ORIGINAL ARTICLE

CKIP-1 serves as a negative regulator and correlates with the degree of differentiation in gastric cancer

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Abstract: Gastric cancer (GC) is one of the most commonly diagnosed malignancies worldwide. CKIP-1 is a casein kinase-2 α-subunit (CK2α) interacting protein. Though previous reports have shown that CKIP-1 plays a critical role in several types of cancers, hardly there are any studies that examined the role of CKIP-1 in the progression of GC. Our present study aimed to investigate the role of CKIP-1 in GC. Results demonstrated low-level expression of CKIP-1 in GC tissues and cell lines. Moreover, knockdown of CKIP-1 promoted cell proliferation, migration, and invasion in GC cell lines, whereas CKIP-1 overexpression inhibited proliferation, migration, and invasion in the cells. Altogether, our data suggests that CKIP-1 may act as a novel tumor suppressor gene in GC and is related to GC differentiation.

Keywords: Gastric cancer, CKIP-1, wound healing, proliferation, migration, invasion

Introduction

Gastric Cancer (GC) is a common malignant tumor of upper digestive tract [1]. GC is the fourth most common type of cancer and its mortality rate ranks second in the world [2]. Lack of effective early detection methods and poor prognosis led to the low survival rate of GC patients. Patients with GC usually lack symptoms in the early stages, and efficient early detection methods are limited, leading to the diagnosis of GC in the advanced stages [3]. Hence, it is necessary to identify and develop effective treatment strategies for patients with advanced GC to improve their prognosis.

Casein kinase-2 interacting protein-1 (CKIP-1) is a pleckstrin homology domain-containing protein that was originally identified as an interacting protein of casein kinase-2 α-subunit (CK2α) [4]. CKIP-1 has been implicated in various functions to date, such as megakaryocytic differentiation, chronic heart failure, cardiac hypertrophy, human lung cancer cell proliferation and apoptosis, and osteoporosis [4-9]. Recent studies have suggested that CKIP-1 acts as a candidate in tumor suppression [10], such as colon cancer [11]. But the role of CKIP-1 in the development of GC is not yet known. Hence, we aimed to find the evidence if CKIP-1 is associated with the progression of colorectal cancer and whether the expression of CKIP-1 is related to the differentiation state.

Materials and methods

Tissue procurement

The archived paraffin blocks of formalin-fixed surgical specimens corresponding to 50 cases of gastric tissues were obtained from the Guizhou Provincial People’s Hospital. Fresh gastric tumor and adjacent normal-appearing gastric mucosal specimens were also obtained from the hospital. The specimens were neither treated by radiotherapy nor chemotherapy. The age of the patients ranged from 20 to 80 years. Patient characteristics were summarized in Table 1.

Antibodies and reagents

CKIP-1 and GADPH antibodies were purchased from Abcam (Cambridge, MA, USA) and Pro-
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Table 1. CKIP-1 expression characteristics patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n=50)</th>
<th>CKIP-1 protein expression</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative (n=16)</td>
<td>Weak (n=23)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>13 (5.3)</td>
<td>15 (41.7)</td>
</tr>
<tr>
<td>Male</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>3 (21.4)</td>
<td>8 (57.1)</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥50</td>
<td>32</td>
<td>8 (25)</td>
<td>17 (53.1)</td>
</tr>
<tr>
<td>&lt;50</td>
<td>18</td>
<td>8 (44.4)</td>
<td>6 (33.3)</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>27</td>
<td>9 (33.3)</td>
<td>12 (44.4)</td>
</tr>
<tr>
<td>No</td>
<td>23</td>
<td>7 (30.4)</td>
<td>11 (47.8)</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I+II</td>
<td>35</td>
<td>6 (17.1)</td>
<td>20 (57.1)</td>
</tr>
<tr>
<td>III+IV</td>
<td>15</td>
<td>10 (66.7)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well and mod</td>
<td>24</td>
<td>2 (83.3)</td>
<td>14 (58.3)</td>
</tr>
<tr>
<td>Poorly</td>
<td>26</td>
<td>14 (53.8)</td>
<td>9 (34.6)</td>
</tr>
</tbody>
</table>

Data are presented as number (%). Fisher’s exact probabilistic method was performed to determine the statistical significance of the relationship of CKIP-1 expression with various variables.

Human gastric cancer cell lines (AGS and MKN45 cells) and human normal gastric epithelial, GES-1 cells were maintained in RPMI 1640 (Hyclone, USA) containing 10% fetal calf serum and cultured at 37°C in 5% CO₂.

**Cell lines**

Human gastric cancer cell lines (AGS and MKN45 cells) and human normal gastric epithelial, GES-1 cells were maintained in RPMI 1640 (Hyclone, USA) containing 10% fetal calf serum and cultured at 37°C in 5% CO₂.

