RESEARCH ARTICLE

Expression of C4.4A is a Potential Independent Prognostic Factor for Patients with Gastric Cancer

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Abstract

C4.4A, a metastasis-associated gene, encodes a glycolipid-anchored membrane protein which is overexpressed in several human malignancies. However, there are few data available on C4.4A expression and its relationship with progression in gastric cancer. Our study was designed to explore the expression of C4.4A in gastric cancer and to correlate it with clinical outcome. C4.4A expression was studied by quantitative real-time RT-PCR and immunohistochemistry for assessment of correlations with clinicopathological factors. C4.4A mRNA expression was significantly up-regulated in gastric cancer as compared with noncancerous tissue (p<0.05), being observed in 107 (88.4%) of the 121 gastric cancer cases by immunohistochemistry. We found that the expression of C4.4A mRNA was correlated with size of the tumor, depth of invasion, lymph node metastasis, distant metastasis and TNM stage. Moreover, patients with overexpression of C4.4A has a significantly worse survival (p<0.05). Further multivariable analysis indicated that the expression of C4.4A was an independent prognostic indicator for gastric cancer (p<0.05). In conclusion, overexpression of C4.4A correlates with metastatic potential of gastric cancer and C4.4A could be a novel independent prognostic marker for predicting outcome.

Keywords: C4.4A - gastric cancer - prognosis - metastasis

Introduction

Gastric cancer is the fourth most common malignancy in the world and the second leading cause of cancer-related deaths (Jemal et al., 2010). Early gastric cancer may have a 5-year survival of over 90% following surgery (Wang et al., 2012). Owing to the vague and non-specific symptoms, gastric cancer is often diagnosed at advanced stages with a 5-year survival rate of <30%, also this kind of carcinoma carries a high risk of recurrence even after radical surgery and adjuvant systemic chemotherapy (Hartgrink et al., 2009). Therefore, to improve the poor prognosis of patients with gastric cancer, biologic markers that can predict prognosis and response toward a specific therapy should be established in the treatment of gastric cancer.

C4.4A is a glycosyl-phosphatidyl-inositol-anchored molecule and belongs, like the urokinase-type plasminogen activator receptor (uPAR), to the Ly6 family (Jacobsen and Ploug, 2008). While uPAR has three cysteine containing domains, C4.4A has two and the third domain is devoid of cysteines. Most other members of the Ly6 family have only one domain (Rosel et al., 1998). The C4.4A protein was first identified in a highly metastasizing rat pancreatic adenocarcinoma cell line (Matzku et al., 1989).
who underwent surgical resection for primary gastric cancer at Department of General Surgery of Huashan Hospital, Fudan University (Shanghai, China) in 2006. No preoperative chemotherapy or radiotherapy had been performed in any of these cases. The clinical records, pathologic reports, and follow-up information were also obtained when available. The corresponding non-tumor tissues served as controls, which were at least 5 cm away from the margin of the tumor and were confirmed as such by two experienced pathologists. The postoperative pathologic staging was determined according to the guidelines of the International Union Against Cancer (UICC) (Omejc et al., 2001; Li et al., 2013). This study was approved by the Ethics Committee of Huashan Hospital, Fudan University (Shanghai, China), and written informed consent was obtained from all the patients for surgery and to use their resected samples for research. Freshly tissue samples were obtained from all of the resected specimens and were rapidly frozen at -80°C for storage until RNA extraction. The remaining specimens were fixed in 10% phosphate-buffered formalin and paraffin-embedded for histological analysis.

**Immunohistochemistry**

The expression of C4.4A protein was investigated by immunostaining using SABC kits (Wuhan Boster Biological Technology, Wuhan, China) according to the manufacturer’s protocol. Sections (4μm thick) of formalin-fixed and paraffin-embedded tumor tissue specimens were prepared for immunohistochemical analysis. Briefly, after blocking endogenous peroxidase activity, antigen retrieval was performed by microwaving the sections. A rabbit polyclonal antibody for human C4.4A (abcam, Cambridge, UK) was applied as primary antibody. After washing with PBS, the slides were incubated with biotinylated secondary antibody and streptavidin-biotin complex. Each incubation step was followed by washing 3 times with PBS. After incubation with diaminobenzidine for about 5–10 minutes, the sections were counterstained with hematoxylin. Negative controls were included by omission of the primary antibody.

