Alterations in enhancer of zeste homolog 2, matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 expression are associated with *ex vivo* and *in vitro* bone metastasis in renal cell carcinoma

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Abstract. Renal cell carcinoma (RCC) has a high potential for bone metastasis; however, the molecular mechanisms underlying this metastasis have remained to be elucidated. The present study aimed to explore the expression levels of enhancer of zeste homolog 2 (EZH2), matrix metalloproteinase-2 (MMP2) and tissue inhibitor of metalloproteinase-2 (TIMP2) as determinants of RCC-associated bone metastasis. Their expression was evaluated in a newly generated RCC cell subline that has a high potential for bone metastasis, in tissue specimens from metastasized bone tissues from patients with RCC and in RCC tissues without metastasis. A total of 25 RCC tissue specimens without metastasis and 13 RCC tissue specimens with bone metastasis were acquired for immunohistochemical analysis of EZH2, MMP2 and TIMP2 protein expression. The expression levels of EZH2, MMP2 and TIMP2 mRNA and protein were analyzed in the ACHN and ACHN-BO5 cell lines using western blot and reverse transcription polymerase chain reaction (PCR) analyses. Methylation-specific PCR was also used to analyze TIMP2 promoter methylation. EZH2 and MMP2 proteins were found to be expressed at higher levels in tissues from patients where RCC had metastasized to the bone as compared with those in RCC patients without metastasis, whereas there was no significant difference in the expression of TIMP2

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protein between the two tissues. Furthermore, the expression of EZH2 protein was correlated with MMP2 expression, but there was no significant correlation between the expression of EZH2 and TIMP2 proteins. The *in vitro* results using cell lines confirmed the *ex vivo* findings, indicating that the expression levels of EZH2 and MMP2 protein and mRNA were higher in ACHN-BO5 cells than those in ACHN cells. By contrast, TIMP2 protein and mRNA expression levels were lower in ACHN-BO5 cells than those in the parental ACHN cells. The TIMP2 promoter was highly methylated in ACHN-BO5 cells compared with that in ACHN cells. Upregulation of EZH2, MMP2 and TIMP2 expression was correlated with metastasis of RCC to bone tissues *ex vivo* and *in vitro*. Further studies are required in order to elucidate the mechanism underlying the altered expression of these genes.

Introduction

Renal cell carcinoma (RCC), which has been suggested to originate from the renal tubules and collecting tube epithelial cells, accounts for 85% of malignant kidney neoplasms and ~2% of all human malignancies (1,2). RCC is a pathologically heterogeneous disease, which can be classified into clear, papillary, granular, spindle and mixed cell subtypes based on certain cytoplasmic features (3). RCC morbidity increases by 2% annually and mortality has reached ~100,000 cases/year worldwide (4). Approximately 30% of patients with RCC develop metastatic disease, most frequently in the lungs, bones or brain (5). A clinical study confirmed that osteolysis represented 30% of the total metastatic disease cases associated with RCC (6). The incidence rate of bone tissue metastasis was higher in autopsy data from patients with RCC (5). The prognosis of RCC patients is influenced by a variety of factors, including tumor size, invasion, metastasis, histological type and nuclear grade (7); the five-year survival rate of patients with RCC was 90% for stage I, 51% for stage II, 22% for stage III and 4.6% for stage IV (8). Therefore, the elucidation of the key factors and molecular mechanisms underlying the metastasis of RCC to bone is required. A previous study by our group established an ACHN cell subline (ACHN-BO5) with high potential of bone