**Plasmids and transfection**

The human CKIP-1 cDNA was sub-cloned into a vector (PLenti CMV Puro Empty) to generate an overexpression vector (PLenti CMV-CKIP-1). The lentiviral expression vector for CKIP-1 shRNA was constructed by inserting shRNA sequence into a scramble shRNA vector (5’-CCTGAGTGACTATGAGAGCTTCTCATGTCACTCAGG-3’ (F) and 5’-CCTGAGTGACTATGAGAGCTTCTCATGTCACTCAGG-3’ (R)). The cells were transfected with jet PRIME (Polyplus, USA) according to the manufacturer’s instructions. Infective lentiviruses were produced by cotransfection of the expression vector and packaging plasmids into 293T cells, which were then added to MKN45 cells in the presence of 8 µg/ml polybrene. By 48 h post-infection, infected cells were selected using 1 µg/ml puromycin for about 14 days to generate the stable transfectants.

**Immunohistochemistry**

CKIP-1 protein expression was detected by immunohistochemical staining in gastric mucosal and cancer tissues. For more perspicuity, the samples were fixed in formalin and embedded in paraffin. The paraffin sections were deparaffinized and subjected to heat-induced epitope retrieval step. Endogenous peroxidase activity was blocked by using 1% hydrogen peroxide in distilled water. To block the non-specific binding, the slides were incubated with 3% bovine serum albumin for 60 min at room temperature. Samples were then incubated for overnight at 4°C with primary antibody (CKIP-1, 1:500). Negative controls were performed by omitting the primary antibody. The expression of CKIP-1 was semi-quantitatively evaluated by using immunohistochemical scoring model that assessed the sum of the extent of staining (1: 0-30%; 2: 30-60%; 3: 60-100%) and intensity (1: weak; 2: moderate; 3: strong). The assessment was made by a single senior pathologist (Y.C).

**Western blotting**

The cells were washed twice with PBS and resuspended in cold RIPA buffer containing 1 mmol/L phenylmethanesulfonyl fluoride and a cocktail of protease inhibitors (dilution, 1:100; Beyotime, Nantong, China). The cell samples were centrifuged at 12,000 rpm at 4°C for 15 min. Supernatants were recovered and total proteins were quantified using BCA Protein Assay kit (Beyotime). Total protein was extracted and separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred onto the PVDF membranes (Millipore, USA). After being blocked for 2 h at room temperature, the membranes were incubated with 1:1000 dilution of polyclonal anti-rabbit CKIP-1 antibody overnight. The proteins were then incubated with the corresponding secondary antibody for 1 h at room temperature. After washing, PVDF membranes were transferred into the Bio-Rad...
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Figure 1. CKIP-1 expression in normal gastric tissue and gastric cancer. Gastric cancer was categorized into high differentiated and low differentiated. Normal gastric tissues and gastric cancer cases were stained using hematoxylin and eosin (H&E), (Magnification, 100×) and immunohistochemistry of CKIP-1 (Magnification, 200×).

Figure 2. The expression of CKIP-1 in normal human gastric epithelial cell line, GES-1 and two human GC cell lines, AGS and MKN45 were determined by WB.

Wound healing scratch assay

For wound healing scratch assay, control cells, overexpression CKIP-1 MKN45 and knockdown CKIP-1 MKN45 cells were seeded in 6-well plates. Cells were cultured for 24 h at 37°C, and then the wound was made by scratching a line across the bottom of the dish on the monolayer of the confluent cells with a sterile P-200 pipette tip. The cells were gently flushed with PBS and supplemented with the medium. The same area of the gap was imaged by using a microscope equipped with a digital camera at 0, 24 and 48 h after scratching was performed.

CCK-8 assay

Cells were seeded in 96-well plates at an initial density of 1×10⁴ cells per well. Cells were cultured for 24, 36 and 48 h at 37°C. Then 100 μl serum free RPMI 1640 containing 10% CCK8 [Dojindo-Biochem (Shanghai, China)] reagent was added in each well, and the cells were cultured for 2 h at 37°C. The cell viability was then detected by using a microplate reader at 450 nm.

Cell migration and invasion

Cell migration assay was carried out using Transwell Permeable Support (Corning Incorporated, Corning, NY, USA). Control cells, Overexpression CKIP-1 MKN45 and Knockdown CKIP-1 MKN45 cells were carefully transfected on the top chamber of each transwell apparatus at a density of 1×10⁶ per ml. Cells were allowed to migrate for 24 h at 37°C. Cells that had penetrated to the bottom side of the membrane were fixed in formalin, stained using 0.1% Crystal violet solution (Beijing Solarbio Technology Co. Ltd, Beijing, China) and counted under a microscope.

Reagent was added in each well, and the cells were cultured for 2 h at 37°C. The cell viability was then detected by using a microplate reader at 450 nm.

Figure 1.

Figure 2.
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Figure 3. Phase-contrast microscopy image of the same wounded area at 0, 24, 48 h after scratching.

Figure 4. Cell numbers were counted in following time points: 24, 36, 48 h. Cell viability was measured using CCK8 assay. The experiment was repeated independently three times. *P<0.05, **P<0.01 (compared with control group).

