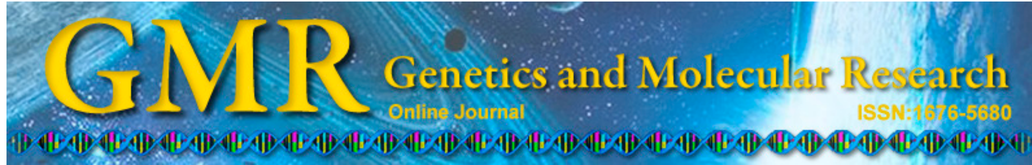

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ZNF797 plays an oncogenic role in gastric cancer

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ABSTRACT. Human cancer cells resemble stem cells in expression signatures leading them to share some features, most notably, self-renewal. A complex network of transcription factors and signaling molecules are required for continuation of this trait. ZNF797 (SALL4) is a zinc finger transcriptional activator crucial for maintenance of self-renewal in stem cells; however, its expression level has not yet been elucidated in gastric tumor cells. Its expression was analyzed to determine this level and probable clinicopathological consequences. SALL4 expression in fresh tumor and distant tumor-free tissues from 46 colorectal samples was compared by real-time polymerase chain reaction. Greater than a 2-fold increase in SALL4 expression was detected in 89.5% of tumors *vs* normal related tissues. SALL4 expression was significantly correlated with tumor cell metastasis to lymph nodes, especially in moderately differentiated tumor samples ($P < 0.05$). Furthermore, higher levels of SALL4 mRNA expression were significantly associated with younger patients with tumor cells in stages I and II ($P < 0.05$). These results indicate a relationship between SALL4 expression and tumor cell metastasis to lymph nodes and consequent progression of tumors to advanced stages III and IV. Along with the promising evidence of its role in self-renewal in various

cancers, SALL4 is introduced as a potentially interesting therapeutic target to reverse a number of aberrations that promote gastric tumor development and maintenance. This result may lead to new approaches for cancer therapy.

Key words: Gastric cancer; SALL4 expression; 5' Nuclease assay

INTRODUCTION

Annually, nearly 600,000 people die from the third most prevalent tumor type in the world, gastric cancer (Pisani et al., 1999). Gastric cancer is also one of the most frequent malignancies in Iran. Several investigators have reported that in about 40-50% of gastric cancer patients who undergo tumor resection, there is a subsequent metastasis through the bloodstream or lymphatic circulation to other organs which decreases their 5-year survival. Therefore, early detection with accurate methods is significantly required (Cenitagoya et al., 1998). The SALL gene family, including four members (SALL1 to SALL4), was initially cloned based on DNA sequence homology to the Drosophila gene spalt (sal) (Kohlhase et al., 1996). Sal is an essential homeotic gene for the development of the fly (Kuhnlein and Schuh, 1996). The human SALL gene family is involved in normal development. Structural properties of human SALL consist of several C2H2 zinc finger domains that can bind DNA and in some cases RNA and proteins (Kohlhase et al., 2002a). In embryonic stem cells (ESCs), SALL4 has significant roles in the maintenance of pluripotency and self-renewal, efficient proliferation/stabilization, and cell fate decision (Zhang et al., 2006; Yuri et al., 2009). It is also engaged in the maintenance of human adult stem cell features (Yang et al., 2010). Depending on ESCs' context, the transcription factor SALL4 activates or represses various transcriptional networks involved in self-renewal and pluripotency by regulating crucial transcription factors and epigenetic modulators. SALL4 is also engaged in the regulation of chromatin remodeling by bridging transcriptional regulation and epigenetic regulation in stem cells (Yang et al., 2008). Having direct interaction with key cell-signaling pathways such as Wnt and TGF-beta, SALL4 can play essential roles in cell fate decision and survival of ESCs (Ma et al., 2006; Shuai et al., 2009). SALL4 is an important regulator of the stemness state and survival, not only in several types of normal stem cells but also in cancer cells and possibly cancer stem cells (Lim et al., 2008). In adults, SALL4 expression is normally restricted to CD34+ hematopoietic stem/progenitor cells (Ma et al., 2006), and in adult mice, SALL4 is also predominantly expressed in testes and ovaries (Kohlhase et al., 2002b). Nonetheless, SALL4 expression is reported in numerous malignancies, such as precursor B-cell lymphoblastic lymphoma (Cui et al., 2006), myelodysplastic syndromes (Ma et al., 2006), acute myeloid leukemia (Shuai et al., 2009), endometriotic samples (Forte et al., 2009), ovarian germ cell tumors (Cao et al., 2009a), all types of testicular germ cell tumors (GCTs) (Cao et al., 2009c), all metastatic seminomas/dysgerminomas and embryonal carcinomas (Cao et al., 2009b), and primary mediastinal yolk sac tumors (Liu et al., 2010). It is suggested that SALL4 may not only be important in the pathogenesis of GCTs, especially to maintain their poorly differentiated status, but can also be used as a highly specific marker to confirm the germ cell origin of a metastatic tumor, due to its sensitivity and specificity (Cao et al., 2009a,b,c). An intricate network of genetic and epigenetic aberrations involving various signaling pathways is responsible for the development of gastric cancer. Having considered its involvement in tumorigenesis, progression, and

