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Original Article

Immunohistochemical expression of LAPTM4B in clear cell renal cell carcinoma by tissue microarray

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Abstract: This study assesses the immunohistochemical expression of LAPTM4B in clear cell renal cell carcinoma (ccRCC) by tissue microarray (TMA) and evaluates its association with pathologic features. Four TMAs were constructed with 59 cases of ccRCC samples for reliability assessment, and Spearman index was used for correlation analysis. Afterwards, LAPTM4B expression was evaluated on TMAs containing 126 samples. An H score was calculated in each spot. TMA can be used to reliably assess the expression levels of LAPTM4B in ccRCC tissues ($P<0.001$). LAPTM4B expression was observed in 80% (103/126) of the tumor samples, but little expressed in controls (35/126). Moreover, LAPTM4B expression was associated with TNM staging ($P=0.016$), lymph node metastasis ($P=0.022$), and vascular invasion ($P=0.009$). LAPTM4B expression had a positive correlation with Ki67 expression ($P=0.006$).

Keywords: LAPTM4B, ccRCC, tissue microarray

Introduction

LAPTM4B (lysosome-associated protein transmembrane 4 beta) is a newly identified cancer-associated gene (NM_018407, Gene ID= 55353) mapped to chromosome 8q22. 1, and encodes a 35 kDa tetratransmembrance glycoprotein. As a potential proto-oncogene, LAPTM4B was initially identified in hepatocellular carcinoma cells and it plays critical roles in proliferation and metastatic potential of various tumors [1]. LAPTM4B protein is upregulated and associated with poor differentiation in human hepatocellular carcinoma [1] and recent, studies found that the LAPTM4B overexpression was involved in carcinogenesis and progression of several solid tumors [2]. It was demonstrated that LAPTM4B is over-expressed in various malignancies including those of the liver, ovary and prostate cancer [3-6]. In addition, Li et al. demonstrated that LAPTM4B is amplified in breast tumors and that it's overexpression by amplification was associated with breast cancer recurrence [7]. Furthermore, studies also showed that LAPTM4B was upregulated in lung adeno-carcinomas and was associated with poor prognosis in non-squamous NSCLCs [8]. The expression of LAPTM4B in kidney cancer remains unknown. Kidney cancer is a kind of adenocarcinoma which derived from renal tubular epithelial cells. Clear cell renal cell carcinoma (ccRCC) is the most common primary renal malignancy occurring in adults, which accounts for approximately 70%-80% of renal cell carcinomas [9]. ccRCC is related with significant morbidity and mortality but, nonetheless, has a wide variance in individual patient outcomes-despite a reasonably uniform histologic appearance [10]. However, the roles LAPTM4B playing in ccRCC tumorigenesis was unclear. In our research we focused on the expression level of LAPTM4B in ccRCC, and relationship between LAPTM4B expression and clinicopathological characteristics of ccRCC patients was also analyzed for further investigation.

Tissue microarray (TMA) technology was first reported by Kononen et al. in 1998 [11], and since then, has been increasingly used in cancer research. It is a potentially important technique for translational research because of its high-throughput parallel molecular profiling at DNA, RNA, and protein levels for large numbers

of samples [12, 13]. TMA has many advantages over conventional techniques, such as high efficiency, uniform reaction conditions, reagent conservation, less damage to donor tissue blocks, and easier automated imaging analysis [12-14]. More researchers are focusing on the reliability of TMA in tumor specific biomarker identification recent years, and most of them demonstrated positive conclusions [15, 16]. On the other hand, some researchers showed that TMA was ineffective to evaluate some antigens such as ATPaseC1 [17].

In the present study, we first evaluated the reliability of using TMA for LAPTM4B expression in ccRCC tissues. In this section, both whole sections and TMAs of 30 ccRCC cancer specimens and 29 adjacent normal tissues were conducted by immunohistochemistry. The correlation between whole slides and 4 TMA slides were calculated by Spearman's test. Afterwards, the expression of LAPTM4B in 126 patients was evaluated by TMA-based immunohistochemistry, and the relationships between LAPTM4B protein expression and clinicopathological variables were investigated.

