Original Article
Musashi1 promotes tumor metastasis and is a prognostic marker for renal carcinoma

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Abstract: Background: Musashi1 (MSI1) has been reported to be involved in cancer development and progression. The biologic role of MSI1 in renal cell carcinoma (RCC), however, remains unknown. Methods: Expression of MSI1 in normal kidney cells and kidney cancer cells were measured by real-time PCR. In addition, MSI1 expression in 20 paired kidney cancer and non-cancerous tissues were quantified using real-time PCR. Furthermore, the expression of MSI1 in 115 kidney cancer samples was detected to analyze the correlations between MSI1 expression and the clinicopathological features of RCC patients. The biological function of MSI1 on tumor cell invasion and migration were explored through wound healing and transwell migration assays. Results: MSI1 was significantly upregulated in renal cancer cells and tissues compared with normal kidney cells and tissues. High levels of MSI1 were positively associated with tumor stage (P=0.002) and distant metastasis (P=0.013) of RCC patients. Patients with higher MSI1 expression had a significantly poorer overall and recurrence-free survival time (P=0.019 and P=0.012, respectively) than patients with low MSI1 expression. Multivariate analysis showed that MSI1 overexpression was an independent prognostic indicator (P=0.009 and P=0.015, respectively) for the survival of RCC patients. Ablation of MSI1 inhibited the invasion and metastasis of renal cancer cells. Conclusion: Our results suggest that MSI1 expression is upregulated in RCC, and that MSI1 plays an important role in promoting cell invasion and metastasis of RCC.

Keywords: MSI1, renal carcinoma, migration, invasion

Introduction
Renal cell carcinoma (RCC) is the most common malignancy of the kidney, accounting for 2%-3% of adult malignancy [1]. Surgery is the most effective treatment of preliminary cancer. The 5-years survival for patients diagnosed with organ-confined disease is approximately 90%, whereas the prognosis of patients with distant metastasis remains poor, with a 5-year survival rate of less than 10% [2]. At present, the assessment and treatment of metastatic RCC remains a challenge. Therefore, novel biomarkers for diagnosis and individualized therapy of RCC would be valuable.

The Musashi (MSI) family has been identified as crucial regulators in various cellular processes via the regulation of translation target gene expression. The MSI family includes MSI1 and MSI2 [3]. MSI1 is a RNA-binding protein which is encoded by regulatory genes located on 11q13, and the molecular scaffold of MSI1 exhibits a high degree of sequence similarity with MSI2, which binds consensus motifs in the 3'untranslated regions of mRNAs, interacts with the poly(A)-binding protein and competes for eukaryotic initiation factor-4G, thereby interfering with translation initiation [4]. The function of MSI1 has been linked to tissue stem cells. Kayahara et al. found that MSI1 was expressed in crypt base columnar cells of mouse small intestine and could be a putative intestinal stem cell and early lineage marker [5]. Wang et al. identified MSI1 as a key determinant of the mammary lineage through its ability to coordinate cell cycle entry and activate the Notch and Wnt pathways [6]. Battelli et al. demonstrated that Msi-1's ability to regulate progenitor maintenance is through the translational inhibition of the cyclin-dependent kinase inhibitor p21WAF-1 in the developing nervous system [7]. Recent studies have shown that MSI1 was overexpressed and involved in the development
of human cancers. Gong et al. found that MSI1 promoted the EMT progression through activation of the Wnt signaling pathway in cervical cancer [8]. In line with this study, Hou et al. suggested that MSI1 could be used as a putative stem cell marker in cervical squamous cell carcinoma and its expression was correlated with poor clinical outcome in cervical cancer patients [9]. Nahas et al. revealed that MSI1 competes with miR130a and -206 for interaction with TAC1 mRNA, to stabilize and increase its translation, which increase breast cancer cells growth [10]. Nikpour et al. reported that MSI1 regulates apoptosis, gene expression and stress granule formation in urothelial carcinoma cells [11]. These investigations suggest that MSI1 has a close relationship with human cancers. However, there is no relevant report regarding MSI1 in renal carcinoma till now.

To explore the biological role of MSI1 in renal carcinoma, we examined the expression of MSI1 in kidney cancer cell lines and kidney cancer tissues, and found that MSI1 expression was upregulated in both renal cancer tissues and cell lines. Moreover, knockdown of MSI1 inhibited the migration and invasion of kidney cancer cells in vitro. Furthermore, MSI1 expression was associated with several clinicopathological factors and showed an unfavorable influence on both disease-free survival and overall survival. Thus, we propose that MSI1 may predict unfavorable outcomes in RCC and promote cancer progression.

