Prognostic Value of Upregulation of Myristoylated Alanine-Rich C-Kinase Substrate in Gastric Cancer

Ruiliang Quan, Zhongliang Ning, Yongcang Wang, Wei Yu, Haixing Zhu

Corresponding Author: Ruiliang Quan, e-mail: qrlahsl6639@aliyun.com
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Background: Accumulating evidence suggests a connection of Myristoylated alanine-rich C-kinase substrate (MARCKS) with several physiological and pathological processes. However, the relevance of MARCKS in gastric cancer (GC) needs to be elucidated.

Material/Methods: The abundance of MARCKS in GC tissues was assessed using techniques of immunohistochemistry (IHC) and quantitative real-time PCR (qRT-PCR). Moreover, the MARCKS expression profile in the TCGA database was analyzed through an online website analysis. We also investigated MARCKS function using cell wounding and Matrigel invasion assays.

Results: TCGA analysis and our data suggest that transcript abundance and protein level of MARCKS was higher in GC tumor samples compared with peri-tumor tissues. There was a remarkable association of upregulated MARCKS with the cell differentiation (P<0.001), T stage (P=0.034), and N stage (P=0.002) followed by advanced TNM phase (P=0.008). Furthermore, it was predicted that higher expression of MARCKS is linked to poor overall survival (P=0.015) and disease-free survival (P=0.020), and that high levels of MARCKS function as an independent prognostic marker, as shown by multivariate Cox regression analysis in prediction of poor overall (HR=0.408; 95% confidence interval=0.247–0.674; P<0.001) and disease-free survival rates (HR=0.525; 95% confidence interval=0.216–0.584; P<0.001). GC cells showed significant reduction in cell migration and invasion upon depletion of MARCKS as noted through Matrigel invasion and cell wounding assays. Further analyses showed that silencing MARCKS impeded the epithelial-mesenchymal transition (EMT).

Conclusions: Our study indicates that elevated expression of MARCKS is significantly associated with metastatic capability of GC cells, and MARCKS overexpression can serve as a biomarker of GC poor prognosis.

MeSH Keywords: Epithelial-Mesenchymal Transition • Prognosis • Stomach Neoplasms

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Background

Gastric cancer (GC) ranks fourth highest among malignant cancer in recent decades, and it remains a major cause of death from cancer worldwide [1,2]. Mortality in GC patients is often associated with relapse and metastasis [3]. Hence, exploring molecular mechanisms of GC metastatic progression can provide insights into new diagnostic and therapeutic targets. Progressive cancer cells show essential alterations in morphology, such as loss of cellular interactions and apicobasal polarity. This feature is termed epithelial-to-mesenchymal transition (EMT) [4,5]. Abnormal activation of EMT mediates cancer cell invasion and metastasis, which is frequently induced by the tumor microenvironment. In addition, accumulating evidence indicates that the invasive and metastatic behavior of GC is correlated with the EMT mechanism [6,7]. MARCKS is a membrane-associated protein initially observed to be a substrate for protein kinase C [8]. MARCKS plays a crucial role in diverse cellular functions such as cytoskeletal control, motility, inflammatory pathway, secretion and exocytosis, neurological function, and development [9–13]. Thus, it is expected that MARCKS influences neoplastic growth through its myriad functions. Emerging evidence corroborates the above conjecture [14–16]. The role of MARCKS in cancer has been preliminarily revealed, but not including GC. Therefore, the aim of the present study was to explore the contribution of MARCKS to GC and to the underlying molecular mechanism.

Material and Methods

Patient information

From January 2012 to November 2012, patients from the First Affiliated Hospital of USTC were enrolled in the study. GC patients who had undergone radical gastrectomy were recruited for participation. None of the patients had previously received treatment for GC. Patients were excluded based on: (1) previous treatment with a preoperative neoadjuvant (radiation or chemotherapy); (2) surgical histological evidence of digestive system disease; and (3) failure to follow up. Informed consent was obtained from all patients, and the study procedures were approved by the Ethics Committee of the First Affiliated Hospital of USTC (Hefei, China).

Meta-analysis of TCGA database

Gene Expression Profiling Interactive Analysis (GEPIA) (http://geopia.cancer-pku.cn/) was used to analyze expression levels of MARCKS using the TCGA database. GEPIA consists of online database tools to analyze gene expression and clinical prognosis data. Additionally, we calculated disease-free survival (DFS) of GC patients using this web tool. All patients with GC were stratified into 2 groups based on MARCKS expression (low expression or high expression) depending on the cut-off values between lower and upper quartiles, and the best performing threshold was used as a cut-off.