Methods and materials

Patients and specimens

Primary tumor specimens from 126 patients who underwent surgical resection and were diagnosed as clear cell renal cell carcinoma at the Department of Urology Surgery, China-Japan Union Hospital, Jilin University between March 2008 and December 2013. For all patients, histological type of renal cell carcinoma was determined by the World Health Organization classifications, and pathological staging was based on the international staging system revised in 2008. None of the patients received radiotherapy or chemotherapy before surgical resection, and all patients were treated with routine treatment after the operation. Each patient signed informed consent according to the Helsinki Declaration, and this study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University.

Tissue microarray construction for reliability assessment

30 ccRCC cancer specimens and 29 adjacent normal tissues which obtained from the surgical pathology archives between 2008 and 2013 were randomly selected for variability assessment of TMA staining. Four pure areas were selected and circled from donor blocks by a pathologist. Tissue microarray (TMA) blocks containing duplicate 1.0 mm diameter cores of 10% buffered formalin-fixed paraffin-embedded tissue blocks from each specimen were constructed using a tissue array instrument (Mitogen Minicore, ALPHELYS). TMA blocks was sampled 4 times at different positions, each block contained 1 marker core for TMA orientation. Comparison of whole slides and TMA blocks was conducted to evaluate the reliability of using TMA. Four tissue microarrays (TMAs) which containing 59 spots each were built at the Jilin TMA Engineering Lab using a previously described procedure [18]. The correlation between whole slides and 4 TMA slides were calculated by Spearman's test.

Tissue microarray construction for LAPTM4B expression evaluation

Four TMA slides contained 126 cases of primary ccRCC and matched adjacent normal tissues were conducted. Clinical data including sex, grade, stage, lymph node metastasis and vascular invasion was obtained for all included cases. All cases were primary ccRCC and were reviewed by a pathologist. All new recognized entities including clear cell papillary, translocation carcinomas, etc, were excluded from the analysis.

Immunohistochemistry staining

Tissue sections and microarray sections were cut 4-ized entities including clear cell papillary, transdeparaffinized with xylene and rehydrated in graded alcohol concentrations according to the standard procedures. Then, all the deparaffinized sections were immersed in 0.01 mol/L citrate buffer (pH 6.0) and boiled in a pressure cooker for antigen retrieval for 150 seconds. After cooling down, samples were incubated with 3% hydrogen peroxide (H_2O_2) for 15 min to block endogenous peroxide activity. Subsequently, the sections were incubated at 4°C overnight with primary rabbit polyclonal LAPTM4B antibody (1:200 dilution; purchased from Proteintech Company, 18895-1-AP). In the next day, the sections were rinsed with PBS and incubated for 15 min at room temperature with biotin-labeled secondary antibody followed by horseradish peroxidase conjugated streptavidin for 30 min. At last, sections were treat-