Materials and methods

Ethics statement

The Research Ethics Committees of The Second Affiliated Hospital of Nanchang University approved this protocol and written informed consents were obtained from each patient. The entire study was performed based on the Declaration of Helsinki.

Patients and samples

115 cases of primary RCC samples were collected, and the pathological information was retrieved from the archives of The Second Affiliated Hospital of Nanchang University between January 2005 and December 2010. The histopathological diagnosis of all samples was respectively diagnosed by two pathologists. None of the patients enrolled in this study suffered from other cancers. In addition, 20 paired fresh kidney cancer samples and adjacent normal kidney tissue samples were collected. For the use of these clinical materials for research purposes, informed consent from all patients and approval from the Institute Research Ethics Committee were obtained in accordance with our institutional guidelines.

Cell lines

Human RCC cell lines (786-O and ACHN) and normal kidney cell lines (293T and HK-2) were obtained from the Chinese Academy of Science (Shanghai, China) and cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) in a humidified incubator containing 5% CO$_2$ at 37°C.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from tumor samples and cells with Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Relative expression was calculated via the comparative cycle threshold method and was normalized to the expression of GAPDH. The following primer sequences were used: MSI1, sense, 5'-AGCACGACCCCGGTAAAATG-3', and anti-sense, 5'-GCTCGACGAGGAATGCAAC-3'; GAPDH, sense, 5'-CGAGATCCCTCCAAAAATCAA-3', and anti-sense, 5'-TCACACCCATGACGAACAT-3'. Each experiment was conducted for at least three times.

Cell transfection

MSI1 siRNA (Si-MSI1-1 and Si-MSI1-2) and non-targeting siRNA (Control) were purchased from RiboBio (Guangzhou, Guangdong, China). Lipofectamine 2000 reagent (Invitrogen, USA) were used to transfect the cells. Knockdown was assessed by Real-time PCR after 48 hours of transfection.

Wound healing assay

Briefly, 5×10$^5$ cells were seeded in 6-well plates and incubated overnight. After achieving 90% confluence, the cell monolayer was scratched with a sterile pipette tip to produce a straight line. Photographic images were taken at 0 and 24 h along the scrape line by microscope. Results were expressed as relative scratch width, based the distance migrated relative to the original scratched distance.
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Cell migration and invasion assays

Briefly, $1 \times 10^5$ cells were seeded on a fibronectin-coated polycarbonate membrane insert in a transwell apparatus (Costar). After the cells were incubated for 24 h, the non-invaded cells on the upper membrane surface were removed with a cotton tip, and the cells that passed through the filter were fixed with 4% paraformaldehyde and stained with hematoxylin. For the cell invasion assay, the procedure was similar to the cell migration assay, except that the transwell membranes were pre-coated with 24 mg/ml Matrigel (Corning, USA). Experiments were performed three times.

Western blot analysis

Total cellular proteins were extracted and separated in SDS PAGE gels, and Western blot analysis was performed according to standard procedures. The antibodies used in this study included: anti-MSI1 (1:1000, Abcam) and GAPDH (1:2000, Santa Cruz Biotechnology).

Immunohistochemistry

Paraffin-embedded sections of tumor tissues were stained according to standard protocols with primary antibody against MSI1 (Abcam) followed by staining with biotinylated secondary antibody. After washing any unbound antibodies, the sections were incubated with a buffer containing HRP-conjugated streptavidin followed by addition of substrate solution containing peroxidase. The results were combined to give a mean score for further comparative evaluations. Briefly, the IHC score was determined by combining the score for the percentage of positively-stained tumor cells with the grade of

Figure 1. MSI1 is overexpressed in primary human RCC. A. Real-time PCR analysis of MSI1 expression in 20 paired human RCC tissues (T) and the adjacent normal kidney tissues (ANT) from the same patient. B. MSI1 expression in two RCC cell lines (786-O and ACHN) and normal kidney cell lines (293T and HK-2). C. Kaplan-Meier overall survival curves for patients with RCC indicating the correlation of MSI1 overexpression with worse overall survival and recurrence-free survival rates.
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The staining intensity. The percentages of positively-stained tumor cells were scored as follows: 0, no positive tumor cells; 1, <10%; 2, 10%-35%; 3, 35%-75%; 4, >75%. The staining intensities were graded as follows: 1, no staining; 2, weak staining (light yellow); 3, moderate staining (yellow-brown); 4, strong staining (brown). The IHC score ≥6 was defined as high expression and SI<6 was defined as low expression.

