Heterogeneous nuclear ribonucleoprotein K is overexpressed and associated with poor prognosis in gastric cancer

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Abstract. Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is one of the major pre-mRNA-binding proteins, that is involved in translational modifications. In our previous studies, we found that hnRNP K is associated with human gastric cancer. The protein levels of hnRNP K were detected in cell lines and tissue microarrays. The correlation between hnRNP K expression and patient survival rate was evaluated by Kaplan-Meier survival analysis. In addition, we also detected hnRNP K expression in preoperative and postoperative serum samples from patients with gastric cancer, and serum samples from healthy volunteers. We found that hnRNP K was overexpressed in the gastric cancer cell lines. The levels of hnRNP K were significantly elevated in the gastric cancer tissues compared with that noted in the tumor-adjacent gastric mucosal and normal gastric mucosal samples, and hnRNP K expression was found to correlate with tumor stage and lymph node metastasis. However, the level of serum hnRNP K did not differ significantly between gastric cancer patients and healthy volunteers. We also found that patients whose tumors showed elevated expression of hnRNP K had poor survival. The present study suggests that hnRNP K is a promising tissue biomarker for diagnosing gastric cancer and is a prognostic indicator for patients with gastric cancer.

Introduction

Gastric cancer is an aggressive disease that still has a daunting impact on global health. Seventy-three percent of gastric cancer cases are diagnosed in Asia, with almost 50% of the world’s cases diagnosed in China (1,2). Despite an overall decline in incidence and mortality over the past decade, gastric cancer remains the fourth most common type of cancer and is the second leading cause of tumor-related death worldwide (3). Currently, diagnosis of gastric cancer is made by gastroscopic biopsy. Patients often evade detection until they have obvious symptoms, resulting in diagnosis at the middle or advanced stage. Due to the poor prognosis of gastric cancer, early diagnosis is essential. Surgical resection, chemotherapy and radiotherapy, the main methods of treatment for gastric cancer, have improved survival (4). Unfortunately, treatment of advanced or metastatic gastric cancer has seen little progress, and the median overall survival (OS) in this group remains <1 year (5). The mechanisms of occurrence and development of gastric cancer remain unclear.

Tumor biomarkers are the ‘hot spot’ of cancer research. Tumor markers are often involved in the development of cancer, and perform an important function in the process of tumor evolution. Therefore, they have potential as targets for tumor therapy. CA19-9 and CEA are common serum biomarkers for gastrointestinal tumors, but low sensitivity and specificity limit their clinical usefulness (6). Therefore, it is important to find molecular markers with high sensitivity and specificity to diagnose gastric cancer earlier.

In our previous study, heterogeneous nuclear ribonucleoprotein K (hnRNP K), a potential human gastric carcinoma-associated antigen, was found in gastric cancer using serologic proteome analysis (SERPA) (7). hnRNP K was found to be upregulated in various types of human tumors, including colon (8), lung (9), breast, liver (10), esophageal (11), oral squamous cell (12) and nasopharyngeal cancers (13). Barboro et al reported that the association between androgen...
receptor (AR) and hnRNP K expression plays an important role in the progression of prostate cancer and has potential prognostic value (14). Very little is known concerning the behavior of hnRNP K in gastric cancer. Only Zhao et al has reported that hnRNP K is expressed at a higher level in gastric carcinoma with \( H. pylori \) L-form (Hp-L) infection than in gastric cancer without Hp-L infection (15). In the present study, we examined the expression of hnRNP K in gastric cancer tissue microarrays, cultured cell lines and serum samples, and evaluated the relationship between the survival rate of gastric cancer patients and hnRNP K expression.

**Materials and methods**

**Ethics statement.** The present study was approved by the Ethics Committee of the University of South China. The patients and healthy volunteers provided signed informed consent. The present study was conducted in accordance with the Declaration of Helsinki.

**Cell culture and cell immunochemical staining.** Gastric cancer cell lines MGC-803 and SGC-7901 and normal gastric mucosal epithelial GES-1 cells were cultured in RPMI-1640 medium containing heat-inactivated 15% fetal bovine serum (FBS) on 6-well plate with a cover slide. Cells were cultured for three days at 37°C in 5% CO₂.

