Overexpression of ZNF797 (SALL4) gene in gastric cancer tissue, gastric cancer stem cell and MKN-45 cell line

ABSTRACT

Gastric cancer (GC) is one of the most common types of cancer and leading causes of mortality worldwide. Recent studies have shown that ZNF797 plays an important role in the development and progression of different cancers. In the present study, ZNF797 expression was examined in different grades of primary human gastric cancer, gastric cancer stem cell (GCS) and MKN-45 cell line cell line. Gastric cancer tissue samples were collected from 37 patients with gastric cancer. Furthermore, MKN-45 cell line, GCS and 10 normal gastric tissue samples were prepared. Expression of ZNF797 at mRNA and protein levels was considered using Real-time PCR, flow cytometry, immunohistochemistry and western blot methods. Expression of ZNF797 was significantly increased in 46% of patients (3.351±2.94; p<0.05) with gastric cancer. We also observed a significant increase in expression of ZNF797 in GCS (4.31±0.04; P<0.005). The mRNA expression level of ZNF797 in MKN-45 gastric cancer cell line was 2.81±0.07. Furthermore, ZNF797 expression was significantly correlated with tumor grade (p<0.01) and disease stage (p<0.01). ZNF797 may be involved in GC development and progression and can be considered as a prognosis factor for the diagnosis of this cancer, especially at the early stages. The oncogenic role of ZNF797 indicates that it may be a potential target for cancer therapy.

Key words: ZNF797, Cancer stem cell, MKN-45, expression analysis, real-time PCR.

INTRODUCTION

Gastric cancer is one of the most common types of cancer throughout the world, which has accounted for 3 to 10% of all cancer-related deaths (Bozzetti et al., 1999; Schwartz, 1996). Approximately, 90% of all gastric tumors are malignant, and gastric adenocarcinoma contains 95% of the total number of malignancies (Schwartz, 1996). The absence of early pathognomonic symptoms leads to a delayed diagnosis of the disease. As a result, 80 to 90% of patients with gastric adenocarcinoma present with locally advanced or metastatic tumors that have poor rates of respectability (Dicken et al., 2005; Parkin et al., 2001). Recent studies have indicated that at least 80% of patients with gastric cancer are being detected in advanced stages (Haidari et al., 2012; Malekzadeh et al., 2009). Gastric cancer not only represents heterogeneity in histopathological findings, but also multiple cellular and molecular pathways are involved in disease development (Dicken et al., 2005). Therefore, identification of these cellular and molecular mechanisms and genetic factors involved in gastric cancer development is valuable for the rapid diagnosis and treatment of the disease.

ZNF797 is a member of the SALL (Spalt-like transcription factor 4) gene family (SALL1 to SALL4), which has been reported to play an oncogenic role in gastric cancer (Forghanifard et al., 2013; Yang, 2018). It is originally cloned based on DNA sequence homology to Drosophila gene spalt (sal) and has two major isoforms: ZNF797A and ZNF797B (Forghanifard et al., 2013; Yang et al., 2011; Yang...
et al., 2012). ZNF797 is a zinc finger transcription factor which plays a critical role in the maintenance of pluripotency and self-renewal of embryonic stem cells through the regulation of NANOG, OCT4 and SOX2M (Xiong, 2014; Xiong et al., 2015). Human ZNF797 contains a single C2HC zinc finger adjacent the N-terminus and also multiple C2H2 zinc fingers in the middle portion of and at the C-terminus of the protein (Yang, 2018). C2H2 Zinc finger domains can bind to DNA, and in some cases, RNA and proteins. ZNF797 regulates Oct4 expression through binding to the highly conserved domain of Oct4 (Xiong et al., 2015). ZNF797 mutations can be associated with several abnormalities such as Okihiro syndrome (Duane-radial ray syndrome), Holt–Oram syndrome, acro-renal-ocular syndrome, and thalidomide embryopathy (Forghanifard et al., 2013; Miettinen et al., 2014; Wang et al., 2013; Yang, 2018). Overexpression of ZNF797 has been reported in patients with primary acute myeloid leukemia (Cui et al., 2006), myelodysplastic syndrome (Zhang et al., 2014), colorectal and breast cancers, as well as Wilms tumors and germ cell tumor (Cao et al., 2009; Cui et al., 2006; Han et al., 2014; Li et al., 2013; Liu et al., 2014; Miettinen et al., 2014; Rodriguez et al., 2014; Wang et al., 2013; Zhang et al., 2014; Zhang et al., 2015). Recent evidences have indicated that ZNF797 not only is an important marker of non-teratomatous germ cell tumors, but also it is expressed in other tumors such as gastric adenocarcinoma (Forghanifard et al., 2013; Rodriguez et al., 2014; Ushiku et al., 2010). Ushiku et al. (2010) found that ZNF797 expression in gastric cancer represents the retro-differentiation of tumor cells to precursor cells and regain the fetal phenotype. Furthermore, they found that overexpression of ZNF797 was correlated to hepatoid and fetal gut-like gastric tumors (Ushiku et al., 2010).

