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ORIGINAL RESEARCH

Disregulation of miR-216a and miR-217 in Gastric Cancer and Their Clinical Significance

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Abstract
Objective The majority of gastric cancer (GC) diagnoses occur at the middle or late stage of the disease, indicating that finding novel biomarkers that could be detectable at earlier stage is urgently needed. Accumulating studies have shown that microRNAs, a class of tiny single-stranded RNAs, play important roles in multiple biological processes including cancer development. The present study aimed to evaluate the effect of miR-216a and miR-217 in GC.
Material and Methods The real-time quantitative reverse-transcription PCR was exploited to identify and compare the expression levels of miR-216a and miR-217 in 37 pairs of samples of gastric cancer tissue and adjacent normal tissue. Superimposed on this, the potential relationship between miR-216a/217 levels and clinicopathological parameters in patients suffering GC was explored.
Results The results obtained from this study showed that the miR-216a is significantly upregulated in gastric cancer tissues, compared with adjacent normal tissues, but the altered expression of miR-217 was not significant. For miR-216a/217, no significant correlations were detected between expression levels of these miRNAs and clinical and pathological characteristics of patients.
Conclusion This prospective study proposes that upregulation of miR-216a might represent an important mechanism for the development of gastric cancer.

Keywords miR-216a · miR-217 · Gastric cancer · Expression analysis · qPCR

Introduction

Gastric cancer (GC) is the fourth most common cancer in men and fifth in women and has become the second leading cause of cancer-related death worldwide, with a frequency that shows diversity across geographically distributed populations [1]. In Iran, gastric cancer is the most common fatal cancer and this malignancy is the first and second leading cause of cancer-related deaths in Iranian men and women, respectively [2]. In recent years, GC accounted for only 2 % of cancer deaths. This shows that the mortality rate of GC has decreased dramatically. Although the reason for the unusual decrease in GC incidence is unclear, but some parameters such as drop-in the incidence of *Helicobacter pylori*, advances in food preservation methods, general improvement in nutritional status, and consumption of fresh fruits and vegetables as hygiene and health improvements can explain this phenomenon [3]. Despite the declining incidence during the past half century, GC still remains a disease with high mortality rate and unfortunately, the lack of early pathognomonic symptoms delays the diagnosis. In fact, the disease is often diagnosed in advanced stages and is associated with a poor prognosis for

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patients. Therefore, the search for diagnostic strategies such as identification of predictive, early diagnostic, and prognostic biomarkers is essential and can help diagnose the disease at an early stage, treatment response evaluation, and possibly targeted therapy [4].

One of the potential biomarkers for gastric cancer is microRNAs (miRNAs). The first evidence for microRNAs as regulatory RNAs was provided by Ambros and colleagues by the discovery of the lin-4 and let-7 in *Caenorhabditis elegans* [5]. The involvement of this class of non-coding RNAs in the development of cancer was first demonstrated in 2002. Since this time, cancer-related functions of miRNAs have been extensively studied [6]. By definition, miRNAs are ~22 nucleotide small single-stranded non-coding RNAs that post-transcriptionally control gene expression via either inducing translational silencing or causing degradation of the mRNA of the targeted genes. These tiny regulators of gene expression act through binding in a sequence-specific manner to the 3′UTR of mRNA [7–9]. Each miRNA potentially regulate multiple target genes [10]. To date, about 2000 miRNAs have been identified in the human genome and have been recorded in the miRDB [11].

miRNAs have a variety of roles in cancer development, tumor progression, and metastasis. Interestingly, there are numerous studies showing the downregulation or upregulation of miRNAs in a variety of cancers, including gastric cancer [12]. In the present study, real-time quantitative reverse-transcription PCR was exploited to identify and compare the expression levels of miR-216a and miR-217 in gastric cancer and adjacent normal cells. Furthermore, this study aimed to evaluate the potential relationship between miR-216a/217 levels and clinicopathological and also prognostic outcomes in patients suffering gastric cancer. It should be noted that the miR-216a and miR-217 are clustered miRNAs and located on human chromosome 2p16.1 within intron 2 of the non-protein coding transcript AC011306.2 [13].

Materials and Methods

Cohorts of Gastric Cancer

In this cohort study, 37 pairs of matched gastric tumors and noncancerous tissue samples from gastrectomy were collected from patients (24 men, 13 women) that referred to Emam Reza and Sina hospitals (Tabriz, Iran) in the period from August 2013 to January2015. The tissue samples were obtained from resected surgical specimens and were quickly frozen in liquid nitrogen until RNA extraction. Surgical pathologic staging was determined according to the World Health Organization (WHO) Classifications [14].Written informed consent was obtained from each patient. The Ethics Committee of Imam-Reza Hospital approved the use of these collected tissues. Table 1 provides a summary of clinicopathological features of all patients.