The reactivity degree of C4.4A in each immunostained slide was assessed independently by two pathologists without knowledge of any of the clinicopathologic data. The percentage scoring of the immunoreactive tumor cells was as follows: 0 (0%), 1 (1%-10%), 2 (11%-50%), and 3 (>50%). The staining intensity was visually scored and stratified as follows: 0 (negative), 1 (weak, if it was a blush), and 2 (strong, if it was obviously positive at original magnification ×20). A final score was obtained for each case by multiplying the percentage and the intensity score. Therefore, tumors with multiplied score exceeding 4 were recorded as positive immunoreactivity to C4.4A; all the other scores were considered to be negative.

**Quantitative real-time RT-PCR**

Total RNA was extracted from frozen tissue samples with TRIzol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instructions. A cDNA synthesis was carried out with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocols. A quantitative PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). Expression analysis was performed in triplicate for each sample. The GAPDH gene was used as the normalization control. The C4.4A mRNA expression level was quantified using the ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primers used in this study were: C4.4A forward, 5’ TCCCGCTGTAACTCTGACC 3’, C4.4A reverse, 5’ TCAACCTGGGCTCCTCCTAC’; GAPDH forward, 5’ CACCACCTCTCCACCTTTTGG 3’; GAPDH reverse, 5’ CCACCACCTGGTGTGCTTAG 3’. Three independent experiments were performed in triplicate.

**Statistical analysis**

Statistical analyses were carried out using Stata 8.0 software (Stata, College Station, TX, USA). For statistical analysis, the patients were classified into high and low C4.4A mRNA expression groups, based on whether they were above or below the mean value (high C4.4A expression group, n=67; low C4.4A expression group, n=54). The difference between C4.4A mRNA expression in tumor tissue and that in corresponding non-tumor tissue was evaluated using t-test. The χ²-test or Fisher’s exact test was used to analyze the associations between C4.4A expression and clinicopathological features of gastric cancer. Survival curves were estimated by the Kaplan-Meier method and the difference between survival curves was analyzed by a log-rank test. Survival data were evaluated using univariate and multivariate Cox regression analysis. Differences among groups were considered significant when \( p<0.05 \).

**Results**

**Demographic characteristics**

In this study, there were 75 men and 46 women with a mean age of 55 years (range, 37-88 years). 77 cases had lymphatic metastasis and 7 patients had distant metastasis. We examined tissue specimens from all the 121 primary tumors. All deaths were attributable to gastric cancer. Patient characteristics are detailed in Table 1.

**Immunohistochemical staining for C4.4A**

To investigate whether C4.4A gene was upregulated in gastric cancer, we first examined C4.4A protein expression in paired gastric cancerous and non-cancerous tissues by immunohistochemical staining. C4.4A expression was found in the plasma membrane or in the cytoplasm. The results revealed that C4.4A was observed in 88.4% (107/112) of gastric carcinoma tissues with no immunoreactivity in control mucosa cells (Figure 1A and 1B). In addition, we also found that C4.4A expression was significantly increased in lymph nodes with metastasis (Figure 1C).

**Evaluation of C4.4A mRNA by quantitative real-time RT-PCR**

We further analyzed C4.4A mRNA levels in these paired cancerous and non-cancerous tissues by
The amount of C4.4A mRNA was normalized using the endogenous reference GAPDH. The real-time RT-PCR results are shown in Figure 2. Gastric cancer samples demonstrate pronounced high levels of C4.4A mRNA expression when compared with normal gastric tissue (<0.05).