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aggressiveness of various tumors, our aim in this study was to analyze SALL4 expression and its impact on clinicopathological features in gastric cancer.

MATERIAL AND METHODS

Fresh tumor and distant tumor-free gastric tissues were obtained during gastric surgery from 46 patients who had not received any other therapeutic intervention such as chemo- or radiotherapy. After collection, the specimens were immediately treated with RNA later and stored at -20°C until extraction. Histopathological characteristics such as tumor size, location, and differentiation grading were recorded.

cDNA synthesis and quantitative RT-PCR

RNA extractions and cDNA syntheses were performed. Quantitative real-time PCR was carried out using SYBR green PCR Master Mix (Fermentas, Lithuania), containing ROX as a reference dye on a Stratagene Mx-3000P real-time thermocycler (Stratagene, La Jolla, CA, USA) with the primers presented in Table 1. 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 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression applying the comparative threshold cycle method. The PCR efficiencies for GAPDH and SALL4 were verified by generating related standard curves. The relative levels of SALL4 gene expression were compared based on fluorescence intensity changes of samples from tumor *vs* corresponding normal tissues. A more than 2-fold increase in expression was considered overexpression, while a more than 2-fold decrease was considered underexpression. The range between those two values was interpreted as no change or normal expression. All experiments were performed in triplicate.

Table 1. Primer sequence.

	Forward primer sequence	Reverse primer sequence
SALL4	CCAAAGGCAACTTAAAGGTTCAC	CCGTGAAGACCAATGAGATCTC
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATATCCACT

Statistical analysis

Data were analyzed using the SPSS 19.9 statistical package (SPSS, Chicago, IL, USA). Depending on requirements, either the χ^2 or Fisher exact test was applied to assess the correlations between gene expression and various histopathological features. An independent sample *t*-test and ANOVA were also used to correlate gene expression levels and different categorical data. $P < 0.05$ was considered to be statistically significant.

RESULTS

Fresh-frozen gastric tumors and corresponding normal-margin specimens of 46 newly diagnosed patients were obtained during surgery prior to any other treatments, ensuring that histopathological characteristics of gastric tissues were not affected by therapeutic intervention. According to microscopy, all tumor samples contained more than 75% tumor cells, with

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rare or no infiltrating cells. This procedure helped to ensure precise evaluation of gene expression in tumor cells compared to normal cells. SALL4 expression was analyzed by reverse transcription and real-time PCR amplification. The mean age ± standard deviation (SD) of the enrolled patients was 53.80 ± 14.89 years (age range = 21-86). Clinicopathological features of the patients are presented in Table 2.

Table 2. Clinicopathological features of the patients and correlation with ZNF797.

