IJHOSCR

Original Article

International Journal of Hematology-Oncology and Stem Cell Research

The Expression Analysis of *MEST1* and *GJA1* Genes in Gastric Cancer in Association with Clinicopathological Characteristics

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Received: 20, Jul, 2022
Accepted: 06, Nov, 2023

ABSTRACT

Background: Gastric cancer is an invasive cancer, which is usually diagnosed in advanced stages. However, the markers affecting its progression, and invasion are of great importance in its diagnosis and treatment. The current research aimed to study the correlation of genes that contributed to epithelial-mesenchymal transition (EMT), *Mest1,* and *GjA1*, with some clinicopathological specifications in gastric cancer patients to better comprehend the functions of these genes in this tumor.

Materials and Methods: RNA was extracted from the tumor, and normal tissues and cDNA were synthesized. Then, by designing specific primers for *Gja1* and *Mest1* genes, their expressions were studied by RT-PCR. The data was analyzed by GraphPad Prism 8 software.

Results: Significant differences among the expressions of mentioned genes associated with clinicopathological variables of gastric cancer patients, including tumor size, grade, stage, metastasis, and lymphatic invasion were seen.

Conclusion: The obtained data showed the important role of EMT-related genes, *Gja1* and *Mest1* in the clinical progression of the tumor. Further studies with larger sample sizes are required to confirm these genes as biomarker candidates for detecting gastric cancer.

Keywords: Gene; Expression; Gastric; Cancer; Mest1; GjA1

INTRODUCTION

One of the most common cancer types is gastric cancer which affects a large number of people each year[1]. There is an increasing prevalence of this disease among men, with a higher incidence among men than women[2]. Diagnosis of the disease occurs after metastasis, at an advanced stage when symptoms are frequently absent. This significantly diminishes the likelihood of effective treatment[3]. Epithelial– mesenchymal transition (EMT) is one of the most important and necessary processes in the metastasis process, which is associated with decreased cell adhesion followed by increased cell migration[4]. Meanwhile, changes in the expression of some genes are very important factors in EMT. There are several studies suggesting that type I EMT proteins and genes could have a functional role in type III EMT, such as FGF-10[5,6]. Recently, our study indicated different expressions of VIM and fibronectin genes in different colorectal cancer lines, and expressions of Scinderin and Gelsolin genes in gastric cancer[7]. Mesoderm-specific transcript (MEST) is a type I EMT transcription factor. The high expression of *MEST* in EMT in breast cancer was shown via a switching promoter,

and the involvement of this transcription factor has been reported in EMT[8, 9]. The pseudogenes of Mest1 are located on the short arm of chromosomes 3 and 4, as well as the long arm of chromosomes 6 and 15. This gene is highly expressed in the early embryo [10].

The GJA gene family encodes connexin (Cx) or gap junction proteins. These proteins act as cell channels, connexons, and binding channels[11]. It was documented that these channel structures play a role in a variety of cellular responses that occur in pathological states. Prior studies have demonstrated the involvement of the Mest1 and GJA1 genes in extracellular matrix (EMT), indicating their potential contribution to cancer metastasis [12]. Assuming these genes are involved in the EMT, the current research was conducted to evaluate the relationship between the expression levels of those genes and clinicopathological specifications in gastric cancer patients.

MTERIALS AND METHODS

The present study was approved by the ethics committee of Tehran University of Medical Sciences (NO, IR.IAU.PS.REC.1397.313). The current research was designed as a case/control study. All samples (including 30 tumors and 30 normal adjacent fresh frozen tissues) stored in -80°C for further procedures and demographic, morphological, and clinical data were recorded.

RNA Extraction and cDNA Synthesis

Cellular RNA extraction was performed using RNA extraction kit (Sinaclon Cop, Iran) based on manufacturer's instructions. To determine the purity and concentration of extracted RNA, the study made use of Nanodrop (Nanodrop Spectrophotometr, Thermo scientific) and 1% agarose gel electrophoresis. BioFact kit was applied to synthesize cDNA. Thermal program included 94 **°C** for 4 min, 94 °C for 30s, 62 °C for 30s, and 72 °C for 30s.

