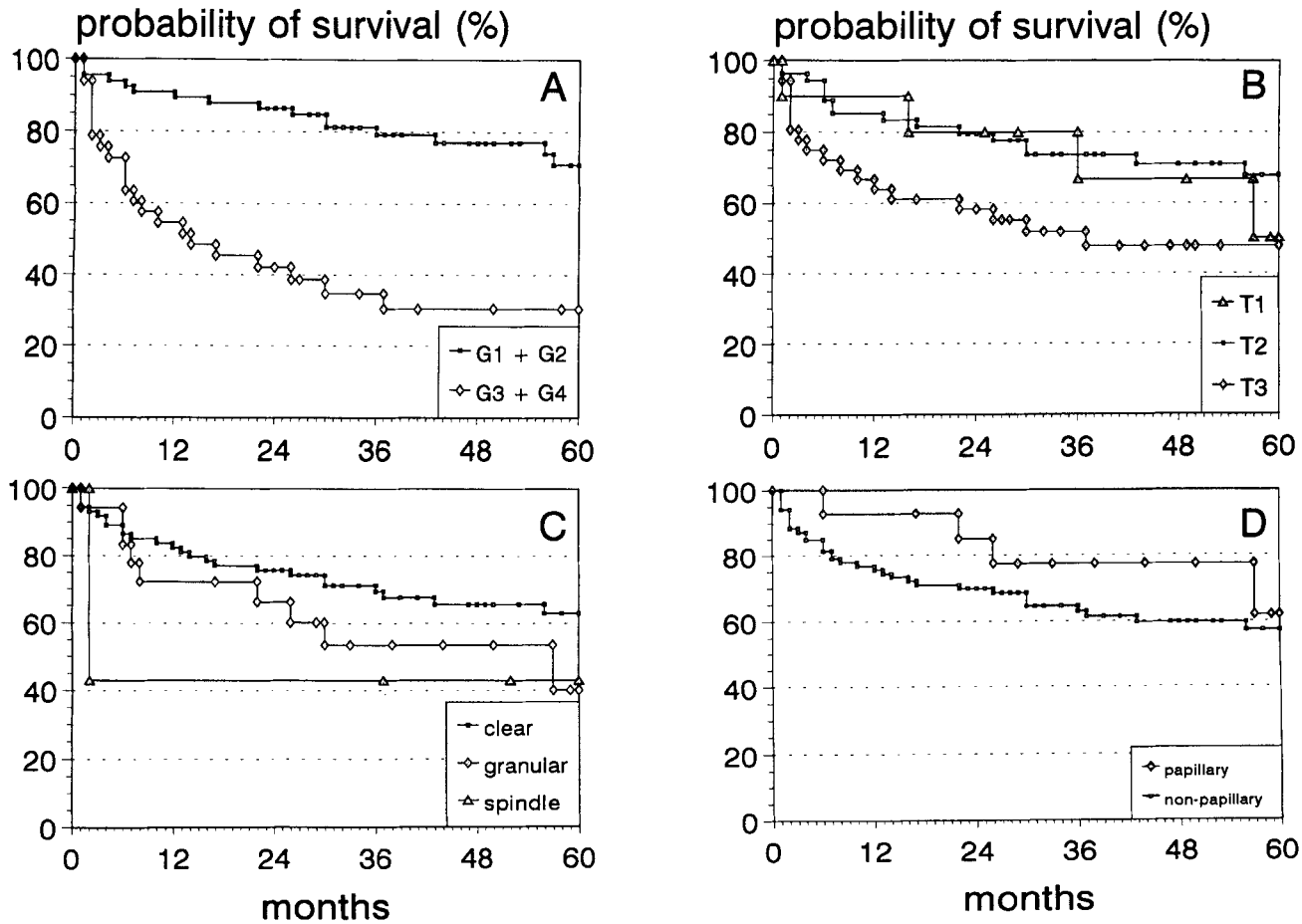

FIGURE 3 – Kaplan-Meier survival analysis of RCC, comparing grade 1 + 2 with grade 3 + 4 *(a)*, stage T1, T2 and T3 *(b)*, clear-, granular- and spindle-cell type *(c)* and papillary and non-papillary tumour architecture *(d)*.

It must be emphasized that our studies were designed to estimate the validity of immunohistochemically detectable p53 protein as an indicator of prognosis. Since not all mutations in the *p53* gene result in protein production and we cannot completely exclude the detection of wild-type protein, our results allow, at best, only limited conclusions regarding mutations in the *p53* gene. This may also explain the discrepancy between our results (immunoreactive p53 in 32%) and those of Torigoe *et al.* (1992), who found mutations in only 10% of cases.

The papillary type of RCC is reported to exhibit trisomy of the short arm of chromosome 17 (Kovacs *et al.*, 1989; Ogawa *et al.*, 1992) which harbours the *p53* gene. At the outset of the study, we hypothesized that mutation of this gene might be involved in the development of this relatively rare type of RCC. However, our results do not support this hypothesis. Although we could examine only 14 cases of papillary RCC, 6 (43%) were p53-positive and did not differ significantly from non-papillary tumours. Allelic loss at chromosome 17p has been found more often in granular-cell tumours (Ogawa *et al.*, 1992). As indicated by our results, this is not reflected in a higher percentage of p53-immunoreactive tumours in this category as compared to other cell types. The specific association of chromosome 17p loss with granular-cell type was recently challenged by Reiter *et al.* (1993), as they also found a substantial number of clear-cell carcinomas with deletions on chromosome 17p. These authors observed a p53 mutation in 33% of the RCC cell lines tested. They concluded that abnormalities of the *p53* gene are common in RCC and may be involved in progression of the tumour.

In a number of tumours, the appearance of a p53 mutation is regarded as a late event in tumorigenesis, resulting in a shift towards more malignant behavior (Harris and Holstein, 1993; Kaklamanis *et al.*, 1993). Our data do not support a similar mechanism for RCC, since p53 immunoreactivity is not elevated in tumours of higher grade or in a higher T-category. Furthermore, among very small RCCs, diameter 2.5 cm or less (by some regarded as adenomas) p53 positivity was comparable to that in larger tumours.

Of the other pathological parameters, nuclear grade in particular was significantly correlated with survival. The distinction between grade 2 and grade 3 represents a major impact on prognosis. These results confirm previous findings regarding the importance of nuclear grading of RCC (Fuhrman *et al.*, 1982; Gelb *et al.*, 1993). Stage is a well-established prognostic factor (Fuhrman *et al.*, 1982): extension into fatty tissue and/or the renal vein (T3) resulted in a significantly worse survival. There was no difference in prognosis between T1 and T2 cases (differing only in size), in accordance with our finding that tumour size has no prognostic implications. Survival of patients with granular-cell tumours was not different from that of patients with clear-cell tumours, although there was a tendency towards a worse prognosis for the former. This is in agreement with previous work suggesting a worse prognosis for patients with granular-cell tumours, the latter feature, however, not being independent of nuclear grade (Fuhrman *et al.*, 1982).

In conclusion, p53 immunoreactivity in RCC does not correlate with any subgroup of RCC, has no impact on prognosis and therefore is of no use in defining RCC prognosis. The best microscopical variable remains nuclear grade, which has a strong impact on prognosis.

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