The present study aims to consider ZNF797 expression in the tissue samples of 37 patients with gastric cancer tissue as compared with healthy individuals. Furthermore, ZNF797 mRNA level is comparatively examined in different grades of human gastric carcinoma, gastric cancer stem cell (GCSC), as well as MKN-45 gastric carcinoma cell line.

METHODS AND MATERIALS

Study population

This study was approved by the ethics committee of Tehran University of medical sciences and written informative consent was obtained from all participants at the department of cancer research in Imam Khomeini and Labbafinejad hospitals. Gastric cancer tissue specimens were obtained from 37 patients (with a mean age of 38±9 years) who had not received any treatment just before surgery (Table 1). Gastric cancer was confirmed by surgical findings and pathological examinations. Clinical and pathological findings of patients, including disease stage, tumor grading, metastasis status, lymph node involvement, and etc. were recorded. Inclusion criteria for patients were: (i) patients with known gastric cancer; and (ii) complete clinical and pathologic information. Patients who met the following criteria were excluded from the study: (i) the presence of tumor in other places; (ii) previous treatments such as chemotherapy or radiotherapy; and (iii) history of other chronic diseases like diabetes mellitus, liver disease, and etc. All specimens were collected and transferred into RNA later Reagent (Qiagen, USA) and kept at 4°C. The tissue samples were washed with PBS and cut into smaller pieces and stored at −80°C until use.

MKN-45 cell culture

The human gastric adenocarcinoma cell line MKN-45 (NCBI NO: C615) was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 µg/ml penicillin in a humidified incubator containing 5% CO2 at 37°C.

Cell sorting by CD44 positive selection

The MKN-45 cell line was passed through a MACS column [Miltenyi Biotec, UK], including CD44 monoclonal antibody, thereby CD44+ cells were separated from other cell, and then they were cultured. The isolation of cells was performed according to the method of the articles written by Gao et al. (2012).

Flow-cytometry analysis

The CD44 positive cells separated from MKN-45 cell line in passage 2 (10^5-10^6 cells) were used for identification of phenotypic markers by flow-cytometry. For fluorescent antibody cell surface staining, cells were washed with HBSS + 2% BSA two times and incubated with the specific antibody at concentrations recommended by the respective manufacturer. Cells were incubated for 20 min and analyzed under flow-cytometry. The antibodies used were: CD90, CD44, CD133, CD34 and CD45 (abcam, UK). Primary antibodies were added to PBS at the concentrations recommended by the manufacturer. Incubation was performed for 30 min. Cells were then washed twice in PBS. Corresponding secondary antibodies with fluorescent conjugates were subsequently diluted in PBS at the concentrations suggested by the manufacturer instructions. Incubate was performed for 20 min and cells were analyzed using flow-cytometry (Becton Dickinson, Germany and BD biosciences Inc).
Table 1: Histological and clinical data.

