Original Article
Concurrent alterations of RAGE, RECK, and MMP9 protein expression are relevant to Epstein-Barr virus infection, metastasis, and survival in nasopharyngeal carcinoma

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Abstract: This study aimed to concurrently investigate the expressions of receptor for advanced glycation end products (RAGE), reversion-inducing cysteine-rich protein with Kazal motifs (RECK) and matrix metalloproteinase 9 (MMP9) in nasopharyngeal carcinoma (NPC) and their correlations with clinicopathological properties. Using immunohistochemistry, we found that RECK expression was downregulated in NPC tissues compared with chronic nasopharyngitis (CNT) tissues, while RAGE and MMP9 expressions were upregulated. We further found that RECK expression level was inversely correlated with MMP9 expression level in NPC, whereas RAGE expression level was positively correlated with MMP9 expression level. Moreover, aberrant expressions of these proteins had a positive correlation with the titers of EBVCA-IgA, lymphatic metastasis, recurrence and survival. Together, these findings suggest that dysregulations of RECK and RAGE expressions may be collectively involved in tumor progression of NPC by regulating MMP9 expression and that they may be a good prognostic predictors for NPC.

Keywords: Nasopharyngeal carcinoma, RAGE, RECK, MMP9

Introduction
Nasopharyngeal carcinoma (NPC) is endemic in Southeast Asia and shows strongly aggressive and metastatic biological properties. NPC has a higher incidence of invasion and metastasis at the time of diagnosis compared with other head and neck cancers. Recurrence and metastasis are the most common causes of treatment failure and mortality, and they are main unfavorable prognostic factors in patient with malignancy including NPC [1].

The formation of tumor invasion and metastasis is a multigene and multistep process with the participation of various metastasis-related genes. Degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is a crucial mechanism in tumor invasion and metastasis. Studies have demonstrated that MMPs are the common and essential target effectors for many oncogenes and tumor suppressor genes facilitating tumor invasion and metastasis [2]. MMP9, a key member of the MMPs, plays a vital role in cancer metastasis process. Previous studies have shown that MMP9 overexpression was correlated with reduction in survival and induction of tumor invasion and metastasis in NPC [3, 4]. The reversion-inducing cysteine-rich protein with Kazal motifs (RECK), a negative regulator of MMPs, can inhibit tumor invasion and metastasis as a metastasis suppressor gene [5]. It has been reported that RECK is significantly downregulated and closely relate to invasion, metastasis and prognosis in many cancers [5-8]. The receptor for advanced glycation end-products (RAGE) is a transmembrane receptor of the immunoglobulin superfamily and abnormally expressed in many malignant tumors [9, 10].
Taguchi et al. [11] reported that RAGE was closely linked to tumor growth and metastasis through the induction of MMP9 and MMP2 activity in glioma cell. To date, few studies have been reported concerning the relationship between RECK or RAGE and NPC [12, 13], including our previous study with in situ hybridization [14]. However, to our knowledge, no reports have looked for the simultaneously aberrant expressions of RECK, RAGE and MMP9 in primary NPC tissues and their potential roles in clinical evaluation of NPC.

Based on these findings, we raise a new view on the mechanism of the high metastatic property of NPC: RECK and RAGE might contribute to tumor progression and metastasis, as well as carcinogenesis, of NPC by the induction of MMP9. To confirm and elucidate our view on metastasis, we investigated synchronously the expressions of RECK, RAGE and MMP9 by immunohistochemical analysis in the specimens of NPC patients and clarified the relationship between them. In addition, we explored correlations of these expressions with clinical characteristics including patient outcome.

