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THE ROLE OF p53 ISOFORMS' EXPRESSION AND p53 MUTATION STATUS IN RENAL CELL CANCER PROGNOSIS

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Running title: Significance of p53 isoforms in renal cell cancer

ABSTRACT

OBJECTIVES: To analyze p53 mutations and gene expression of p53, Δ40p53 and Δ133p53 isoforms in RCC tissues and normal adjacent tissue (NAT) and to associate them to clinical features and outcome.

PATIENTS AND METHODS: Forty-one randomly selected patients, with primary, previously untreated RCC, with complete clinicopathohistological data were analyzed. NAT samples were available for 37 cases. Expression of p53, Δ40p53 and Δ133p53 was determined using RT-qPCR. A functional yeast-based assay was performed to analyze p53 mutations.

RESULTS: More than half (56.1%) of patients harbored functional p53 mutations, and they were significantly younger than those with wild type (WT) p53 (P=0.032). Expression of p53, Δ40p53 and Δ133p53 was upregulated in mutant (MT) p53 RCC compared to WT p53 RCC tissues. However, there was no difference in expression of these isoforms between MT p53 RCC tissues and NAT. Expression of Δ133p53 was significantly downregulated in WT p53 tissues compared to NAT (P=0.006). Patients that harbored functional p53 mutation had better overall survival (hazard ratio 4.32, 95% confidence interval 1.46-18.82, P=0.006). Multivariate analysis demonstrated that tumor stage and p53 mutation might be used as independent prognostic marker for overall survival in RCC patients.

CONCLUSIONS: Our findings support the specific events in the carcinogenesis of RCC. p53 isoforms can be differentially expressed depending on p53 mutational status.

Keywords: p53, p53 isoforms, renal cell cancer, p53 mutation

1. INTRODUCTION

Renal cell cancer (RCC) represents 2-3% of all cancers, with the highest incidence in Western countries [1,2]. Over the last two decades the incidence of RCC is increased, mostly due to increased detection of tumors by ultrasound and computed tomography [3]. The Tumor, Nodes, Metastasis (TNM) staging system is used to assess the anatomic extent of disease [4]. The most common histologic subtype is clear cell RCC which is believed to account for 80-90% of all RCCs [4]. Despite of all available prognostic markers, it seems that RCC follows an unpredictable disease course [5]. To improve the prognosis of the disease course, a better understanding of critical genes associated with disease progression is required.

The p53 tumor suppressor protein is critical in the control of cell growth and the maintenance of genomic stability [6]. In contrast to other tumors, p53 is rarely mutated in RCCs suggesting that p53 function might be suppressed by other mechanisms [7-9]. p53 encodes 12 different isoforms that differ in their N- and C-terminus due to alternative splicing, promotor or translation initiation site [10]. The sum of their activities determines the p53-mediated cell response in a given tissue [6,11-13]. They have structural differences, different subcellular localization, they exert different effects on p53-mediated gene expression while some isoforms exhibit some functions independent of p53 [12,14-16]. The Δ40p53 isoform lacks the first 39 amino acids and has lost the first transactivating domain but still retains the second one and the entire DNA binding domain. It has reduced ability to activate transcription of p53 mediated genes by itself, but it can form complexes with p53 and modulate p53-dependent gene expression in a positive and negative manner depending on its relative levels and cellular context [10,17]. Δ133p53 and Δ160p53 isoforms, produced from internal promoter P2, lack the first 132 and 159 amino acids, respectively and have lost both transactivating domains and a part of the first conserved cysteine box of the DNA binding domain. Δ133p53 forms heterocomplex with p53 and hence modulates gene expression in p53-independent way [13].

The expression of p53 isoforms has been shown to be dysregulated in several human cancer types, so we assume that in RCC p53 isoforms might participate in p53 inactivation, in tumor initiation and progression. The aim of this study was to analyze p53 mutation status in RCC tissues and mRNA expression of p53, Δ40p53 and Δ133p53 isoforms in RCC tissues and normal adjacent tissues (NAT), and to associate this information with clinical features and outcome.

