Original Article
Co-expression of RAGE and HMGB1 is associated with cancer progression and poor patient outcome of prostate cancer

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Abstract: Receptor for advanced glycation end products (RAGE), along with its ligand high mobility group box 1 (HMGB1), is believed to play an important role in prostate cancer. The aim of this retrospective study was to investigate the expression of RAGE and HMGB1 and their clinical impact on prostate cancer progression and prognosis. The expression of RAGE and HMGB1 was assessed by immunohistochemistry in cancer lesions from 85 confirmed prostate cancer cases. We determined the potential association between the expression level of these two proteins and the clinicopathological features and overall patient survival. RAGE and HMGB1 were expressed in 78.8% (67/85) and 68.2% (58/85) cases of prostate cancer, respectively, and in the majority (54/85) of cases, these two proteins were co-expressed. There was a strong correlation between RAGE and HMGB1 expressions (P<0.001). The expression of RAGE, HMGB1 and their co-expression were all associated with advanced tumor clinical stage (P<0.05 for all). RAGE expression was also associated with the prostate specific antigen (PSA) level (P=0.014). However, neither the individual expression of those genes nor their co-expression was significantly related with age or Gleason score. The co-expression of RAGE and HMGB1 was associated with poor overall survival in patients with stage III and IV prostate cancer (P=0.047). These results suggest that the expression of RAGE and HMGB1 is associated with the progression and poor prognosis of prostate cancer. RAGE and HMGB1 could be new prognostic biomarkers for prostate cancer as well as molecular target for novel forms of therapies.

Keywords: RAGE, HMGB1, prostate cancer, progression, prognosis

Introduction
Prostate cancer is one of the most common cancers in males, especially in aged males. In 2008, prostate cancer was the second most diagnosed cancer and the sixth leading cause of cancer death among males, accounting for 14% of the total new cancer cases and 6% of the death caused by cancer [1]. In China, with the growing average life expectancy, the western life style and diet, and the improved detection rate, the incidence of prostate cancer has shown a steep increase in the last decade [2, 3].

Prostate cancer has a natural course that is different from many other human tumors. Most early-stage prostate cancers are latent and only approximately 25% of them will become aggressive and life-threatening [4]. However, currently, it is difficult to predict the progression of early stage cancers [4-7], which will have a great impact on therapeutic decisions.

It has been reported that the role of RAGE (receptor for advanced glycation end products) and its ligands play a role in the development and progression of cancer [8-10]. RAGE is a cell surface molecule and multi-ligands receptor, belonging to the immunoglobulin superfamily. It has several ligands, including HMGB1 (high mobility group box 1), several members of the calcium-binding S100 family of proteins, some species of AGEs, and β-sheet fibrillar material.
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such as amyloid-β, serum amyloid A, immunoglobulin light chains, transthyretin, and prions, among others [8, 11]. RAGE was first reported in 1992 [12] and has been suggested to be involved in several diseases, including diabetes, cancers, inflammation, cardiovascular disease, chronic kidney disease, and Alzheimer’s disease [13]. Abnormal expression of RAGE and its ligands has been reported in a number of cancers, including prostatic, colorectal, pancreatic, lung, oral squamous cell cancers and these molecules may be involved in cancer invasion and metastasis [8-10, 14, 15]. HMGB1, also known as amphoterin, is an abundant non-histone component of chromatin [16, 17] and can be released into the extracellular to bind to RAGE [8]. The interaction between RAGE and HMGB1 triggers the activation of key cell signaling pathways, such as NF-κB, p38, p44/42 MAPKs, resulting in the cancer progression and metastasis [8-10, 18-24].