Factor	N (%)	P value
Gender		0.030
Female	20 (43.5%)	
Male	26 (56.5%)	
Grade		0.020
PD	1 (2.2%)	
WD	29 (63%)	
MD	16 (34.8%)	
Tumor invasion		0.53
T1, T2	8 (17.4%)	
T3, T4	38 (82.6%)	
Stage		0.39
I/II	34 (73.9%)	
III/IV	12 (26.1%)	
Lymph node metastasis		0.047
N0	33 (71.7%)	
N1	9 (19.6%)	
N2	4 (8.7%)	

PD, WD, and MD = poorly, well, and moderately differentiated, respectively.

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Upregulation of SALL4 in gastric samples

We compared SALL4 mRNA expression in 46 tumor specimens to their paired normal specimens by quantitative real-time RT-PCR. Significant overexpression of SALL4 mRNA was detected in 40 of 46 tumor specimens (87%, $P < 0.0001$). The minimum and maximum mRNA expression changes were -5.28- and 14.30-fold, respectively (5.33 ± 3.59). Furthermore, the related means and standard deviations were 6.37 ± 2.36 in patients with SALL4 overexpression and 1.61 ± 2.42 in other patients. Figure 1 schematically compares expression levels in the two groups.

Association of SALL4 expression with clinicopathological variables

We observed SALL4 expression correlated with multiple indices of poor prognosis. SALL4 expression was significantly associated with tumor cell metastasis to lymph nodes ($P < 0.05$). Of nine patients with lymph node metastasis, all (100%) overexpressed SALL4 (6.45 ± 1.38), while in 15.2% of patients (5 of 33) without metastasis to lymph nodes, SALL4 was not overexpressed (4.68 ± 3.72). Additional, expression of SALL4 was associated with the grade of tumor cell differentiation. SALL4 overexpression was significantly correlated with gender ($P = 0.030$, correlation coefficient: 0.384). SALL4 gene expression was significantly higher in males than in females (5.25 ± 3.60 and 3.96 ± 2.45, respectively). In advanced tumor stages (stages III and IV), a significant inverse correlation was observed between SALL4 overexpression and the number of involved lymph nodes ($P = 0.006$, correlation coefficient: -0.737). In addition, in tumor samples without invasion to adventitia (T1, T2), SALL4 gene expression significantly correlated with patient age ($P = 0.033$, correlation coefficient: -0.967).

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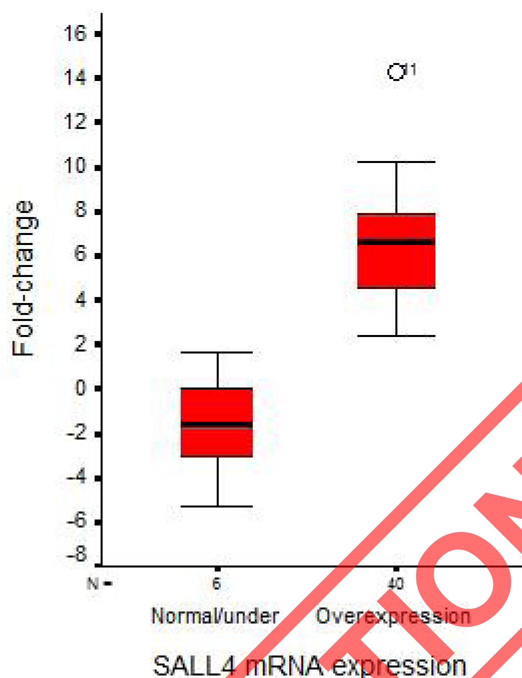

Figure 1. Schematically compared SALL4 mRNA expression levels in tumor specimens to their paired normal specimens by quantitative real-time RT-PCR.