| C4.4A mRNA expression | High (n=67) | Low (n=54) | p value |
|------------------------|-------------|-------------|---------|
| Age(years) ≥65          | 52          | 29          | 23      |
| <65                    | 69          | 38          | 31      |
| Gender Male             | 75          | 45          | 30      |
| Female                 | 46          | 22          | 24      |
| Tumor size(cm) ≥5       | 53          | 34          | 19      |
| <5                     | 68          | 33          | 35      |
| Tumor location Upper    | 22          | 13          | 9       |
| Middle                 | 28          | 16          | 12      |
| Lower                  | 71          | 38          | 33      |
| Depth of invasion T1+T2 | 21          | 7           | 14      |
| T3+T4                  | 100         | 60          | 40      |
| TNM stage I+II          | 51          | 15          | 36      |
| III+IV                 | 70          | 52          | 18      |
| Lymph node metastasis Present | 77 | 53 | 24 |
| Absent                 | 44          | 14          | 30      |
| Distant metastasis Present | 7 | 4 | 3 |
| Absent                 | 114         | 63          | 51      |

*Upper vs Lower; *Middle vs Lower; *Upper and Middle vs Lower

**Correlations between C4.4A mRNA expression and clinicopathologic features**

Clinicopathologic and demographic features of the 121 gastric cancer patients and association with C4.4A mRNA expression are shown in Table 1. High expression of C4.4A was significantly correlated with tumor size (p<0.01), depth of invasion (p<0.01), TNM stage (p<0.01), presence of lymph node metastasis (p<0.01) and presence of distant metastasis (p<0.01). No significant difference was detected in distribution according to age, gender and tumor location between the two C4.4A expression groups.

**Relationship between C4.4A and survival**

In the univariate survival analysis (Table 2), there were no differences in the survival between above 65 years old and below 65 years old, among gastric cardia cancer, corpus cancer and antrum cancer. It was identified...
that C4.4A expression, gender, the size of tumor, depth of tumor invasion, tumor TNM stage, lymph node metastasis and distant metastasis were significantly associated with prognosis of gastric cancers. C4.4A expression above the median was associated with decreased survival in the overall analysis (Figure 3). Furthermore, the multivariate analysis by entered into a Cox proportional hazards model indicated that the status of higher C4.4A expression remained a significant and independent prognostic factor for survival after adjusting for the other factors in Table 3 (relative risk 1.61, \( p=0.03 \)).

**Discussion**

C4.4A glycoprotein was first identified in a highly metastasizing rat pancreatic adenocarcinoma line (Matzku et al., 1989). The possible importance of the protein as a tumor marker was indicated by the restricted expression in normal tissues and upregulation of C4.4A mRNA in different tumors (Rosel et al., 1998; Seiter et al., 2001; Wurfel et al., 2001; Fletcher et al., 2003). In this study, we reported for the first time that C4.4A expression was significantly up-regulated in gastric cancer at both mRNA and protein levels. High C4.4A expression was positively associated with tumor size, depth of invasion, TNM stages, lymph nodes metastasis, and distant metastasis. The 5-year overall survival rate of patients with high C4.4A expression was significantly decreased as compared with low C4.4A expression. Furthermore, multivariate analysis indicated that C4.4A served as an independent factor for predicting poor prognosis of patients with gastric cancer.

In normal human tissues, C4.4A mRNA is present in placental tissue, skin, esophagus, and peripheral blood leukocytes, but not in other tissues (Konishi et al., 2010). Although the physiological function of the C4.4A protein is largely unknown, up-regulation of C4.4A expression is observed during the wound healing process of migrating keratinocytes and urothelial cell (Smith et al., 2001; Hansen et al., 2004). Recently, C4.4A mRNA was detected in cancer cell lines of different origin including melanoma, breast, bladder and renal cell carcinoma as well as in tumor tissues of malignant melanoma, breast cancer, lung carcinoma and lung tumor derived metastases, and primary and metastatic transitional cell carcinoma of urothelial cell origin (Seiter et al., 2001; Smith et al., 2001; Wurfel et al., 2001; Fletcher et al., 2003). Some researchers developed three antibodies against the C4.4A protein. C4.4A-119 and C4.4-277 antibodies detected 70-kDa C4.4A, mainly in the cytoplasm, irrespective of intra-tumoral location. The C4.4A GPI-M antibody reacted with the membranous ~40-kDa C4.4A, exclusively at the tumor invasive front (Yamamoto et al., 2013). In our study, C4.4A expression was found in the cytoplasm, especially in cell membrane. And C4.4A was also observed in metastatic lymph nodes.

