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ORIGINAL ARTICLE

Retrospective analysis of a large patient sample to determine p53 and Ki67 expressions in renal cell carcinoma

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Summary

Purpose: *This study aimed to investigate the expression of p53 and Ki67 genes in renal cell carcinoma (RCC) and its possible clinical value.*

Methods: *A retrospective analysis of clinical data from 1239 patients with RCC was performed to explore the relationship between the expression of Ki67 and p53 proteins and tumor stage, grade and prognosis.*

Results: *p53 expression was not significantly correlated with TNM stage and Fuhrman grade (p>0.05); Ki67 expression was significantly correlated with TNM stage and Fuhrman grade (p<0.05). Kaplan-Meier and log-rank survival rate results showed that the prognosis of Ki67 and p53 double-positive group was significantly inferior to the single-positive and negative group (p<0.001). In the multivariate Cox risk regression analysis model, TNM stage, relative risk/RR=3.196, p<0.001), Fuhrman grade (RR=3.196, p<0.001) and Ki67 and p53 double-positive [Ki67 (+) p53 (+), RR=3.196, p<0.001] were significantly correlated with tumor prognosis, and independent predictors of the patient disease-free survival (DFS).*

Conclusion: *The combined detection of p53 and Ki67 expressions, which are superior to single marker, could be used to improve significantly the accuracy of prognosis of RCC patients.*

Key words: *Ki67, p53, prognosis, renal cell carcinoma*

Introduction

RCC is one of the most common malignant tumors of the urinary system, accounting for approximately 2.6% of all human malignancies [1]. More than 23,000 new RCC cases are recorded every year in China; in the USA, 49,096 new RCC cases have been documented, with 11,033 deaths in 2009 [2,3]. In the past 20 years, studies have demonstrated a close link between the p53 and RCC at molecular level. Studies have also shown that the positive correlation of p53 with lymph node and distant metastasis is significantly higher than that without metastasis [4]; this result suggested that p53 mutation is a terminal event of tumorigenesis, and closely related to the biological behavior of the tumor. Girgin et al. [5] and Gurova et al. [6] showed that positive p53 is an independent adverse prognostic factor for p53-positive patients.

Dijkhuizen et al. [7] found that the p53 mutation rate has no evident relationship with cell type, histological type and tumor grade. Uhlman et al. [8] also were not able to prove a relationship of p53 with tumor stage. Vasavada et al. [9] and Papadopoulos et al. [10] also did not find any correlation between p53 and prediction of the clinical disease course and prognosis. These conflicting studies have shown that the value of p53 as a predictor of clinical prognosis is inconsistent, possibly due to the small sample size of each study, offering no strong statistical power. Ki67 is a nuclear antigen expressed in proliferating cells. Ki67 has a short half-life; as such, the detection of this antigen is more reliable than that of a long half-life proliferating cell nuclear antigen. Stud-

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ies on RCC [1], lymphomas [2], gastric cancer [3], colorectal cancer [4], bladder cancer [5], and breast cancer [6] have shown that overexpression of Ki67 antigen is correlated with the biological behavior and prognosis of these malignancies.

When the majority of patients with RCC show typical clinical symptoms, the tumor is at an advanced stage, and 30% of the patients have metastatic disease. Although many patients with early RCC may be cured by surgery, approximately 50% of them are diagnosed with or finally progress to metastatic RCC. Hence, effective treatment should be administered postoperatively to patients with RCC to avoid overtreatment, and eradicate micrometastases at an early stage. The pathogenesis of RCC is unclear; biological behavior and prognosis are also difficult to predict. Studies have further shown that p53 and Ki67 are closely related to the prognosis of RCC [11-13].

In this study, the clinical data of a large sample covering a period of 10 years were assessed to clarify the clinical value of p53 and Ki67 protein expression in RCC.

Methods

General information

This study was conducted in accordance with the declaration of Helsinki and after approval from the Ethics Committee of Fuzhou General Hospital. Written informed consent was obtained from all patients.

The data of 1,239 patients with RCC who underwent surgical treatment from January 2000 to December 2012 in Fuzhou General Hospital, Nanjing Military Area Command, People's Liberation Army of China were collected. Among these patients, 858 were males, and 381 females (M/F ratio 2:1). Age ranged from 21 to 89 years (mean 53.9±19.2). All of the patients pathologically diagnosed with RCC underwent radical nephrectomy or partial resection of the kidney. Surgical specimens were then subjected to routine p53 and Ki67 immunohistochemical staining. According to TNM staging system (AJCC standard 2009), 861 (69.5%) T1 stage cases, 162 (13.1%) T2 cases, and 216 (17.4%) T3 cases were identified. According to the Fuhrman grading system, recommended by WHO, 93 (7.5%) G1 cases, 573 (46.2%) G2 cases, 432 (34.9%) G3 cases, and 141 (11.4%) G4 cases were determined. The patients were followed up for an average of 54.9 months (±13.42 standard error/SE), and the median follow-up time was 56.8 months (range 13-168). Among the 1.239 patients, 201 (16.2%) died of RCC, including 66 (40.7%) T2 patients and 69 (31.9%) T3 patients.