### Materials and methods

#### Patients and tissue samples

A total of 90 pathological specimens were selected from January 2004 to January 2006 at Zhongshan Hospital of Xiamen University (Xiamen, China), including 60 cases of NPC tissues and 30 cases of chronic nasopharyngitis (CNT) tissues. All specimens were confirmed by histopathological examination. No patients had received radiotherapy and chemotherapy before biopsy. Sixty patients with NPC encompassed 30 cases with cervical lymph node metastasis and 30 cases without cervical metastasis.
lymph node metastasis according to the results of ultrasonic guided fine needle aspiration cytology and radiology (CT, MRI or PET scan). They comprised 45 men and 15 women with age from 17 to 76 years (median, 45.8 years). The tumor-node-metastasis (TNM) classification was defined according to the World Health Organization (WHO) 2005 NPC staging system [15]. Patients with NPC underwent radical radiotherapy and concurrent chemotherapy after histopathological diagnosis and were followed up. The end of follow-up periods of this study was defined as 9 October 2013. The clinicopathological data of NPC patients are shown in Table 1. In addition, specimens of CNT were obtained from 30 healthy persons suspected of having cancer at above hospital. These cases included 17 men and 13 women with age range between 20 and 63 years (mean age 40.6 years). Prior consents of the patients and ethical approval for the study from the Medical Ethics Committee of above hospital were obtained.

**Antibodies and immunoreagents**

Mouse anti-human RECK monoclonal antibody, goat anti-human RAGE polyclonal antibody and secondary antibody kits for RAGE were purchased from R & D Systems, Inc. (Minneapolis, MN, USA). Mouse anti-human MMP9 monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibody kits for RECK and MMP9 was purchased from Fuzhou New Biological Technology Development Co., Ltd. (Fuzhou, China). Primary antibodies against RECK and MMP9 were diluted to 1:200; Primary antibody against RAGE was diluted to 1:20. Negative controls were performed using an isotype IgG instead of the primary antibody. A known positive slide (colon carcinoma specimen) was used as a positive control.

**Immunohistochemistry**

**RECK and MMP9 expression (Elivision method):** Serial paraffin sections (4 μm-thick) were deparaffinized and rehydrated. After washing in water (2 × 3 min each), high temperature and high pressure antigen retrieval for MMP9 was performed using 10 mM citrate buffer (PH 6.0) in a pressure cooker for 2 min at 100°C, while antigen retrieval for RECK was performed in 1 mM EDTA (PH 9.0). After blocking of endogenous peroxidase activity and washing in water (3 × 3 min each), sections were incubated overnight at 4°C with primary antibody against RECK or MMP9. Followed by washing in PBS (3 × 3 min each), sections were incubated for 20 min at room temperature with DAB enhancer. Followed by washing in PBS (3 × 3 min each), sections were incubated with secondary antibody for 30 min at room temperature and then washed in PBS (3 × 3 min each). Thereafter, the sections were visualized with DAB, briefly washed in water, counterstained with hematoxylin-eosin, returned blue using lithium carbonate, dehydrated in ethanol, mounted with resinene, and subsequently analyzed using a bright field microscope.

**RAGE expression (R & D reagent kit):** Before incubating with primary antibody, the steps were the same as above except that antigen retrieval for RAGE was performed in 10 mM citrate buffer (PH 6.0). The following steps were as follows: the slides were added with non-immune serum of the same animal source, incubated for 15 min at room temperature, drained the excess serum, added with avidin blocking reagent for 15 min at room temperature, rinsed with PBS for 5 min, drained the slides, and then added with biotin blocking reagent for 15 min at room temperature. Followed by rinsing with PBS for 5 min, slides were incubated overnight at 4°C with primary antibody against RAGE. Followed by rinsing with PBS (3 × 15 min each), slides were incubated with secondary antibody for 60 min at room temperature. After rinsing in PBS (3 × 15 min each), sections were incubated with HSS-HRP for 30 min at room temperature. After three 2-min washes in PBS, the subsequent steps were described as same as above Elivision method.