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metastasis compared with that of ACHN cells in animals in order to aid the elucidation of the underlying molecular mechanisms (9). In this subline, the pathological karyokinesis was significantly increased, which indicated that the malignant phenotype of the ACHN subline was higher than that of the parental ACHN cells. Following five passages of in vitro culture, the subline was named 'ACHN-BO5'. Subsequently, the gene alterations responsible for the high potential of bone metastasis were investigated using a complementary DNA (cDNA) microarray analysis to compare ACHN-BO5 cells with the parental ACHN cells. Alterations in the expression of enhancer of zeste homolog 2 (EZH2) and matrix metalloproteinase-2 (MMP2) were detected in ACHN-BO5 cells. EZH2 is involved in maintaining the transcriptional repressive state in cells and mutation of EZH2 causes Weaver syndrome (10), a congenital disorder associated with rapid growth beginning in the prenatal period, resulting in a characteristic facial appearance and certain skeletal features (11). In addition, studies have demonstrated that altered EZH2 expression promotes human cancer development (12-16). Proteins of the MMP family degrade or break down the extracellular matrix during normal physiological processes, including embryonic development and tissue remodeling, but also have a significant role in tumor metastasis (17). By contrast, tissue inhibitor of metalloproteinase-2 (TIMP2) is a natural matrix metalloproteinase inhibitor. Therefore, these proteins may have a role in mediating the metastasis of RCC to bone. In the present study, the expression of EZH2, MMP2 and TIMP2 were evaluated in RCC tissue specimens with or without bone metastasis and in ACHN-BO5 and ACHN cells to elucidate the correlation between their expression and metastasis in bone. Methylation-specific PCR analysis of TIMP2 promoter methylation was also performed. The results of the present study may aid the elucidation of the mechanisms underlying RCC metastasis and provide potential therapeutic targets for the prevention or treatment of RCC metastasis.

Materials and methods

Cell lines and culture. The human renal carcinoma cell line ACHN was obtained from the China Center of Type Culture Collection (Wuhan, China). The ACHN-B05 cell line was a subline of ACHN with high potential of bone metastasis, established in a previous study by our group (9). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies) at 37°C in a humidified incubator with 5% CO₂.

Tissue specimens and immunohistochemistry. Primary renal cancer biopsy specimens (n=25) and biopsies of renal cancer that had metastasized to bone tissues (n=13) were obtained from The Tongji Hospital affiliated with Huazhong University of Science and Technology (Wuhan, China) between March 2010 and April 2013. The normal control renal biopsy specimens were obtained from adjacent normal tissues. The present study was approved by the ethics committee of Tongji Hospital affiliated with Huazhong University of Science and Technology. Written informed consent was obtained

from all patients or their family. All tissues were fixed in 4% paraformaldehyde solution (Boster Biological Technology, Ltd., Wuhan, China) for 20 min at room temperature and embedded into paraffin using a routine tissue process (18). Tissue sections (4- μ M thick) were prepared from the paraffin blocks and mounted onto glass slides. For immunohistochemical analysis, tissue sections were deparaffinized and rehydrated in water. The sections were heated in a pressure cooker (121°C, 4 min) in a citric acid buffer (Boster Biological Technology, Ltd.) for antigen retrieval and then incubated with 3% H₂O₂/phosphate-buffered saline (PBS; Boster Biological Technology, Ltd.) at room temperature for 30 min to block potential peroxidase activity. Following incubation with 20% normal serum (Boster Biological Technology, Ltd.) for 30 min, the sections were further incubated with primary antibodies: Mouse monoclonal immunoglobulin G₁ (IgG₁) anti-MMP2 antibody at a dilution of 1:800 (sc-13594; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), goat polyclonal IgG anti-TIMP2 antibody at a dilution of 1:600 (sc-6835; Santa Cruz Biotechnology Inc.) or an goat polyclonal IgG anti-EZH2 antibody at a dilution of 1:100 (E6906; Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. The following day, the sections were washed three times with PBS and subsequently incubated with the secondary antibodies (anti-mouse IgG and anti-goat IgG horseradish peroxidase; Boster Biological Technology, Ltd.) for 1 h at 37°C. A color reaction was performed using 3,3'-diaminobenzidine (Boster Biological Technology, Ltd.) as the chromogen. Diluted Sav-HRP conjugates were applied to the sections on the slides and incubated in a humidified chamber at room temperature for 30 min (protected from the light). Slides were washed with PBS twice, for 5 min each. DAB substrate solution (freshly made just before use: 0.05% DAB - 0.015% H₂O₂ in PBS) was applied to the sections on the slides to reveal the color of antibody staining. The stained tissue sections were independently reviewed and scored under an Olympus CKX31 inverted microscope (Olympus Corp., Tokyo, Japan) by two investigators. The statistical results were analyzed using Sigmaplot 11.0 software (Systat Software, Inc., Chicago, IL, USA).