Overexpression of RAGE and HMGB1 have been observed in prostate cancer [14, 15, 25-29]. Ishiguro et al. found that untreated primary prostate cancer tissue and hormone-refractory prostate cancer tissue showed significantly higher RAGE and HMGB1 mRNA expression than normal prostate tissues. In addition, they detected RAGE and HMGB1 mRNA expression in all three commonly used prostate cancer cell lines, DU145, PC-3 and LNCaP, with DU145 the highest of the three lines [14]. Kuniyasu et al. described that RAGE production was enhanced in metastatic compared to non-metastatic prostate cancer, where it was co-expressed with HMGB1 [15]. We also reported RAGE overexpression at both RNA and protein level in Chinese prostate cancer samples [29]. In this retrospective study, we analyzed the expression of RAGE and HMGB1 in a larger series of Chinese prostate cancer samples using immunohistochemistry (IHC) and explored its relationship with the clinicopathologic parameters and overall survival. We found that the expression of RAGE and HMGB1 was associated with tumor progression and poor prognosis of prostate cancer.

Materials and methods

Samples

A total of eighty-five cases of prostate cancer, including forty-two radical prostatectomy and forty-three needle biopsy samples, were included in this study. Hematoxylin and eosin stained slides from all cases were reviewed to confirm the diagnosis of prostate cancer by two independent pathologists (TZ and XZ). Cases received pre-operative hormone therapy, chemotherapy and radiotherapy, or with a diagnosis of diabetes, chronic kidney, cardiovascular and Alzheimer’s disease as well as other cancers had been excluded. Clinical information about the cases was described in Table 1. TNM staging was made before any treatment according to American Joint Committee on Cancer (AJCC), 7th edition TNM-system. Prostate specific antigen (PSA) was assessed before biopsy and any treatment. Thirty histologically benign prostatic hyperplasia (BPH) diagnosed in the same period were randomly chosen as controls. This study was approved by Medical Ethics Committee of Nanfang hospital, and the tissue samples were used with the informed written consent from the patients or their family members.

Immunohistochemical staining and scoring

Consecutive 4 μm sections were cut from formalin-fixed paraffin-embedded tissue blocks. Before IHC staining, histological features in hematoxylin and eosin stained slides were reviewed by pathologists. Sections were immu-

Table 1. The demography of cancer cases

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>10 (11.8)</td>
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<tr>
<td>≥60</td>
<td>75 (88.2)</td>
</tr>
<tr>
<td>PSA (ng/ml)</td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>22 (25.9)</td>
</tr>
<tr>
<td>≥20</td>
<td>63 (74.1)</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
</tr>
<tr>
<td>&lt;7</td>
<td>28 (32.9)</td>
</tr>
<tr>
<td>≥7</td>
<td>35 (41.2)</td>
</tr>
<tr>
<td>&gt;7</td>
<td>22 (25.9)</td>
</tr>
<tr>
<td>T stage</td>
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<tr>
<td>T1-T2</td>
<td>36 (42.4)</td>
</tr>
<tr>
<td>T3-T4</td>
<td>49 (57.6)</td>
</tr>
<tr>
<td>N stage</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>47 (55.3)</td>
</tr>
<tr>
<td>N1</td>
<td>38 (44.7)</td>
</tr>
<tr>
<td>Distant metastases</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>44 (51.8)</td>
</tr>
<tr>
<td>Yes</td>
<td>41 (48.2)</td>
</tr>
</tbody>
</table>
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nostained by anti-RAGE and anti-HMGB1 antibodies (Abcam, Cambridge, UK) using the immunoperoxidase technique. Briefly, after deparaffinization in xylene, slides were rehydrated in decreasing concentrations of ethanol and then heated for antigens retrieval. 0.3% H$_2$O$_2$ was applied to block the endogenous peroxidase and goat serum albumin (Zymed, South San Francisco, USA) was used to reduce nonspecific antibody binding. Anti-RAGE antibody was used at 1:300 and anti-HMGB1 antibody was used at 1:250 dilution. After incubation at 4°C overnight with the primary antibody, slides were briefly washed in PBS and incubated with biotinylated secondary antibody. Then the sections were incubated with Horseradish Peroxidase (HRP) followed by incubations with 3,3′-diaminobenzidine (DAB) working solution for color reactions. Finally, slides were counterstained with hematoxylin and mounted. The primary antibody was replaced by appropriate serum for negative control. Colon cancer and normal liver samples were used as positive control tissues for RAGE and HMGB1, respectively.

