Original Article
Up-regulation of E-cadherin by saRNA inhibits the migration and invasion of renal carcinoma cells

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Abstract: Previous studies have reported that double stranded RNAs (dsRNAs) have a potent ability to induce gene expression by targeting its promoter in cancer cells, which is called RNA activation (RNAa). In the present study, we have identified that a candidate dsRNA (dsEcad-215) could stimulate E-cadherin mRNA and protein expression via RNAa in renal cell carcinoma (RCC). Because the expression level of E-cadherin was down-regulated in RCC tissues compared to adjacent non-tumor tissues, dsEcad-215 was subsequently transfected into the RCC cell lines ACHN and 786-O. Expectedly, our results indicated that transfection of dsEcad-215 readily inhibited cell migration and invasion. In addition, several critical EMT-promoting genes (ZEB-1 and Vimentin) were down-regulated, while the anti-EMT gene β-catenin was up-regulated both at the mRNA and protein levels after dsEcad-215 transfection, suggesting that an enhanced E-cadherin level by dsEcad-215 suppressed EMT to inhibit cell motility. Collectively, our findings provide a potential effective therapeutic strategy for RCC, and dsEcad-215 might act as an alternative anti-cancer metastasis drug.

Keywords: saRNA, E-cadherin, renal cell carcinoma, RNA activation, metastasis

Introduction

Renal cell carcinoma (RCC) is one of the most common malignant tumors in the urinary system, and clear cell RCC represents the main histological type. In recent years, the incidence and the mortality rates of renal cell carcinoma have increased gradually, with an expected 66,800 newly-diagnosed cases and 23,400 deaths in China in 2015 [1]. Currently, nearly 40% of all RCC patients die from advanced stages with local or systematic metastasis [2]. Although the tumor can be completely removed by surgery, it commonly recurs during follow-up. As RCC is resistant to current treatment regimens, patients with metastatic RCC have poor prognoses [3]. Therefore, it is urgent to identify novel therapeutic targets, including non-coding RNAs (ncRNAs) in RCC, in order to develop more effective treatment options for this fatal disease.

Previous studies have reported that E-cadherin was low expressed in RCC [4]. Moreover, the deregulation of cell-to-cell adhesion molecules facilitates RCC invasion and metastasis [5]. However, as a vital cell adhesion molecule, E-cadherin maintains the integrity of epithelial cells. Reduced E-cadherin expression or mutation results in a functional loss of the cadherin complex, and leads to the metastasis of epithelial malignancies [6, 7]. Furthermore, the epithelial-to-mesenchymal transition (EMT) is a process that plays crucial roles in cancer metastases and invasiveness at an early stage [8, 9]. Thus, the reactivation of E-cadherin in RCC may contribute to RCC cell suppression.

As we know, RNA activation (RNAa) is a recently discovered method to up-regulate specific gene expressions [10, 11]. Because of its unique ability to enhance endogenous gene expression, RNAa may act as a potential novel therapeutic strategy for RCC. Inspiringly, it has been shown that a candidate small activating RNA (saRNA) (dsEcad-215) possesses a potent capacity to stimulate E-cadherin expression by targeting its promoter at sequence positions at -215 relative to the transcription start site (TSS) in prostate cancer and bladder cancer cells [10,
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However, whether it can up-regulate E-cadherin in RCC cell lines and then inhibit cell invasion and migration remains unclear.

In the present study, we first examined E-cadherin expression in RCC tissues, and then transfected dsEcad-215 into RCC cells to examine its effects in vitro. Our results showed that E-cadherin was decreased in RCC tissues. Furthermore, dsEcad-215 had a potent ability to induce E-cadherin expression by targeting its promoter region, and inhibiting RCC cells migration and invasion.

Materials and methods

Tissue samples

From August 2016 to November 2016, paired RCC and adjacent normal tissue specimens were collected from 15 renal cancer patients who were operated on in the Department of Urology in the Ruijin Hospital affiliated to the
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Medical School of Shanghai Jiaotong University (Shanghai, China). The samples were obtained from patients undergoing RCC resection. All cases were confirmed RCC by surgical pathology. Each sample for the experiment was immediately obtained from the operative specimen and frozen in liquid nitrogen and stored at -80°C until use. Following approval by the institutional ethics review board in Ruijin Hospital and after getting written informed consent from the patients, their specimens were obtained.

dsRNAs

In this study, all the RNA duplexes which possess 2-nucleotide 3' overhangs were chemically synthesized by RiboBio Co., Ltd. (Guangzhou, China). A small interfering RNA (siEcad) purchased from RiboBio (Guangzhou, China) was used to silence E-cadherin expression. We used a nonspecific control dsRNA (dsControl) which lacks significant homology to all known human sequences for a negative control [10, 13]. The sequences of all the custom RNAs are listed in Table 1.