DNA extraction and methylation-specific PCR (MSP). Genomic DNA was extracted from ACHN and ACHN-BO5 cell lines using a Genomic DNA extraction kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. DNA concentration, purity and integrity were measured using a spectrophotometer (Gilford 250; Gilford Instrument Laboratories, Inc., Oberlin, OH, USA) and gel electrophoresis. For MSP, genomic DNA samples (2 μ g) were denatured with sodium hydroxide chemically modified with sodium bisulfite (Boster Biological Technology, Ltd.). An MSP primer for the amplification of the TIMP2 promoter was designed using Methprime software (http://www.urogene.org/methprimer/index1.html). The methylation primers of the TIMP2 promoter were as follows: Forward, 5'-TTTTATTGTAGGAAAGGTCGA-3' and reverse, 5'-GAAATCATAAAACAACGCGTA-3', which amplified a 159-bp PCR product. The demethylation primers of the TIMP2 promoter used were: Forward, 5'-GAAGGAATATTTTATTGTAGGAAAGGTT-3' and reverse, 5'-TATAACACAAAATCATAAAACAACAACATA-3', which amplified a 176-bp PCR product. PCR amplification

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Gene	Sequences	Size of PCR products (bp)		
EZH2	5'-GTGGAGAGATTATTTCTCAAGATG-3' 5'-CCGACATACTTCAGGGCATCAGCC-3'	289		
MMP2	5'-GAGAACCAAAGTCTGAAGAG-3' 5'-GGAGTGAGAATGCTGATTAG-3'	207		
TIMP2	5'-CCTCGGCCTTTC CTGCAAT-3' 5'-TATCTACACGGCCCCCTCCT-3'	89		
GAPDH	5'-GAAGGTGAAGGTCGGAGTC-3' 5'-GAAGATGGTGATGGGATTTC-3'	226		

PCR, polymerase chain reaction; EZH2, enhancer of zeste homolog 2; MMP2, matrix metalloproteinase-2; TIMP2, tissue inhibitor of metalloproteinase-2.

occurred in a final volume of 25 μ l containing: 2.5 μ l PCR buffer, 2 μ l MgCl₂, 2.5 μ l deoxynucleotide triphosphate mixture, 1 μ l of each primer, 5 μ l modified DNA template, 10.85 μ l sterilized deionized water and 0.15 μ l Taq enzyme (Takara Bio, Inc., Otsu, Japan). The PCR conditions were set at a pre-denaturation temperature of 94°C for 5 min followed by 45 cycles of 94°C for 30 sec, 60°C (methylated) or 55°C (demethylated) for 45 sec, 75°C for 40 sec and a final extension at 73°C for 5 min. Genomic DNA chemically modified with ss1 methylation enzyme (New England Biolabs, Shanghai, China) and bisulfite salts (Sigma-Aldrich) was used as a positive control. Deionized water was used as a negative control. PCR products were isolated using agarose gel electrophoresis and imaged under an ultraviolet lamp (XX15B, Spectronics Corp., Westbury, NY, USA).

RNA isolation and semi-quantitative RT-PCR. The expression levels of EZH2, MMP2 and TIMP2 mRNA were evaluated using RT-PCR analysis. Briefly, total cellular RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies) and reverse-transcribed to cDNA using the First Strand cDNA Synthesis kit (Rever Tra Ace- α ; ToYoBo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The primer sequences of genes and fragment sizes are indicated in Table I. The PCR conditions were as follows: Pre-denaturation at 94°C for 5 min and 32 cycles of 94°C for 45 sec; 52.5°C, 56°C and 54°C as the annealing temperature for 1 min, 72°C for 90 sec and a final extension at 72°C for 10 min. The PCR product was subsequently sequenced and identified by gel electrophoresis.