Primer Design

β-actin gene was used as control. Primer designs for *GjA1* and *Mest1* genes were done using Primer3 software[13]. Table 1 shows the designed primer sequences

RT-PCR

The expression levels of the studied genes were assessed using RT-PCR technique. In this study, cybergreen kit (Biofact) and Real Time PCR device (Rotor-Gene Q) were used. The final reaction volume was 20 µl (10 µl of cybergreen, 7 µl double distilled water, 1 µl forward primer, 1 µl reverse primer, and 1 µl cDNA). At the end of reaction, the melting curve of the products was plotted from 55 to 95 ° C and the presence of a specific peak point in the curve was investigated

Statistical analyses

$2^{-\Delta\Delta C}$T method was used to evaluate the relative change in the expression levels of the studied genes. Quantitative and qualitative variables were presented as mean±SD and percentage (%), respectively. The value of delta ct was used for comparing tumor and normal adjacent tissues. To evaluate the significant changes in expression levels of *GjA1* and *Mest1* genes in tumor and normal tissues t-test was used. The probability levels of p <0.05 was considered statistically significant. GraphPad Prism V.8 software (San Diego, California, USA) was used to analyze the data.

RESULTS

Patients' demographic, clinical, and morphological data

Table 2 shows the demographic, clinical, and morphological characteristics of patients.

Table 1: The sequences of primers for *GjA1*, *Mest1*, and *β-actin* genes

Genes	Sequences
GjA1-F	5'- TCCAGTCACCCATGTTGCC-3'
GjA1-R	5'- CTGACTGCCTGAACTTGCCTT-3'
Mest1-F	5'- AAGACTCTGTGGGTGTGGTT-3'
Mest1-R	5'- AGGATGAGGGAGTGGTGGG-3'
β-actin-F	5'- CACCATTGGCAATGAGCGGTTC-3'
β-actin-R	5'- AGGTCTTTGCGGATGTCCACGT-3'

Table 2: Patients' demographic, clinical, and morphological data

Attributes	Levels	Patient n (%)
Age		
	<65	18 (64.3)
	≥65	10 (35.7)
Stage		
	I	11 (36.7)
	II	8 (26.7)
	III	4 (13.3)
	IV	3 (10.0)
Gender		
	Male	23 (76.7)
	Female	7 (13.3)
M stage		
	0	25 (83.3)
	1	(13.3)
Grade		
	I	5(16.7)
	II	15(50.0)
	III	8 (26.7)
	IV	2 (6.7)

Mest1 and GJA1 expressions in normal and tumor cells

There was no significant difference in the expressions of *GjA1* and *Mest1* genes between gastric tumor and normal adjacent samples (*P*> 0.05). The expression level of *GjA1* gene in normal (1.02± 0.05) and in tumor (1.08± 0.06) tissues did not show a statistically significant difference. Furthermore, the expression of *Mest1* gene was 0.99±3 0.03 and 1.01± 0.04 in normal and tumor tissues, respectively, indicating that there was no significant difference (Figure 1).

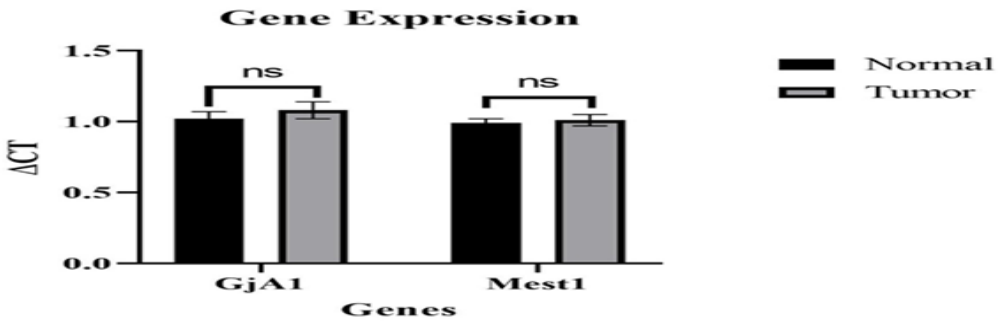

Figure 1: Comparing *Mest1* and *GjA1* gene expressions between tumor and normal tissues. ns: non-significant