All slides stained with RAGE and HMGB1 were assessed by two independent pathologists (TZ and XZ). The scoring system included the extent and intensity of staining. The extent score was graded as: 0, less than 5% of tumor cells stained; 1, 5 to 25% stained; 2, 25 to 50% stained; and 3, more than 50% stained. The staining intensity was scored as: 0, negative; 1, weak; 2 moderate; 3, strong. Final scores (range, from 0 to 9) were obtained by multiplying staining extents and intensities. Final scores were described based on the following: 0, no expression; 1 to 3, weak expression; 4 to 6, moderate expression; and 7 to 9, strong expression. For statistical analysis, no expression and weak expression were combined and defined as negative expression (−), moderate and strong expressions were combined for positive expression (+).

Figure 1. Representative immunohistochemistry images of the expression of RAGE and HMGB1 in BPH and prostate cancer samples. (A-D) Negative expression of RAGE (A and B) and HMGB1 (C and D) in BPH (case 919347). (E-H) Positive expression of RAGE (E and F) and HMGB1 (G and H) in BPH (case 906875). (I-L) Positive expression of RAGE (I and J) and HMGB1 (K and L) in prostate cancer (case 600491). (A, C, E, G, I, K) ×200; (B, D, F, H, J, L) ×400.

Statistical analyses

SPSS13.0 software was employed for statistical analysis. Statistical difference of data from categorized groups was assessed by two-tailed χ² test. Spearman rank correlation was used to analyze the correlation of RAGE and HMGB1 expression. The Kaplan-Meier method was used to estimate the correlation of overall survival with RAGE, HMGB1 and their co-expres-
Table 2. Correlation of clinicopathologic parameters with RAGE and HMGB1 expressions

<table>
<thead>
<tr>
<th>Clinicopathologic parameters</th>
<th>RAGE expression</th>
<th>P value</th>
<th>HMGB1 expression</th>
<th>P value</th>
<th>Co-expression RAGE (+)/HMGB1 (+)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>1</td>
<td>9</td>
<td>0.681</td>
<td></td>
<td></td>
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<td>≥60</td>
<td>17</td>
<td>58</td>
<td>0.016</td>
<td></td>
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<tr>
<td>PSA (ng/ml)</td>
<td>Median</td>
<td>19.25</td>
<td>57.17</td>
<td>0.016</td>
<td>27.90</td>
<td>55.77</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>30.58</td>
<td>46.43</td>
<td></td>
<td>36.36</td>
<td>46.09</td>
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<tr>
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<td>0.234</td>
<td>13</td>
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<td>2</td>
<td>20</td>
<td></td>
<td>4</td>
<td>18</td>
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<tr>
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<td>23</td>
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<td>17</td>
<td>19</td>
</tr>
<tr>
<td></td>
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<td>5</td>
<td>44</td>
<td></td>
<td>10</td>
<td>39</td>
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<tr>
<td>N stage</td>
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<td>15</td>
<td>32</td>
<td>0.007</td>
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<td>3</td>
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<td>6</td>
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<tr>
<td>Distant metastases</td>
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<td>14</td>
<td>30</td>
<td>0.013</td>
<td>19</td>
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<td></td>
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<td>4</td>
<td>37</td>
<td></td>
<td>8</td>
<td>33</td>
</tr>
</tbody>
</table>

The level of significance was defined as \( p < 0.05 \).

**Results**

**Overexpression of RAGE and HMGB1 in prostate cancer**

The average diagnostic ages with standard deviations of BPH and prostate cancer patients were 69.63 ± 8.24 and 70.15 ± 9.60 years respectively (Table 1) and there was no significant difference between the two groups (\( P = 0.792 \)). RAGE staining was detected predominantly in the cytoplasm and membrane and HMGB1 signals were localized in the nucleus and cytoplasm in prostate cancer cells. Positive expressions of RAGE and HMGB1 also could be detected in luminal and basal cells in some BPH tissues but not as strong as in prostate cancer cells (Figure 1).