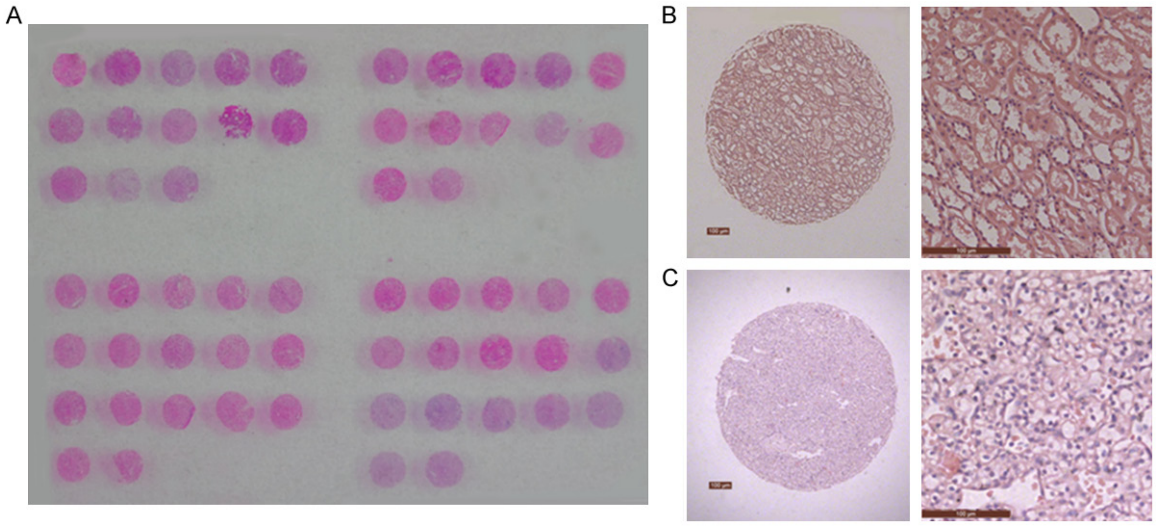

Figure 1. Hematoxylin and eosin staining was conducted to tissue microarray. A. A microarray slide that consisted of 59 spots. B. Adjacent normal tissues of ccRCC magnified by 50-fold and 200-fold. C. Primary ccRCC cores magnified by 50-fold and 200-fold.

Table 1. Comparison of overall interobserver agreement for the assessment of LAPTM4B across 4 TMAs and whole slides

LAPTM4B Expression	Tumor Median H-scores	Spearman Index	
		r	P
Whole Slides	7.83 (3-9)		
TMA-1	7.53 (3-9)	0.79	<0.001
TMA-2	8.05 (3-9)	0.65	<0.001
TMA-3	7.48 (3-9)	0.80	<0.001
TMA-4	7.64 (3-9)	0.82	<0.001

ed with 3, 30-diaminobenzidine tetrahydrochloride (Maixin, China) for 5 min, then counterstained with hematoxylin for 60 sec and mounted with neutral balsam. Immunohistochemistry was performed using an immunohistochemistry kit (Maixin, China) according to the manufacturer's instructions. Negative controls were performed by replacing the primary antibody with normal rabbit serum. The positive controls were hepatocellular carcinomas with positive expression of LAPTM4B protein.

Staining evaluation

For LAPTM4B protein expression degree was based on the percentage of positive stained cancer cells and the staining intensity. The percentage of immunoreactive cells was scored as follows: 0-5%, 0; 5%-30%, 1; 30-60%, 2; 60%-100%, 3. The staining intensity was rated as follows: 0, negative staining; 1, weak staining; 2, moderate staining; 3, intense staining. The overall score of LAPTM4B expression was the product of scores of the staining intensity and the percentage of positive stained cancer cells. A total staining score of 0-9 was calculated and divided into two groups: a low-expression group with an overall score between 0 and 3 and a high-expression group with an overall score between 3 and 9. Tissue specimens were examined separately by two pathologists under double-blinded conditions without prior knowledge of the clinical outcomes of the specimens. The independent scores assigned by the two pathologists were combined as a final score. In cases of significant disagreement, the contradictory scores were reviewed between the two pathologists by discussion.

Ki67 straining

Ki67 antibody was purchased from Maixin Corporation, China. The staining procedure was conducted as described above. Two observers scored the staining results. Ki67 staining of tumor cells was read as negative (<10% of cells show staining), or positive (>10% of cells show staining).

Statistical analysis

SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis.

