RECK overexpression reduces invasive ability in ameloblastoma cells

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BACKGROUND: Ameloblastoma is a frequent odonto-genic neoplasm characterized by local invasiveness and high risk of recurrence. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is a tumor suppressor that inhibits metastasis and angiogenesis. The aim of this study was to investigate effects of RECK overexpression on invasive potential in ameloblastoma cells.

METHODS: Lentiviral vectors containing human RECK gene were created and subsequently stably transfected into immortalized ameloblastoma cell line hTERT⁺-AM. Functional characteristics of hTERT⁺-AM cells with stable RECK overexpression included proliferation, migration, invasion, and regulation of matrix metalloproteinases (MMP)-2, MMP-9 measured by zymography or commercially available assays.

RESULTS: The stable and higher expression of RECK mRNA and protein (P < 0.01) was detected in RECK-transfected hTERT⁺-AM cells. RECK overexpression caused a decrease in migration and invasion (P < 0.01) for hTERT⁺-AM cells and a decrease in activity of MMP-2, MMP-9 (P < 0.01). Proliferation was not affected by RECK overexpression (P > 0.05).

CONCLUSIONS: Overexpression of RECK gene significantly inhibited cell invasive ability of hTERT⁺-AM cells, suggesting RECK may be a new target for ameloblastoma treatment.


Keywords: ameloblastoma; invasion; matrix metalloproteinase; reversion-inducing cysteine-rich protein with Kazal motifs

Introduction

Ameloblastoma is a common benign odontogenic tumor, which is a borderline tumor with locally invasive, accounting for about 36% of all odontogenic tumors in China (1). It has been increasingly unable to accept patients, because of many times of recurrence and repeated surgical treatment to patients resulting in severe facial deformity and psychological trauma (2). Therefore, prevention and control of ameloblastoma invasion is a clinical problem to be resolved.

Matrix metalloproteinases (MMPs) are a family of zinc and calcium-dependent proteolytic enzymes, which play an important role in matrix degradation during the tumor growth and invasion. MMP-2, as an important member of MMPs, can degrade type IV collagen, one of the major components of the basement membrane, resulting in the promotion of tumor invasion and metastasis. Previously, we have found that siRNA targeting of MMP-2 mRNA using a plasmid-based system effectively inhibited the activity of MMP-2 in ameloblastoma cells, which subsequently resulted in reduced ameloblastoma cell invasiveness in vitro (3). In addition, our previous study have shown the inhibited MMP-2 activity results in reduced ameloblastoma cell in vitro adhesion to ECM and in vivo invasiveness (4). Thus, inhibition of MMP-2 activity can suppress the local invasiveness of ameloblastoma, and MMP-2 activity is in relation to the invasive capacity of ameloblastoma.

Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is a new MMP suppressor gene found in recent years (5). RECK can inhibit at least three kinds of MMPs (MMP-2, MMP-9, and MT1-MMP) in the post-transcriptional level (6). It is shown the membrane surface-anchored RECK inhibits the secretion of pro-MMP-2 protein. The anchored and soluble RECK protein may inhibit MMP-2, MMP-9, and MT1-MMP activity. RECK protein can inhibit the activation of pro-MMP-2, inhibiting the activity of MT1-MMP to suppress conversion process from pro-MMP-2 to MMP-2. Meanwhile, RECK may directly inhibit pro-MMP-2 activation in the last step. Thus, there is a strong role for RECK to inhibit MMP-2 activity.
Studies have shown that abnormal expression of RECK is common in malignant tumors such as liver cancer (7), cervical cancer (8), and other tumors, associated with tumor invasion and metastasis and prognosis (9). Both ameloblastoma and keratocystic odontogenic tumor (KCOT) are odontogenic tumors; nevertheless, ameloblastoma has significantly more invasive ability than KCOT (1). Meanwhile, we have demonstrated that the expression of RECK in ameloblastoma is significantly lower than in keratocystic odontogenic tumor (10). Our recent studies have shown that down-regulated RECK and up-regulated MMP-2 are associated with ameloblastoma biological behavior, and RECK may regulate MMP-2 in the post-transcriptional level to participate in ameloblastoma invasion, recurrence, and malignant (10).

Due to these findings, we investigated RECK function in ameloblastoma cells. The aim of this study was to investigate the functional effects of the regulation of RECK by stable transfection in ameloblastoma cell lines. An additional aim was to investigate to discuss the functions of RECK in maintaining the cellular proteolytic balance.

