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Selected morphologic features influencing the prognosis of conventional renal cell carcinomas co-expressing P53 and MDM2

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Renal cell carcinoma is the most deadly of common urologic malignancies. The classical prognostic factors, including tumor type, grade and stage, as well as performance status of the patient, offer important information, but there is a need for new biomarkers which could improve the quality of prognostication. It has been proposed that tumors co-expressing P53 and MDM2 could represent a specific, more aggressive subgroup. The aim of the study was to explore this hypothesis using tissue microarrays, using two different anti-P53 antibodies. The material analyzed consisted of 470 cases of renal clear cell carcinoma. Reaction for P53 was positive in 15.1 or 13.2% of cases, depending on the antibody used. Reaction for MDM2 was positive in 37.9% of cases; 6.5 or 5.3% of cases coexpressed P53 and MDM2. Both P53-positive and double P53/MDM2-positive cases were higher grade and more likely to contain a sarcomatoid component, but their stage was similar to negative cases. PAb1081 P53-positive MDM2-positive cases were larger than the rest of the tumors (7.6 cm vs. 6.1 cm, $p < 0.001$). Our data support the hypothesis of prognostic significance of P53, and double P53/MDM2 positivity, yet further studies are needed to clarify the issue.

Key words: renal cell carcinoma, prognostic factors, P53, MDM2.

Introduction

Renal cell carcinoma (RCC) constitutes approximately 9% of human cancers and is the most deadly of urological malignancies. Recently, interest in the biology of RCC, especially its most frequent variant clear cell RCC (CCRCC), has increased considerably. This increased interest is partly due to the introduction of new methods of treatment, such as targeted drugs or alternative surgery. Prognostic factors of CCRCC include stage, grade and histologic type; many biomarkers have been studied, but few of them have yet become of any practical importance [1, 2].

TP53, possibly the best known tumor suppressor gene, is mutated in over 50% of human cancers, and in some cases (e.g. high grade urothelial carcinoma, high grade serous ovarian carcinoma) is the main driving force behind this neoplastic process. Tumors bearing *TP53* mutation usually express detectable amounts of P53 protein; immunohistochemistry is thus often used as a surrogate for direct detection of *TP53* mutation. The mutation of *TP53*, however, is not the only mechanism leading to increased P53 expression. In CCRCC, *TP53* mutation is much less frequent; however, P53 expression is detectable at the histochemical level [3]. The significance of the latter observation in CCRCC remains controversial. It has

been proposed that CCRCCs co-expressing P53 and MDM2 may constitute a distinct, more aggressive group of CCRCCs [4-6]. The aim of this study was to compare the P53/MDM2-positive carcinomas with all other CCRCCs in regard to their basic morphologic features known to influence prognosis. In addition, the use of two different P53-specific antibodies allowed for a more in-depth look at expression and co-expression patterns in the tumor material studied.

Material and methods

The material studied was retrieved from the Department of Pathomorphology archives. Cases were reviewed by an expert urologic pathologist and reclassified according to the most recent WHO classification [7]. For the present study, only unequivocal conventional (clear cell) carcinomas were chosen. The tumors were graded according to the International Society of Urological Pathologists, which uses a modification of the Fuhrman method [8], referred to as the Fuhrman method from this point forward. The presence of sarcomatoid components and necrosis was observed, and in accordance with Delahunt *et al.* [9], tumor grade that took necrosis into consideration was assigned.

For each case, a slide containing well-preserved and representative tumor tissue was selected and a respective area was marked for study. Corresponding blocks were used to construct a tissue microarray (TMA) using Tissue MicroArrayer MTA-1 (Beecher Instruments Inc., Sun Prairie, USA). From each donor block, three 0.6 mm cylinders were selected. The acceptor paraffin blocks were prepared noting the location of each cylinder, and 3-μm thick sections were cut.