2. PATIENTS AND METHODS

2.1. Patients' data

We prospectively analyzed 41 randomly selected patients with primary, previously untreated RCC, with complete clinicopathohistological data. Inclusion criteria were as followed: 1) histologically proven RCC (all cell types of RCC are eligible), 2) patients with all stages of disease according to TNM classification system, 3) patients were older than 18 years, 4) obtained written informed consent for the storage and use of their tissue and clinical information in this study. Thirty-seven patients underwent radical (90.2 %), and four patients partial nephrectomy (9.8%) at the University Hospital Centre Zagreb, Croatia, from November 2010 until October 2013. Four patients (9.7%) had metastatic disease at the beginning of the study and thirteen patients (31.7%) had locally advanced RCC defined by TNM classification (T3/T4, N0, M0). This study complied with the Helsinki Declaration and was approved by the ethical committee from the University Hospital Centre Zagreb, Zagreb. Tissue samples were collected immediately after nephrectomy and evaluated by a pathologist; areas of histologically normal adjacent renal cell tissue were available for 37 cases. All clinical information was collected prospectively from hospital information system and anonymized.

2.2. RNA extraction and RT-qPCR

Total RNA was extracted from 50-100 mg of tissue using TRIzol reagent (Thermo Fisher Scientific, USA) and subsequent RNA clean-up through RNeasy Mini Kit (Qiagen, USA) including DNase I treatment according to the manufacturer's instructions. Total RNA was quantified and purity assessed using the BioSpec-nano Micro-volume UV-Vis Spectrophotometer (Shimadzu, Japan). RNA was reversely transcribed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Absolute copy numbers were determined using the standard curve method by qPCR using the ABI Prism 7300 Detection System (Thermo Fisher Scientific). Reactions were performed in a final volume of 25 µl using the TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific) under standard thermal cycling conditions (50°C for 2 min and 95°C for 10 min followed by 50 cycles at 95°C for 15 s and 60°C for 1 min). Primers and probes sequences (Metabion, Germany) are given in Supplementary Table S1. The absolute copies numbers were calculated from standard curves generated from serial dilutions of linearized plasmid construct carrying the amplicon with known concentration to allow copy numbers determination, and normalized to the average levels of housekeeping gene TATA box-binding protein (*TBP*). Reactions were conducted in duplicates.

2.3. Functional Analysis of Separate Allele in Yeast (FASAY)

We have adapted, and *ad hoc* modified the well-established FASAY assay [18], also known as Gap Repair Assay [18], in order to screen 41 patients for TP53 status. Briefly, cDNA obtained from RCC patients was used as template for a two-step PCR approach to amplify the *TP53* gene. The first-step PCR was performed using 25 µg of cDNA, the Go-Taq G2 Green Master Mix (Promega, USA), p53-Ex2.1-Fw (GTCACTGCCATGGAGGAGCCGCA) and p53-P4 (ACCCTTTTTGGACTTCAGGTGGCTGGAGTG) primers (Eurofins Genomics, Germany). PCR products were diluted 1:400, and used for the second-step nested PCR using again Go-

Taq G2 Green Master Mix but p53-P3 (ATTTGATGCTGTCCCCGGACGATATTGAAC) and p53-Ex10-Rv (CTTCCCAGCCTGGGCATCCTTG) more internal primers. cDNAs from MCF7 (WT p53) and MDA-MB-231 (only expressing the R280K p53 mutant allele) cells were used as negative and positive controls, respectively.

Five or 10 µl of the second-step nested PCR product was added to 1.5 µl of double digested pRDI22 yeast expression plasmid (CEN/ARS, LEU2) and co-transformed in yeast using the Lithium acetate method [20] in the yeast reporter strain yIG397 that contains the ADE2 reporter gene under the control of a p53 Responsive Element. LEU2+ yeast colonies were selected on synthetic plates lacking leucine and containing limiting amount of adenine (5 mg/L). Transformant colonies were white in case of expression of WT p53, whereas MT p53 colonies appeared red. Colonies were counted, and red colony frequency (RCF) was calculated. Given the overall higher amount of background red colonies due to the two-step PCR approach (about 15-20%), samples with an RCF higher than 60% were considered heterozygous for *TP53*. Samples with an RCF below 40% were scored as homozygous wild-type, while samples with almost all red colonies (RCF = 90-100%) were considered homozygous/hemizygous *TP53* mutants. Lastly, samples with an RCF score between 40 and 60% were uncertain and were re-analyzed for a more accurate screening. However, heterozygous samples were considered mutant due to the fact that heterozygous state is often transient and the inactivation of the wild-type allele is likely to happen. Moreover, many MT p53 proteins gain oncogenic activity which overcome the tumor suppressor activity of the remaining WT p53 allele[21].