As we know, C4.4A is a structural homolog of uPAR. For decades the uPA system has been thought to drive tumor progression by mediating directed extracellular proteolysis on the surface of migrating or invading cells. Intervention with this proteolysis by targeting of uPAR has been proposed to represent a novel approach for inhibiting tumor progression (Ma and Tao, 2012). C4.4A may play an important role in the tumor invasion and metastasis. Seiter (2001) found that normal human skin weakly expresses C4.4A. Melanocytes and nevi are negative, but up to 60% of primary malignant melanoma and 100% of lymph node and skin metastases of melanoma are C4.4A positive. Hansen (2008) observed that normal squamous esophageal epithelium shows a strong cell surface associated C4.4A expression in the suprabasal layers, whereas basal cells are negative. Upon transition to dysplasia and carcinoma in situ the expression of C4.4A is abruptly and coordinately weakened. C4.4A expression reappeared in tumor cells located at the invasive front and local lymph node metastases. Konishi (2010) also found that strong membranous staining of C4.4A at the invasive front of colorectal cancer tumors and at the frontier of metastatic lesions to lymph node and lung. A significant difference in 5-year overall survival was found between samples with high and low expression of C4.4A mRNA. Combined with our research, we considered that C4.4A expression could play a key role in promoting tumor invasion and metastasis of gastric cancer.

C4.4A was supposed to be the receptor of uPAR and to play a role similar to uPAR. However, the precise mechanism of how C4.4A is induced at the invasive front is not known. Interestingly, C4.4A was identified by SILAC (Stable Isotope Labeling by Amino acids in Cell culture) technique as a substrate to ADAM10 and ADAM17, which indicates that C4.4A contributes to tumor progression is related to ADAM10 and ADAM17 (Esselens et al., 2008). Paret (2005) showed that C4.4A bound Laminin-5 (LN5) and supported cell migration. As

Table 3. Multivariate Analysis for Survival by the Cox Proportional Hazard Regression Model

| Variables               | Relative risk | 95% CI       | \( p \) value |
|-------------------------|---------------|--------------|--------------|
| C4.4A expression        | 1.61          | 1.06-2.68    | 0.03         |
| Gender                  | 1.31          | 0.88-1.93    | 0.21         |
| Tumor size              | 1.47          | 0.89-2.41    | 0.14         |
| Depth of invasion       | 2.13          | 1.53-4.89    | 0.02         |
| TNM stage               | 3.40          | 2.44-6.63    | <0.01        |
| Lymph node metastasis   | 1.84          | 1.07-2.32    | 0.01         |
| Distant metastasis      | 2.68          | 2.08-3.40    | <0.01        |

CI: confidence interval
LN5 binds to collagen VII, one possibility is that C4.4A may also be involved in forming an anchor between the cell surface and the collagen matrix (Frank and Carter, 2004). Matrix metalloproteinase-9 (MMP-9) can degrade collagen IV, V, XI and XVI. MMP-9 was a prognostic indicator for survival in gastric cancer (Gao et al., 2013). C4.4A may promote tumor metastasis through MMP-9. It was also reported that cleavage of LN5 by MT1-MMP or MMP19 facilitates cell migration (Udayakumar et al., 2003; Sadowski et al., 2005). When tumor invades tissues and new vessels are not forming, hypoxia may appear to promote C4.4A expression to enhance tumor cell migration. A recent study showed that C4.4A transcription is activated by C/EBPβ transcription factor (Fries et al., 2007), which fibroblasts produce in response to anoxic conditions (Mondino et al., 1999).

In conclusion, our findings indicated for the first time that C4.4A was overexpressed in gastric cancer, and C4.4A expression in gastric cancer was associated with clinicopathological factors and prognosis. The high C4.4A expression, directly contributing to tumor development and progression, was shown as a novel independent prognostic marker for predicting the outcome of gastric cancer. This observation invites for further clinical studies of the significance of this molecule in a large group of gastric cancer. And the mechanisms of C4.4A in the tumor invasion and metastasis are also needed to be explored.

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