For immunohistochemistry, a standard staining protocol was used. Briefly, the slides were dewaxed, rehydrated and incubated in 3% peroxide solution for 10 minutes to block endogenous peroxidase activity. Antigen retrieval was carried out by microwaving in citrate buffer (0.2% citric acid titrated to pH 6.0 with 2N NaOH) 3 times for 5 minutes each at 750 W. The primary antibodies are listed in Table I. The Lab Vision detection system (Thermo Fisher Scientific, Waltham, USA) was used. The chromogen used was 3-amino-9-ethylcarbazole. The slides were counterstained with Mayer hematoxylin (Thermo Fisher Scientific, Waltham, USA) and coverslipped. The cases were classified as positive or negative by one of the authors (M.H.) without knowledge of the clinicopathologic parameters and the results of scoring were introduced into an Excel spreadsheet (Microsoft Corp., Redmond, USA). Cases lost from the TMAs were excluded from the study. Student t-statistics, χ^2 and ANOVA tests were used when appropriate. Correlations were measured by Pearson's and gamma correlation coefficients. The statistical analysis was done with Statistica 10 PL (StatSoft Inc., Tulsa, USA) and P values less than 0.05 were considered significant.

Table I. Antibodies used in the study

Specificity	Dilution	Manufacturer	Clone
MDM2	1/50	Novocastra	1B10
P53	1/200	DAKO	DO-7
P53	1/50	Novocastra	PAb1801

Table II. Concordance of P53 stains

	PAb1081	
DO-7	**negative**	**positive**
negative	377	22
positive	31	40
	$\gamma = 0.91$	

Results

The material under study was obtained from 470 cases of CCRCC. There were 280 (59.6%) males and 190 (40.4%) females. The mean age was 61.3 years (range 26 to 92; SD 10.59). Females in this group were slightly older than males (62.3 vs. 60.7) yet this was not statistically significant. In 200 cases (42.6%) the tumor was stage pT1, in 41 cases (8.7%) pT2, in 223 cases (47.4%) pT3 and in 3 cases (0.6%) pT4, while in 3 cases (0.6%) no information about the stage was available. The average diameter of the lesions was 6.2 cm (range 0.8 to 26 cm, SD 3.3) and the size of the tumors in both sexes was very similar (6.3 cm in females versus 6.2 cm in males, $p > 0.05$). At presentation, 5 cases showed lymph node metastases and 2 additional distant metastases. Because of the low number of such cases, metastatic disease is not analyzed in detail in this manuscript.

A sarcomatoid component was present in 29 cases (6.2%). Fuhrman grade was G1 in 140 cases (29.8%), G2 in 169 cases (36%), G3 in 112 cases (23.8%) and G4 in 49 cases (10.4%). Necrosis was present in 109 cases (23.2%). Tumor grade according to Delahunt *et al.* [9] was G1 in 283 cases (60.2%), G2 in 91 cases (19.4%), G3 in 54 cases (11.5%) and G4 in 42 cases (8.9%).

A positive reaction for P53 using the PAb1081 antibody was observed in 62 cases (13.2%). Reaction for P53 using the DO-7 antibody was positive in 71 cases (15.1%). The relationship between these two reactions is shown in Table II. Reaction for MDM2 was positive in 178 cases (37.9%). The relationship between P53 and MDM2 stains is shown in Table III.

Table III. Concordance of P53 and MDM2 positivity

		MDM2	
P53		NEGATIVE	POSITIVE
DO-7	negative	252	147
	positive	40	31
		$\gamma = 0.14$	
PAb1081	negative	255	153
	positive	37	25
		$\gamma = 0.06$	

The cases that were positive for P53 immunohistochemistry using the PAb1081 antibody tended to be of a significantly higher grade, according to the standard system of stratification as well as the method proposed by Delahunt *et al.* (both $p << 0.01$). Remarkably, a sarcomatoid component was more frequently observed in these same cases ($p << 0.01$). There was no relationship between P53 expression and pT stage. Interestingly, 2 out of 5 lymph node positive cases showed P53 expression when stained with PAb1081 ($p < 0.05$), but not when stained with DO-7. The tumors showing necrosis were more likely to be P53-positive with DO-7 (48%, $p < 0.05$) but not PAb1081. None of the above relationships were present for MDM2 staining. The immunopositive and immunonegative tumors did not differ significantly in their diameter, nor was a difference in patients' age noted; however, MDM2-positive cases tended to be slightly larger, yet this was not a significant difference (6.4 vs. 6.1, $p < 0.08$).