<table>
<thead>
<tr>
<th></th>
<th>Total (n=37)</th>
<th>High ZNF797 (n=17)</th>
<th>Medium ZNF797 (n=16)</th>
<th>Low ZNF797 (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men/woman</td>
<td>21/16</td>
<td>10/7</td>
<td>12/4</td>
<td>2/2</td>
</tr>
<tr>
<td>Tumor grade I</td>
<td>17</td>
<td>12</td>
<td>5</td>
<td>---</td>
</tr>
<tr>
<td>Tumor grade II</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>---</td>
</tr>
<tr>
<td>Tumor grade III</td>
<td>10</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Tumor grade I/II</td>
<td>20</td>
<td>16</td>
<td>4</td>
<td>---</td>
</tr>
<tr>
<td>Tumor grade III/IV</td>
<td>17</td>
<td>3</td>
<td>12</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2: Primer sequence for ZNF797.

<table>
<thead>
<tr>
<th>Sequence (5'→3')</th>
<th>Product length</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZNF797 Forward primer: AGGAATTTCGTGGCGGAGG</td>
<td>308bp</td>
<td>61</td>
</tr>
<tr>
<td>Reverse primer: TGAAGAACTCCGGACACGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH Forward primer: CCCTTCATTGACCTCAACTACATG</td>
<td>115bp</td>
<td>59</td>
</tr>
<tr>
<td>Reverse primer: GGAGTTCCATTGATGACAAGC</td>
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</tr>
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Quantitative RT-PCR

RNA was extracted using Qiagen RNeasy kit. Complimentary DNA (cDNA) was synthesized from 1 µg of RNA, according to instructions in the cDNA synthesis kit (TAKARA, JAPAN). Real-time PCR analysis was performed by SYBR green PCR Master Mix (TAKARA, JAPAN), on a Rotor-Gene Q, real-time thermocycler (Qiagen, USA), and Real-time RT-PCR primers for ZNF797 and GAPDH were designed by Allele ID software 6.0 (PREMIER Biosoft, CA, USA) (Table 2). The thermal profile included 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 57°C, and 45 s at 72°C. Data were normalized to GAPDH expression applying the comparative threshold cycle method. The PCR efficiencies for ZNF797 and GAPDH were verified by generating related standard curves. The level of relative for ZNF797 expression was compared based on fluorescence intensity changes of samples from endometriosis as compared with compliant normal tissues. A more than two-fold increase in expression was considered to be over-expression, while a more-than-two-fold decrease was considered to be under-expression. The range between those two values was interpreted as no change or normal expression. All experiments were performed in triplicate.

Immunohistochemistry

Immunohistochemistry (IHC) analysis was performed as previously described by Qiao et al. (2002). Briefly, paraffin embedded tissue sections were deparaffinized and rehydrated. After that, slides were treated with 3% H2O2 for 10 min and 2% BSA for 30 min at room temperature before incubation overnight with 2 µg/ml monoclonal Anti-Sall4 antibody (ZNF797) (abcam, UK) antibody at 4°C. Slides were washed 3 times in PBS and then incubated with an HRP-linked anti-mouse secondary antibody for 30 min. The slides were again washed 3 times with PBS, followed by chromogen detection with DAB for 10 min, and hematoxylin counterstaining.

Western blot analysis

Samples were collected and lysed with standard RIPA buffer. After centrifugation at 40,000 g at 4°C for 45 min, the supernatant was subjected to SDS-PAGE analysis. 100 µg of protein from each sample (n=2), CD44 positive GCSCs (n=1) and MKN-45 gastric cancer cell line (n=1) were subjected to 12% SDS-PAGE. Proteins were transferred to the nitrocellulose membrane (Millipore, USA) using Western-Blot technique followed by blocking using 5% bovine serum albumin (BSA, Sigma, Germany) for 60 min at room temperature with shaking. The membranes were then incubated with Mouse anti-SALL4 (abcam, UK) antibody (1:1000 dilutions) for 1 h followed by the incubation of membranes with HRP conjugated anti-mouse antibody at RT with mild shaking. Enhanced chemiluminescence (ECL)
western blotting system (GE Healthcare, USA) was used to develop the membrane on high performance chemiluminescence film (GE Healthcare) according to the company guidelines. After each step, the membrane was washed with PBS (Qiao et al., 2002; Taubert et al., 2007).