**Immunohistochemical analysis**

The staining was evaluated independently by two experienced pathologist (D-N Zhou and P Yin) using double-blind method. Each section was observed randomly in five non-overlapping fields (more than 200 tumor cells per field) with high-power microscopy. Immunoreactivity was assessed in a semiquantitative method based on the intensity and proportion of stained cells. The intensity score was as follows: 0, no staining or yellowish staining; 1, yellow staining; 2, brown staining. The proportion score was as follows: 0, < 10%; 1, 10-50%; 2, > 50%. The

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**Table 1**

<table>
<thead>
<tr>
<th><strong>Stage</strong></th>
<th><strong>Number of Patients</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>5</td>
</tr>
<tr>
<td>T2</td>
<td>15</td>
</tr>
<tr>
<td>T3</td>
<td>20</td>
</tr>
<tr>
<td>T4</td>
<td>2</td>
</tr>
</tbody>
</table>

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Table 2. Expression of RECK, RAGE and MMP9 protein in different tissues

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cases</th>
<th>RECK N (%)</th>
<th>P</th>
<th>RAGE N (%)</th>
<th>P</th>
<th>MMP9 N (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT</td>
<td>30</td>
<td>20 (66.7)</td>
<td></td>
<td>13 (43.3)</td>
<td></td>
<td>14 (46.7)</td>
<td></td>
</tr>
<tr>
<td>NPC</td>
<td>60</td>
<td>16 (26.7)</td>
<td>0.000*</td>
<td>43 (71.7)</td>
<td>0.009*</td>
<td>47 (78.3)</td>
<td>0.002*</td>
</tr>
</tbody>
</table>

N, number of positive expression; Asterisk denotes significant P value (compared with CNT).

The sum of intensity and proportion scores was used as the final scores for RECK, RAGE and MMP9 immunoreactivity. For statistical analysis, final staining scores of 0-1 and 2-4 were respectively classified as negative and positive expression.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The relationship between the levels of RECK, RAGE and MMP9 expression and clinicopathological characteristics was analyzed using the Chi-square or Fisher's exact test. The correlation between variables was analyzed using Spearman's rank correlation. Overall survival (OS) and disease-free survival (DFS) curves were calculated using the Kaplan-Meier method and compared by log-rank test. Multivariate survival analysis with Cox's proportional hazard regression model was used to evaluate the independent prognostic factors. Statistical significance was defined by a P < 0.05.

Results

Expression of RECK, RAGE and MMP9 in NPC and CNT

Representative examples of RECK, RAGE and MMP9 staining in NPC and CNT were shown in Figure 1A-H. RECK staining was mainly localized in cytoplasm and part of stroma of tumor cells (Figure 1C and 1D); RAGE staining in cytoplasm and nucleus (Figure 1E and 1F); MMP9 staining in cytomembrane and cytoplasm (Figure 1G and 1H). The percentages of NPC samples with positive expression of RECK, RAGE and MMP9 were 26.7% (16/60), 71.7% (43/60) and 78.3% (47/60), respectively. The percentages of CNT samples with positive expression of RECK, RAGE and MMP9 were 66.7% (20/30), 43.3% (13/30) and 46.7% (14/30), respectively. The expression level of RECK in NPC was significantly downregulated compared with CNT (P < 0.05), whereas the expression levels of RAGE and MMP9 in NPC were significantly upregulated compared with CNT (P < 0.05, Table 2).

Relationships of RECK, RAGE or MMP9 expression with clinicopathological parameters of NPC

The relationships between clinicopathological parameters and RECK, RAGE or MMP9 expression levels in NPC patients were summarized in Table 1. Decreased RECK expression and increased RAGE and MMP9 expressions had no correlation with gender, age, T stage and clinical stage (P > 0.05), whereas they had a significant correlation with the titers of EBVCA-IgA, cervical lymph node metastasis, tumor recurrence and the post-treatment survival time of NPC patients (P < 0.05). The percentages of positive RAGE and MMP9 expression in group with cervical lymph node metastasis, tumor recurrence and shorter survival time were significantly higher than that in group without cervical lymph node metastasis, tumor recurrence and with prolonged survival time. Nevertheless, the situation of RECK is contrary.