Cells were washed three times in phosphate-buffered saline (PBS), fixed for 15 min in 10% formaldehyde, and then again washed three times with PBS. The S-P immunohistochemical kit was purchased from Fujian Maixin Biological Technology Ltd. (Fujian, China). The cells were then incubated 10 min with peroxidase blocking solution and washed three times with PBS. Cells were incubated at 37°C for 10 min with enough non-immunologic animal serum to block non-specific binding sites, and then anti-hnRNP K antibody (diluted 1:1,500; ab-32969; Abcam Trade Company) was added and incubated at 4°C overnight with rabbit anti-hnRNP K antibody (diluted 1:2,000; ab-32969), washed with TBST three times 5 min each time, and incubated with the appropriate secondary antibody (goat anti-rabbit IgG; diluted 1:3,000) for 1 h. The samples were then washed with TBST three times again for 5 min each time and enhanced chemiluminescence (ECL) western blotting detection reagents were used to visualize the target proteins (both from KeyGen Biotech, Nanjing, China).

**Tissue microarrays and immunohistochemistry.** Human gastric cancer tissue microarrays (TMA) were obtained from Dr Yongjun Wu (Department of Pathology, The First People's Hospital of Xiangtan, Hunan, China). The specimens were biopsy or gastric cancer resection specimens collected from year 2003 to 2006, including 199 gastric cancer, 31 tumor-adjacent gastric mucosal and 98 normal gastric mucosal specimens. None of the patients received preoperative radiotherapy or chemotherapy.

We placed the paraffin sections at 55°C constant temperature in an incubator for one night, and then they were dewaxed with xylene, and washed three times with PBS. After that an antigen retrieval step was performed. Immunohistochemistry (IHC) was performed according to S-P kit instructions. Immunohistochemical reactions were developed in freshly prepared 3,3'-diamino-benzidine tetrahydrochloride (DAB kit; Fujian Maixin Biological Technology Ltd.) for immune complex visualization. Finally, the TMA IHC was evaluated by light microscopic examination, and the intensity of immunostaining was assessed by two independent professors of pathology. We selected five different high magnifications, counting the total number of cells and hnRNP K-positive cells. The results were evaluated based on both the intensity of immunostaining and the positive cell percentage. The intensity of immunostaining in each core was scored 0-3 (0, negative; 1, weak, 2, moderate; and 3, strong). In addition, the samples were scored into four groups based on the percentage of positively stained cells: 0-25% positivity scored as 1, 26-50% as 2, 51-75% as 3, and 76-100% as 4. The staining intensity score and the percentage immunoreactivity score were then calculated to obtain a composite score (composite score = intensity score x percentage score); 0-5 was defined as low expression, and 6-12 was defined as high expression (16).

**Western blot analysis.** Total cell proteins from the MGC-803, SGC-7901 and GES-1 cells were quantified using a BCA protein assay kit. Then, the samples were separated by 10% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes, and then blocked with Tris-buffered saline Tween-20 (TBST) containing 5% non-fat milk for 1 h, and washed with TBST three times. Then, the samples were probed at 4°C overnight with rabbit anti-hnRNP K antibody (diluted 1:2,000; ab-32969), washed with TBST three times 5 min each time, and incubated with the appropriate secondary antibody (goat anti-rabbit IgG; diluted 1:3,000) for 1 h. The samples were then washed with TBST three times again for 5 min each time and enhanced chemiluminescence (ECL) western blotting detection reagents were used to visualize the target proteins (both from KeyGen Biotech, Nanjing, China).

**Statistical analysis.** Data are reported as mean ± SD. The differences between two groups were compared using Chi-square tests, t-test, one-way ANOVA, Kaplan-Meier survival analysis and log-rank test were performed to determine survival differences between the different groups. Factors associated with the outcomes were evaluated by Cox multivariate regression analysis. Statistical analysis was carried out using SPSS version 17.0 software program (SPSS, Inc., Chicago, IL, USA). All analyses were regarded as statistically significant at the P<0.05 level, and all P-values were two-tailed.

**Results**

**Higher expression of hnRNP K in human gastric cancer cell lines.** To evaluate the expression of hnRNP K between gastric cancer and normal gastric mucosal epithelial cells, we performed cell immunochemical staining analysis in two gastric cancer cell lines (MGC-803 and SGC-7901) and a normal gastric mucosal epithelial cell line (GES-1).
Obviously, the intensity of immunostaining in the MGC-803 and SGC-7901 cells was stronger than that found in the GES-1 cells (Fig. 1A). We also performed western blot analysis in the three cell lines. Compared with the normal gastric mucosal epithelial cell line GES-1, hnRNP K protein expression was significantly higher in the gastric cancer cell lines MGC-803 and SGC-7901 (P<0.05; Fig. 1B).