Immunohistochemistry analysis

Paraffin-embedded 4-um tissue sections were used for IHC staining of MARCKS following the protocol previously described [9]. The ratio of IHC-positive tumor cells was scored as follows: none (score 0); ≤50% positively stained carcinoma cells (score 1), 50–75% positively stained carcinoma cells (score 2), and ≥75% positively stained carcinoma cells (score 3). The IHC-positive tumor cells were clustered as follows: absent: (score 0), poor: (score 1), intermediate: (score 2), and strong: (score 3). Staining index (score 0 to 9) was calculated by multiplying the proportion intensity counts by the staining intensity counts. The strength of staining was grouped as follows: poor expression (0 to 4) and elevated expression (5 to 9). IHC results were separately analyzed by 2 expert pathologists using a binocular microscope.

Immunoblotting

Cellular lysates were obtained from tissues in RIPA buffer containing protease inhibitors. The total protein concentration of cells was assessed by the bicinchoninic acid (BCA) assay. Proteins were resolved using protein gel and subsequently blotted onto PVDF membranes. After incubating the membrane in primary antibodies directed against MARCKS (cat: ab52616, Abcam, Cambridge, UK), E-cadherin (cat: ab40772, Abcam, Cambridge, UK), N-cadherin (cat: ab18203, Abcam, Cambridge, UK), vimentin (cat: ab195877, Abcam, Cambridge, UK), or GAPDH (cat: ab181602, Abcam, Cambridge, UK), the membrane was washed in TBST and subsequently incubated in horseradish peroxidase-conjugated secondary antibody (cat: ab172730, Abcam, Cambridge, UK). Proteins of interest were detected using chemiluminescence using. GAPDH was used as a loading control.

Real-time quantitative PCR (RT-PCR)

TRizol reagent was used for total RNA extraction from tissues and cells following the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Isolated RNA was reverse-transcribed using the cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA samples were used to study gene expression through RT-PCR by using the SYBR Green PCR Master Mix (Applied Biosystems) on the 7900 Real-Time PCR System (Applied Biosystems) The following primer pair was used for MARCKS: forward 5’-AGCCGCTTACGAGGAGG-3’ and reverse 5’-TTGGCGAAGTAAGGAGG-3’. Human GAPDH was used for endogenous control. Relative quantification was calculated by the fold change formula.
Cell culture and transfection

BGS-837 and SGC-7901 were procured from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS; HyClone, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were grown in an atmosphere of 5% humidified CO₂ at 37°C. The siRNA-mediated MARCKS depletion was performed for 24–96 h using MARCKS siRNA (HSS180966) and negative control siRNA from Invitrogen (Carlsbad, CA, USA).

Transwell assay

To assess the invasive ability of cancer cells, we performed BioCoat Matrigel invasion assays (BD Biosciences) following the manufacturer’s instructions. In brief, cell suspensions were incubated in serum-free medium for 24 h and transferred to the upper chamber (1×10⁵ cells/well). Then, we added 500 μl complete medium to the lower chamber. After incubating at 37°C for 24 h, the chamber was taken out, and the invaded cells were fixed by using methyl alcohol for 20 min and stained using 0.1% crystal violet stain for 15 mins. Then, the number of invaded cells was counted under a light microscope.

Wound-healing assays

Wound-healing assay was performed to study cancer cell mobility. Cells were cultured to obtain a confluent monolayer in 12-well culture plates followed by scratching a wound using sterile 200-μl pipette tips. PBS washing was performed to remove any cellular debris. We used an inverted phase-contrast microscope at 200× magnification to capture images at the beginning (0 h) and after 12 h of wounding. The average cell migration distance indicated the extent of cellular migration.

Nude mouse tumorigenesis model

Cells (1–5×10⁶) were isolated by trypsin, followed by 6 PBS washes before being subcutaneously injected into the backs of 6-week-old nude mice provided by Nanjing Experimental Animal Center (Nanjing, China), along with injection of 200 ml PBS. Both groups contained 6 mice each. The mice were reared in a sterile animal facility with free access to water. After 4 weeks, mice were sacrificed and the size of tumor nodules was measured. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Anhui Medical University (protocol number AMU18-020).

Statistical analysis

We used SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) for all statistical analyses. Significance between groups was determined using the paired 2-tailed t test. The χ² test was used to analyze the relationship of MARCKS expression level with clinic-pathologic characteristics. Kaplan-Meier plots enumerated for survival curves and the log-rank test together with survival data were examined through multivariate Cox regression analysis. P<0.05 was considered significant.