RNA Extraction and Real-Time Quantitative Reverse-Transcription PCR

Total RNA from tissue samples was extracted using TRIzol reagent (Invitrogen). Quality and quantity of extracted total RNA was assessed using the Picodrop-100 spectrophotometer (PicoDrop, Hinxton, UK). cDNA was synthesized by PrimeScript RT reagent kit (TaKaRa, Bio Inc., Shiga, Japan) using 2 μg of total RNA as template. Briefly, 2 μg of total RNA containing small RNA extracted from tissue samples was first polyadenylated by poly (A) polymerase and then reverse transcribed to cDNA using a mixture of oligo (dT) adaptor provided in the kit. Real-time PCR was carried out with a SYBR Green supermix kit (Parsgengan, Iran). The PCR condition for miR-216a was 95 °C × 1 min, followed by 40 cycles of 95 °C × 5 s, 62 °C × 20 s, and 72 °C × 30 s. Then the final extension followed by 72 °C × 2 min and ramped from 67 to 97 °C to obtain the melting curve. The

Table 1 Selected features of participants according to GC and relationships between miR-216a and miR-217 expression levels in cancer tissue samples (n = 37)

Clinical parameters	Number	miR-216a ΔCt	p value	miR-217 ΔCt	p value
Gender					
Male	24	0.03 ± 0.05	0.15	0.04 ± 0.07	0.24
Female	13	0.04 ± 0.01		0.04 ± 0.05	
Age					
< 66	17	0.06 ± 0.01	0.22	0.07 ± 0.03	0.28
≥ 66	20	0.02 ± 0.02		0.05 ± 0.02	
Histological grade					
Poorly differentiated	12	0.06 ± 0.01	0.17	0.06 ± 0.02	0.37
Moderately differentiated	9	0.02 ± 0.04		0.04 ± 0.03	
Highly differentiated	16	0.05 ± 0.02		0.03 ± 0.05	
Histological type					
Intestinal	26	0.03 ± 0.06	0.36	0.08 ± 0.03	0.41
Diffuse	11	0.05 ± 0.02		0.05 ± 0.02	

PCR condition for miR-217 was same as miR-216a except denaturation and annealing steps that was 95 °C × 10 s and 61.5 °C × 25 s, respectively. The U6 snRNA was used as normalized control. Each of the experiments was performed in triplicate. The relative expression of miR-216a and miR-217 in tumor samples and adjacent non-tumor tissues was calculated using the comparative Ct method. Fold change in miRNA expression was calculated according to the equation $2^{-\Delta\Delta CT}$.

Data Analysis

The real-time qRT-PCR results were analyzed using LinReg software. The relative expression analysis of miR-216a and miR-217 was performed by a randomization test using the Relative Expression Software Tool (REST) 2009. Statistical analysis was performed with SPSS Version 21 statistic software package. The Kruskal and Mann-Whitney tests were used for statistical analysis in each of the diagnostic groups. The statistically significant level was set at $\alpha = 0.05$ two-side. All the data were presented as means ± standard deviation (SD). The association between miR-216a and miR-217 expression and clinicopathological features in samples from patients with gastric cancer was analyzed using the Spearman rank correlation calculation test. Receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC) were used to evaluate the feasibility of using miR-216a and miR-217 as a diagnostic biomarker for the detection of gastric cancer.

Results

Expression Levels of miR-216a and miR-217

Because there has not been any report on the expression of miR-216a and miR-217 and their relation to GC, we explored expression profiling of these two miRNAs in stomach biopsies from GC patients. To conduct the miR-216a/miR-217 expression levels in tumor and adjacent normal tissues, we compared the expression levels of these miRNAs in gastric cancer tissues and matched corresponding adjacent tissues ($n = 37$). Our data showed that miR-216a was significantly increased in gastric tumor samples but the expression level of miR-217 was not significant ($p < 0.05$). Differences of miR-216a and miR-217 expression levels were illustrated in Figs. 1 and 2, respectively.

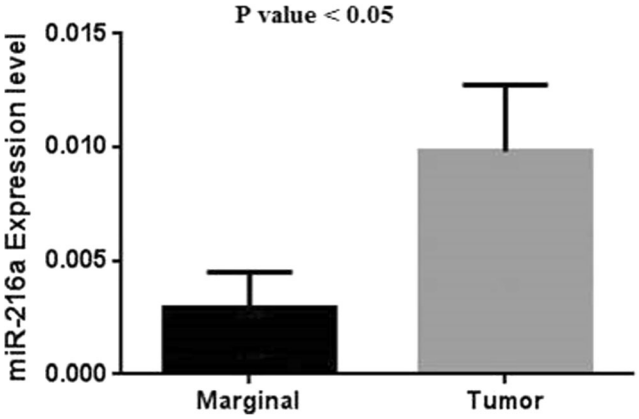

Fig. 1 miR-216a is overexpressed in gastric tumor tissues. The analysis of the miR-216a expression level was performed in gastric tumor tissues ($n = 37$) and matched noncancerous tissues. The miR-216a significantly increased in gastric tumor samples compared with that in matched noncancerous tissues

