ORIGINAL ARTICLE

Matrix metalloproteidase 2 (MMP2) mediates MHC class I polypeptide-related sequence A (MICA) shedding in renal cell carcinoma


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Abstract

Introduction: The MHC class I chain-related molecule A (MICA) is a ligand for the natural killer group 2, member D (NKG2D) immunoreceptor activation. The engagement of tumor cell surface MICA to NKG2D stimulates the NK and T cell antitumor immunity. Shedding of MICA by tumor cells facilitates tumor immune evasion, which might partially contribute to tumor progression.

Material and methods: Immunohistochemistry was performed on both normal and neoplastic renal tissues. Human renal carcinoma cell lines 786-0 and ACHIN were transfected and target sequences to silence human MMP2 by shRNA expression were established. The degree of MICA shedding was measured and quantitative real-time PCR and Western-blot analysis were performed.

Results: The membrane type matrix metalloproteinase 2 (MMP2) mediated the MICA shedding, which was blocked by suppression of MMP2 expression. Concomitantly, MMP2 overexpression enhanced the MICA shedding, indicating that MMP2 was involved in the renal cell carcinoma-associated proteolytic release of soluble MICA (sMICA), which facilitated the tumor immune escape.

Conclusions: These findings suggested that MMP2 might be a new potential target for tumor immune therapy. Elucidation of the mechanisms by which tumors shed MICA could be of great importance for cancer treatment in order to reinforce the NK and T cell antitumor immunity.

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Keywords
Renal cell carcinoma; MICA; MMP2; NKG2D

Abbreviations: RCC, renal cell carcinoma; PCR, polymerase chain reaction; 786-0, cultured cells of human renal cell cancer; ACHN, adenocarcinoma cell line, human kidney; MMP2, matrix metalloproteinase 2; MICA, MHC class I chain-related molecule A; NKG2D, natural killer group 2, member D.


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Matrix metalloproteinase 2 (MMP2) mediates the elimination of MHC class I polypeptide-related sequence A (MICA) in renal cancer

**Resumen**

**Introducción:** La molécula A relacionada con la cadena de clase I del CMH (MICA) es un ligando del grupo 2 de células asesinas naturales, y miembro D (NKG2D) de la activación de inmunorreceptores. La unión de la superficie de la célula tumoral MICA con NKG2D estimula las células asesinas (NK) y la inmunidad de las células T antitumorales. La eliminación de MICA por las células tumorales facilita la evasión inmune del tumor, lo cual podría contribuir en parte a la progresión tumoral.

**Materiales y métodos:** Se aplicó inmunohistoquímica tanto en tejidos renales normales como neoplásicos. Se transfirieron las líneas de células tumorales de riñón humano 786-O y ACHN, y se establecieron secuencias objetiva para silenciar la MMP2 humana a través de la expresión ARNhc. Se midió el grado de eliminación de MICA y se llevó a cabo una PCR cuantitativa en tiempo real, así como el análisis de Western blot.

**Resultados:** La membrana de tipo metalloproteína de matriz-2 (MMP2) mediaba la eliminación de MICA, que se bloqueaba por la supresión de la expresión de MMP2. Simultáneamente, la sobreeexpresión de MMP2 aumentaba la eliminación de MICA, lo cual indica que MMP2 interviene en la liberación proteolítica de MICA soluble (sMICA) asociada al carcinoma de células renales, que facilita el escape tumoral inmune.

**Conclusiones:** Dichos resultados sugieren que MMP2 podría ser un nuevo objetivo potencial para la terapia inmune de tumores. Un esclarecimiento de los mecanismos por los que los tumores eliminan MICA podría ser de gran importancia para el tratamiento del cáncer, para así reforzar las NK y la inmunidad antitumoral de las células T.

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**Introduction**

Renal cell carcinoma (RCC) is the seventh most common cancer in China and was predicted to result in nearly 13,000 deaths in 2011. Surgery is the first line of treatment for this cancer, where a successful resection often results in a long-term disease-free status. Although the overall survival rate for this cancer is more than 60% over 5 years, approximately 30% of the patients who have a diagnosis of localized RCC develop metastatic recurrence. Therefore, developing new approaches to treat such aggressive cancers represents a major challenge for clinicians and medical researchers.

Natural killer group 2, member D (NKG2D) is a c-type lectin-like activating immunoreceptor. MICA (MHC class I chain-related molecule A), located within the HLA locus, is the ligand for NKG2D. NKG2D has three extracellular domains, including: α1, α2, and α3. When NKG2D binds to MICA, they constitute an immune-surveillance system for protecting the host from malignant cells and viruses. MICA is expressed in most human epithelial tumors but it is generally absent in normal tissues. Cleavage of tumor-associated MICA from the cell surface is thought to be important for the tumor immune escape. Some studies showed that the soluble MICA (sMICA) level was higher in the cancer patients’ sera as compared to that of the healthy controls. Therefore, they postulated that the accumulation of sMICA in serum might lead to the down-regulation of NKG2D through facilitation of NKG2D internalization and lysosomal degradation. These results indicated that sMICA might be an indicator for cancer prognosis.

