ORIGINAL ARTICLE

Differential expression of two genes Oct-4 and MUC5AC associates with poor outcome in patients with gastric cancer

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Summary
Gastric cancer (GC) is the most frequent leading cause of cancer-associated mortality worldwide that is linked to poor prognosis due to the lack of appropriate biomarkers. Our aim was to evaluate the MUC5AC and Oct-4 expression levels in GC and to assess their association with clinical factors. Immunohistochemical analysis (IHC) and qRT-PCR were performed in GC patients to examine the MUC5AC and Oct-4 expression levels. The mRNA level of MUC5AC was significantly decreased in tumour tissues compared with non-cancerous tissues (1.11 ± 0.69 vs 3.7 ± 0.71; P = .024). On the other hand, Oct-4 mRNA level was upregulated in tumour tissues as compared to normal tissues (2.86 ± 0.78 vs 0.87 ± 0.54; P = .0015). Decreased expression of MUC5AC was detected in 27 patients (67.5%), while high to moderate expression levels were observed in 13 cases (32.5%), but in normal tissues the expression levels of MUC5AC were increased (P = .001). The decreased expression of MUC5AC was associated with aggressive tumour characteristics, such as TNM stage (P = .023), histologic type (P = .012) and lymph node metastasis (P = .001). High expression of Oct-4 was detected in 24 tumour tissues (60%), while 16 cases (40%) showed low expression level. Increased Oct-4 expression was correlated with clinicopathological characteristics such as TNM stage (P = .002), histologic type (P = .008) and lymph node metastasis (P = .001). Our results showed that high Oct-4 expression and the reduction of MUC5AC expression may be involved in the progression and an unfavorable prognosis of GC.

KEYWORDS
Gastric cancer, IHC, MUC5AC, Oct-4, QRT-PCR

1 | INTRODUCTION

Despite development of therapeutic strategies, the clinical outcome of gastric cancer (GC) remains poor. However, effective prognostic/predictive markers for multimodal therapy are currently required. Mucins are a group of extremely glycosylated extracellular ingredients of the mucosal layer. Mucins have been described to be linked to protection, lubrication and hydration of the external surface of human epithelial tissue layers. It has been reported that expression and glycosylation of mucins are associated with carcinogenesis including invasion, proliferation and regulation of tumour cells.1-3 MUC5AC mucin has protective role in the surface of the stomach from chemical, microbial and, mechanical damage.4 MUC5AC was reported to be implicated in gastric carcinogenesis with Lowe level of expression. Moreover, it has been indicated that MUC5AC expression level can be linked to severity of GC.5-7 Downregulation of MUC5AC expression has been determined in certain neoplastic lesions and might be appeared in advanced GC.8-11 Oct-4 has been revealed that it associated with the
development, transformation, and metastasis of the tumours.\textsuperscript{12} In addition, in a study by Yasuda et al.,\textsuperscript{13} have reported the high expression of Oct-4 on sporadic colorectal cancer. Moreover, another study by Wen et al.\textsuperscript{14} indicated the upregulation of Oct-4 was upregulated in metaplastic pancreatic ducts compared with normal acini and pancreatic carcinoma. Furthermore, many markers for early diagnosis of the various cancers earlier have conducted\textsuperscript{15-17} Therefore, this study was aimed to investigate the clinical significance of MUC5AC and Oct-4 expression in patients with GC in an Iranian population.

2 | RESULTS

2.1 | MRNA levels of MUC5AC and Oct-4

The mRNAs levels of markers were determined in GC tissues and non-cancerous tissues using qRT-PCR. The mRNA level of MUC5AC was significantly decreased in tumour tissues compared with non-cancerous tissues ($1.11 \pm 0.69$ vs $3.7 \pm 0.71$; $P = .024$; Figures 1 and 2). In addition, Oct-4 mRNA in tumour tissues as compared to normal tissues was upregulated ($2.86 \pm 0.78$ vs $0.87 \pm 0.54$; $P = .0015$; Figures 1 and 2).

2.2 | Protein expression level of MUC5AC and Oct-4

Results showed low MUC5AC immunoexpression in 27 patients from 40 cases (67.5%), whereas high to moderate expression levels were detected in 13 cases from 40 patients (32.5%; Figure 3). MUC5AC immunoexpression was decreased significantly in normal tissues ($P = .001$). Moreover, MUC5AC expression in cancer tissues decreases significantly with advanced TNM stage ($P = .023$), histologic type (poor differentiation, $P = .012$), lymph node metastasis ($P = .001$), (Table 1). High expression of Oct-4 was detected in 24 tumour tissues (60%), while 16 cases (40%) showed low expression level (Figure 3). High Oct-4
In GC tissues, low level of MUC5AC expression was strongly associated with advanced TNM stage, lymph node metastasis, and high tumour size. They expressed that low expression level of MUC5AC might be associated with gastric carcinogenesis that is in agreement with our study. Previous studies demonstrated that the decreased MUC5AC might be associated with gastric carcinogenesis and its expression is remarkably downregulated in GC. There has been a positive association between low MUC5AC expression and poor survival in patients with gastric cancer. On the other hand, it was detected that MUC5AC expression level can be a favourable prognostic factor in early gastric cancer that indicates an inverse association of MUC5AC with the invasiveness of GC cells.