Materials and methods

Cell culture

Ameloblastoma is a benign tumor, and it is difficult to establish the relevant cell lines, so there are few available cell lines currently. The human immortalized ameloblastoma cell line hTERT+-AM was a gift from Dr. Qian Tao (Department of Oral and maxillofacial Surgery, Guanghua School and Hospital of Stomatology and Institute of Stomatological Research, Sun Yat-sen University, Guangzhou, China). The paper about establishing the cell line hTERT+-AM had been published (11). Cell cultures were maintained in α-MEM supplemented with 10% fetal bovine serum (FBS) and 200 μg/ml G418 (Gibco, Grand Island, NY, USA) in a humidified incubator of 5% CO2 at 37°C with 5% CO2. The study was approved by the Institute Research Ethics Committee at the Sun Yat-sen University.

Vector and infection

The lentiviral vector pLV.EX3d.P-puro-EF1A-RECK-IRES-eGFP (Lenti-RECK-eGFP/puro) containing full-length human RECK was produced by Cyagen Biosciences (Guangzhou, China) and validated by sequencing. HTERT+-AM cells were infected with lentiviral particles containing Lenti-RECK-eGFP/puro or Lenti-eGFP/puro. The infected cells were selected with puromycin and purified by picking colonies and viewed under a fluorescent microscope (Leica, Solms, Germany).

Quantitative real-time RT-PCR

Total RNA was isolated from transfected or untransfected cells with TRIzol reagent (Invitrogen, San Diego, CA, USA). RNA was quantified spectrophotometrically (NanoDrop 2000, Waltham, MA, USA). One microgram of total RNA was reverse transcribed to cDNA using the Primerscript RT reagent (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer’s protocol, and the resulting cDNA was diluted to 10-fold. Specific primers for human RECK: F: 5’-GCTGGCAATTTGGTGCTCTA-3’ and R: 5’-GGGTAAGTGCCCATTCGTG-3’, and GAPDH: F: 5’-GCACCCTCAAGGCTGAGAC-3’ and R: 5’-TGGTGAGACGCCAGTGGA-3’, were designed using Primer 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized by TaKaRa Inc. GAPDH was used to normalize the amount of total human cDNA. Reactions were carried out in a Roche Light Cycler 480 System (Roche, Mannheim, Germany) using a SYBR Premix ExTaq kit (TaKaRa) according to the manufacturer’s instructions. Data were calculated by the 2-ΔΔCT method (12) and presented as fold change compared with a reference (defined as 1.0-fold).

Western blot analysis

Cell protein fractions were prepared using RIPA buffer (Beyotime, Shanghai, China). Protein concentrations were determined by the BCA Protein Assay Kit (Beyotime). Equivalent amounts of protein (50 mg for MMPs blots and 40 mg for RECK blots) were resolved by SDS-PAGE (8–10%) and transferred to polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked for 1 h in 5% non-fat milk and probed overnight with the following primary antibodies: anti-RECK (rabbit monoclonal antibody) at 1:1000 from Cell Signaling Technology (CST, Danvers, MA, USA). Expression of GAPDH was used as a loading control and detected with anti-GAPDH from CST. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (1:3000; CST) served as secondary antibody. Bands were detected by enhanced chemiluminescence (ECL Western Blotting Substrate; Thermo Scientific, Wilmington, DE, USA) in a G:BOX Chemi XT4 system (Syngene, Cambridge, UK).

Immunocytochemistry

The cellular localization of exogenous RECK was confirmed using immunocytochemistry with anti-RECK primary antibody (CST) and an SABC-AP kit (Boster, Wuhan, Hubei, China) according to the manufacturer’s instructions. All of the images were photographed using an inverted microscope.

Cell viability

One thousand cells per well were seeded in a 96-well plate and grown in a humidified incubator of 5% CO2 at 37°C. Cell viability of fivefold samples was measured after 24, 72, and 120 h using the CCK-8 test (Cell Counting Kit-8; Dojindo, Kumamoto, Kyushu, Japan) according to the manufacturer’s instructions.