2.4.Statistical analysis

Normality of data was tested using D'Agostino and Pearson Omnibus test. Continuous variables were log transformed prior to analyses to distribute the data normally. ANOVA and Student's t-test were used to determine the difference of p53 isoforms mRNA expression between various subgroups. Spearman's correlation coefficient was used to calculate the correlation among p53

isoforms' expression. Isoforms' expression was dichotomized into "low" and "high" by a median value. The relationship between p53 isoforms' expression and p53 mutation status to clinical parameters was interrogated using chi-square test. Overall survival (OS) was determined with Kaplan-Meier method and log-rank test, while Cox proportional-hazards regression model was used for multivariate analysis. Statistical analyses were performed using MedCalc for Windows, version 17.6 (MedCalc Software, Belgium). Two-tailed $P<0.05$ was considered to be significant.

3. RESULTS

3.1. p53 mutation status in RCC patients

For the analysis of p53 mutation status we used the FASAY that can distinguish inactivating mutations from functionally silent mutations. Table 1 summarizes the clinicopathological features of the cohort, as well as the results of p53 mutation analysis. More than half of the patients (56.1 %) harbored non-functional p53 mutations (including both homozygous (36.6%) and heterozygous (19.5%) mutants). Of note, p53 mutant cancers were associated with younger age at diagnosis (median 60 for MT p53 vs 69 years for WT p53, P=0.032).

3.2. Low expression of p53 isoforms in RCC

To determine gene expression of p53 isoforms in RCC, the mRNA expression of p53, Δ40p53 and Δ133p53 was analyzed by RT-qPCR in 41 tumors and 37 matched NATs. As shown in Supplement Table 2, Δ40p53 and Δ133p53 had remarkably weak expression compared to p53 - the p53 median expression was 8.85 times higher than the median expression of Δ40p53 and 16.32 times higher than the median expression of Δ133p53 isoform. Also, we observed significantly lower Δ133p53 expression in RCC tissues compared to NATs (P=0.002) (Figure 1). In addition, the expression of each isoform within NATs and cancer tissues regardless of

p53 mutation status was highly associated with one another (Spearman's rank correlation coefficients ranged from 0.700 to 0.886, all P values < 0.05).

3.3.The expression of p53 isoforms in RCC is associated with p53 mutational status

We have further analyzed whether the expression of p53 isoforms is associated with p53 mutation status in RCC tissues (Supplementary Table S2). Cancer tissues that harbored p53 mutations had a significantly higher expression of p53, Δ40p53 and particularly Δ133p53 isoforms (P=0.0009, P=0.004 and P=0.0008, respectively) compared to WT p53 tumors (Figure 2A.). When compared with NATs separately in WT p53 and MT p53 RCCs, only in WT p53 cancer tissues Δ133p53 expression was significantly lower (P=0.006) (Figure 2B). We detected no significant alteration of isoform expression in MT p53 tumors compared to NATs (Figure 2C).

3.4.The association between p53 isoforms expression and clinical features

Table 2 summarizes the association between p53 isoforms' expression and clinical features. There was no significant difference in the expression of either p53, Δ40p53 or Δ133p53 in relation to gender, presence of symptoms, Fuhrman grade, histologic type, lymph node metastases, distant metastases and tumor stage according to TNM classification system. However, higher mRNA expression of p53 and Δ40p53 was associated with larger tumor size (P=0.044 and P=0.021, respectively) and more often renal capsular invasion (P=0.040 and P=0.013, respectively). Younger patients had higher expression of Δ133p53 isoform (P=0.032).

3.5.The association of p53 isoforms expression and p53 mutational status with OS

Next, we have investigated the connection between p53 isoform expression and OS. Median follow-up was 45 months (range 2 to 72 months). The median values for p53, Δ40p53 and Δ133p53 were used to fractionate samples into two groups, a high-expressing group, and a low-expressing group. There was no difference in OS regarding the level of p53, Δ40p53 and

Δ133p53 expression (Figure 3A-C). However, we observed difference in OS of patients harboring WT p53 compared to those with p53 mutations (hazard ratio 4.32, 95% confidence interval (CI) 1.46-18.82, P=0.006) (Figure 3D). A median OS of 27 months (95% CI: 13.0-56.8 months) was observed in patients with WT p53 while it was 53 months (95% CI: 37.4-60 months) in patients with MT p53. In the univariate analysis, Fuhrman grade, tumor stage, tumor size and p53 mutation status were statistically significant prognostic markers for OS (Table 3). In the multivariate model, only tumor stage and p53 mutation status can be considered as independent prognostic marker (Table 3).