Immunohistochemistry

Surgical specimens were fixed with 4% formaldehyde, embedded in paraffin and consecutively cut at 4 μm thickness.

The slices were routinely dewaxed with water, incubated in 3% H_2O_2 for 5-10 min at room temperature to block endogenous peroxidase activity and washed thrice with phosphate buffer for 5 min. The primary antibody working solution was added dropwise at 37°C and incubated for 1 h. The slices were then washed thrice with phosphate buffer for 5 min. Then, horseradish peroxidase (HRP) polymer (enzyme-labeled secondary antibody) was added dropwise and incubated for 30 min at room temperature. The slices were then washed thrice with the buffer for 5 min: DAB (3,3' –diaminobenzidine) staining was then performed. Tap water was used to rinse the specimens thoroughly; afterward, the slices were restained, dehydrated, hyalinized and mounted.

p53 and Ki67 monoclonal antibodies (Invitrogen Biotech Corp, USA) were used;

Determination of protein expression

p53 and Ki67 positive cells showed stained brownish-yellow granules in the nucleus (Figure 1). No tumor cell staining or ≤15% cell staining was considered as p53 and Ki67 negative; >15% of the tumor cells staining was considered as positive [14].

Statistics

Data were processed using SPSS software 13, (SPSS Inc, Chihago, IL, USA). Chi-square test, Kaplan-Meier method, log-rank test and univariate and multivariate Cox proportional hazard regression models were used for statistical analyse. Statistical significance was set at $p<0.05$.

Results

p53 and Ki67 protein expression and clinicopathological factors

Positive p53 expression was observed in 651 (59.8%) cases and positive Ki67 expression in 591 (47.7%) cases. Positive Ki67 and p53 protein ex-

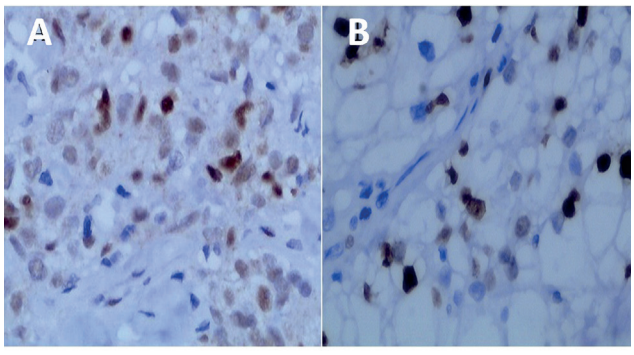

Figure 1. A: p53 protein: immunohistochemical staining (×400). **B:** Ki67 protein: immunohistochemical staining (×400).

Table 1. The relationship between p53, Ki67 expression and clinicopathological parameters in patients with renal cell carcinoma

		p53			*Ki67*		
Parameters	*N*	-	+	*p-value*	-	+	*p-value*
Gender				0.920			0.350
Male	858	408	450		441	417	
Female	381	180	201		207	174	
Age (years)				0.083			0.400
≥60	879	431	448		453	426	
<60	360	157	203		195	165	
TNM stage				0.132			<0.001
1a+b	861	405	456		483	378	
2a+b	162	69	93		66	96	
3a- c	216	114	102		99	117	
Fuhrman grade				0.316			<0.001
G1-2	666	321	345		131	91	
G3-4	573	267	306		90	101	

pression were not significantly correlated with gender or age (p>0.05). p53 protein expression was not significantly correlated with TNM stage and Fuhrman grade (p>0.05). However, Ki67 protein expression was significantly correlated with TNM stage and Fuhrman grade (p<0.001; Table 1).

Survival analysis

On the basis of Ki67 and p53 expressions, we divided the patients into 4 groups: (1): double-negative group [Ki67 (-) and p53 (-)]; (2): single-positive group [Ki67 (-) and p53 (+)]; (3): single-positive group [Ki67 (+) and p53 (-)]; and (4): double-positive group [Ki67 (+) and p53 (+)]. The DFS of the double-positive group was significantly shorter compared with the single-positive group (p=0.02) and the double-negative group (p=0.003; Figure 2).