Correlation Between miR-216a/miR-217 Expression Levels and Clinicopathological Characteristics in Patients with Gastric Cancer

We further evaluated the relationship between the expression levels of miR-216a/miR-217 in the patients' gastric cancer tissues and clinicopathological characteristics of the patients using non-parametric test. However, no significant correlations were detected between expression levels of these miRNAs and clinicopathological characteristics, including age and gender of patients, histological grade, and histological type of tumors (Table 1).

Capability of miR-216a as a GC Tumor Marker

To evaluate the sensitivity and specificity of miR-216a expression levels as tumor marker in GC patients, ROC curves were constructed and the area under the curve was calculated. The values of the AUC were 0.72 out of 1 for the miR-216a assay

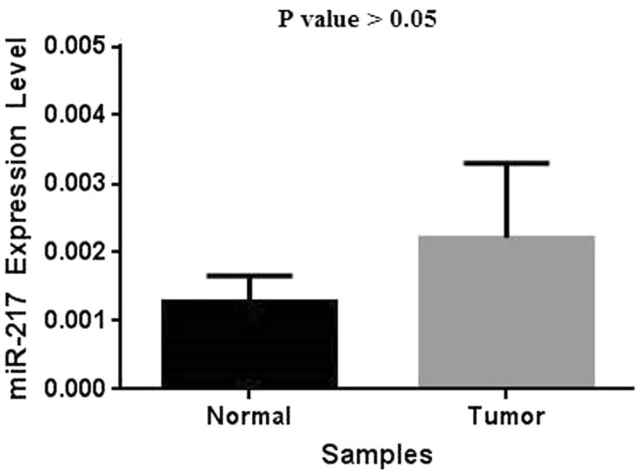

Fig. 2 miR-217 levels were non-significantly increased in GC cells

(Fig. 3). Because the increased expression of miR-217 was not significant, we did not calculate ROC plot AUCs for this miRNA.

Discussion

Gastric cancer has become one of the major causes of cancer-related mortality with more than 700,000 deaths every year worldwide [15] including Iran; so early detection and diagnosis can improve the management and prognosis of this malignancy. Finding the genes that are differentially expressed in gastric cancer is indispensable for exact diagnosis, molecular characterization of the tumor, informed surgical and making decisions regarding adjuvant chemotherapy, designing new therapeutic strategies such as novel biological treatment, and explanation of biological behavior of tumor in molecular level for most accurate prognosis [16]. Alteration on miRNA expression in cancer and correlation between miRNA expression profiles with clinical parameters of malignancy could potentially be used in the diagnosis and therapies of different forms of cancer [17]. As a consequence, exact determination of the level of expression of miRNAs in a tumor tissue is an essential parameter to describing the crucial roles of these tiny non-coding RNAs in multiple biological processes, including development, proliferation, differentiation, apoptosis, invasion, and metastasis [18]. A number of miRNAs that are deregulated in gastric cancer have been revealed by expression analysis in gastric cancer tissues compared with normal tissues and have shown that these classes of RNAs are closely associated with GC [19]. However, there have been no reports describing the biologic role of miR-216a/miR-217 in GC. This is the first expression study of these two miRNAs on gastric cancer. In this prospective study, we demonstrate that increased levels of miR-216a may have an oncogenic role in GC. Here, we have provided some evidences that show the role of miR-216a/miR-217 in cancer progression based on previous studies.

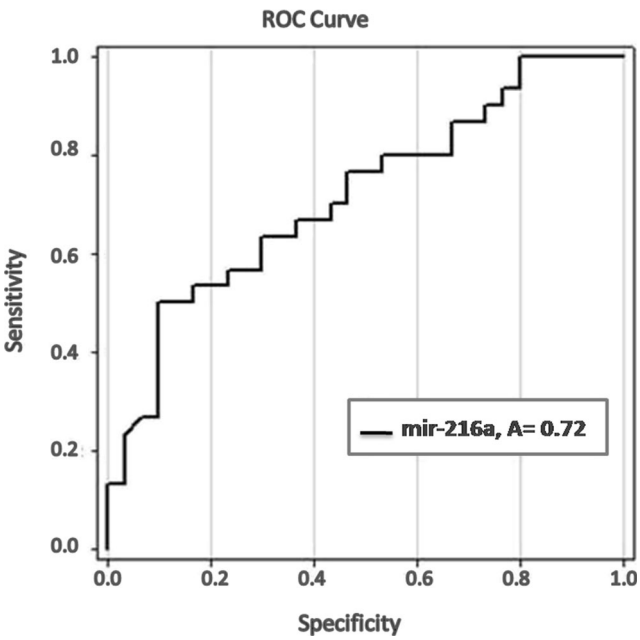

Fig. 3 Diagnostic performance of the expression data of miR-216a represents the AUC-ROC is 0.72 out of 1. ROC receiver operating characteristics, AUC area under the ROC curve