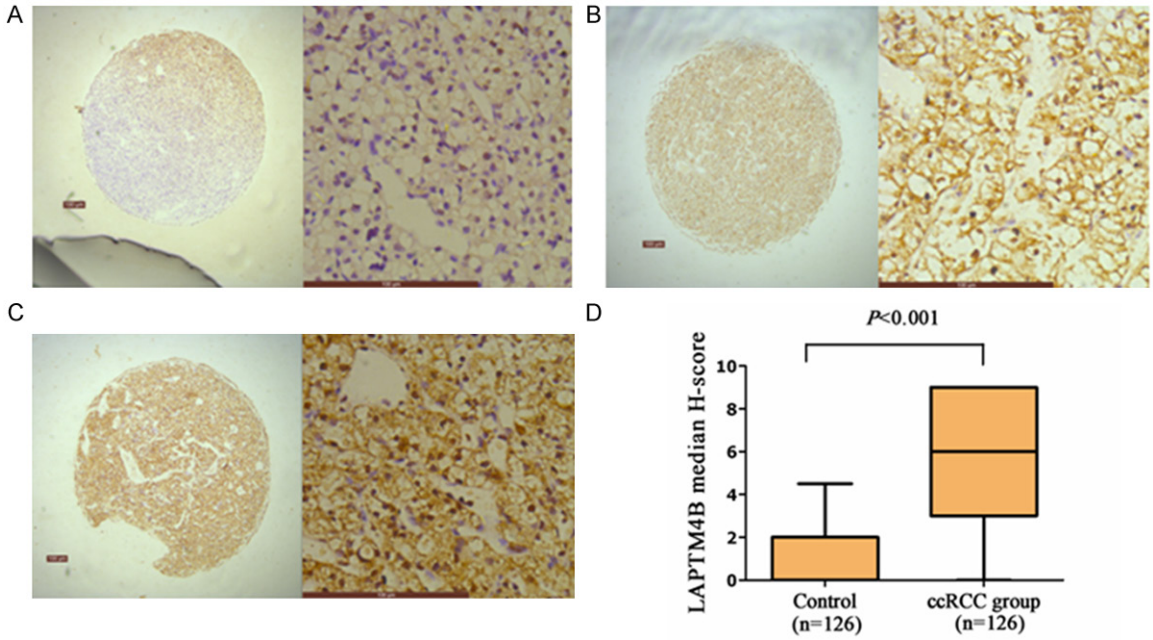

Figure 2. Expression of LAPTM4B was increased in ccRCC tissues by IHC (magnified by 50-fold and 400-fold). Representative micrographs of weak (A), moderate (B) and strong (C) staining of LAPTM4B in ccRCC tissues. (D) Different expression of LAPTM4B in ccRCC tissues and matched adjacent noncancerous tissue.

The correlation between whole slides and 4 TMA slides were calculated by Spearman's test. Paired t test was used to compare the LAPTM4B protein expression level between tumor and normal specimens. The chi-squared test or the Fisher's exact was used to examine the association between LAPTM4B protein expression and various patients' clinicopathologic factors. *P* value <0.05 was considered to be statistical significance.

Results

Variability assessment of TMA staining

4 TMA slides containing 59 spots were constructed (**Figure 1**). Median Scores are calculated and Spearman index are performed by SPSS18.0. **Table 1** shows Spearman values for overall interobserver agreement in the assessment of LAPTM4B on whole slides and on each TMA slide. Our comparison shows a significant positive correlation trend for better agreement with whole slides and TMAs (P<0.001). Agreement among the 4 slides of TMAs was, in general, relatively high. Our outcome indicates that TMA can be used to reliably assess the expression levels of LAPTM4B in ccRCC tissues.

Up-regulation of LAPTM4B protein in ccRCC

In order to detect the expression of LAPTM4B in clinical ccRCC samples, a cohort of 126 matched paraffin-embedded ccRCC normal/cancer specimens were collected to construct tissue microarray. Immunohistochemical analysis was performed on the TMA slides. Overall, LAPTM4B showed little expression in normal kidney cortex tissue, but was extensively expressed in the primary ccRCC, and mainly localized within the cytoplasm or on the cell membrane (**Figure 2A-C**). LAPTM4B was frequently observed in ccRCC tissues in comparison with matched adjacent noncancerous tissue (**Figure 2D**) (P<0.001).

Relationship between LAPTM4B expression and clinicopathological characteristics

We analyzed the relationship between LAPTM-4B expression and clinicopathological characteristics of ccRCC patients (**Table 2**). LAPTM4B is dominantly high expressed in ccRCC samples (103/126). Among the samples, higher LAPTM4B expression was more frequently observed in later TNM staging (III+IV) ccRCCs compared with earlier ones (I+II) (P=0.016).