The association of Mest1 and GJA1 gene expressions with Tumor size

The expressions of *Mest1* and *GjA1* genes were studied in 3 tumor groups with tumor sizes ≤3cm, 3-6cm, and ≥6 cm. There were significant differences in the expressions of *GjA1* and *Mest1* genes between different tumor sizes (*P* <0.0001). The higher expression of *GjA1* gene was observed in tumors smaller than 3 cm and larger than 6 cm compared to 3-6 cm tumors. On the other hand, *Mest1* expression was lower in the tumors larger than 6 cm, and maximum expression of *Mest1* gene was observed in 3-6 cm tumors (Figure. 2).

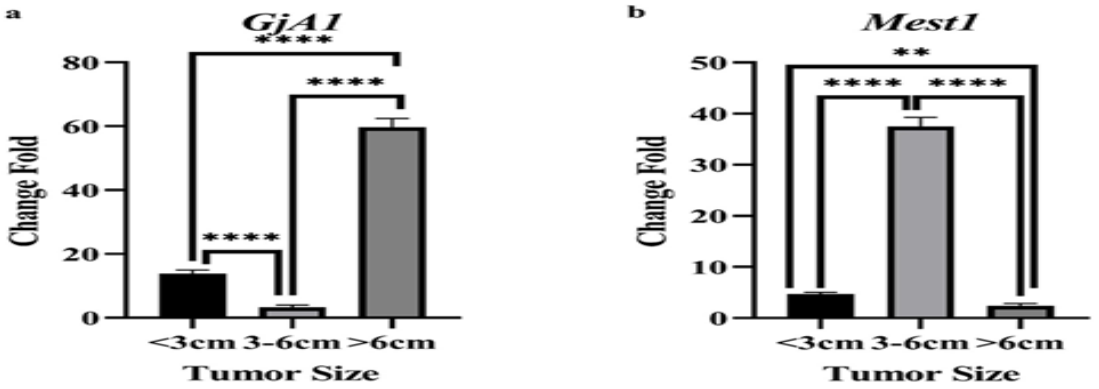

Figure 2: Comparing *GjA1* (a) and *Mest1* (b) gene expressions among gastric cancer tumors with different sizes. ****$P<0.0001$, **$P<0.01$.

The association of Mest1 and GJA1 gene expressions with tumor grade

The expressions of *GjA1* and *Mest1* genes were examined in gastric tumors with different grades. The results are presented in Figure 3, suggesting significant differences (*P* <0.0001). The highest expression of *Mest1* gene was observed in grade III gastric tumor, and the lowest in grade IV. However, the highest expression of *GjA1* gene with the progression of gastric cancer was observed in grade IV gastric cancer and the lowest in grade I (Figure 3).

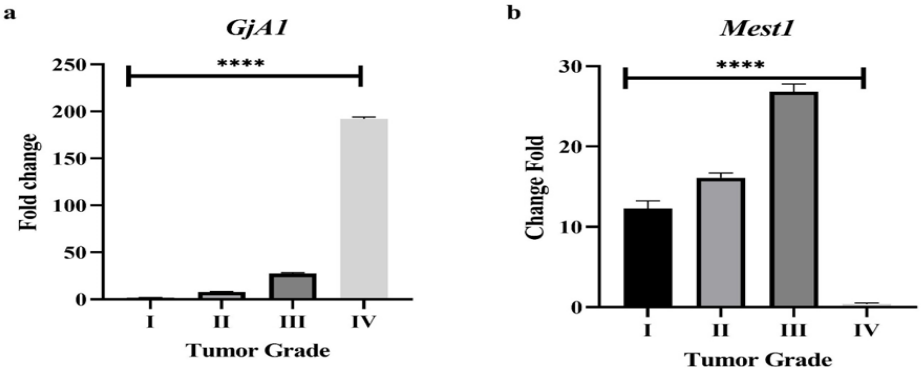

Figure 3. Comparing *Mest1* and *GjA1* gene expressions among different grades of gastric tumors

The association of Mest1 and GJA1 gene expressions with metastasis

A comparison between the expression ratio of *Mest1* and *GjA1* genes in normal and tumor tissues in terms of presence or absence of metastasis is demonstrated in Figure 6. T-test showed a significant difference between two groups (*P* <0.0001). The results indicated that under metastatic conditions, the expression of GjA1 decreased while that of Mest1 increased (Figure 4).