Cell migration and invasion assay

Cell motility was assessed using a wound healing assay (13). Briefly, transfected and untransfected cells were separately seeded in 6-well plates. After 24 h, cells were wounded by a 10-μl sterile pipette tip and washed using PBS to remove cellular debris and allowed to migrate in α-MEM without serum for 24 h. Wound closure and cell migration images were photographed using an inverted microscope. Results were expressed as a migration index—that is, the distance migrated by RECK-transfected relative
to the distance migrated by negative-control (or Blank) cells. Experiments were carried out in triplicate.

Cell invasion assay was measured using 24-well transwells (8-μm pore size; BD Sciences, Franklin Lakes, NJ, USA) coated with matrigel (1 mg/ml; BD Biosciences). Briefly, top chambers were filled with 1 × 10^5 cells per 200 μl α-MEM without serum, and bottom wells obtained were filled with 600 μl α-MEM containing 10% FBS as chemoattractant. After a 24-h incubation period in a humidified atmosphere of 5% CO₂ at 37°C, the non-invading cells were carefully removed using a cotton swab. Then, the cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet, imaged, and counted under an inverted microscope in six randomly selected fields. Experiments were carried out in triplicate.

Gelatin zymography

Equal amounts of total proteins extracts from transfected and untransfected cells were separated on 10% SDS-PAGE gels using a MMP Zymography Electrophoretic Analysis kit (Genmed Scientifics, Arlington, MA, USA) according to the manufacturer’s instructions. Clear zones of gelatin lysis against a blue background stain indicated the presence of MMPs. Each lysis zone within a given sample lane was analyzed using Image J software (National Institute of Health, Bethesda, MD, USA). All optical density measurements were made among samples on the same gel to ensure comparability.

Statistical analysis

Data are shown as mean ± standard deviation (SD). The difference between treatment and control cells was examined using the one-way ANOVA in measurement data and chi-square in enumeration data, and P values of < 0.05 were regarded as statistically significant. All statistical analyses were conducted using the SPSS software package (version 16.0; SPSS, Armonk, NY, USA).

Results

**RECK overexpression after RECK stable transfection in ameloblastoma cell line**

Previous study has shown that both RECK protein and mRNA expression of tissue were significantly lower in ameloblastoma than in KCOT (10). Given that RECK may participate in the invasion of ameloblastoma, we established a cell culture model using human ameloblastoma cells. HTERT+-AM cells were stably transfected with Lenti-RECK-eGFP/puro or the corresponding vector without insert. The expression of RECK protein and mRNA in transfected and untransfected cells was shown in Fig. 1. Immunocytochemistry detection confirmed that RECK was positive in the RECK-transfected cells, and the RECK was located in the cytoplasm and the cytomembrane (Fig. 2).

**Effect of RECK status on viability and invasiveness of ameloblastoma cells**

To characterize the effect of RECK overexpression in ameloblastoma cells, we detected the viability and invasiveness of hTERT+-AM cells. Firstly, CCK-8 test was performed to evaluate the effect of RECK on proliferation of hTERT+-AM cells. Our results showed that RECK overexpression did not obviously influence cell viability after 24, 72, and 120 h (Fig. 3). Next, wound healing and matrigel invasion assays were carried out to evaluate the effect of RECK on invasiveness of transfected and untransfected cells. Wound healing assays showed that up-regulation of RECK caused a suppression of cell migration in hTERT+-AM cells (Fig. 4). Furthermore, transfection of the RECK-expressing vector, but not the control vector, markedly inhibited hTERT+-AM cell invasiveness (Fig. 4). Taken together, our results suggest that RECK is a negative regulator for migration and invasion of ameloblastoma cells.

**RECK overexpression in ameloblastoma cells attenuates MMP-2 and MMP-9 activity**

We have previously shown that the protein and mRNA expression of MMP-2 were higher in ameloblastoma than in KCOT (10). This prompted us to analyze the effect of RECK on the activity of MMP-2 and MMP-9 in hTERT+-AM cells. MMP expression was rather weak so that gelatinolytic activity could be detected in bands around 72 kDa (pro-MMP-2) and 92 kDa (pro-MMP-9). Pro-MMP-2 was significantly reduced in RECK-transfected cells compared to vector-transfected cells or untransfected cells (Fig. 5). Besides, RECK-transfected cells exhibited lower MMP-9 activity than vector-transfected cells or control cells (Fig. 5).
Discussion