Table 2. Correlation between expression of LAPTM4B and clinical pathologic features of clear cell renal cell carcinoma

Variable	Patients' No. (n=126)	LAPTM4B Expression (%)		*P* value
		Low (n=23)	High (n=103)	
Age (years)				
≤50	28	9 (39.1)	21 (20.4)	0.210
>50	98	14 (60.9)	84 (79.6)	
Gender				
Male	74	12 (52.2)	64 (62.1)	0.139
Female	52	11 (47.8)	39 (37.9)	
Tumor size (cm)				
≤5	52	8 (34.8)	44 (43.7)	0.155
>5	74	15 (65.2)	59 (56.3)	
TNM stage				
I+II	77	17 (73.9)	60 (58.3)	0.016*
III+IV	49	6 (26.1)	43 (41.7)	
Lymph node metastasis				
Yes	29	5 (21.7)	24 (23.3)	0.022*
No	97	18 (78.3)	79 (76.7)	
Vascular invasion				
Yes	44	6 (26.1)	38 (36.9)	0.009**
No	82	17 (73.9)	65 (63.1)	
Ki67				
Positive	47	6 (26.1)	41 (39.8)	0.006**
Negative	79	17 (73.9)	62 (60.2)	

Furthermore, LAPTM4B expression was associated with lymph node invasion (P=0.022), vascular invasion (P=0.009) and Ki67 expression (P=0.006) (**Table 2**).

Discussion

TMA technique offers the advantage of evaluating a large number of cases under the same immunohistochemistry conditions. Due to the heterogeneity of biomarkers expression within same tumor, the use of TMAs instead of whole-tissue sections is criticized for its limitation in current studies. In our research we assessed the consistency between the whole sections and TMAs based on this consideration. Four TMA slides were constructed, which contained 59 spots each. The whole-tissue sections from the same patients stained by immunohistochemistry were compared with TMAs. It showed significant positive correlation between them (P<0.001), which is consistent with other reports that showed viability and validation of TMAs [15, 16, 19].

LAPTM4B was originally identified as an HCC-associated gene that belongs to the mammalian LAPTM family [20]. Studies have shown that LAPTM4B is up-regulated in many human cancers and may have roles in the disease progression of malignant cells and multidrug resistance [21, 22]. LAPTM4B over-expression has been identified as a new predictor of epithelial ovarian carcinoma metastasis and as an important prognostic marker of metastatic ovarian tumors [23, 24]. However, the expression of LAPTM4B in clear cell renal cell carcinoma remains unknown. Furthermore, the relationship between LAPTM4B expression and its prognostic value in ccRCC remains unclear. It is demonstrated that almost 80% ccRCCs to express LAPTM4B in 90% or more of tumor cells. In addition, our results indicate that LAPTM4B expression is up-regulated in metastatic ccRCC patients compared with non-metastatic patients. A clinicopathological association analysis indicates that high expression in tissue sections is strongly associated with high TNM staging and vascular invasion. Besides, knockdown of LAPTM4B expression is suggested to inhibited cell growth, induced cellular apoptosis and decreased cellular autophagy via NRF2-mediated stress response pathway in lung cancer cells [8], which suggest that LAPTM4B might be a proto-oncogene in lung cancer. Ki67 is a nuclear antigen associated with cell proliferation, and it was reported that nuclear staining of Ki67 was significantly associated with disease-specific survival of (DSS) in ccRCC cohort [25, 26]. In this study, we found a highly positive significant relationship between the expression of LAPTM4B and Ki67. It provided a clue that LAPTM4B might be a proto-oncogene in ccRCC, and the involved signal pathways would be the ongoing tissue. The lack of following-up survival data is another weakness in the study given the nature of the cohort as part of consultation cases for which no additional data were obtained. Taking into consideration the

association between histologic grade and prognosis, future studies should focus on the predictive value of LAPTM4B in ccRCC.

In summary, it was demonstrated that the use of tissue microarrays for semiquantitative evaluation of LAPTM4B expression in ccRCC was variable. LAPTM4B was over-expressed in ccRCC, in most cases at high levels. Nevertheless, higher LAPTM4B expression in ccRCC patients was associated with TNM staging, lymph node metastasis, vascular invasion and Ki67 expression level of ccRCC. These results indicate that LAPTM4B may serve as a novel potential prognosis biomarker for ccRCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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