On the other hand, the matrix metalloproteinase (MMP) family represents a group of 26 zinc-dependent endopeptidases in human, which degrade components of the extracellular matrix, such as collagens and gelatin. MMPs are noteworthy due to their involvement in a large number of physiological and pathological processes, including oncogenesis. MMPs are classified based on their substrate specificities and biological characteristics. Inhibitors of the broad-spectrum metalloproteinases, including the MMPs, have been shown to interfere with MHC shedding.

A previous study demonstrated that MMP2 (gelatinase A) was the member of gelatinases which was present in large quantities in cancer tissues. The MMP2 cleaved proteins, solubilized pericellular matrix components, and shed cellular ectodomains. However, there have been few studies that showed that MICA was expressed in renal carcinoma and that sMICAs were detected in renal carcinoma patients’ sera. Therefore, we asked whether MMP2 activity mediated the MICA cleavage in renal carcinoma.

Our studies showed that MICA shedding occurred not only in poorly differentiated tumors, but also in well-differentiated tumors as well as the tumors in their early stage. The sMICA releasing was significantly suppressed by an MMP2 shRNA and was increased by MMP2 overexpression, suggesting that MMP2 might be critically involved in the MICA proteolytic shedding.

**Materials and methods**

**Human tissue specimens**

Paraffin-embedded specimens, including carcinoma and normal samples, were prepared after the surgical excision in
the Department of Urology, Shanghai Tenth People’s Hospital, Tongji University, from 2010 to 2012. The Ethics Committee at Tongji University approved the specimen collection. All tissue samples were fixed in 10% neutral formalin for 24 h. The 4-μm-thick sections were cut for immunohistochemical procedures.

Cell line and cell culture

The human renal carcinoma cell lines, 786-O and ACHN, were purchased from Cell Bank Type Culture Collection of Chinese Academy of Sciences (CBTCCCAS, Shanghai, China). The 786-O and ACHN cells were propagated in RPMI-1640 medium (HyClone) and Dulbecco’s modified Eagle medium (DMEM) (Gibco), respectively. The medium was supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were cultured at 37 °C in 5% CO2.

Immunohistochemistry

Expression of MICA was analyzed in a two-step procedure using an EnvisionTM Detection kit (DAKO). Briefly, deparaffinized tissue sections were incubated with 1% H2O2 in methanol for 30 min at room temperature to block the endogenous peroxidase activity, followed by incubation with 1.5% normal goat serum at 37°C for 30 min to prevent nonspecific binding. Incubation with anti-MICA antibody (dilution of 1:40; Abcam) was performed at 4°C overnight using predetermined optimal dilution. As negative controls, sections were incubated with 1.5% normal goat sera. Sections were then incubated with anti-rabbit immunoglobulin for 60 min and the reaction products were visualized according to the procedure of the EnvisionTM Detection kit. The sections were further counterstained with Mayer’s solution (WAKO Pure Chemical Industries). Using a Nikon microscope (Eclipse Ti-U), a semiquantitative method was used to evaluate the degree of immunostaining, which included — (no expression), + (weak), ++ (moderate), and +++ (high) on a cell-by-cell basis in five microscopic fields (100× magnification), as previously described.14

RNAi with shRNA infection

The shRNA against MMP2 were cloned in the pMKO.1 retrovirus vector and the MMP2 cDNA was cloned into the pBabe-puro retrovirus vector for overexpression. The plasmids were transfected by CaCl2 in HEK 293T cells. Forty-eight hours after the transfection, supernatants were collected, filtered, and used to infect the 786-O and ACHN cells in the 6-well plates at a 70% confluency with 4 μg/ml of polybrene (Sigma). Seventy-two hours after the infection, the cells were harvested for further analysis. The target sequences to silence human MMP2 by shRNA expression were established using techniques previously described.15

MICA shedding assay

The 786-O and ACHN cells were seeded in a 6-well plate at a density of 4 × 10^5 per well. Each well contained a total of 2 ml of complete medium. After 24 h, cells were infected with retrovirus vector and were cultured for another 72 h. The supernatant was collected and filtered through a 0.45-μm filter. The amount of soluble MICA was measured in the supernatant using human MICA DuoSet sandwich ELISA kit (R&D Systems). The measurement of the degree of MICA shedding in cells as previously described.16 All samples were assayed in triplicates.