Correlation between RECK, RAGE and MMP9 expression in NPC

The correlation of RECK, RAGE and MMP9 expression in all NPC patients was assessed by Spearman correlation analysis (Table 3A and 3B). There was a significant correlation of RECK and RAGE expression with MMP9 expression. RECK expression was negatively correlated...
with MMP9 expression ($R = -0.415, P = 0.001$), while RAGE expression was positively correlated with MMP9 expression ($R = 0.388, P = 0.002$). However, no significant correlation between RECK expression and RAGE expression was seen ($R = -0.123, P = 0.350$).

**Relevance of RECK, RAGE or MMP9 expression with NPC patient’s survival**

We examined the post-treatment OS and DFS of 60 NPC patients according to the expression levels of RECK, RAGE and MMP9. Figure 2A and 2B show the survival curves of the patients according to positive or negative RECK expression. Patients with RECK-positive tumours exhibited higher OS and DFS than patients with RECK-negative tumors ($P = 0.018; P = 0.016$). Figure 2C-F show the survival curves of the patients according to positive or negative RAGE and MMP9 expression. Patients with RAGE-positive and MMP9-positive tumors exhibited lower OS and DFS than patients with RAGE-negative and MMP9-negative tumors ($P = 0.011; P = 0.007; P = 0.008; P = 0.013$). We next performed multivariate analysis including age, gender, T stage, nodal metastasis, clinical stage, the titers of EBVCA-IgA, recurrence, RECK expression, RAGE expression, and MMP9 expression for OS and DFS. The results revealed that RECK, RAGE and MMP9 expression levels and nodal status were independent prognostic factors for NPC (Table 4).

**Discussion**

RECK gene is located on chromosome 9p13-p12. RECK is expressed in a variety of normal human tissues and non-neoplastic cell lines, whereas its expression is diminished or absent in malignant tissues and malignant cell lines. The restoration of RECK expression in tumor cell lines resulted in the suppression of proteolytic activity of MMP9 and further decreased the ability of invasion and metastasis [6, 16]. The RECK gene has been shown to be an important factor affecting tumor invasion, metastasis, and angiogenesis in many human cancers, and it has acted as an independent prognostic indicator for cancer patients [6, 17, 18].

To date, few studies have been reported concerning the relationship between RECK and NPC. Liu et al [12] found that Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) induced downregulation of RECK expression in NPC cell line, which was a critical step for LMP1-induced tumor metastasis. Similarly, our present study detected downregulation of RECK expression was correlated with higher titers of EBVCA-IgA. Our previous research found that aberrant expression of RECK mRNA was association with metastasis, recurrence, and prognosis of NPC patients [14]. In this report, we revealed that RECK protein expression level was significantly downregulated in primary NPC samples compared to CNT samples, suggesting that RECK might be relevant to the development of NPC. We also revealed that the downregulated expression of RECK was closely linked to cervical lymph node metastasis in NPC, indicating that RECK might play an important role in lymphatic metastasis of NPC as a metastasis suppressor. This investigation was the first to discover that RECK protein expression was connected with the clinical outcome of NPC. NPC patients with tumor recurrence and post-treatment survival time below five years had a lower level of RECK positive expression, indicating that RECK protein could serve as a good prognostic marker for NPC.

RAGE gene is located on chromosome 6p21.3. RAGE is a transmembrane receptor of the immunoglobulin superfamily, which is expre-
RAGE, RECK, and MMP9 in nasopharyngeal cancer

This study first determined the potential influence of altered expressions of RECK, RAGE and MMP9 proteins on the survival rate of NPC. RECK expression level in NPC was inversely correlated with patient's OS and DFS; RAGE and MMP9 expression levels in NPC were consistently correlated with patient’s OS and DFS. Our findings present the evidence that decreased expression of RECK and increased expressions of RAGE and MMP9 may predict a higher risk of metastasis, recurrence and shortened survival in NPC.