There is a strong correlation between MUC5AC expression and gastric carcinogenesis and its expression is remarkably downregulated in GC. Furthermore, it has been revealed that MUC5AC expression level can be linked to severity of GC. Previous studies demonstrated that the decreased MUC5AC might be associated with gastric carcinogenesis that is in agreement with our study. The overexpression of MUC5AC has been observed in colorectal and breast cancer, with association with lymph node and distant metastasis and deeper invasion. The potential prognostic value of MUC5AC expression in GC is contradictory. There has been a positive association between low MUC5AC expression and poor survival in patients with gastric cancer. In the present study, the mRNA and protein expression level of MUC5AC was significantly decreased in tumour tissues as compared to non-cancerous tissues. On the other hand, our findings demonstrated that the mRNA and protein expression level of Oct-4 was upregulated in GC tissues compared with normal tissues.

In addition, low expression of MUC5AC and high expression of Oct-4 were significantly associated with advanced TNM stage, poorly differentiated, and lymph node metastasis.

Previous studies have shown downregulation of MUC5AC expression in certain neoplastic lesions and advanced GC. Furthermore, the overexpression of MUC5AC has been observed in some malignancies such as colorectal and breast cancer.

There have been studies that reported that Oct4 is overexpressed in many kinds of cancers, such as, lung cancer, oral squamous cell carcinoma, GC, breast cancer, and esophageal cancer and ectopic expression may play a role in the improvement of disease. Nevertheless, there is little relation between MUC5AC and Oct4 with clinical factors in GC, and contradictory findings have been reported. In addition to clinical outcome, lymph node and distant metastasis has been shown to be important factors for prognostic in GC.

There is a positive correlation between MUC5AC expression and gastric carcinogenesis and its expression is remarkably downregulated in GC. Furthermore, it has been revealed that MUC5AC expression level can be linked to severity of GC. Previous studies demonstrated that the decreased MUC5AC might be associated with gastric carcinogenesis that is in agreement with our study. The overexpression of MUC5AC has been observed in colorectal and breast cancer, with association with lymph node and distant metastasis and deeper invasion. The potential prognostic value of MUC5AC expression in GC is contradictory. There has been a positive association between low MUC5AC expression and poor survival in patients with gastric cancer. On the other hand, it was detected that MUC5AC expression level can be a favourable prognostic factor in early gastric cancer that indicates an inverse association of MUC5AC with the invasiveness of GC cells.

Foveolar expression of MUC5AC was reported to be correlated with longer survival. Kim et al. demonstrated that the low level of MUC5AC expression was significantly associated with progression of T stage, lymphovascular invasion, lymph node metastases and high tumour size. They expressed that low expression level of MUC5AC is an independent poor prognostic factor in diffuse-types of GC, and in another study by Kocer et al. showed that decreased MUC5AC expression is linked to prognosis of colorectal cancer, indicating the existence of an aggressive form. Moreover, invasive depth and lymph node metastasis were showed to be prognostic markers in the advanced tumour subgroup. Concordant with other studies, our results exhibited that decreased expression of MUC5AC was correlated with poor overall survival.

Additionally, retained MUC5AC expression was published to be correlated with extended survival in early GC, and the depth of invasion. Another study indicated an important prognostic value only in poorly differentiated adenocarcinoma. Our data indicated that the expression of Oct-4 has a remarkable relationship with lymph node metastasis, TNM stage, and differentiation and high expression this marker was associated with lymph node metastasis, advanced TNM stage, histologic differentiation.

3 | DISCUSSION

In the present study, the mRNA and protein expression level of MUC5AC were estimated using the Kaplan–Meier method and compared by the log-rank test.
In parallel, Kumar et al., stated the role of Oct-4 in tumourigenesis and malignant transformation. Recent reports have revealed the expression of Oct4 in cancer stem-like cells and its correlation with primitive and aggressive tumour phenotype. The high expression of Oct-4 has been documented to be associated with differentiation status, but not with sex, age, TNM stage, tumour size, lymph node metastasis, and depth of invasion in GC. Oct4 expression was significantly increased in GC tissues and its overexpression was found to be correlated with poor clinical prognosis. Overexpression of Oct-4 has also been observed in sporadic colorectal cancer than those in adjacent normal colonic epithelium.
FIGURE 4  Survival analyses for Oct-4: Overall survival between patients with expression levels of Oct-4 were estimated using the Kaplan–Meier method and compared by the log rank test