Detailed expression data were presented in Table 2. Positive expression of RAGE was detected in 78.8% (67/85) of prostate cancer and 46.7% (14/30) of BPH samples respectively and it was significantly higher in prostate cancer than BPH cases (\( P = 0.002 \)). HMGB1 expression was positive in 68.2% (58/85) of prostate cancer and in 33.3% (10/30) of BPH cases and its expression was also significantly higher in prostate cancer than BPH cases (\( P = 0.001 \)). 63.5% (54/85) of prostate cancer and 13.3% (4/30) of BPH samples expressed both RAGE and HMGB1 with significant difference between prostate cancer and BPH cases (\( P < 0.0001 \)). There was a strong correlation between RAGE and HMGB1 expressions with correlation coefficient of 0.512 (\( P < 0.001 \)).

**Correlations of RAGE and HMGB1 expression with advanced clinical stage**

High-level expression of both RAGE and HMGB1 was individually significantly associated with T stage, lymph node metastasis and distant metastases (Table 2). Furthermore, high level RAGE expression was correlated with high diagnostic PSA level (\( P < 0.05 \)). Both RAGE and HMGB1 expression was not significantly related to the diagnostic age (\(< 60 \text{ vs } ≥60\)) or Gleason score. The co-expression of RAGE and HMGB1 also showed a significant positive correlation with T stage, lymph node metastasis and distant metastases (Table 2).

**Correlations of RAGE and HMGB1 expression with patient outcome**

To explore the association of RAGE and HMGB1 expression with patient outcome, we only included patients with clinical stage III and IV cancers, due to the short follow-up period and the generally slow progression nature of prostate cancer. These patients received regular treatment according to prostate cancer diagnosis and treatment guideline of China, including...
Figure 2. Kaplan-Meier survival curves for RAGE (A), HMGB1 (B) alone and co-expression (C). Survival analysis was performed for RAGE in a cohort of 7 negative expression patients and 51 positive expression patients using log-rank test P=0.359 (A); for HMGB1 in a cohort of 15 negative expression patients and 43 positive expression patients, using a log-rank test P=0.082 (B); and for co-expression of RAGE and HMGB1 in a cohort of 17 negative co-expression patients and 41 positive co-expression patients. Log-rank test: P=0.047 (C).
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radical prostatectomy (8 cases), hormonal therapy (52 cases), radiotherapy (5 cases) and chemotherapy (2 cases), single or in combination. Fifty-eight patients with stage III and IV cancers were underwent overall survival analysis, with average follow-up of 47.9 months. Thirty-six of the 58 patients were still alive at the end of the follow-up period. In Kaplan-Meier survival analysis, there was statistically significant decreased overall survival rate for cases with co-expression of RAGE and HMGB1 compared with the rest of patients ($P=0.047$). Neither RAGE nor HMGB1 alone was associated with overall survival rate (Figure 2).

Discussion

Finding new abnormal gene expression in prostate cancer will help us to predict prognosis and identify novel therapeutic targets. In previous studies, both RAGE and its ligand HMGB1 had been found overexpression in prostate cancer tissue and cell lines [14, 15, 25-29]. In the present study, we analyzed the expression of HMGB1 and RAGE in Chinese prostate cancer samples and correlated them with both clinicopathologic parameters and patient outcome.

We previously demonstrated RAGE overexpression at both RNA and protein level in Chinese prostate cancer samples [29]. In this study, we not only confirmed our previous observation of RAGE overexpression in a larger series of prostate cancer samples, but also detected significantly higher expression levels of HMGB1 and more cases with co-expression of these two proteins in prostate cancer samples compared with BPH cases. High frequency co-expression of HMGB1 and RAGE have been previously reported [14, 15], but not in Chinese prostate cancer.

We found that high-level expression of RAGE and HMGB1 was significantly associated with aggressive features of prostate cancer, including T stage, lymph node metastasis and distant metastases, individually and in combination. While previous studies have showed the correlations of HMGB1 expression with T stage in Chinese prostate cancer [26] and the expression of both proteins with cancer metastasis in Japanese prostate cancer [15], this study firstly demonstrated that the expression level of both genes are correlated with all the features (T, N and M) associated with a clinical advanced disease.