Results

MARCKS level in cancerous and noncancerous gastric tissues

We mined the TCGA database for the status of MARCKS in human GC. Bioinformatics analysis revealed a higher level of MARCKS mRNA in GC tissues compared with normal controls (Figure 1A). Additionally, GC patients with low MARCKS expression showed improved disease-free and overall survival rates (P=0.0033 for OS; P=0.0053 for DFS; Figure 1B, 1C). To confirm the TCGA results, RT-PCR for MARCKS mRNA was performed in 20 tumor and peri-tumor tissue samples. As depicted in Figure 2A, MARCKS mRNA was highly expressed in the GC tissues (P<0.001). Similarly, immunohistochemistry staining indicated that MARCKS was primarily localized in the cytoplasm and membrane of cells (Figure 2B). MARCKS protein was observed in the majority of GC tissues and in far fewer of the noncancerous GC tissues.

Overexpression of MARCKS is correlated with stage of cancer and patient survival

A total of 219 patients with GC who had undergone radical gastrectomy with median follow-up time of 52 months were recruited for participation. The MARCKS protein expression in tumor tissues from 219 patients with GC was detected by immunohistochemistry analysis. In addition, we investigated the correlation between the profile of MARCKS expression and clinicopathological indexes. The expression of MARCKS was significantly associated with cellular differentiation (P<0.001), pathological T (P=0.034), lymphatic metastasis (P=0.002), and TNM stages (P=0.008) (Table 1). Kaplan-Meier curves for OS (P<0.001) and DFS (P<0.001) were compared to determine the association between the expression and prognostic value of MARCKS. Patients with high-expression tumors generally had a worse prognosis than patients with low-expression tumors (Figure 3; P<0.001 for OS and DFS). Multivariate Cox regression analysis was used to assess the prognostic effect of MARCKS abundance in GC cells. The result showed that elevated MARCKS level significantly reduced OS (P<0.001) and DFS (P<0.001) in GC patients compared to their counterparts (Table 2). MARCKS was therefore identified as an independent prognostic factor.
The effect of abundance of MARCKS on migration and invasion of gastric cancer cell lines in vitro and in vivo

To further assess the effect of MARCKS in GC, a siRNA-mediated knockdown of MARCKS was used. Wound-healing assay was used to determine the effect of MARCKS on cell mobility and invasion ability. MARCKS-depleted cells showed significantly less migration than the control group (P<0.001) (Figure 4). These results indicate that the migratory capacity of GC cells was suppressed by knockdown of MARCKS. Transwell cell migration assay showed that the si-MARCKS groups had a markedly lower invasive capacity compared to the control groups, indicating that MARCKS might increase the abilities of cell motility and invasion (Figure 5).

Overall, MARCKS provides a proliferative advantage to gastric cancer cells in vitro. The nude mouse transplantation tumor experiments showed that GC cells successfully formed tumor nodules on the subcutaneous tissue of nude mice. As shown in Figure 6, the si-MARCKS group had significantly smaller tumor nodules compared to the control group. Combined with the in vitro assays, these results suggest that MARCKS acts as a catalyst for migration and invasion in gastric cancer cells.

MARCKS aggravates gastric cancer progression via EMT

We detected the levels of EMT markers (E-cadherin, N-cadherin, and vimentin) through immunoblotting to validate the role of...
MARCKS in neoplastic metastasis. The results showed that MARCKS inhibition decreased the levels of N-cadherin and vimentin, but the level of E-cadherin was slightly higher (Figure 7). To summarize, our results suggest that MARCKS enhances GC progression via the EMT mechanism.

### Discussion

Accumulating evidence suggests that altered MARCKS expression is correlated with oncogenic and neoplastic progression [17]. For example, MARCKS potentiates metastasis in non-small-cell lung cancer [15,16]. Similarly, Dorris and colleagues
have shown MARCKS can be a potential new target for prostate cancer treatment, as it regulates important features of prostate cancer cell motility [18]. However, reports from Bickeböller et al. show that MARCKS is a mediator of apoptosis in microsatellite-stable colorectal cancer cells, thus adding a novel tumor-suppressing function to already defined functions of MARCKS. MARCKS was found to be associated with adverse outcome in microsatellite-stable cancers [19]. These studies suggest that MARCKS has a broad spectrum of roles in a variety of cancers. Here, we demonstrated the role of MARCKS in GC tumorigenesis and progression. First, we demonstrated upregulation of MARCKS in the tumors and peri-tumor samples from patients with GC, which agrees with the analysis of the TCGA database. Our study also shows that MARCKS expression is associated with tumor differentiation and advanced TNM stage. Finally, multivariate Cox regression analysis proved that MARCKS is an independent predictor of prognosis in patients with GC. We found that MARCKS is a potential prognostic biomarker or therapeutic target for patients with GC, and MARCKS expression