Table 1. Sequences for dsRNAs used in present study

<table>
<thead>
<tr>
<th>RNAs</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsControl Sense</td>
<td>ACU ACU GAG UGA CAG UAG A [dT] [dT]</td>
</tr>
<tr>
<td>dsControl Antisense</td>
<td>UCU ACU GUC ACU CAG UAG U [dT] [dT]</td>
</tr>
<tr>
<td>dsEcad-215 Sense</td>
<td>AAC CGU GCA GGU CCC AUA A [dTdT]</td>
</tr>
<tr>
<td>dsEcad-215 Antisense</td>
<td>UUA UGG GAC CUG CAC GGU U [dTdT]</td>
</tr>
<tr>
<td>siEcad Sense</td>
<td>GGC CUG AAG UGA CUC GUA A [dT] [dT]</td>
</tr>
<tr>
<td>siEcad Antisense</td>
<td>UUA CGA GUC ACU UCA GCC C [dT] [dT]</td>
</tr>
</tbody>
</table>

Table 2. Sequences for real time quantitative PCR primers used in present study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (F)</td>
<td>TCCCATCACCATCTTCCA</td>
</tr>
<tr>
<td>GAPDH (R)</td>
<td>CATCACGCCACAGTTTCC</td>
</tr>
<tr>
<td>E-cadherin (F)</td>
<td>ACCGAGGAGGAAAGCCAGTTTCC</td>
</tr>
<tr>
<td>E-cadherin (R)</td>
<td>AGCGAGGAGGAAAGCCAGTTTCC</td>
</tr>
<tr>
<td>β-catenin (F)</td>
<td>GGAAGATGGGATCAAACTG</td>
</tr>
<tr>
<td>β-catenin (R)</td>
<td>TCCGCTCTGACCTGAGAA</td>
</tr>
<tr>
<td>ZEB1 (F)</td>
<td>ACTCTGATCTCAACGCC</td>
</tr>
<tr>
<td>ZEB1 (R)</td>
<td>TGTCACATGATAGGGCTT</td>
</tr>
<tr>
<td>Vimentin (F)</td>
<td>GATGCCTGAGATGGAGAAA</td>
</tr>
<tr>
<td>Vimentin (R)</td>
<td>GGCAGATTTAATATTGAGA</td>
</tr>
</tbody>
</table>

F, forward; R, reverse; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. CDK4/6, cyclin-dependent kinase 4/6.

RNA isolation and quantitative real-time PCR

By using TRIzol reagent (Invitrogen, Massachusetts, USA) according to the manufacturer’s instructions, total cellular RNA was extracted from the RCC cells. 500 ng RNA was reversely transcribed into cDNA according to the directions offered by the Takara reverse transcription kit (Takara, Liaoning, China). Then the cDNA was amplified by SYBR Premix Ex Taq II (Takara, Liaoning, China) conducted on the Mx3000P instrument (Stratagene, California, USA). All the primers included in this study were offered by Invitrogen (Shanghai, China) and listed in Table 2. The 2^{ΔΔCt} method was used to calculate the relative expression of the target genes’ mRNA; GAPDH was used as an internal control. All the experiments were repeated three times.

Protein extraction and Western blotting analysis

Using a RIPA lysis buffer supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland), all the cells were gathered, and the total proteins were extracted. After the protein concentrations were calculated, 50 µg protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. 5% bovine serum albumin (BSA) (Sigma-Aldrich, California, USA) was used to block the nonspecific binding for 2 h at room temperature. The membrane was then incubated with primary
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antibodies at 4°C overnight, which included GAPDH (1/500) (Boster, Wuhan, China), E-cadherin (1/1000) (BD Biosciences, New York, USA), β-catenin (1/500) (Boster, Wuhan, China), Vimentin (1/500) (Boster, Wuhan, China) and ZEB1 (1/1000) (Cell Signaling Technology, Boston, USA). After a few washes, the membranes were incubated with a relevant secondary antibody and tested using an enhanced chemiluminescence (ECL) assay kit (Millipore, Massachusetts, USA).