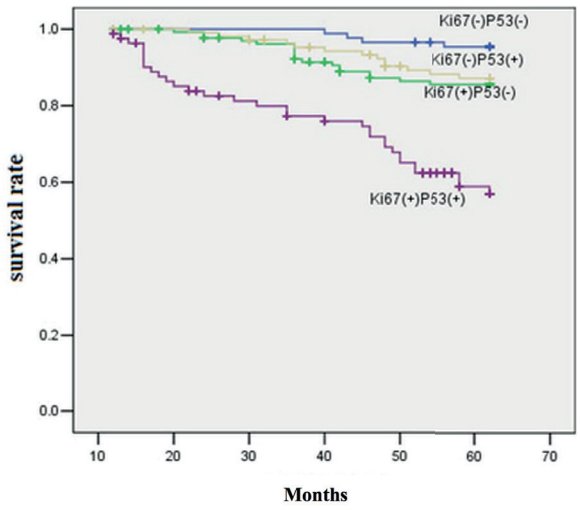

Figure 2. Kaplan-Meier survival of patients with renal cell carcinoma according to Ki67 and p53 expression. DFS of the double-positive group was significantly shorter compared with the single-positive group (p=0.02) and the double-negative group (p=0.003).

Cox proportional hazard regression analysis

In univariate Cox regression model, TNM stage, Fuhrman grade, p53-positive expression, and Ki67-positive expression were significant predictors of prognosis of patients with RCC (Table 2, left side). All of these variables were combined and introduced to the multivariate Cox regression model. Introduced were also p53 and Ki67 double-positive vs single positive+negative (Table 2, right). The results showed that only TNM stage, Fuhrman grade and p53 and Ki67-double positive values were significantly correlated with tumor prognosis.

Discussion

Pathological type, TNM stage and Fuhrman grade have been considered as effective predictors in the current clinical prognosis of RCC [14]. However, the results are not as satisfactory as expected when the prognosis of patients was predicted in terms of these variables; for instance, approximately 30% of the patients with localized disease developed recurrence postoperatively [15], when RCC exhibits distant metastasis and the median survival time ranges from one year to two years

Table 2. Renal cell carcinoma Cox proportional hazards regression analysis

	Univariate Cox model		*Multivariate Cox model*	
	RR (95%CI)	*p-value*	*RR (95%CI)*	*p-value*
T stage	2.263 (2.262-3.036)	<0.001	3.196 (2.719-3.757)	<0.001
Fuhrman grade	1.476 (1.118-1.948)	0.006	2.001 (1.504-2.661)	<0.001
p53	2.544 (1.800-3.442)	<0.001	2.190 (0.911–5.024)	0.087
Ki67	2.257 (1.885-3.398)	<0.001	2.112 (0.739–6.503)	0.099
2 vs 0+1	-	-	3.892 (3.006–4.942)	<0.001

2 double-negative group: [Ki67(-)p53(-)].
1 single-positive group: [Ki67(-)p53(+)/Ki67(+)p53(-)].
0 double-positive group: [Ki67(+ p53(+)]

[16]. Therefore, new RCC markers should be identified to improve the accurate prediction of this disease. Studies have further suggested that a gene closely related to the development of RCC is present, and this gene can accurately predict the prognosis of patients with RCC [11,13,17]. Thus far, no clinical research with large number of patients has been performed to verify this gene; furthermore, no single or combined molecular markers have been recommended for the prediction of the prognosis of RCC [16].

In this study, data of 1,239 patients with RCC covering a period of more than 10 years were collected; the data of these patients were also used to determine and analyze the relationship between the expressions of p53 and Ki67 and the disease prognosis. The results showed that p53 and Ki67 protein expressions were closely related to prognosis because the double-positive expressions of p53 and Ki67 proteins indicated that patients could have worse prognosis.

The results also showed that a positive p53 expression was present in 651 of 1,239 (59.8%) cases of RCC; this is significantly higher than that obtained in a previous study [18], possibly because of p53-positive cut-off value selection and sample selection. The positive expression rate of Ki67 was 47.7%, which is similar to that presented in previous reports [19,20]. The data indicated that the expressions of p53 and Ki67 are closely related to the DFS of Ki67 and p53 double-positive expression group was significantly lower than that of the single-positive group and the double-negative group. Based on the results of multivariate Cox regression analysis, tumor grade, stage and double-positive expression of p53 and Ki67 were independent predictive factors of PFS. However, differences were observed between the results of the present study compared with those in a previous study [21], in which overexpression of p53 protein could not provide information to predict the prognosis of RCC. However, Kim et al. [21] argued that the positive Ki67 expression cannot be considered in the prediction of the prognosis of patients with RCC. This difference may be attributed to the enrollment of pT1 and pT3 patients in the present study, whereas Bui et al. [20] enrolled only pT3 patients. These authors also excluded some patients with distant metastases because they did not accept surgical operation. By contrast, Kim et al. [12] revealed that approximately 49% of their patients exhibited distant metastases on presentation. In the survival analysis, DFS was used to investigate the predictive value of p53, and Ki67, whereas Bui et al. [20] used the overall survival time.

In summary, the combined detection of p53 and Ki67 protein expressions in RCC proved to have clinical significance. Positive p53 and Ki67 protein expressions were correlated with disease prognosis, showing poor survival. We believe that p53 and Ki67 double-positive expression could be used as independent predictor of disease-specific prognosis of RCC and may provide the basis of individualised adjuvant treatment for each patient.

Acknowledgements

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