Roughly 95% of all malignant gastric neoplasms are adenocarcinoma, which are cell growths that occur in the epithelial cells [20]. In the other hand, metastasis causes most majorities of gastric cancer-related deaths. In fact, lymph node metastasis is a major prognostic factor in GC [21]. One of the best known changes prior to the metastasis process is the epithelial-mesenchymal transition (EMT) that converts epithelial cells into mesenchymal cells. This conversion that also occurs during embryonic development as a normal embryological process, frequently involved in cancer invasion and metastases. In fact, the EMT can be an initial step in the invasion and metastasis of solid tumors and is related to therapeutic resistance. Increasing numbers of miRs including miR216a and miR-217 have been showed to act as critical modulators of the EMT. The miR-216 and miR-217 have been demonstrated to promote the EMT [22, 23]. Therefore, based on previous studies, it appears that miR-216a, and likely miR-217, is not only valuable for further inspection, and also might become potential therapeutic targets in the future, but it may also serve as an effective prognostic biomarker for predicting the metastasis. Superimposed on this our study showed that the values of the AUC were 0.72 for the miR-216a assay and this indicated that the miR-216a may be used as potential diagnostic and probably predictive and prognostic biomarker for GC.

The other pathway relevant to the microRNA-216a/217 is transforming growth factor β (TGF-β) pathway. TGF-β plays important roles in progression of malignant tumor. Clinical studies showed the positive correlation of TGF-β1 expression with invasion and poor prognosis in gastric carcinoma [24]. The relationship between overexpression of microRNA-216a/217 and TGF-β pathway has been studied by Xia et al. This group demonstrated that the treatment of hepatocellular carcinoma (HCC) cells by TGF-β induces overexpression of miR-216a/217 in these cells. Based on this study, on the one hand, overexpression of miR-216a/217 targets mothers against decapentaplegic homolog 7 (SMAD7) which has been shown to be a TGF-β receptor type 1 (TGFBR1) antagonist. On the other hand, inhibition of SMAD7 led to increases in expression of the TGF-β pathway member, TGFBR1. This pattern of regulation indicated that upregulation of miR-216a/217 may provide a positive feedback regulation mechanism for the TGF-β pathway through SMAD7 as target. Based on

these interactions, it seems that relationship between miRNAs and TGF-β acts via autoregulatory feedback loops and affects the destination of tumor cells [25].

The another mechanism apart from TGF-β pathway through SMAD7 that may be involved in the pathogenesis of gastric cancer is reduced expression of phosphatase and tensin homolog (PTEN). Documented evidence has been shown that PTEN is one of the functional downstream targets of miR-216a/217 [26]. Downregulation of PTEN by these two miRs results in activation of Akt pathway. Genetic alterations in this pathway in gastric carcinoma have often been demonstrated and commonly activated in advanced gastric carcinoma [27]. Based on these findings, we hypothesize that enhanced expression of miR-216a/217 in GC might leads to downregulation of PTEN and more active signaling through the PI3K/Akt pathway. It is also worthwhile remembering that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is one of the important signaling pathway that exerts multiple biological activities including promotion of cell growth, cell immortalization, cell proliferation, cell metabolism, angiogenesis, metastasis, and also chemotherapy resistance [28]. As mentioned above, upregulation of miR-216a/217 leads to the activation of the TGF-β Pathway. In the other hand, documented evidence has showed that TGF-β activates Akt by inducing the miR-216a and miR-217. Thus, PTEN might be a link between TGF-β and Akt activation [29]. However, the mechanism by which TGF-β activates Akt has not been entirely clarified; further studies can confirm the miR-216a/217 as mediators of chemoresistance through the PI3K/Akt pathway in gastric carcinoma. In line with this hypothesis, the miR-216a/217 might be a novel candidate target in the advancement of resistance to chemotherapy in GC. Taken together, further evaluations such as expression analysis of miR-216a/217 in resistant cells of gastric carcinoma and metastatic positive tissues can be a complementary material for our study.

Conclusion

In conclusion, several thousands of miRNAs have been showed to be related to GC. One problem related to using these miRNAs as biological markers for determining cancer status is the variability among different patients, even with the same type of cancer. So it is impossible to use only one marker as a dependable method. For this reason, just the combination of multiple miRNAs could be effective for diagnostic, prognostic, and targeted therapy purposes [30].

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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