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DISCUSSION

Expression analysis of SALL4 in normal and tumor gastric tissues revealed overexpression of SALL4 in a nearly 90% of gastric samples. We conclude from this result that increased expression of this stem cell transcription factor contributes to gastric tumorigenesis. Analysis of different aspects of SALL4 may help us to understand how SALL4 functions in gastric cancer pathogenesis. SALL4, a member of the spalt family, is a homeotic gene originally identified in *Drosophila* as a transcription factor required for development (Frei et al., 1988). Having essential roles in development in mammals, SALL4 is vital for the maintenance of pluripotency, self-renewal, efficient proliferation/stabilization, and cell fate decision of ESCs (Elling et al., 2006; Zhang et al., 2006; Yuri et al., 2009). SALL4 is also involved in the maintenance of human adult stem cell properties (Yang et al., 2010). In stem cells, SALL4 functions as both an activator and a repressor of gene transcription depending on the cell context. It suppresses important differentiation genes and activates key pluripotency genes (Elling et al., 2006; Lim et al., 2008). It acts by regulating critical transcription factors and epigenetic modulators to activate or repress different transcriptional networks in self-renewal and pluripotency in ESCs, probably by recognizing specific DNA binding sites (Lim et al., 2008; Lu et al., 2009). The transcriptional network of SALL4/OCT4/Nanog is crucial for the maintenance of "stemness" of ESCs (Lim et al., 2008), whereas SALL4, as a core factor, plays a dominant role in this regulatory network (Yang et al., 2010). It activates OCT4 as a transcriptional factor and interacts with Nanog in a protein-protein complex (Wu et al., 2006; Liang et al., 2008). Due to

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the same genomic binding sites of SALL4 and Nanog, reciprocal regulation of these proteins is suggested in governing pluripotency and self-renewal of ESCs (Wu et al., 2006). Interestingly, overexpression of OCT4 and Nanog has been shown in a gastric cell line and samples (Zhang et al., 2010; Huang et al., 2012). These data, along with SALL4 overexpression, which is reported here, lead us to hypothesize the existence of similar activated regulatory transcriptional networks in gastric cancer playing essential roles in proliferation and self-renewal characteristics of tumor cells. Considering the existence of the stemness regulatory network of SALL4/OCT4/Nanog in gastric cells, we suggest that the pathogenesis of gastric malignancy maybe related to abundance and contribution of stem cell-like cells in the tumors. Our results support the hypothesis of cancer stem cell-based tumor development and progression.

Chromatin remodeling plays essential roles in stem cell fate decisions. SALL4 may regulate this process by connecting transcriptional regulation and epigenetic regulation in stem cells (Yang et al., 2008). SALL4 can recruit either the Mi-2/nucleosome remodeling and deacetylase (NuRD) complex, epigenetic repressor, or histone methyltransferase complex to specific promoter regions, resulting in histone deacetylation or H3K4 trimethylation, respectively. By balancing the interactions with both complexes, it can repress or activate the transcription of target genes (Lu et al., 2009). SALL4 increases the trimethylation levels of histones H3-K4 and H3-K79 in the Bmi-1 promoter in association with a methyltransferase *in vivo* (Yang et al., 2007). Bmi-1 is a component of a polycomb group (PcG) multiprotein complex required to maintain the transcriptionally repressive state of many genes, including Hox genes, throughout development (Rajasekhar and Begemann, 2007). Our data showed SALL4 overexpression in significant correlation with lymph node metastasis, and epithelial mesenchymal transition. Furthermore, Bmi-1 is overexpressed in gastric samples associated with degree of tumor size, depth of invasion, lymph node metastasis and worse prognosis of carcinomas, in gastric cancer (Lu et al., 2012). Combining these data and results, we propose that the role of SALL4 in lymph node metastasis may be due to its role in epigenetic modulation of Bmi-1, leading to repression of the genes involved in epithelial cell differentiation. In conclusion, this study elucidates the clinical importance of stem cell marker SALL4 expression at the mRNA level for the first time in gastric cancer. We have shown SALL4 to be a new molecular marker of metastatic tumor. Its oncogenic role is confirmed by its overexpression in gastric cancer and its correlation with lymph node metastasis, a determinant of poor prognosis. SALL4 overexpression may indicate the existence of a stemness regulatory network in gastric cells. This can support the hypothesis of cancer stem cell-based tumor development and progression in gastric cancer. Connecting the epigenetic and genetic programs and contributing in different signaling pathways, SALL4 is expected to initiate a cascade resulting in tumor invasion and metastasis.

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