Until now, very few studies have been reported concerning the relationship between RAGE and NPC. Tsuji et al [13] found that EBV LMP1-induced RAGE expression enhanced lymph node metastasis through the induction of angiogenesis in NPC. Similarly, our present study found upregulation of RECK expression was correlated with higher titers of EBVCA-IgA. In this report, we revealed that RAGE protein expression level was significantly upregulated in primary NPC samples compared to CNT samples, suggesting that RAGE might be connected with the development of NPC. We also revealed that the upregulated expression of RAGE was closely linked to cervical lymph node metastasis in NPC, indicating that RAGE might play an important role in lymphatic metastasis of NPC as a metastasis promoter. This investigation was the first to discover that RAGE protein expression was connected with the clinical outcome of NPC. NPC patients with tumor recurrence and post-treatment survival time below five years had a higher level of RAGE positive expression, indicating that RECK protein could act as a useful prognostic marker for NPC.

This study first determined the potential influence of altered expressions of RECK, RAGE and MMP9 proteins on the survival rate of NPC. RECK expression level in NPC was inversely correlated with patient’s OS and DFS; RAGE and MMP9 expression levels in NPC were consistently correlated with patient’s OS and DFS. Our findings present the evidence that decreased expression of RECK and increased expressions of RAGE and MMP9 may predict a higher risk of metastasis, recurrence and shortened survival in NPC.

Table 4. Multivariate survival analysis of prognostic factors in patients with NPC

<table>
<thead>
<tr>
<th>Variables</th>
<th>Overall survival</th>
<th></th>
<th></th>
<th>Disease-free survival</th>
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<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>P</td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Nodal metastasis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Yes vs. No</td>
<td>2.039</td>
<td>1.247-3.334</td>
<td>0.005</td>
<td>2.478</td>
<td>1.339-4.584</td>
</tr>
<tr>
<td>RECK expression</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Positive vs. Negative</td>
<td>1.843</td>
<td>1.068-3.179</td>
<td>0.028</td>
<td>4.889</td>
<td>0.059-0.843</td>
</tr>
<tr>
<td>RAGE expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Positive vs. Negative</td>
<td>6.081</td>
<td>1.832-20.180</td>
<td>0.003</td>
<td>5.735</td>
<td>1.922-17.141</td>
</tr>
<tr>
<td>MMP9 expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive vs. Negative</td>
<td>2.450</td>
<td>1.379-4.354</td>
<td>0.002</td>
<td>2.708</td>
<td>1.475-4.971</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval.
the activity of MMP9 and affects invasive and metastatic ability of human cancer cells [26-28]. Combined with the above results, we conjecture that RECK and RAGE have a certain interaction with MMP9 in the process of NPC invasion and metastasis.

In the current work, we simultaneously analyzed the role and correlation of RECK, RAGE and MMP9 protein expression in NPC subjects. These findings indicate that aberrant expressions of RAGE, RECK and MMP9 are concurrently related with EBV infection, metastasis and poor prognosis of NPC. Taken together, we prompt that RECK and RAGE gene are involved in tumor invasion and metastasis of NPC, and MMP9 is the final target effector. Alterations of RECK and RAGE expression collectively upregulate the expression level of MMP9, and then enhance the hydrolytic ability of MMP9, thereby facilitate tumor cells invasion and metastasis. Further studies will focus on the mechanism of their interaction.

In conclusion, we have identified that coexistent dysregulations of RAGE, RECK and MMP9 in NPC tissues correlate to EBV infection, metastasis, recurrence and poor survival of NPC patients. MMP9 may be the co-regulatory target of RAGE and RECK gene when they attribute to tumor progression and metastasis development of NPC.

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Disclosure of conflict of interest

None.

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