Statistical analysis

All data were analyzed for statistical significance using SPSS 18.0 (USA). The χ²-test was applied to the examination of correlation between MSI1 expression and clinicopathological characteristics. The Kaplan-Meier method was used to analyze the overall survival rate and recurrence-free survival time. Cox regression was used for multivariate analysis. Values were expressed as the mean ± SD from at least three independent experiments. P<0.05 was considered significant.

Results

MSI1 expression is increased in RCC tissues and cell lines

To assess the expression of MSI1 in RCC, we collected twenty paired RCC tissues and adjacent normal kidney tissues, and two RCC cell lines (786-O and ACHN) and normal kidney cell lines (293T and HK-2) and performed real-time PCR. RCC tissues showed higher expression levels of MSI1 compared with adjacent normal kidney tissues (Figure 1A), in addition, MSI1 expression was significantly higher in RCC cell lines compared with normal kidney cell lines (Figure 1B). The representative immunostaining of MSI1 in RCC tissues was shown in Figure 2.

Overexpression of MSI1 is associated with clinicopathological features in RCC patients

We next analyzed the correlation between the expression of MSI1 and clinicopathological characteristics of RCC patients. As summarized in Table 1, high expression of MSI1 was significantly associated with tumor stage (P=0.002) and distant metastasis (P=0.013). However, there were no significant associations between MSI1 expression and age, gender, tumor size, and tumor grade. Moreover, the expression level of MSI1 expression was significantly associated with the overall survival (P=0.019) and recurrence-free survival (P=0.012) of RCC patients, as patients with lower levels of expression had better survival than those with higher levels of MSI1 expression. Multivariate Cox regression analysis showed that high expression of MSI1 was a poor independent prognostic factor for RCC patients (P=0.009 and P=0.015, respectively) (Table 2).

Knock-down of MSI1 suppresses RCC cells migration and invasion

To examine the effect of MSI1 on cancer cell migration, we performed transwell assay and...
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Table 2. Multivariate cox regression analysis of overall survival and recurrence-free survival

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Overall survival</th>
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<th>Recurrence-free survival</th>
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<td></td>
<td>Hazard Ratio (95% CI)</td>
<td>P</td>
<td>Hazard Ratio (95% CI)</td>
<td>P</td>
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<tr>
<td>Age (&gt;55 y vs ≤55 y)</td>
<td>1.447 (0.151-4.389)</td>
<td>0.428</td>
<td>1.650 (0.748-3.286)</td>
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<tr>
<td>Sex (Male vs Female)</td>
<td>1.249 (0.495-7.926)</td>
<td>0.387</td>
<td>1.105 (0.567-7.730)</td>
<td>0.313</td>
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<td>Tumor Stage (T3-T4 vs T1-T2)</td>
<td>2.679 (1.244-5.409)</td>
<td>0.021</td>
<td>2.155 (1.248-5.802)</td>
<td>0.019</td>
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<td>LN metastasis (yes vs no)</td>
<td>2.937 (1.519-6.556)</td>
<td>0.018</td>
<td>3.345 (1.721-6.547)</td>
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<td>Distal metastasis (yes vs no)</td>
<td>3.895 (1.035-9.725)</td>
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<td>3.844 (1.284-12.826)</td>
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<td>Tumor size (&gt;4 cm vs ≤4 cm)</td>
<td>2.842 (0.351-8.298)</td>
<td>0.270</td>
<td>2.456 (0.815-9.646)</td>
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<td>Fuhrman grade (III-IV vs I-II)</td>
<td>1.324 (0.154-3.454)</td>
<td>0.509</td>
<td>1.525 (0.637-4.624)</td>
<td>0.554</td>
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<tr>
<td>MSI1 expression (high vs low)</td>
<td>2.625 (1.528-5.387)</td>
<td>0.009</td>
<td>3.163 (0.339-4.542)</td>
<td>0.015</td>
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Figure 3. MSI1 is involved in the invasion and metastasis of RCC cells. A, B. Western blotting analysis of MSI1 expression in vector and MSI1-silencing 786-O and ACHN cells; GAPDH was used as a loading control. C, D. Representative photos of wound-healing assays and transwell invasion assay in control and MSI1-shRNA RCC cells. Bars represent the mean ± SD of three independent experiments. *P<0.05.