Analysis of P53-positive, MDM2-positive cases showed similar tendencies to the ones shown above for P53-positive cases: double immunopositive tumors were higher grade (both in the standard and Delahunt system) and were more likely to show sarcomatoid components, but they did not differ in their stage. Interestingly, PAb1081 P53-positive, MDM2-positive tumors were significantly larger than the rest of the tumors studied (7.6 cm vs. 6.1 cm, $p < 0.001$); this difference was much less evident for DO-7 P53-positive, MDM2-positive cases (6.6 cm vs. 6.2 cm, non-significant).

Discussion

Renal cell carcinoma constitutes a heterogeneous group of diseases, differing in their genetics, morphology and clinical features. The best established morphologic prognostic factors include tumor type and stage, presence of sarcomatoid components and for some subtypes, histologic grade [1]. The grading system for CCRCC has been revised recently [8], with necrosis being defined as an additional poor prognostic factor. Therefore, the presence of necrosis has been proposed as an additional criterion for grading [9]. Although much effort has been made to identify further prognostic biomarkers, so far they have been of little use in clinical practice [1, 10].

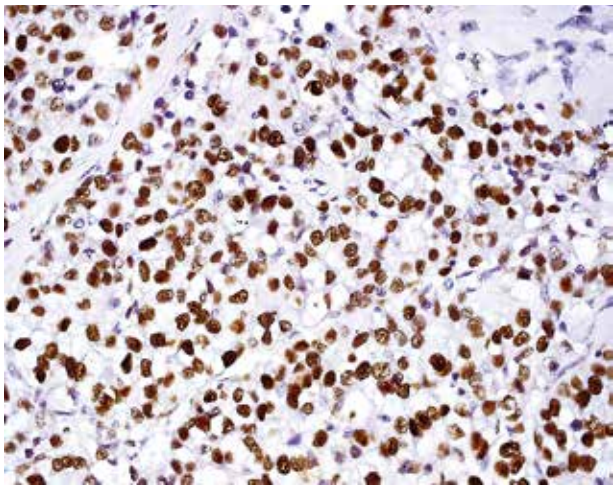

Fig. 1. Strong positive nuclear reaction for P53 in clear cell renal cell carcinoma (DO-7 antibody). Immunohistochemistry, original magnification 400×

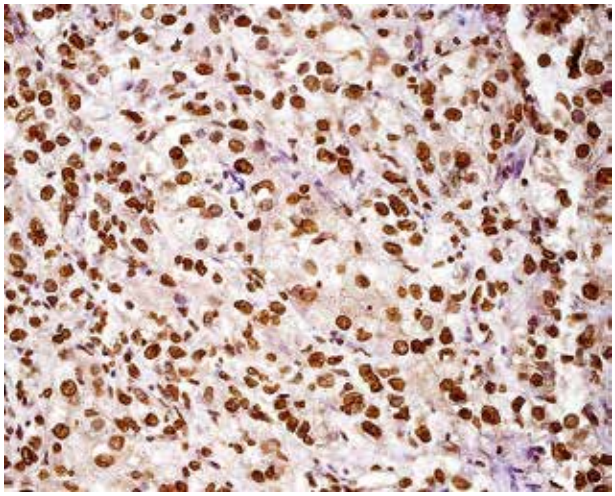

Fig. 2. Strong positive nuclear reaction for MDM2 in clear cell renal cell carcinoma. Immunohistochemistry, original magnification 400×

Inactivating mutations in the *TP53* gene are frequently observed in many human cancers, especially carcinomas. Its gene product participates in cell damage sensing, proliferation and apoptosis, and is often referred to as the 'guardian of the genome'. The role of *TP53* mutations and their effects on protein expression in RCC have been studied for years, yet their prognostic role remains controversial. The *TP53* mutation rate in RCC is low [3]; nevertheless, a significant proportion of cases express its protein product. Most of the studies analyzing P53's role in RCC have been done using immunohistochemical methods of detection.