Statistical analysis

SPSS 20.0 software was used to collect all the data. The data were given as mean ± standard deviation. One-way ANOVA was used to analyze the significance between groups. Significance was determined at P<0.05.

Results

Patient characteristics and CKIP-1 protein expression

To investigate the properties of gastric carcinoma that are associated with CKIP-1 expression, we identified 50 surgical specimens which were well characterized for clinicopathological parameters, including sex, age at diagnosis, metastatic state, TNM stage II and differentiation (Table 1).

Results revealed no correlation of CKIP-1 level with sex, age, and metastatic state (P>0.05). Meanwhile, strong CKIP-1 staining was detected in well and moderately differentiated gastric cancer than that in poorly differentiated gastric cancer (33.3% vs 11.5%, P<0.01). In this case, the expression of CKIP-1 protein was significantly correlated with the differentiation in the gastric cancer (P<0.01). Loss of expression of CKIP-1 or loss of its expression pattern may lead to poor differentiation. The CKIP-1 expressions of specimens of stages I or II also differed from those with stages III or IV (P<0.01). The expression of CKIP-1 in GC depends on the stage of GC, where high differentiated GC showed high expression, while low differentiated GC showed low expression (Figure 1).

Expression of CKIP-1 in gastric cancer cell lines

We examined the expression of CKIP-1 in GC cell lines, AGS (moderately differentiated adenocarcinoma) and MKN45 (poorly differentiated adenocarcinoma), and GES-1 as a normal gastric epithelial cell line. The expression of CKIP-1 was significantly elevated in normal gastric epithelial cell line, GES-1 compared with the cancer cell lines, AGS and MKN-45 (Figure 2).

Statistical analysis

SPSS 20.0 software was used to collect all the data. The data were given as mean ± standard deviation. One-way ANOVA was used to analyze the significance between groups. Significance was determined at P<0.05.

Results

Patient characteristics and CKIP-1 protein expression

To investigate the properties of gastric carcinoma that are associated with CKIP-1 expression, we identified 50 surgical specimens which were well characterized for clinicopa-
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Also a significant elevation was observed in moderately differentiated adenocarcinoma cells, AGS, than poorly differentiated adenocarcinoma cells, MKN-45 (Figure 2).

Wound healing of MKN45 cells in vitro

We examined whether CKIP-1 could regulate cell motility using a wound healing assay (Figure 3). Results showed that the migration rate of overexpression of CKIP-1 cells was significantly lower than control cells at 24 and 48 hours. Meanwhile, knockdown of CKIP-1 cells was significantly higher than control cells.

CKIP-1 affects MKN45 cell proliferation

Cell proliferation was measured by recording changes in absorbance (optical density, OD) at 450 nm wavelength. Results showed that overexpression of CKIP-1 significantly inhibited MKN45 cell proliferation at 24, 36 and 48 hours (Figure 4). In addition, we also found that knockdown of CKIP-1 in MKN45 cells resulted in a greater percentage of cell proliferation (Figure 4).

Effects of CKIP-1 on MKN45 cell migration and invasion

Transwell assay showed that CKIP-1 overexpression decreased cell migration and invasion of MKN45 cells, while knockdown of CKIP-1 increased cell migration and invasion of MKN45 cells (Figures 5A, 5B, 6A and 6B).

Discussion

GC is still one of the most common malignant tumors, and the 5-year survival rate of GC is reported to be less than 10% [12]. Notably, many genetic changes are involved in the invasion and metastasis of cancer cells [13].

CKIP-1 has been reported to regulate several important proteins that control cell survival, such as CK2, Akt, IFP53 and Smurf1. All these proteins are related to human cancer [14]. Besides, several important pathways of CKIP-1 negative regulation, for example, TGF-β/BMP
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and PI3K/Akt signaling, participate in the regulation of tumor stem cells [15, 16]. Recent studies have demonstrated CKIP-1 as a tumor suppressor protein in various cancers, such as lung cancer [17]. However, the role of CKIP-1 expression in GC is still unknown. Our results have demonstrated that the expression of CKIP-1 was downregulated in GC tissues and cells. Decreased expression of CKIP-1 was related to decreased differentiation status of GC. Furthermore, the protein expression of CKIP-1 was lower in GC cell lines (AGS and MKN45 cell lines), especially in the poorly differentiated adenocarcinoma cells. MKN-45 was very low. The CKIP-1 expression in cells and tissues was correlated with the degree of differentiation in GC. We next studied the functional role of CKIP-1 in GC cells. CKIP-1 overexpression repressed gastric cell proliferation, migration and invasion, while knockdown of CKIP-1 expression promoted cell proliferation, migration and invasion. Taken together, the present study provided direct evidence that CKIP-1 was an important tumor suppressor protein and correlated with the degree of differentiation in GC.

Acknowledgements

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Disclosure of conflict of interest

None.

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References