hnRNP K is overexpressed in gastric cancer tissues. To confirm whether hnRNP K expression is elevated in human gastric cancer tissues, we analyzed the level of hnRNP K protein by IHC in the human gastric carcinoma tissue microarrays (TMAs). The hnRNP K expression in gastric cancer tissues was significantly higher compared with that found in the tumor-adjacent gastric mucosal and normal gastric mucosal specimens. Both in the gastric cancer and non-gastric carcinoma tissues, hnRNP K was localized in the nucleus and showed low staining in the cytoplasm. However, we found that hnRNP K expression was significantly elevated in the gastric glandular neck epithelium when compared to that in the other glandular epithelium (Fig. 2). According to various studies, gastric gland stem cells localize in the gastric neck glands,
YANG et al.: hnRNP K IS OVEREXPRESSED IN GASTRIC CANCER

where they have the ability to proliferate and differentiate. This indicates that elevated expression of hnRNP K promotes the ability of gastric cancer cells to grow and proliferate.

We also evaluated the relationship between the clinico-pathological parameters of the gastric cancer patients and the nRNP K expression level. We found that there was no correlation between the protein expression level and gender, age, degree of differentiation or tumor size. However, hnRNP K expression was significantly elevated in the group with lymph node metastasis than that found in the group without lymph node metastasis, and was also higher in stage III and IV than stage I and II samples (Table I; fig. 3).

Relationship between the survival rate of gastric cancer patients and hnRNP K expression and clinicopathological characteristics. One hundred ninety-nine patients with gastric cancer were followed-up for 8 years. There were 34 cases lost to follow-up and 121 patients died during this time; overall survival (OS) was 26.7%. Twenty-eight of the 53 (52.8%) patients with low expression of hnRNP K died; while 93 of 112 (83.0%) patients with high hnRNP K died. The death rate of the high hnRNP K group was 1.57 times higher than that in the low hnRNP K group. We found that patients with elevated hnRNP K expression in cancer cells had poorer survival compared with those patients with low hnRNP K expression (log-rank=62.339, P=0.000; fig. 4). The mean survival time of patients with low hnRNP K expression was 52.277 months and the median survival time was 61 months, while the median survival time in the cohort with higher hnRNP K expression was 20.657 months.

Cox multivariate analysis indicated that the degree of differentiation and lymph node metastasis were associated with poor survival. Moreover, the risk of death in the poorly differentiated group was 2.203 times (death ratio=1/HR) higher than that of the moderately and well differentiated groups. The mortality risk in the lymph node metastasis group was 1.976 times higher than that in the patients with no lymph node metastasis (Table II).

Table I. Relationship between the expression levels of hnRNP K and clinicopathological characteristics of the patients.

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Expression of hnRNP K</th>
<th>P-value (two-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low n (%)</td>
<td>High n (%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>126 49 (38.9) 77 (61.1)</td>
<td>0.790</td>
</tr>
<tr>
<td>Female</td>
<td>73 27 (40.0) 46 (60.0)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>83 31 (37.3) 52 (62.7)</td>
<td>0.836</td>
</tr>
<tr>
<td>≥50</td>
<td>116 45 (38.8) 71 (61.2)</td>
<td></td>
</tr>
<tr>
<td>Normal gastric mucosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-adjacent gastric mucosa</td>
<td>98 82 (83.7) 16 (16.3)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WDAC</td>
<td>88 37 (42.0) 51 (58.0)</td>
<td>0.498</td>
</tr>
<tr>
<td>MDAC</td>
<td>58 22 (37.9) 36 (62.1)</td>
<td></td>
</tr>
<tr>
<td>PDAC</td>
<td>53 17 (32.1) 36 (67.9)</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>65 39 (60.0) 26 (40.0)</td>
<td>0.000</td>
</tr>
<tr>
<td>Yes</td>
<td>134 37 (27.6) 97 (72.4)</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>96 47 (49.0) 49 (51.0)</td>
<td>0.003</td>
</tr>
<tr>
<td>III-IV</td>
<td>103 29 (28.2) 74 (71.8)</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;3.0</td>
<td>114 45 (39.5) 69 (60.5)</td>
<td>0.666</td>
</tr>
<tr>
<td>≤3.0</td>
<td>85 31 (36.5) 54 (63.5)</td>
<td></td>
</tr>
</tbody>
</table>

*Normal gastric mucosal vs. tumor-adjacent gastric mucosal tissues. TNM, tumor-node-metastasis. WDAC, well-differentiated adenocarcinoma; MDAC, moderately differentiated adenocarcinoma; PDAC, poorly differentiated adenocarcinoma.
To evaluate the expression level of serum hnRNP K between gastric cancer patients and normal controls, we measured the levels of hnRNP K in serum samples by ELISA. Most serum samples showed low expression of hnRNP K. There was no significant difference between the gastric cancer patients and the healthy volunteers or between the preoperative and postoperative groups (Table III). We believe that hnRNP K is not a suitable circulating tumor biomarker for detecting gastric cancer.