Protein extraction and western blot analysis. Cells were collected and lysed with a pre-cooled cell lysis buffer (Boster Biological Technology, Ltd.) containing 100 mM Tris-HCl, 500 mM EDTA, 20 mM NaCl, and 10% SDS. The protein concentration was determined using a Bicinchoninic Acid Assay kit (Sigma-Aldrich, Irvine, Scotland). Briefly, an equal amount of protein sample was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (A-FIT Biosciences, Beijing, China). For western blot analysis, the membranes were incubated in 5% skimmed milk/PBS at room temperature for 1 h and then further incubated with

anti-MMP2 antibody at a dilution of 1:800, anti-TIMP2 antibody at a dilution of 1:600 or anti-EZH2 antibody at a dilution of 1:100 at 4°C overnight. The following day, the membranes were washed three times with PBS-Tween-20 and incubated with a horseradish peroxidase-conjugated secondary antibody at a dilution of 1:7,500 at room temperature for 2 h. Immunoreactive proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions and exposed to x-ray films (Kodak, Rochester, NY, USA). The expression levels of these proteins were normalized to an internal control, GAPDH.

Statistical analysis. Values are presented as the mean \pm standard deviation of three independent experiments. All statistical analyses were performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA). The expression levels of EZH2, MMP2, TIMP2 mRNA and protein in ACHN and ACHN-B05 cells were compared using one-way analysis of variance. Differences in TIMP2 promoter methylation were analyzed using a χ^2 test. A Pearson's correlation test was used to analyze the associations between different groups. P<0.05 was considered to indicate a statistically significant difference between values.

Results

Differential expression of EZH2, MMP2 and TIMP2 proteins in tissues of patients with RCC as well as ACHN and ACHN-B05 cells. In the present study, the expression of EZH2, MMP2 and TIMP2 proteins in tissues from patients with RCC with and without bone metastasis were evaluated by immunohistochemical analysis. The expression of EZH2 protein was higher in tissues from patients with RCC that had metastasized to the bone than in tissues of patients with RCC without metastasis (P=0.031; Fig. 1A-D). Analogously, expression levels of MMP2 protein were also higher in tissues from patients where RCC had metastatized than those in patients with RCC without metastasis (P=0.047; Fig. 2A-D). By contrast, there were no significant differences in the expression of TIMP2 protein between the tissue types (P=0.2932; Fig. 3A-D). Furthermore, the expression of EZH2 and MMP2 proteins were found to be



Figure 1. Immunohistochemical detection of EZH2 proteins in tissue sections of renal cancer patients with or without bone metastasis (magnification, x400). (A) EZH2-control; (B) EZH2 expression in RCC tissue; (C) EZH2 expression in tissue of patients with RCC with bone metastasis; (D) quantification of immunohistochemical analysis, comparing EZH2 expression score in tissues of RCC with and without bone metastasis. Images are representative of three independent experiments. EZH2, enhancer of zeste homolog 2; RCC, renal cell carcinoma.



Figure 2. Immunohistochemical detection of MMP2 protein expression in tissue sections of renal cancer patients with or without bone metastasis (magnification, x400). (A) MMP2 control; (B) MMP2 expression in RCC tissue; (C) MMP2 expression an RCC tissue specimen with bone metastasis; (D) quantification of immunohistochemical analysis, comparing MMP2 expression levels in RCC tissues with and without bone metastasis. Images are representative of three independent experiments. MMP2, matrix metalloproteinase-2; RCC, renal cell carcinoma.

correlated (r=0.6652; P=0.0131; Fig. 4A), whereas there was no significant correlation between EZH2 and TIMP2 protein expression (r=-0.5484; P=0.0523; Fig. 4B).