**Statistical analysis**

All data were analyzed using the SPSS software. Correlation between ZNF797 expression in various stages and grades was assessed by Pearson's correlation test. The ANOVA, Tukey test was applied to compare gene expression patterns between all groups. A P value <0.05 was considered to be statistically significant.

**RESULTS**

**Isolation and identification of cancer stem cells**

Gastric cancer stem cells (CD44 positive cell) were successfully isolated from MKN-45 gastric adenocarcinoma cell line after positive immune-selection to deplete CD44 cells (Figure 1). The morphological appearance of gastric cancer stem cells (CD44 positive cell) is different from MKN-45 cell line (Figure 1). Gastric cancer stem cells (CD44 positive cell) appeared as cuboidal-shaped cells with scant cytoplasm and granules around the nuclei (Figure 1B). As compared to the MKN-45 cell line (Figure 1A) derived from the adenocarcinoma patients, gastric cancer stem cells had same proliferation capacity. Flow-cytometry analyses showed that gastric cancer stem cells were strongly positive for surface markers CD44, CD105, CD90, but negative for CD45 and CD34.

**ZNF797 expression**

The expression of ZNF797 in CD44 positive gastric cancer stem cells, MKN-45 cell line, normal and cancerous gastric tissues was analyzed using RT-PCR (Figure 2). ZNF797 was highly expressed in gastric adenocarcinoma samples and CD44 positive GCSCs (Figure 2A), while it was downregulated in tissue samples of normal groups (Figure 2B). Hematoxylin and Eosin staining of gastric cancer and normal tissues can be seen in Figure 2C and D. IHC analysis showed increased expression of ZNF797 in gastric cancer tissues of the patients (Figure 2E), while it was negative in control group (Figure 2F). Western blot analysis also shown increased expression of ZNF797 in gastric tissue (Figure 2G).

**ZNF797 mRNA expression in different disease stages and grades**

There was a significant difference in expression of ZNF797 in patients with different disease stages and grades. Twenty patients (54%) had disease stage I/II (Figure 3), and 17 patients (46%) had disease stage III and IV. ZNF797 gene expression by about 3.934 indicates an early stage, and expression of about 1.762 represents the advanced stages of gastric cancer (Figure 3). The mean expression of ZNF797 mRNA levels in patients with disease stage I/II was significantly higher than that in patients with disease stage III/IV (p<0.001; Figure 3).

There was also a significant difference in the mean expression of ZNF797 mRNA levels between patients with different disease grades (p<0.01). Seventeen patients (46%) were in grade I, 10 (27%) patients in grade II, and 10 patients (27%) in grade III (Figure 3). The mean expression of ZNF797 mRNA levels in grade I was significantly higher than that in patients with disease grades II and III (p<0.01; Figure 3).

**DISCUSSION**

Gastric carcinoma is one of the most common malignant cancers in the world which is often associated with poor prognosis (Xiong, 2014; Yang et al., 2011). Tumor metastasis and recurrence is the major obstacle for long-term survival of patients with gastric carcinoma (Haidari et al., 2012, Malekzadeh et al. 2009). Therefore, early diagnosis and treatment of this disease is crucial to improve survival rate in patients. Furthermore, it is important to screen the genes involved in the process of cancer development and progression.