4. DISCUSSION

The p53 regulates different cellular responses to stress including DNA repair, cell cycle arrest, proliferation, senescence, differentiation, cell migration and cell death, hence maintaining cell integrity. Therefore, p53 mutations, the most common genetic changes in human cancers, often are associated with worse disease outcome. Of note, p53 isoforms have distinct and independent roles in cancer. Several clinical studies have reported that p53 isoforms are abnormally expressed in human cancers suggesting that they could contribute to cancer formation and progression [22,23]. Two independent studies have determined the expression of p53 isoforms in RCC tissues so far, using semi-quantitative analysis of p53 isoforms levels. However, in both studies, p53 isoforms' expression has not been associated with clinical features and outcome [24,25]. Also, many studies have analyzed p53 mutation status using immunostaining and sequencing methods, but none of them examined the functional mutation status of p53 in RCC tissues [26,27]. The aim of this study was to determine the expression of p53, Δ40p53 and Δ133p53 isoforms in RCC and NATs, to determine the frequency of functional p53 mutations, and to associate the differential expression of isoforms and p53 mutational status with clinical features and outcome.

To this aim, we have determined the p53 status in our tumor collection using FASAY and 56.1% of tumors were identified as bearing a mutant p53 protein (either one or both alleles were mutated). The possible explanation for such a high frequency is the fact that most of the other studies have used immunochemical methods to detect p53 mutations [26,27]. In these studies, high-level expression of p53 is used as a surrogate mutation indicator due to abnormally extended half-live of mutant p53. However, in 10% to 20% of mutant p53 cases, tumors may harbor nonsense (truncating) mutations, which can lead to unstable mutant proteins, that will be expressed at low levels and falsely considered as WT p53 [9]. Furthermore, the majority of studies analyzed the central core domain of the gene (exons 4-8 or 5-8), the most common site of p53 mutations. However, approximately 15% of p53 mutations occur outside exons 5 through 8, that is in exons 4, 9, and 10, and therefore, it is likely that there will be some underestimation of p53 mutations in these studies [9].

The results of our study provide information about mRNA expression of p53 and N-truncated isoforms in RCC and NATs by RT-qPCR. Full-length p53 was the most expressed isoform in RCC tissues, while Δ40p53 and Δ133p53 were notably less expressed. This finding is consistent with study of Marabese and co-workers who had found low levels of Δ40p53 and Δ133p53 isoforms in ovarian carcinoma compared to p53 expression [28]. Also, in squamous cell carcinoma of the head and neck, Δ133p53 expression is lower compared to transactivating full-length isoforms of p53 (including p53β) [29].

We didn't observe any significant difference in p53 and Δ40p53 expression in cancer tissues compared to NATs, while Δ133p53 isoform was downregulated in cancer tissues. The lack of overexpression of p53 in RCC could be explained on the basis of the study conducted with transgenic mice exposed to ionizing radiation, which have revealed that p53 is not necessarily upregulated in kidney tissues in response to stress and is less effective in kidney than in other tissues [30]. Moreover, it is well accepted that full-length p53 expression is generally not

modulated at the mRNA level, but rather at protein levels, often due to an array of several post-translational modifications. In contrast to this observation, another RCC study detected upregulation of TAp53 isoforms (p53, p53β and p53ɣ), but no significant alteration of Δ133p53 expression was detected in different tumor stages as compared to normal renal tissue [24]. However, downregulation of Δ133p53β and ɣ was found only in early tumor phases [24]. In another RCC study, Song and collaborators observed the significant upregulation of p53β isoform in tumor samples which correlated with tumor stage. Again, the other isoforms were expressed at different levels in both tumor and normal tissue but without statistical significance [25]. Both studies used semi-quantitative analysis of p53 isoforms expression and did not analyses p53 mutation status.

We have associated the expression of p53 isoforms with p53 mutation status and found that the expression of all examined isoforms was higher in tumor tissues harboring mutant p53. In RCC tissues with WT p53 we have observed downregulation of Δ133p53 isoform when compared to NATs. In tumors harboring MT p53 down-regulation of Δ133p53 was not observed.

As mentioned before, p53 and Δ40p53 are transcribed from P1 promoter, while Δ133p53 expression is driven by alternative promoter P2 [22], which can be activated by p53 and suppressed by Δ40p53 through suppression of p53 functions [31]. It seems that in the cases that retained WT p53, the feedback regulatory loop might be altered, resulting in lower p53 activity on P2 promoter and downregulation of Δ133p53 isoforms. Based on these results, we speculate that dysregulation of p53 isoforms could contribute to cancer formation in WT p53 tissues. In addition, there was a trend of higher OS in patients with higher expression levels of p53 and Δ133p53 isoforms (Figure 3A-C). Analysis of larger sample size might validate p53 isoform expression level as a prognostic biomarker in RCC patients.