Girgin *et al.* observed P53 positivity in 20% of RCC cases studied; however, their study was limit-

ed to grade 3 and 4 cases only. A limitation to the Girgin *et al*. study was the lack of determination of mutation status of *TP53* at the genetic level as only protein expression was studied. Furthermore, an older classification system was used during that study. Also, the cases with P53 expression showed significantly shorter survival, even in multivariate analysis [11]. Additionally, in a small series, Erdem *et al.* [12] found significant relationships between P53 expression, tumor size, renal sinus invasion and Fuhrman grade. Conversely, Baytekin *et al.* observed a negative correlation between P53 expression and both the grade and the stage of RCC tumors. Additionally, they were also unable to see any prognostic significance associated with this marker's expression; indeed only stage appeared important for survival. However, it is important to note that this study only included a relatively small and heterogeneous group of RCCs [13]. Similarly, Kramer *et al.* [14] also failed to show any prognostic significance associated with expression of P53 immunohistochemically. In additional multivariate models, the stage was regarded as the only variable of importance. Likewise, these authors only analyzed a relatively small group of cases, composed not only of clear cell carcinomas but also of other subtypes. In a study by Shvarts *et al.* [15], P53 expression was strongly correlated with survival both in univariate and multivariate analysis. Further, it was indeed the only molecular predictor of survival in the discussed multivariate models. Interestingly, Kankuri *et al.* [16] observed no relationship between P53 expression and either the stage or metastatic spread. Moreover, P53-positive cases tended to be higher grade; however, this relationship was not significant. Interestingly, the significance of P53 expression in RCC may depend on specific histologic type. Exemplified by Zigeuner *et al.* [17], the highest expression was found in papillary carcinoma (over 50% of cases) and lowest in CCRCC (12%) but only in the latter was it observed to significantly influence survival. This was seen both in univariate and multivariate analysis.

The *MDM2* gene product participates in the very same pathway as *TP53*, although with an opposite function, as MDM2 is the main regulator of *TP53* functions and its expression is controlled by a P53-dependent mechanism. Mutations of the *MDM2* gene are seen in a minority of cancers, principally in sarcomas. *MDM2* amplification has been described as a mechanism of sarcomatoid transformation in RCC [18], but this is a rare phenomenon and does not appear to participate in earlier steps involved in carcinogenesis. *MDM2* is located at the 12q13 locus, which is frequently amplified in RCC and is related to poor prognosis [19]. It is important to note that MDM2 presence is linked to poor prognosis; therefore this pathologic mechanism is not a result of gene loss, but its presence and functionality. However, most studies concerning MDM2 in RCC analyze its expression in the context of P53 expression.

The Liverpool group offered an interesting hypothesis, showing that only a subset of RCCs expressing P53 fared worse, namely the cases in which MDM2 was also expressed [4, 5, 20]. The frequency of such co-occurrence has been estimated at about 20% [19]. We decided to explore this idea, analyzing the P53 expression with two antibodies having different specificity as well as comparing P53/MDM2-positive and -negative cases with a number of other established and potential prognostic factors. Moch *et al.*, in an early report, observed a significant correlation between MDM2 and P53 expression; however, only P53 expression appeared to influence the prognosis [21]. Haitel *et al.* [22] discerned more frequent MDM2 as well as P53 expression in higher grade CCRCC. In their material there was no correlation between P53 and tumor stage; however, MDM2 expression was correlated with an increased amount of lymph node metastases. In univariate analysis, both markers were correlated with survival, yet on multivariate analysis, at first, only grade and stage were contributory. Furthermore, combined MDM2/P53 expression improved the performance of the model. Uchida *et al*. found that expression of P53 alone, MDM2 alone, and P53/MDM2 co-expression was related to prognosis; however, MDM2 positivity was observed in less than 2% of cases studied while P53 expression was present in 13% [23].

In conclusion, this study showed that P53 expression was correlated with known prognostic factors including tumor diameter, grade, presence of sarcomatoid components and necrosis. We believe, however, that these results may have been influenced by the use of two separate P53 antibodies, each targeted at a different epitope. Lastly, the significance of MDM2 expression appeared to be much lower in the context of this study.

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