Table III. Serum hnRNP K levels in controls, preoperative gastric cancer patients and paired postoperative gastric cancer patients.

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Serum hnRNP K level (mean ± SD)</th>
<th>P-value  (two-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy volunteers</td>
<td>22</td>
<td>0.984±0.358</td>
<td>0.237^a</td>
</tr>
<tr>
<td>Preoperative group</td>
<td>37</td>
<td>0.908±0.353</td>
<td>0.096^b</td>
</tr>
<tr>
<td>Postoperative group</td>
<td>37</td>
<td>0.783±0.306</td>
<td></td>
</tr>
</tbody>
</table>

^aHealthy volunteers vs. preoperative group. ^bPreoperative vs. postoperative groups.

Discussion

hnRNP K is a RNA-binding protein of the large hnRNP family. hnRNP K was mainly found localized in the nucleus, but recently Thompson et al found it also exists in the cytoplasm and mitochondria (18). hnRNP K contains three repeats of a motif termed the KH domain (for K homology) (19), which could recognize single-stranded DNA or RNA. In addition, hnRNP K has a nuclear-localization signal (NLS) and a nuclear shuttling domain (KNS) which allow transport from the cytoplasm to the nucleus (20). Due to these different structures, hnRNP K is involved in translational modifications, including methylation, sumoylation and phosphorylation. These regulate its interactions with different molecules and influence its functions, such as DNA splicing, chromosome remodeling, transcriptional regulation, mRNA stability, splicing and translation (21). In particular, hnRNP K plays an important role in carcinogenesis. Researchers have shown that hnRNP K activates important genes associated with human tumors, including c-src and c-myc, indicating that hnRNP K participates in cancer development and progression (22). It is active at the chromatin level, and present in greater density near transcribed genes with respect to silent ones. hnRNP K directly binds to the promoter region of the human c-myc gene (23) and was found to promote neoplastic transformation in an eIf4E-dependent manner (24). The expression level of hnRNP K was higher in melanoma, breast and prostate cancers than that in normal control groups (14,25,26). hnRNP K can also suppress apoptosis independent of p53 status by maintaining high levels of endogenous caspase inhibitors (27). Recent studies found that hnRNP K was closely related to non-coding RNA, including lncRNA and miRNA. Moreover, studies have shown that both long (>200 nucleotides) and short ncRNAs have critical regulatory roles in several human diseases, including cancer development and progression (28). Carpenter et al reported that the expression level of hnRNP K is often associated with colorectal cancer staging (29). Wu et al detected elevated protein levels in the cytoplasm of oral squamous cell carcinoma, suggesting that hnRNP K may be an independent prognostic predictor (30). They showed that increased cytoplasmic expression of hnRNP K was associated with poor patient prognosis by multivariate analysis. Even Inoue et al found that the cytoplasmic accumulation of hnRNP K was crucial for its role in the metastasis of fibrosarcoma (31).
There has been little investigation concerning the relationship between hnRNP K and gastric cancer. In the present study, we demonstrated that expression of hnRNP K was higher in gastric cancer cells (MGC-803 and SGC-7901) than in normal gastric mucosal epithelial cells (GES-1) and it was localized in the nucleus. Tissue array immunohistochemistry showed that levels of hnRNP K were significantly elevated in gastric carcinoma tissues than that in tumor-adjacent gastric mucosal or normal gastric mucosal specimens. We evaluated the correlation between the expression of hnRNP K and clinical pathology, and found that it was correlated with lymph node metastasis and tumor stage. Moreover, the survival rate of patients with gastric cancer was associated with the degree of tumor differentiation and lymph node metastasis. Thus, the results supported the observation that patients with elevated expression of hnRNP K have poorer survival compared to those with low hnRNP K expression.

ELISA results showed low expression of hnRNP K in serum of the gastric cancer patients. This suggests that hnRNP K is mainly transported into the nucleus rather than secreted out of cells. There was no significant difference between the gastric cancer patients and healthy volunteers. Comparing the preoperative groups with the postoperative groups, we also found no difference between these two groups. Therefore, hnRNP K is unsuitable as a serum marker in gastric cancer.

We conclude that hnRNP K is upregulated in gastric cancer and is associated with patient survival. Therefore, hnRNP K has the potential as a key biomarker for detection of gastric cancer and is of prognostic value to patients with gastric cancer. However, according to the present study, serum levels may not be useful for measuring this tumor-associated biomarker. We may continue to study the role of hnRNP K in gastric cancer to determine whether it is a promising target for anticancer therapies.

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