**Immunohistochemistry**

Paraffin-embedded tissue sections (5 μm-thick) were prepared, deparaffinized, and rehydrated. To quench endogenous peroxidase activity, the sections were incubated with 3% hydrogen peroxide for 10 min at room temperature. A 0.01M sodium citrate buffer (pH 6.0) was used to carry out antigen retrieval at 95°C for 45 minutes, and then incubated in 5% BSA in a phosphate-buffered saline (PBS) for 10 minutes. After this, the slides were incubated overnight with the primary anti-E-cadherin antibody (Cell Signaling Technology, Boston, USA) at a dilution of 1:300 at 4°C. The next day, the sections were incubated with the secondary antibody for 20 minutes at room temperature and coated using an Envision Dual Labeled Polymer kit (BioGenex, San Ramon, CA) according to the manufacturer’s directions. Haematoxylin was used for nuclear staining.

**Wound healing assay**

The cells were trypsinized and counted after 72 hours’ transfection. The cells were then reseeded in a new 6-well plate. The monolayer cells were scratched manually with a plastic pipette tip, and after two washes with PBS, the wounded cellular monolayer was allowed to heal for 24 h in a serum-free medium. Photographs of the central wound edges per condition were taken at 0 h, 12 h and 24 h after they were scratched using the inverted microscope (Olympus, Tokyo, Japan). Three random non-overlapping areas in each well were pictured.

**Migration and Invasion assay**

Cell migration and invasion was evaluated using a transwell assay. For the cell migration assay, 24 h after transfection, cells at a concentration of 2 × 10^4 were seeded in the top chamber with the non-coated membrane tran-

The statistical analyses were performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). Student’s t-test was used to analyze variables and all the data were presented as the mean ± standard deviation (SD) for these experiments. A P-value < 0.05 was considered to be statistically significant. All experiments were repeated three times.

**Results**

**E-cadherin expression was reduced in RCC tissues compared with normal tissues**

To identify the expression patterns of E-cadherin in clinical RCC tissues, quantitative real-time PCR was performed to assess its level compared with adjacent non-tumor tissues. Results showed that expression level of E-cadherin in tumorous tissues was significantly lower than the corresponding normal tissues (Figure 1A). Immunohistochemical and Western blot analysis (Figure 1B and 1C) further confirmed these results. So, E-cadherin may act as a tumor suppressor in RCC.

**dsEcad-215 activated E-cadherin expression by targeting promoter**

According to the previous study, we speculated that this dsEcad-215 may also activate E-cadherin expression in human RCC cells. Therefore, we transfected synthetic dsEcad-215 into ACHN and 786-O cells, and measured the E-cadherin expression 72 h later. Compared with the mock and dsControl groups, dsEcad-215 caused a significant increase in E-cadherin mRNA (Figure 2B and 2C). This
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induction was further verified by protein analysis (Figure 2D and 2E).

dsEcad-215 inhibited RCC cells migration and invasion depended on the up-regulation of E-cadherin expression

Next, we examined the potential roles of dsEcad-215 on the migration and invasion capacities of RCC cells. The transfection of dsEcad-215 led to retarded wound closing compared with the control groups from 12 h in both ACHN and 786-O cells (Figure 3A and 3B). Then the wound widths of each group were measured and normalized to a corresponding mock baseline. After the ACHN and 786-O cells were scratched at 12 h, the relative distances between the wound edges in the dsEcad-215 groups were significantly shorter than the matched groups (Figure 3C and 3D). Additionally, wound healing in the group co-transfected with siEcad and dsEcad-215 was faster within 24 h compared to the group transfected with dsEcad-215 alone (Figure 3A and 3B). Further quantitative analysis of wound width manifested relative scratch widths in the co-treatment group were remarkably less than the dsEcad-215 transfection groups from 12 h (Figure 3C and 3D).

Next, the transwell assay was conducted to further assess the cells’ migration and invasion abilities. Compared with the mock and dsControl groups, dsEcad-215 had a potent inhibiting effect on the migration of the both ACHN and 786-O cells (Figure 4A). Likewise, the invasion capability of RCC cells transfected with dsEcad-215 was measured using a matrigel invasion chamber assay. As we expected, dsEcad-215 could significantly inhibit their invasion ability compared to the matched groups (Figure 4C). At the same time, the depletion of E-cadherin remarkably restored the cells’ migration capacity in response to ds-
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Ecad-215 treatment (Figure 4A and 4B). Consistent with that, more cells in co-transfection group (dsEcad-215+siEcad) migrated the membranes pre-coated with matrigel compared to the dsEcad-215 group (Figure 4C and 4D). Together, these findings strongly implied that dsEcad-215-inhibited RCC cell migration and invasion was totally dependent on the up-regulation of E-cadherin.