A lack of RECK expression has been noted in several malignant tumors, such as colorectal cancer (14–16), bladder carcinoma (17), gastric cancer (18), and middle-ear squamous cell carcinoma (19), and this lack correlates with a poorer prognosis (20–22). However, our previous study has shown that RECK is down-regulated not only in those cancers, but also in ameloblastoma, which is a benign odontogenic tumor (10). Hence, RECK as a tumor suppressor known to inhibit local invasion is of special interest in ameloblastoma research. Our discoveries of the reduction of local invasion in RECK overexpressing human ameloblastoma cells suggest RECK to play an important role in ameloblastoma tumorigenesis and progression. In this study, Western blot analysis shows that there are weak RECK bands of negative-control and untransfected cells, indicating

Figure 2  The cellular localization of RECK. RECK was only weakly expressed in the NC and Blank group. However, RECK was positive in the RECK-transfected cells, and RECK was located in the cytoplasm and the cytomembrane.

Figure 3  Cell viability after RECK overexpression. Cell viability measured by CCK-8 test after 24, 72, and 120 h did not show any difference between RECK-transfected and negative-control or blank control hTERT+-AM cells.

Figure 4  RECK overexpression represses cell migration and invasion in vitro. (A) Wound healing assays were performed on RECK-transfected, negative-control, and blank control hTERT+-AM cells. One representative experiment is shown. (B) Matrigel invasion assays showed that the cell invasion was significantly inhibited by RECK-transfected hTERT+-AM cells. A histogram shows the relative cell number of six randomly selected fields. All data are shown as the mean ± SD based on three independent experiments. **P < 0.01.
that there is a basal expression of RECK protein in hTERT+-AM cells. RECK mRNA and protein expression were significantly increased after transfection, indicating that RECK transcription and translation are effective in hTERT+-AM cells.

There was basically the same cell viability of our RECK-transfected, negative-control, and untransfected hTERT+-AM cells. This is in step with Rabien et al. (23) who found no effect on cell viability of exogenous human RECK expression in prostate cancer DU-1 45 cells. Nevertheless, Clark et al. (24) found the opposite result that RECK inhibits tumorigenic properties in osteosarcoma SaOS-2 cells. Therefore, further research is needed to prove whether the RECK affects tumor proliferation.

A growing number of studies have found that RECK can inhibit tumor invasion, for example, in cholangiocarcinomacells (25), in nasopharyngeal carcinoma cells (26), and in prostate cancer cells (23). In addition, RECK could inhibit invasion induced by histone deacetylase inhibitor in lung cancer cells (27). Our results of reduced local invasion by RECK in hTERT+-AM cells are the first implication for functional correlation of the tumor suppressor in ameloblastoma. These results once again confirm that RECK is an important factor in tumor formation and progression. Moreover, this is the first time to reveal that RECK may be an inhibitor of local invasion in benign tumor.

Matrix metalloproteinases are major proteolytic enzymes involved in tumor invasion (28). We previously identified that relative expression levels of MMP-2 mRNA were significantly higher in ameloblastoma than in dental sac (3). Besides, MMP-2 expression and activity could be decreased by tissue inhibitor of metalloproteinase-2 (TIMP-2) in an ameloblastoma xenograft model (29) and a primary cultures of ameloblastoma cells (30). The key action of RECK is to inhibit MMPs involved in breakdown of the extracellular matrix (ECM) (31). This study has demonstrated that the results of reduced pro-MMP-9 and reduced pro-MMP-2 in RECK-transfected hTERT+-AM cells are well in line with the literature. The MMP-2 and MMP-9 inhibited by RECK do not display a consistent inhibition across the different tumors studied (32). Our observations suggest that the decrease in MMPs activity in our model of RECK-modified ameloblastoma is responsible for changes in invasive behavior, as pro-/active forms of MMP-2 were higher than MMP-9 in hTERT+-AM cells and would let us assume a more important role for MMP-2 than for MMP-9 or other RECK targets. In view of this, our results disclose a proteolytic dysbalance in human ameloblastoma cells, which can be compensated by exogenous RECK expression. However, RECK-related signaling pathway mechanism in different tumor, especially the RECK upstream regulation, has not been fully elucidated. Some studies begin to expose RECK function in cell signaling (33).

In conclusion, we presume RECK as an important inhibitor could play an important suppression in ameloblastoma local invasion and may become the new targets for prevention and therapy of ameloblastoma. Further research will be an insight into the RECK-mediated mechanism to inhibit invasion of ameloblastoma.

References
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Liang et al.


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