| Variables                               | OS survival | DFS survival |
|-----------------------------------------|-------------|--------------|
|                                         | HR (95% CI) | P            | HR (95% CI) | P            |
| Gender (Male vs. Female)                | 1.003 (0.697–1.443) | 0.987 | 0.968 (0.673–1.392) | 0.968 |
| Age (≤60 vs. >60, years)                | 1.051 (0.748–1.476) | 0.774 | 1.071 (0.761–1.508) | 0.692 |
| Size of tumor (≤4 vs. >4, cm)           | 1.495 (1.000–2.233) | 0.050 | 1.439 (0.968–2.140) | 0.072 |
| Drinking (no vs. yes)                   | 0.737 (0.526–1.033) | 0.077 | 0.780 (0.556–1.093) | 0.149 |
| Differentiation (I–II vs. III–IV)       | 1.026 (0.727–1.448) | 0.883 | 1.000 (0.711–1.409) | 0.990 |
| Vessel (present vs. absent)             | 0.930 (0.660–1.312) | 0.681 | 0.999 (0.709–1.409) | 0.997 |
| Pathological T stage (I–II vs. III–IV)  | 1.308 (0.830–2.064) | 0.247 | 1.317 (0.833–2.081) | 0.238 |
| Pathological N stage (I–II vs. III–IV)  | 1.084 (0.764–1.539) | 0.650 | 1.126 (0.790–1.606) | 0.512 |
| Pathological M stage (M0 vs. M1)        | 1.412 (0.707–2.820) | 0.328 | 1.303 (0.655–2.592) | 0.450 |
| TNM stage (I–II vs. III–IV)             | 0.467 (0.292–0.747) | 0.001* | 0.531 (0.330–0.855) | 0.009* |
| MARCKS (low vs. high)                   | 0.408 (0.247–0.674) | 0.000* | 0.355 (0.216–0.584) | 0.000* |

Table 2. Multivariate Cox regression analyses for overall survival and disease-free survival in GC patients.
Figure 4. Effect of MARCKS on cell migration was observed by wound-healing assay. (A) Inhibition of MARCKS expression suppressed the motility of BGS-837 cells. (B) Inhibition of MARCKS expression suppressed the motility of SGC-7901 cells. n=3 independent experiments.

Figure 5. Effects of MARCKS on cell invasion was detected by Transwell assay (magnification, ×200). The graph indicates the mean ±SD and P-value of the number of cells per high-power fields counted from 3 independent experiments. * P<0.05.

Figure 6. Tumorigenesis ability was detected in vivo by a subcutaneous implantation model in nude mice. n=6 mice each group. * P<0.05.
is significantly enhanced in GC tissue compared with adjacent peri-tumor tissues. Given the correlation between MARCKS and tumor differentiation, we postulated that MARCKS has a crucial function in GC invasion and metastasis. Indeed, our data revealed that MARCKS can further enhance the progression of GC by promoting cell migration and invasion, as observed in both in vitro and in vivo studies. EMT as a cellular phenomenon is marked by migration, metastasis, and invasive properties of cancer cells [20]. Several reports found that cancer cell invasion, migration, and metastasis are triggered by EMT activation [21]. EMT is characterized by downregulation of the epithelial protein E-cadherin, and upregulation of mesenchymal proteins such as N-cadherin and vimentin [22]. In this investigation, we explored the underlying MARCKS-dependent process of GC cell invasiveness. Finally, we analyzed the effects of MARCKS depletion on expression of EMT molecular markers such as E-cadherin, N-cadherin, and vimentin in GC cells. Our study shows that MARCKS knockdown in GC cells results in a morphology with more EMT. GC cells having depleted MARCKS exhibit overexpression of E-cadherin and suppression of vimentin and N-cadherin. Thus, we speculate that MARCKS-mediated regulation of GC tumorigenesis and progression is dependent on the EMT pathway.

Conclusions

We evaluated the expression of MARCKS in 219 cases of GC by using IHC, and found that high MARCKS expression was significantly correlated with worse prognosis of GC. MARCKS is thus a strong candidate prognostic marker of gastric cancer, which could improve treatment for sustained clinical benefit.

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