Quantitative real-time PCR

Total RNA from each group of cells was extracted with Trizol Reagent (Invitrogen). The first strand cDNA was synthesized using SuperScript II RNase H Reverse Transcriptase (Invitrogen) and Oligo (dT) primer (Promega) from 2 μg of total RNA, according to the manufacturer’s instructions. The primer sequences of the MMP2 and GAPDH were as follows: MMP2 forward and reverse primers (5’-TTATTGTGCGACAGTGACA-3’ and 5’-ACACGGCATCAATCTTTCC-3’), and GAPDH forward and reverse primers (5’-GGCTTCTCTCAATGACA-3’ and 5’-ATGATGGCCATGAGGTTCC-3’). The PCR amplification were performed for 40 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 30s, on a Applied Biosystems 7900HT (Applied Biosystems) with 1.0 μl of cDNA and SYBR Green Realtime PCR Master Mix (Takara). At the completion of cycling, the melting curve analysis was performed to establish the specificity of the PCR products. Data were collected and analyzed by SDS2.3 Software (Applied Biosystems). The expression level of each candidate gene was internally normalized against that of the GAPDH. The relative quantitative value was expressed by the 2^{-ΔΔCT} method, representing the amount of the candidate gene expression with the same calibrators. Each experiment was performed in triplicates and repeated three times.

Western blotting

Cells were lysed in RIPA (radioimmunoprecipitation assay) buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS) containing a protease-inhibitor cocktail (Roche) for 30 min on ice. They were then homogenized and centrifuged at 15,000 RPM for 20 min at 4°C. The concentration of cellular proteins was determined with the Protein Quantitative Analysis kit (K3000-BCA Shenergy Biocol). The samples were separated in 10% SDS gels, transferred into PVDF membranes (GE Healthcare Life Sciences), and blocked with TBS (Tris-buffered saline) containing 5% non-fat dried milk. After being incubated with the MICA (1:200 dilution; Abcam) MMP2 (dilution 1:400; Cell Signaling), and β-actin (dilution 1:2000; Abcam) antibodies for 12 h at 4°C, the membranes were subsequently incubated with appropriate secondary antibodies for 30 min at room temperature. The signals were then visualized by enhanced chemiluminescence (ECL).

Statistical analysis

Statistical differences were determined between the treatment groups using one-way ANOVA (analysis of variance). The paired Student’s t-test was used to analyze the results for the statistical significance when only two conditions
were compared. A value of $P < 0.05$ was considered statistically significant.

**Results**

**MICA expression in renal cell carcinoma and normal renal tissue**

Immunohistochemical expression of MICA was examined in renal cell carcinoma and normal renal tissue samples. Tumor cells from renal cell carcinoma displayed an up-regulated expression of MICA (Fig. 1A). From among 30 renal cell carcinomas, 25 (83.3%) were positive for MICA. Expression levels were scored as $-$, $+$, $++$, and $+++\) with the values of 5/30 (16.7%), 10/30 (33.3%), 8/30 (26.7%) and 7/30 (23.3%), respectively. From among 28 normal renal tissues, 18/28 (64.3%), 6/28 (21.4%), 3/28 (10.7%) and 1/28 (3.6%) cases were scored as $-$, $+$, $++$, and $+++\), respectively (Table 1). There was a significant difference in MICA expression between the renal cell carcinoma and normal renal tissues.

**Short hairpin RNA suppression of MMP2 expression inhibited MICA shedding**

To identify the specific MMPs that were involved in MICA shedding, we constructed short hairpin RNA (shRNA) to a specific MMP2 in a lentiviral expression vector. The expression of MMP2 was suppressed in the target cells by up to 70–90% with the specific shRNA as evaluated by Quantitative real-time PCR and Western-blot analysis (Fig. 2A and C). ELISA for MICA shedding showed that the MMP2 silencing had a significant effect on the MICA shedding in 786-O and ACHN cells (Fig. 2B). Further, Western-blot analysis revealed that suppression of MMP2 could increase the surface expression of MICA in 786-O and ACHN cells (Fig. 2C). These results suggested that MMP2 was involved in MICA shedding in the renal carcinoma cells.

**Overexpression of MMP2 enhanced MICA shedding**

To provide further evidence that MMP2 was directly involved in MICA shedding, we overexpressed the active form of human MMP2 in the 786-O and ACHN cells. The expression of MMP2 was increased in the target cells as demonstrated by Quantitative real-time PCR and Western-blot analysis (Fig. 3A and C). The results showed that overexpression of MMP2 significantly increased the MICA shedding (Fig. 3B). Furthermore, Western-blot analysis revealed that overexpression of MMP2 significantly reduced the surface expression of MICA in 786-O and ACHN cells (Fig. 3C).