We also found that high-level RAGE expression was correlated with high diagnostic PSA level, which is consistent with the report of PSA level reduction in LNCaP cells transfected with RAGE RNAi constructs [30]. In a previous report, where HMGB1 protein expression was evaluated in 168 primary prostatectomy tissue samples by IHC, the expression level of HMGB1 was found to be correlated with both Gleason score and preoperative PSA concentration [26]. However, our data show that HMGB1 expression was not associated with Gleason score. While there is a trend that high HMGB1 correlates with high PSA value, it is not statistically significant. This distinctive may due to different samples inclusion criteria or limited sample size in our study. Nevertheless, our data and the previous studies also support deregulation of RAGE and HMGB1 is strongly associated with advanced stage of prostate cancer, which may have impact on patient survival.

Due to the heterogeneity of natural progression of individual prostate cancer cases, it is a major issue to predict the outcome of a prostate cancer diagnosed at an early stage. So far, very limited number of prognostic molecular markers has been identified [31]. Therefore, the most important finding of this study is the association of co-expression of RAGE and HMGB1 with prostate cancer patient outcome. While further investigations with larger number of cases and longer follow-ups are required to confirm our findings, our data indicates the potential to use the co-expression of those proteins as prognostic markers to stratify patient treatment.

There is only one reported study on the prognostic value of HMGB1/ RAGE in prostate cancer and only HMGB1 was included [26]. In that study, HMGB1 protein expression was an independent prognostic factor for biochemical recurrence (BCR)-free survival after radical prostatectomy (RP). In other human cancers, most studies showed that a high-level HMGB1 protein alone or in combination with VEGF-C was linked to poor patient survival rate, including colorectal [32], nasopharyngeal [33] and gastric [34] carcinomas, although it has also been reported that low HMGB1 expression gas-
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...tric cancer cases had a significantly poorer outcome compared with the high HMGB1 group [35]. In colorectal cancer with liver metastases, it has also been reported that serum HMGB1 level correlated with disease progression after radioembolization therapy [36]. In our study, there is a trend of inverse correlation of HMGB1 expression with patient outcome, but it is not statistically significant (P=0.082), potentially due to limited number of cases. However, the co-expression of RAGE and HMGB1 is significantly associated with patient survival, suggesting the expression of those two proteins has additive effect to promote aggressive cancer growth. Co-expression of HMGB1 and RAGE has also been associated with tumor progression and metastasis in gastric [24] and colon cancer [18] as well as glioma [37], although no patient outcome correlation analysis has been done. All those indicated that co-expression of both proteins has a better potential than individually for the potential to be developed as a novel prognostic marker to stratify prostate cancer treatment.

It is still controversial on the association between RAGE expression and patient outcome. Tateno et al. revealed that overexpression of RAGE is related to bad prognosis of esophageal squamous cell carcinoma [38]. However, in colorectal cancer with liver metastases, only HMGB1 but not RAGE expression levels is associated with disease progression after radioembolization therapy [36]. Our data suggest that overexpression of RAGE itself is not associated with bad patient outcome of prostate cancer, but through its effects on prostate cancer progression, it contributes to poor patient outcome in cooperation with HMGB1.

While we showed the clinical correlation between HMGB1/RAGE expression and prostate cancer progression and prognosis, functional studies demonstrated the biological role of those two genes in promoting the survival of prostate cancer cells. Previous studies showed that knockdown of HMGB1 suppressed the growth of LNCaP prostate cancer cells through induction of apoptosis [19] and down-regulation of RAGE expression induced apoptosis and inhibits prostate tumor growth both in vitro and in vivo [30]. Therefore, targeting HMGB1/RAGE pathway may be a novel therapeutic strategy for aggressive prostate cancer.

Conclusion

In summary, this study demonstrates the relationship between RAGE and HMGB1 expression and the disease progression in Chinese prostate cancer. In addition to confirm that RAGE and HMGB1 abnormality are involved in prostate cancer progression, we found that co-expression of RAGE and HMGB1 associated with prostate cancer progression better than each protein individually, thus only co-expression of RAGE and HMGB1 is correlated with poor patient outcome. Therefore, the co-expression of RAGE and HMGB1 has the potential to be used as a prognostic marker to help stratifying prostate cancer into indolent or aggressive groups.

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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References

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