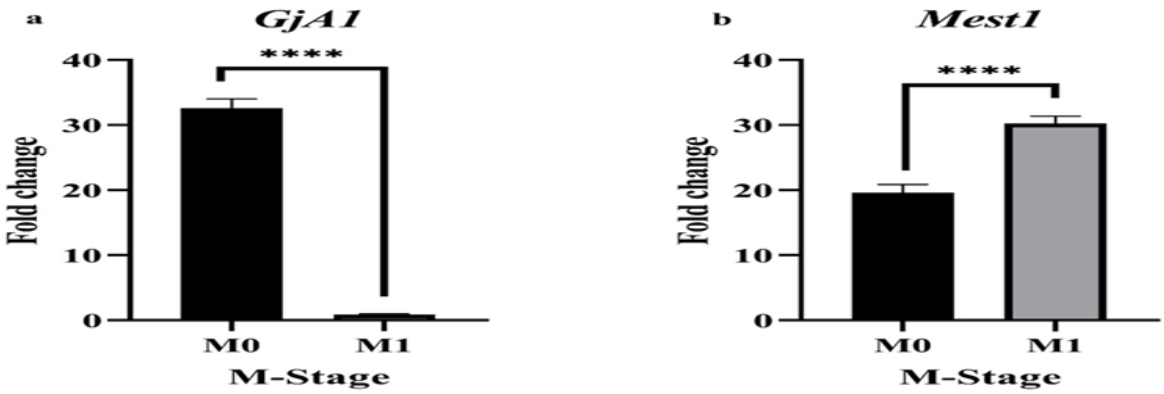

Figure 4. Comparing mean expression levels of *GjA1* (a) and *Mest1* (b) genes in tumor tissues under metastatic (M1) and non-metastatic (M0) conditions

The association of Mest1 and GJA1 gene expressions with disease stage

The expressions of *Mest1* and *GjA1* genes were studied based on clinical stages (I-IV). The results indicated *Mest1* and *GJA1* genes differently expressed in different stages of gastric cancer (*p* <0.0001). *GjA1* gene overexpressed with gastric cancer progression and culminated in stage III. However, it was dramatically reduced in stage IV. On the other hand, *Mest1* expression decreased with the progression of gastric cancer. Its highest expression was observed in stage I and the lowest in stage IV (Figure 5).

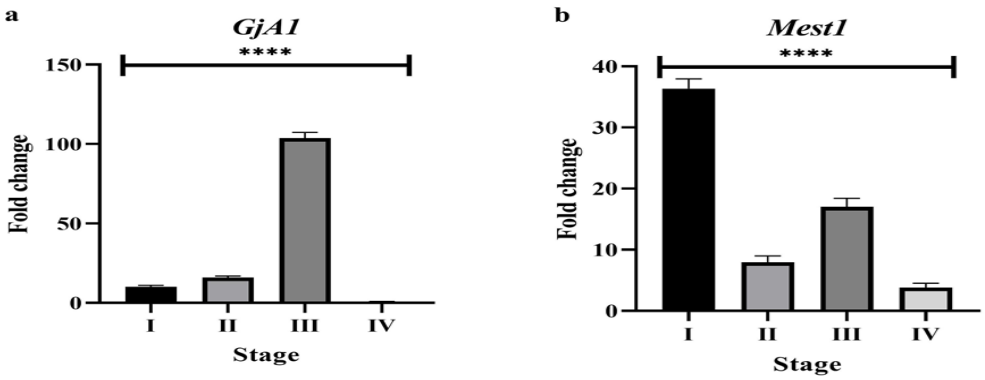

Figure 5. Expression of *GjA1* (a) and *Mest1* (b) genes in different stages of gastric cancer

The association of Mest1 and GJA1 gene expressions with lymphatic invasion

GjA1 and Mest1 gene expressions were compared in terms of lymphatic invasion using t-test, and the results showed significant differences (*P* <0.0001). The GjA1 gene overexpressed and led to the development of lymphatic invasion in the presence of it. The Mest1 gene, however, expressed itself at its maximum level in the absence of lymphatic invasion and reduced in the presence of it (Figure 6).