One of the clinically most interesting findings in this study is the association of p53 mutant status with OS. Patients that harbored p53 mutation tumors had longer OS compared with patients with WT p53 tumors. This finding could be explained by the fact that patients with MT p53 tumors were significantly younger and might have less aggressive tumors (lower tumor stage) than those with WT p53 tumors since higher Fuhrman grade and tumor stage also have negative impact on OS (Figure 3D, Table 3). Also, they had higher levels of p53, Δ40p53 and Δ133p53 isoform expression in RCC tissues and no difference in expression of isoforms in RCCs compared to NATs, suggesting that patients that have lower and dysregulated expression of p53 isoforms could have unfavorable clinical outcome even though they retained WT p53.

These observations confirm the result of a previous study showing that silent p53 mutations or mutations in noncoding regions are associated with cancer formation probably because they lead to unbalanced p53 isoforms expression despite expressing WT p53 [12]. Altogether, our results suggest that the prognostic value of isoforms depend on p53 mutation status and the cancer type.

The results of our research reveal specific combinations of isoforms expression and p53 mutation status providing additional support for specific events in kidney carcinogenesis. These findings suggest that p53 function can be lost either by specific p53 isoforms' expression or by mutations.

Our study has limitations; we analyzed RNA expression rather than protein levels on a modest number of samples. At present, RT-qPCR represents the best method to specifically detect p53 isoforms expression due to the lack of available isoform-specific antibodies. Using RT-qPCR we have focused to distinguish the expression level of N-terminal variants. However, we weren't able to differentiate specifically all p53 isoforms due to excessively long amplicons for RT-qPCR and complex gene organization.

5. CONCLUSIONS

This study provides critical information on the mRNA expression level of N-terminal isoforms in RCC in relation to p53 functional mutation status. Tumors with WT p53 had lower expression of p53, Δ40p53 and Δ133p53 isoforms comparing to MT p53 harboring tumors, and down-regulation of Δ133p53 isoforms comparing to NAT. Our results underline the importance of considering both p53 mutational status and p53 isoforms' expression in RCC clinical studies. Further studies are needed to determine the role of p53 isoform network in RCC carcinogenesis.

FUNDING

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CONFLICT OF INTEREST

None.

LIST OF ABBREVIATIONS

Abbreviation	Term
RCC	renal cell cancer
NAT	normal adjacent tissue
WT p53	wild-type p53
MT p53	mutant p53
OS	overall survival
RT-qPCR	reverse transcription quantitative polymerase chain reaction

RCF	red colony frequency
TBP	TATA binding protein
FASAY	functional analysis of separate allele in yeast

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FIGURE LEGENDS

Figure 1. Expression of p53, Δ40p53 and Δ133p53 isoforms in RCC tissues and NAT samples. Absolute quantification of p53, Δ40p53 and Δ133p53 by RT–qPCR in 41 tumors and 37 NATs. Results are shown as absolute copy numbers, expressed per standard input total RNA normalized to the average levels of housekeeping gene TATA box-binding protein (*TBP*). Values represent the median and interquartile range. Significant P values are shown in bold.

Figure 2. Expression of p53, Δ40p53 and Δ133p53 isoforms in renal cell cancer tissues in association with p53 mutational status. Absolute quantification of p53, Δ40p53 and Δ133p53 isoforms by RT-qPCR in (A) 41 RCC tissues and (B,C) 37 matched RCC tissues and NAT in (B) wild type p53 tumors and (C) mutant p53 tumors. Results are shown as absolute copy numbers, expressed per standard input total

RNA normalized to the average levels of housekeeping gene *TBP*. T-test was used for testing difference between two groups. Values represent median and interquartile range. Significant P values are shown in bold.

Figure 3. Kaplan-Meier plots depicting the impact of p53 isoforms' expression and p53 mutational status on OS of patients with renal cell cancer. High (full line) or low (dotted line) expression of p53 (A), Δ40p53 (B) and Δ133p53 (C) in 41 cases. Analysis of cases with MT (dotted line) or WT (full line) in 41 cases (D). The log rank (Mantel-Cox) test P values are shown. Tick marks indicate censored cases.