Western blot analysis was used to investigate the expression levels of EZH2 protein in ACHN and ACHN-BO5 cells. EZH2 protein expression levels were demonstrated to be higher in ACHN-BO5 cells, a sub-line of ACHN with a higher potential for metastasis to the bone, than those in the parental ACHN cells (P<0.05), suggesting that EZH2 protein may be involved in mediating the metastasis of RCC to bone (Fig. 5A). In addition, the expression levels of MMP2 protein were higher in ACHN-BO5 cells than those in the parental ACHN cells (P<0.05). By contrast, TIMP2 protein expression levels were lower in ACHN-BO5 cells than those in the parental ACHN cells (P<0.05; Fig. 5B).

EZH2, MMP2 and TIMP2 mRNA expression levels differ between ACHN and ACHN-B05 cells. RT-PCR analysis was



Figure 3. Immunohistochemical analysis of TIMP2 protein expression in tissue sections of renal cancer patients with or without bone metastasis (magnification, x400). (A) TIMP2 control; (B) TIMP2 expression in RCC tissue; (C) TIMP2 expression in RCC with bone metastasis; (D) quantification of immunohistochemical analysis results, comparing TIMP2 expression score in tissues of RCC with and without bone metastasis. Images are representative of three independent experiments. TIMP2, tissue inhibitor of metalloproteinase-2; RCC, renal cell carcinoma.



Figure 4. Evaluation of the correlation between EZH2 expression and MMP2 and TIMP2 protein in tissue sections of patients with RCC with or without bone metastasis using Spearman's test. (A) Correlation between MMP2 and EZH2 expression; (B) correlation between TIMP2 and EZH2 expression. EZH2, enhancer of zeste homolog 2; MMP2, matrix metalloproteinase-2; TIMP2, tissue inhibitor of metalloproteinase-2; RCC, renal cell carcinoma.



Figure 5. Western blot analysis of the expression levels of EZH2, MMP2 and TIMP2 proteins in ACHN and ACHN-BO5 cells. The graphs represent the quantified data from the western blot analyses. (A) Protein expression levels of EZH2; (B) protein expression levels of MMP2 and TIMP2. Values are presented as the mean \pm standard deviation (n=3). *P<0.05 vs. ACHN cells. EZH2, enhancer of zeste homolog 2; MMP2, matrix metalloproteinase-2; TIMP2, tissue inhibitor of metalloproteinase-2.





Figure 7. Methylation of the *TIMP2* promoter assessed using methylation-specific PCR analysis. TIMP2-M is the PCR product obtained using a methylated primer and represents methylation of the *TIMP2* promoter, while TIMP2-U was produced using a demethylated primer and represents the demethylated TIMP2 promoter. Values are presented as the mean \pm standard deviation (n=3). *P<0.05 vs. ACHN cells. TIMP2, tissue inhibitor of metalloproteinase-2; PCR, polymerase chain reaction.

Figure 6. Expression of EZH2, MMP2 and TIMP2 mRNA in ACHN and ACHN-BO5 cell lines detected using semi-quantitative reverse transcription polymerase chain reaction. Values are presented as the mean ± standard deviation (n=3). *P<0.05 vs. ACHN cells. EZH2, enhancer of zeste homolog 2; MMP2, matrix metalloproteinase-2; TIMP2, tissue inhibitor of metalloproteinase-2; mRNA, messenger RNA.

performed in order to evaluate whether the altered expression of these three proteins occurred at the transcriptional level in the ACHN and ACHN-BO5 cell lines. The expression levels of EZH2 and MMP2 mRNA were higher in ACHN-BO5 cells than those in ACHN cells, whereas the expression levels of TIMP2 mRNA were lower in ACHN-BO5 cells than those in ACHN cells (Fig. 6).

TIMP2 is more highly methylated in ACHN-BO5 cells. The potential mechanism underlying the downregulated expression of TIMP2 mRNA in ACHN-BO5 cells was assessed using MSP. The TIMP2 promoter was more highly methylated in ACHN-BO5 cells than in ACHN cells (Fig. 7).