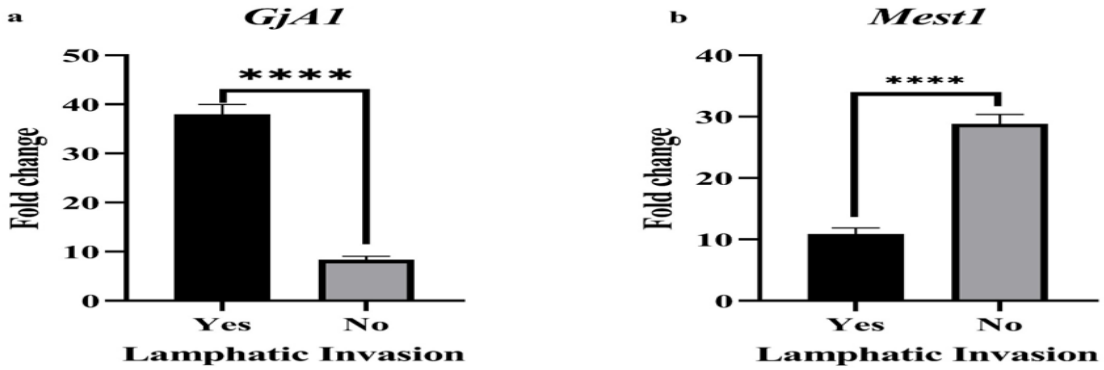

Figure 6. Comparing the expression levels of *GjA1* (a) and *Mest1* (b) genes in tumor tissues under the presence and absence of lymphatic invasion

Correlational analysis

Non-significant correlations were observed among the expressions of GjA1 and Mest1 genes in normal (r=0.211, 95% CI=-0.3865 to 0.5564, P=0.696) and tumor (r=0.227, 95% CI=-0.6334 to 0.3810, **P**=0.591) tissues (Figure 7).

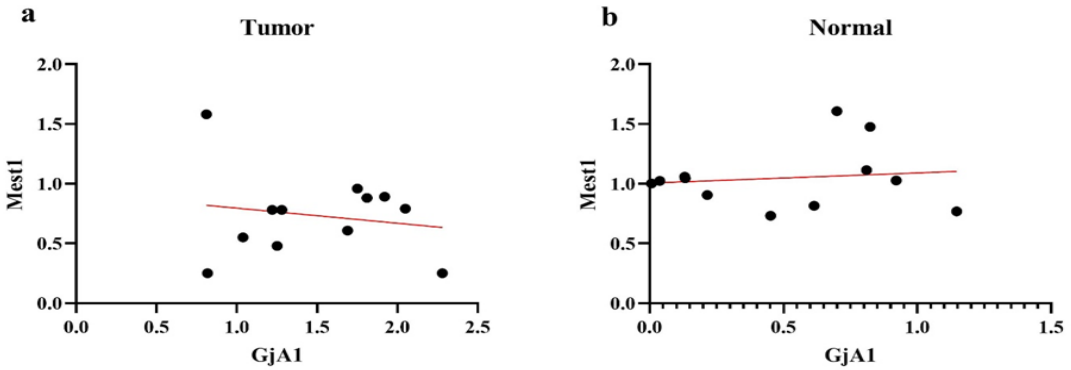

Figure 7: Correlation analysis between GjA1 and Mest1 gene expressions in a) gastric tumor and b) normal tissues