dsEcad-215 regulated EMT-associated genes mainly by enforcing E-cadherin expression

We then assessed the effects of dsEcad-215 transfection on the expression of some essential genes associated with the EMT process in RCC cells. Compared to the mock and dsControl groups, dsEcad-215 significantly up-regulated β-catenin expression, and down-regulated ZEB1 and Vimentin expressions in both ACHN and 786-O cells at the transcriptional level, respectively (Figure 5A and 5B). Moreover, Western blot analysis also revealed a potent increase in β-catenin, and a decrease in ZEB1 and Vimentin protein levels of the two kind cells after the dsEcad-215 transfection (Figure 5C). These data indicated that following the up-regulation of E-cadherin, dsEcad-215 could also affect the EMT-associated gene expression of RCC cells.

Next, to investigate whether E-cadherin was mainly responsible for tumor suppression after dsEcad-215 transfection, we used RNA interference technology to knockout the expression of E-cadherin in the RCC cells (Figure 2B-E). Compared to the dsEcad-215 group, the mRNA of β-catenin was down-regulated, whereas ZEB1 and Vimentin mRNA were up-regulated after the co-treatment of siEcad for 72 h in both cell lines (Figure 5A and 5B). Additionally, an immunoblot analysis further proved those findings (Figure 5C). Taken together, these data confirmed that dsEcad-215 manipulated EMT-associated genes largely via stimulating E-cadherin expression.

Discussion

Gene activation mediated by small ncRNAs, called RNAa, is a promising discovery, which has been identified in many cancer cell lines [10, 14, 15]. As a potently positive gene regulation approach, it can reactivate a functional
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silenced or mutant tumor suppressor in cancer patients [16]. With a greater understanding of RNAa’s molecular mechanisms, RNAa-based research tools and therapeutic strategies have been applied [17, 18].

In the present study, we initially found that the anti-oncogene E-cadherin was decreased in human RCC tissues compared with paired normal renal tissues. Subsequently, we found that dsEcad-215, a synthetic dsRNA, could induce E-cadherin expression by interacting with a promoter in human RCC ACHN and 786-O cells. Moreover, cell migration and invasion abilities were inhibited after the transfection of dsEcad-215. By this mechanism, several critical EMT-promoting genes (ZEB-1 and Vimentin) were down-regulated, while the anti-EMT genes β-catenin was up-regulated after dsEcad-215 treatments. Most importantly, a significant difference of function and protein levels were reversed after co-transfection of siEcad, indicating that dsEcad-215’s antitumor effects were dependent on the up-regulation of E-cadherin expression.

The most common characteristic of EMT is that the tumor cells undergo morphological changes and gain migratory and invasive properties [19]. Here, the transfection of dsEcad-215 could suppress EMT via increasing the expression level of E-cadherin and β-catenin, as well as decreasing ZEB-1 and Vimentin. The complex of E-cadherin/β-catenin can regulate cell adhesion [20]. Loss function of the complex led to reduced cell adhesion and elevated tumor metastasis [21]. As we know, the ZEB-1 is a transcriptional repressor of E-cadherin [22] and acts as a crucial contributor to EMT [23]. But the exact relationship among dsEcad-215, E-cadherin and ZEB-1 remains unknown. As a type III intermediate filament protein, vimentin...
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is mainly found in mesenchymal cells and migratory epithelial cells [24]. The overexpression of vimentin thought to be a typical phenotype for EMT, and metastasis was seen in RCC [25]. Based on our study, dsEcad-215 could profoundly inhibit the EMT process, helping to retard tumor progression.

At present, cancer metastases frequently occur in RCC patients, and the treatment of RCC has become increasingly difficult. It has been reported that reduced expression of E-cadherin may be an important biological marker for the invasion and metastasis of RCC [26]. The E-cadherin down-regulation finally results in the tumor cells breaking away from the original site and the subsequent occurrence of tumor invasion and metastasis [27]. So, we believe that reactivation of E-cadherin by RNAa would undoubtedly help to suppress RCC metastasis.

Taken together, this research demonstrated that the activation of E-cadherin by a promoter-targeted E-cadherin saRNA significantly inhibited RCC cell migration and invasion. Our study showed that targeted activation of E-cadherin is a promising therapeutic method for human RCC and should be paid more attention in a preclinical setting.

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Disclosure of conflict of interest

None.

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