Discussion

Cancer metastasis is a complex process, where cancer cells migrate from their site of origin and invade other parts of the body via the bloodstream, lymphatic system or direct extension (19). Molecularly, tumor cells gain gene transcription capabilities and express various proteins and enzymes in order to degrade extracellular matrix proteins and invade adjacent tissues (20). However, the distant metastasis of various types of human cancer indicates preferences for certain organs; therefore, certain types of cancer tend to spread to particular organs and tissues (19). For example, breast cancer preferentially metastasizes to bone and lung tissue (21). RCC frequently metastasizes to the lungs, bone or brain, whereas the brain is most commonly the distant site of metastasis of melanoma (22). Therefore, a study of organ-specific RCC metastasis may aid in the prevention of RCC progression. In a previous study by our group, an RCC cell line (ACHN-BO5) with high potential for metastasis to the bone was generated (9). These cells exhibited a significantly enhanced invasion and proliferation capacity in vitro, compared to that of the parental ACHN cells. The present study investigated whether cell adhesion molecules, including EZH2, MMP2 and TIMP2, contributed to alterations in the phenotype of tumor cells. Expression levels of EZH2 and MMP2 mRNA and protein were higher in ACHN-BO5 cells than those in ACHN cells, whereas the expression of TIMP2 mRNA and protein was lower in ACHN-BO5 cells than that in ACHN cells. MSP data indicated that the downregulated expression of TIMP2 may be due to the methylation of the TIMP2 promoter. Furthermore, the expression of these proteins was evaluated in tissue specimens from patients with RCC that had metastasized to the bone and patients with RCC without metastasis. The results confirmed those of the in vitro investigations, indicating that the expression of EZH2 and MMP2 protein was higher in tissues from patients with RCC that had metastasized to the bone than that in tissues from renal cancer patients without metastasis; and that there was no significant difference in the expression of TIMP2 protein between the two types of tissue.

The epigenetic modification enzyme, EZH2, has a homologous structure to the *Drosophila* E(z) gene, and was identified in the 1990s (23). Cardoso *et al* (24) demonstrated that the EZH2 protein was a key member of the polycomb group gene family and was able to modulate cell proliferation and the signaling pathway via the suvar3-9, enhancer of zeste, trtharax domain. The EZH2 protein is involved in mediating the development, metastasis, invasiveness and prognosis of various types of human cancer (23-32). Therefore, EZH2 is of interest in basic and clinical studies of cancer. Several studies have demonstrated that EZH2 protein is highly expressed in RCC tissues (33-36), the level of which is associated with RCC dedifferentiation, suggesting that the EZH2 protein may contribute to RCC progression and metastasis. More recently, studies have indicated that EZH2 protein is highly expressed in prostate cancer tissues, which are also associated with a high rate of metastasis to the bone (16,37,38). Knockdown of EZH2 expression in metastatic bone tumors leads to atrophy of the metastatic bone foci and a reduction in or end to bone destruction (39), suggesting that EZH2 protein has a role in mediating metastasis to the bone.

MMPs, including MMP2, are required for degradation of the extracellular matrix and are specifically inhibited by the TIMPs. Therefore, these two families of proteins have significant roles in tumor metastasis. Numerous studies have confirmed that alterations in the balance of MMPs/TIMPs may lead to the metastasis of human cancers to the bone (40-43). Further studies have demonstrated that a downregulation of TIMP2 expression was associated with methylation of the TIMP2 promoter (44-50). Of note, EZH2 contains histone methyltransferase activity, which is able to silence genes via the methylation of H3 histone lysine 27 (51). It was therefore hypothesized that overexpression of EZH2 protein may lead to epigenetic silencing of TIMP2 expression. The results of the present study confirmed that there was an enhanced level of TIMP2 promoter methylation in ACHN-BO5 cells compared to that in ACHN cells. However, the results of the ex vivo investigations did not demonstrate an association between EZH2 expression and reduced TIMP2 protein, which does not support the hypothesis that EZH2 protein may be able to cause epigenetic silencing of TIMP2 expression. Due to the small sample size in the present study, further investigations are required in order to confirm this hypothesis.

Further studies are required to elucidate the molecular mechanisms underlying how the expression of EZH2, MMP2 and TIMP2 is altered in RCC tissues or cells with high potential for bone metastasis.

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