Transwell assay subsequently confirmed that MSI1 knockdown could reduce the migrated cell number of both cancer cells (Figure 3D). These results suggested that MSI1 plays a role in promoting cell migration in RCC.

Discussion

In this study, we examined the expression of MSI1 in RCC tissues and kidney cancer cell lines, for the first time found that the expression of MSI1 is upregulated in RCC tissues and kidney cancer cell lines in compared with that in adjacent noncancerous tissues and normal kidney cells, respectively. Higher MSI1 expression was correlated with advanced clinical stage, tumor metastasis, and poor overall survival and recurrence-free survival. Moreover, downregulation of MSI1 suppresses kidney cancer cell migration and invasion. Taken together, our findings identify MSI1 as a central component in the development of renal carcinoma.

The MSI1 gene is a member of the well conserved MSI family, which distributed in the stem cell compartment of neural, pancreatic and epithelial tissues during mammals evolution [12]. Previous studies have identified that MSI1 regulates mRNA translation and influences multiple biological

wound healing assay in MSI1 silenced RCC cells (Figure 3A, 3B). As shown in Figure 3C, compared with cells that were transfected with empty vector, MSI1 knockdown strongly restrained the motility of 786-O and ACHN cells.
processes, including maintenance of stem cell identity. For example, MSI1 is an important regulator of the perikarya of CNS stem-like cells and non-oligodendroglial progenitor cells [13]. Ablation of MSI1 significantly reduced the proportion of mature oligodendrocytes generated from OP cells in vitro and in vivo during myelination [14]. Several studies also identified MSI1 as a master regulator in cancer progression and development. Akasaka et al. showed that MSI1 was expressed in the proliferative regions of human antrum and its decreased expression in intestinal metaplasia [15]. Kanemura et al. demonstrated that MSI1 is a versatile marker in determining the cellular origin, malignancy, and proliferative activity of glioma cells [16]. Rezza et al. found that overexpression of MSI1 induces tumorigenesis through Wnt and Notch activation in intestinal epithelium [17]. Our data showed that MSI1 was overexpressed in RCC tissues and kidney cancer cell lines compared to that in normal kidney tissues and normal kidney cells, and that MSI1 expression was correlated with tumor stage, indicating that MSI1 may play crucial roles in RCC development.

Moreover, by using RNAi in renal cancer cell lines to inhibit the expression of MSI1, we found that knockdown of MSI1 inhibited kidney cancer cell migration and invasion. In addition, patients with high MSI1 expression were more likely to have metastatic diseases. Our results were supported by previous studies concerning the role of MSI1 in tumor metastasis. Pastó et al. proposed that NOTCH3 signaling regulates MUSASHI-1 expression in metastatic colorectal cancer cells and contributes to epithelial-mesenchymal transition and metastasis [18]. Oskarsson et al. revealed that tenasin C enhances the expression of stem cell signaling components, MSI1 and LGR5, which promotes the survival and outgrowth of pulmonary micro metastases [19]. By examining the expression of MSI1 and PYGO2 in esophageal squamous cell carcinoma, Moghbeli et al. observed a significant correlation between the Ms1 and PYGO2 overexpressed cases and depth of tumor invasion [20]. Together, these studies suggest that MSI1 may contribute to the metastasis of human cancers.

The prognostic roles of MSI1 have been investigated in several malignancies. Liu et al. reported that high MSI1 expression might be closely related to the carcinogenesis, progression, clinical biological behaviors, and prognosis of gallbladder adenocarcinoma [21]. Wang et al. found that MSI1 regulates breast tumor cell proliferation and is a prognostic indicator of poor survival [22]. Similarly, our results demonstrated that patients with high expression of MSI1 have shorter overall and recurrence-free survival compared to that with lower levels of expression. Multivariate analysis also showed that high MSI1 expression is an independent prognostic indicator for the prognosis of RCC patients. Thus, these findings suggest that MSI1 could be used as a valuable prognostic factor for human cancers.

In summary, our experiments provided evidence that MSI1 may contribute to the progression and metastasis of renal carcinoma, and that MSI1 may be a valuable bio-marker for the diagnosis and therapy of renal carcinoma.

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Disclosure of conflict of interest

None.

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