SALL4 is a newly identified oncogene which promotes tumorigenesis, tumor growth and metastasis through the regulation of several downstream genes (Tatetsu et al., 2016). It is a transcription factor that plays an important role in embryonic development and self-renewal of embryonic stem cells. Some studies found increased expression of SALL4 in various cancers such as Yolk Sac tumor, breast cancer, testicular germ tumors and non-small cell lung carcinomas (Cao et al., 2009; Cui et al., 2006; Han et al., 2014; Li et al., 2013; Liu et al., 2014; Miettinen et al., 2014; Rodriguez et al., 2014; Ushiku et al., 2010; Wang et al., 2013; Xiong et al., 2015; Yanagihara et al., 2015; Zhang et al., 2015). Nevertheless, ZNF797 function in gastric cancer and gastric cancer stem cells has not been fully elucidated (Han et al., 2014; Liu et al., 2014; Zhang et al., 2014). In this study, we considered the expression of ZNF797 in both protein and mRNA levels in CD44 positive gastric cancer stem cell, MKN-45 cell line, as well as normal and gastric cancer tissues using real-time quantitative PCR, western blot assay, and immunohistochemical staining methods. Our findings showed overexpression of ZNF797 in CD44 positive gastric, MKN-45 cell line and gastric cancer tissue. We found overexpression of ZNF797 in 46% of gastric cancer specimens and 43.24% of culture medium. Our findings are in agreement with the results of previous studies that reported upregulation of ZNF797 and its oncogenic role in...
cancer development and progression. For example, Liu et al. (2014) found upregulation of ZNF797 mRNA in 103 cases with gastric cancer. They also reported that overexpression of ZNF797 was associated with a poor gastric cancer prognosis. Another study reported increased expression of SALL4 in 86% of patients with breast tumors (Dirican and Akkiprik, 2016). Zhang et al. (2014) demonstrated that SALL4 was abnormally expressed at both mRNA and protein levels in human gastric cancer tissues. Additionally, SALL4 level was significantly associated with lymph node metastasis (Zhang et al., 2014). A more recent study has reported that increased expression of SALL4 promotes the motility, migration, and invasion abilities of gastric cancer cells in vitro (Zhang et al., 2018). In another study, Yuan et al. (2016) demonstrated that SALL4 knockdown greatly inhibited the proliferation, migration and invasion of gastric cancer cells. This result indicates that SALL4 promotes gastric cancer progression, representing a new target for gastric cancer therapy. Similarly, Jiang and Wang (2018) showed that knockdown of SALL4 by miR-16 is associated with cancer cells proliferation and migration.

Our data have also showed that increased expression of ZNF797 in gastric cancer tissue is associated with the disease stage and grade. Patients with diseases stage I/II and grade I had significantly higher ZNF797 expression as compared with patients with disease stage III/IV and grades II and III. These data indicate that ZNF797 may be involved in gastric cancer development and progression and, therefore, can be considered as a potential prognostic biomarker for patients with gastric cancer, especially at early stages. Several studies showed the relationship between ZNF797 expression with clinical and pathological findings of different cancers. For example, Deng et al. (2015) showed that increased expression of SALL4 in intrahepatic cholangiocarcinoma was associated with more frequent lymph nodal metastasis, vascular and nerve invasion, and shorter overall survival. Ushiku et al. (2010) observed that ZNF797 overexpression was significantly associated with older age, male sex, intestinal-type histology, and synchronous hepatic metastasis in gastric carcinoma (Ushiku et al., 2010). Similarly, our finding showed that ZNF797 mRNA level in male patients was relatively higher than in female patients; however, larger sample size is necessary to confirm this relationship.

Although some studies have shown increased expression of ZNF797 in different cancer types, the exact mechanism of its action on cancer progression and metastasis is in not well-understood. Recent evidence has indicated that SALL4 overexpression activates TGF-β/SMAD signaling pathway and subsequently epithelial–mesenchymal transition in gastric cancer cells which in turn promotes the metastasis of gastric cancer (Zhang et al., 2018). Using gastric cancer stem cells, MKN-45 gastric adenocarcinoma cell line and human gastric tumor samples, we showed that ZNF797 is tumorigenic and serves as an oncogenic factor. Therefore, according to these data, SALL4 plays oncogenic roles in cancer development and progression and can be considered as a new target for gastric cancer therapy.

**Conclusion**

Our results demonstrated that ZNF797 expression in both mRNA and protein level was significantly increased in gastric cancer tissues as compared with normal specimens. Furthermore, the expression of ZNF797 in gastric cancer stem cells was significantly higher than in normal gastric tissues. We also found a close relationship between ZNF797 expression and gastric cancer stage and degree. These results suggest that ZNF797 may be involved in the development and progression of gastric cancer and can be considered as a prognosis factor for the diagnosis of this cancer, especially at the early stages. The oncogenic role of ZNF797 indicates that it may be a potential target for cancer therapy.

**ACKNOWLEDGEMENTS**

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