DISCUSSION

As far as the authors of the present study are concerned, studies on the clinical significance of the GJA gene are very limited. Hence, it is critical to consider the clinical implications of this gene family in the context of gastric cancer. Cx43 connexin, which is abundant in gastric epithelial cells [14], is encoded by the GJA1 gene. It is a crucial protein in establishing channels and cell-to-cell connections [15]. *GJA1* expression in association with tumor size was significantly different between the normal and tumor tissues; the overexpression of this gene was observed by increasing gastric tumor size. Various assumptions and possible mechanisms of Cx43 roles in gastric cancer were suggested [16, 17, 11, 18]. Cx43 can induce gastric cancer associated with *Helicobacter pylori* via Vac-A-induced cell death [19]. Tang et al. [20] found that this protein's aberrant expression could increase gastric tumor metastasis via hetero-cellular gap junction channels. Thus, Cx43 upregulation can lead to increase the gastric cancer tumor sensitivity to chemotherapy and there is a relationship *GjA1* expression level and gastric cancer [18]. However, in our research, no significant differences were seen regarding *GjA1* gene expression between tumor and normal tissues, which is contrary to the findings of Liu et al. [21]. However, the overexpression of that gene was shown in the higher grades of the disease.

Metastasis is a complex process involving several mechanisms that are poorly understood. Genetic and biochemical elements determining the acquisition of invasion phenotype, and systematic proliferation of cancer cells[22] were extensively studied. For that, EMT has been suggested as an important contributor[23]. Mesenchymal markers, including FSP1, vimentin, and desmin were reported overexpressed in carcinoma cells[24]. These are usually observed on the invasive front of primary tumors and eventually introduce the later stages of the metastasis cascade[25]. EMT induction is vital for the progression of carcinoma to metastatic state [26.]

The decrement of *GjA1* expression and non-significant difference in *Mest1* gene expression were observed in metastatic tumors compared to non-metastatic tumors. Loss of expression of the GJA gene family was shown to be an important event in cancer invasion and metastasis[27]. However, the role of connexins in malignancy is unclear, as it has not yet been determined whether the expression of connexins is necessary for metastasis or not. However, our results showed that *GjA1* was down-regulated in the metastatic tumors, which is in line with the above hypothesis. Loss of GJA expression may facilitate local invasion of cancerous tumors as reduced cell to cell connections may be contributed in cell segregation [28]. The current study is, to the best knowledge of the authors, the first to show that *GjA1* expression is reduced in metastatic gastric cancer cells and that there is no change in *Mest1* gene expression during metastasis.

A significant difference was seen in the expressions of *GjA1* and *Mest1* genes in relation to the stage of cancer. These results are consistent with the results reported by Kamyabayashi et al.[28], where they stated that the expression of connexins decreased in the early stages of skin cancer in mice and increased in the later stages, causing the cancer to metastasize to the lymph nodes. However, the current research results suggest that decreased *Mest1* expression, and increased *GjA1* expression occur in tumors that metastasize to lymph nodes. Decreased expression of *Mest1* and loss of imprinting of this gene were reported in head and neck cancers [29]. The protein encodes by *PEG1/MEST* pertains to α/β hydrolase family. The exact functions of that gene unknown, because the catalytic properties of this class of enzymes vary widely and include haloalkanes, lipids, and epoxies[30]. However, it has been reported that it reacts with some of the biological substances affected the growth mesoderm cells. Connexins function in the invasion of cancer cells to lymphatic tissues by forming gap connections between tumor and endothelial cells in the lymph nodes[28]. The results of the current study confirm the results obtained in previous studies [31].

CONCLUSION

Mest1 downregulation and GJA1 upregulation both contribute to gastric cancer metastasis. It would appear that the formation of cellular connections in malignancies is significantly

influenced by the GJA1 protein. The present study offers valuable insights into the involvement of the Mest1 and GjA-1 genes in the metastasis of gastric cancer. However, further studies are required to fully understand the mechanism of tumor metastasis in gastric cancer. In this regard, genomics and transcriptomic studies can pave the way for identifying many genes.

ACKNOWLEDGMENTS

We would like to thank and appreciate the efforts of the staff of Imam Khomeini Hospital.

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