Wen et al. have showed the upregulation of Oct-4 in metaplastic pancreatic ducts compared with normal acini and pancreatic carcinoma, and this expression is related to a large tumour size and vascular invasion. Jiang et al. demonstrated that increased expression of Oct-4 could be efficiently related to tumour size, tumour location, TNM stage, lymph node metastasis, and depth of invasion in GC. Oct-4 expression has been documented to be related to a large tumour size and vascular invasion, and it was also identified as an independent predictor of poor prognosis in patients with HCC. Oct-4 overexpression has been reported to be markedly correlated with large tumour size and vascular invasion. Furthermore, its increased expression was shown to be an independent predictor of postoperative recurrence and poor prognosis of human hepatocellular carcinoma. Jiang et al. have also reported that by shorter overall survival high Oct-4 expression have proved as a poor prognosis and overexpression of Oct-4 has been shown to be significant for poor 5-year survival rate of patients. MUC5AC expression, and Oct-4 expression using multivariate Cox proportional hazards model, were independent predictor of overall survival.

In summary, a limited number of samples have been investigated and sample size need be expanded in further. Current results revealed that Oct-4 and MUC5AC may have a key role in the progression and tumorigenesis of GC.

4 | METHODS

4.1 | Patients and specimens

All protocols were in accordance with the Declaration of Helsinki. All participating patients signed the consent forms. Forty GC tissues and the matched normal tissues were collected from the patients who underwent partial or total gastrectomy in Tehran, Mashhad and Tabriz between 2010 and 2014. All the patients received standard gastrectomy with lymphadenectomy (D2 lymphadenectomy) by surgeons. No patients had received preoperative treatment prior to the tissue harvest.

Sixteen male and 24 female, with a mean age of 59.35 years were selected for this study (range 37-85). Specimens were transported to the pathology laboratory, and stored at ~80°C. In addition, 4-μm-thick tissue sections were provided for investigation. Pathological staging and grading were done according to the criteria of the American Joint Commission on Cancer.

4.2 | Extraction of RNA and qRT-PCR

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the constructor’s protocol. cDNA synthesis, quantification of specific mRNA were then performed. Real-time quantitative RT-PCR reactions were performed using SYBR Green master mix kit on Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The sequences for the forward and reverse primers of MUC5AC were 5’-TGCCATCACCCATCTGCC-3’ and 5’-ACCACATCCAGGTCGTCGCT-3’. The primer sequences Oct4 were: 5’-GAC AAC AAT GAG AAC CTT CAG GAG A-3’ and 5’-CTG GCG CCG GTT ACA GAA CCA-3’. GAPDH was used as a reference gene for normalization, and fold changes were calculated using the 2-∆ΔCT method.

4.3 | Immunohistochemistry method

After deparaffinization and rehydration of tissues, the sections were incubated in 0.5% H2O2 in methanol for 20 minutes to inactive endogenous peroxidase activity at room temperature. Sections were microwave treated in citrate buffer (0.01 mol/L, pH 6.0). Moreover, the sections were incubated with the primary antibodies (Rabbit polyclonal anti-Oct4 1:200, mouse monoclonal anti-MUC5AC, 1:200; Abcam, Cambridge, UK) at 4°C. Furthermore, the sections were next incubated with the biotinylated rabbit antimouse IgG and avidin–biotin peroxidase according to the manufacturer’s procedure. The brown color indicative of peroxidase activity was developed by incubating the sections with 3, 3’diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA) in PBS with

| TABLE 3 | Multivariate analysis for overall survival by Cox proportional hazards model |
|-----------------|-----------------|--------|---------|
| Clinicopathological characteristics | HR | 95% CI | P value |
| Gender | 0.80 | 0.723-2.15 | .73 |
| Age | 0.742 | 0.382-1.617 | .624 |
| TNM stage | 3.25 | 1.73-10.53 | .001 |
| Histologic type | 2.66 | 1.45-8.12 | .012 |
| Lymph node metastasis | 2.57 | 1.60-7.16 | .019 |
| MUC5AC expression | 3.2 | 1.754-10.21 | .001 |
| Oct-4 expression | 2.95 | 1.41-8.37 | .003 |
0.05% H₂O₂ for 5 minutes at room temperature. Number of positive stained cell ≤5%, scored 0; between 6% and 25%, scored 1; between 26% and 50%, scored 2; between 51% and 75%, scored 3; >75%, scored 4. IHC staining intensity of markers was categorized as follows: negative, weak staining (1+), and moderate (2+) and strong staining (3+, 4+).

4.4 | Statistical analysis

Software of SPSS version 17.0 for Windows (SPSS Inc, Chicago, IL, USA) was applied for analysis of variables. Correlation between expression levels of markers and clinicopathological factors were assessed using the Fisher’s exact test. The Kaplan–Meier and the log-rank test analysis were applied for survival analysis. Prognostic values of clinicopathological factors were evaluated by multivariate Cox regression analysis. Differences were considered statistically significant when P was less than 0.05.

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DISCLOSURE

The authors have no conflicts of interest to declare in association with this work.

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