**Discussion**

MICA is a major ligand for NKG2D receptor activation. It is generally expressed at the surface of the infected cells as well as in many of the transformed human cells. The tumor cells expressing high levels of MICA were targeted by natural killer (NK) cells, CD8$^+$ T cells, and $\gamma\delta$ T cells, for elimination. In the present study, we evaluated the MICA protein expression in 30 cases of renal cell carcinoma and 28 cases of renal normal tissues. We found a significantly higher level of MICA expression in the renal cell carcinoma as compared to that of the normal renal tissues. However, we did not find any significant difference in the expression of MICA among the renal cell carcinoma samples regardless of their different stages. These results suggested that the MICA

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Figure 2  Inhibition of MMP2 expression or activity blocked MICA shedding. (A) Quantitative real-time PCR showed shRNA suppression of MMP2 expression in 786-O and ACHN cells. (B) ELISA showed the degree of MICA shedding by 786-O and ACHN cells with the suppression of MMP2 expression. (C) Western blot analysis confirmed the inhibition of MMP2 expression and cell surface MICA expression by shRNA in 786-O and ACHN cells at the protein level. Data represented the results from three independent experiments (*<P<0.05 when compared with control).

Figure 3  Overexpression of MMP2 increased the constitutive MICA shedding. (A) Quantitative real-time PCR showed the MMP2 expression in 786-O and ACHN cells. (B) ELISA showed that the over expression of MMP2 significantly increased the constitutive MICA shedding in 786-O and ACHN cells. (C) Western blots showed that an overexpression of MMP2 reduced cell surface MICA expression in the 786-O and ACHN cells. These data represented the results from three independent experiments (*<P<0.05 when compared with control).
protein was involved in the anti-renal cell carcinoma immunity. However, the innate immune cells could not effectively kill the renal cell carcinoma cells, which suggested the possibility for a dysfunction in the NKG2D–MICA-mediated immune surveillance.

It has been reported that sMICA was associated with systemic down-regulation of NKG2D surface expression in the NK, CD8+ T, and γδ T cells. Following sMICA binding to NKG2D on the surface of NK cells, the complex is internalized and the cytotoxic function is reduced. Therefore, a reduction of MICA expression on the cell surface might weaken the strength of the immune response. Many clinical studies have shown that a high serum level of sMICA was strongly correlated with a poor clinical outcome in the patients with various types of cancer. The studies have also shown that the prevention of MICA shedding inhibited tumor initiation in the animal models. Therefore, restoration of NKG2D–MICA-mediated immune surveillance could potentially represent an auxiliary strategy for cancer therapy. However, the exact mechanism by which MICA is shed from the tumor cells remains unclear. Lu et al. observed a higher serum level of sMICA in the patients with osteosarcoma as compared to that of the healthy individuals. In this study, we found that most of the early stage and well-differentiated renal cell carcinoma samples expressed MICA in their stroma. We hypothesized that MICA shedding might have occurred during the early stages of renal cell carcinoma, where the innate immune cells could not remove the malignant cells in time.

Matrix metalloproteinases (MMPs) are Ca2+- and Zn2+-dependent endoproteinases that are classified into interstitial collagenses, stromelysins, and gelatinases based on the substrate specificity. The matrix metalloproteinase 2 (MMP2) is a gelatinase, which is also called type IV collagenase. MMP2 is a cell surface-associated type IV collagen with the capacity to degrade ECM molecules, including: gelatins, fibronectin, and collagen I, which is highly expressed and secreted by the invasive human renal cell carcinoma cells. This could suggest that MMP2 is a key factor for tumor growth, angiogenesis, metastasis, and cellular invasion. Several reports have indicated that the inhibition of MMP2 expression and activity decreased cell migration and invasion in human breast cancer cells.

Therefore, MMP2 has been considered to be a target for anticancer drug development. Our studies showed that the MMP2 shRNA, a specific RNAi for MMP2, increased the membrane-anchored MICA expression and decreased the sMICA release. The MMP2 overexpression decreased the membrane-anchored MICA expression and increased the sMICA release. These results suggested a potential role for MMP2 in MICA shedding. We assumed that MMP2 might have hydrolysed MICA on the renal cell carcinoma cell surface, released the soluble MICA, and ultimately undermined the NKG2D–MICA mediated immune surveillance. This suggested that MMP2 might play a role in MICA shedding in renal cell carcinoma cells, aside from its important role in invasion, metastasis, and angiogenesis processes, allowing these cells to evade the immune attack.

In conclusion, our study provided evidence that MICA represented an MMP2 cleavage product that was formed during the early stage of renal cell carcinoma. Therefore, blocking of the MMP2 activity might represent a potential cancer treatment by targeting renal cell carcinoma tumor invasion and immune escape.

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Conflict of interest
The authors declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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