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EVALUATION OF SERUM MIRNA-217 AND MIRNA-143-3P AS POTENTIAL BIOMARKERS FOR ESOPHAGEAL ACHALASIA

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Abstract

The use of a low-risk and convenient diagnostic method is important in the early diagnosis of achalasia and could prevent the side effect of delay in initiation of treatment. According to our previous NGS study, two miRNAs, hsa-miR-217 and hsa-miR-143-3p were differentially expressed in achalasia esophageal tissue. The present study aimed to assess two microRNAs, hsa-miR-217 and hsa-miR-143-3p as serum biomarkers for early detection of achalasia. Serum was collected from patients with achalasia and non-achalasia participants. The relative expression of hsa-miR-217 and hsa-miR-143-3p were determined using quantitative real-time polymerase chain reaction. The expression of hsa-miR-217 was different in the serum of patients with achalasia and controls, but this difference was not significant (p-value = 0.572). Besides, the results showed that the serum expression of miRNA-143-3p was not significantly different between the patients with achalasia and the control group (p-value = 0.366). Our findings could not support serum miRNA-217 and miRNA-143-3p as potential diagnostic biomarkers for achalasia.

Keywords: Achalasia, biomarker, microRNA, miRNA-217, miRNA-143-3p

INTRODUCTION

Primary esophageal achalasia is a neuromuscular disorder characterized by incomplete relaxation of the lower esophageal sphincter in the absence of regular contractions, followed by dysphagia [1]. Studies reported the annual incidence of achalasia of 1 in 100,000

individuals and the prevalence of 10 per 100,000 [1-3]. Although, there appear to be significant differences between international regions and within countries [4]. The histological study demonstrates the loss of myenteric neurons in the distal esophagus, especially in the lower esophageal sphincter, but still, the main mechanism of this reduction is unknown [5]. The progression of achalasia causes irreversible esophageal motility disorder, and the accumulation of food that is not transferred to the stomach causes numerous complications [6].

Lack of timely diagnosis and delay in initiation of treatment may cause inflammation of the esophageal mucosa, which is a risk factor for esophageal cancer seen in 16 to 28 times higher than the control group [6, 7]. Numerous factors such as infectious, immune, and genetic factors may play an important role in the pathogenesis of this neurodegenerative disease [8]. A complete cure for achalasia is not possible. The goal of treatment is to reduce symptoms and improve esophageal emptying to prevent progression toward mega esophagus and esophageal cancer [9]. Early diagnosis is important to achieve this therapeutic goal. High-resolution manometry is a gold standard for diagnosis and other methods like endoscopy and barium swallow radiography are used to rule out other disorders [10]. The use of a low-risk and convenient diagnostic method is important in diagnosing achalasia. There is an increasing interest in the early detection of diseases using microRNA (miRNA) biomarkers.

MicroRNAs (miRNAs) are intracellular small non-coding RNA molecules that regulate post-transcriptional gene expression and their protein synthesis [11]. MiRNAs play a role in various physiological and pathophysiological processes of the body. Although most miRNAs are in the cell microenvironment, some miRNAs are in the extracellular environment, including various biological fluids, as circulating miRNAs or extracellular miRNAs [12]. The high stability of circulating microRNAs such as serum or plasma makes them potentially suitable as non-invasive biomarkers for diagnosis or prognosis [13]. Circulating miRNAs as non-invasive biomarkers have the prospect of early detection, quality improvement of treatment, and monitoring the progression of many diseases.[14, 15]. Biochemical studies showed that miRNAs are stable under conditions of RNase activity, pH changes, and extreme temperatures. Besides, circulating miRNAs expression is special in different diseases. The expression characteristics of these circulating miRNAs in serum, plasma, and other body fluids increase their potential as minimally invasive biomarkers in the diagnosis and monitoring of a variety of diseases [16].

According to our previous NGS study, two miRNAs, hsa-miR-217 and hsa-miR-143-3p were differentially expressed in achalasia esophageal tissue [17]. The present study aimed to assess these two microRNAs, hsa-miR-217 and hsa-miR-143-3p as serum biomarkers for detection of achalasia. In this study, the expression of miR-217 and miR-143 were evaluated in the serum of patients with achalasia compared with non-achalasia by quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).

MATERIALS AND METHODS

Participants and sampling

This study was performed on 32 participants referred to the clinic of the gastrointestinal disease of Shariati Hospital in Tehran. We enrolled 16 patients with primary esophageal achalasia (cases) who came to the clinic for regular follow-up. The control group was

randomly selected from 16 participants who were referred to the same clinic. All cases and controls matched by age (±5 years) and sex. Blood samples were taken from cases and controls in the morning after fasting at night. After that blood samples were gathered in serum separation tubes and stored at 4 ° C. The serum was isolated from whole blood in less than 24 hours and stored at -80 ° C until miRNA extraction. The concentration of miRNA in serum samples is very small so, we used a constant volume of serum for miRNA extraction.

Extraction of serum total RNA

Total RNA which included miRNA was extracted from 400 µl of the thawed serum using the TRIzol Reagent according to the modified manufacturer instructions (Invitrogen, Sweden). During the extraction of total RNA, 20 µl proteinase K (CinnaGen cat #PR891631, Iran) was added to each sample to allow protein digestion. A spectrophotometer (Denovix DS-11, USA) was used to measure the quality of total RNA and the optical density at 260/280 nm. The RNA was stored at -80°C until use.

Quantification of miRNAs levels using qRT-PCR

The qRT-PCR was performed to detect expression levels of miRNA-217 and miRNA-143-3p in 33 serum specimens of achalasia and non-achalasia patients by ABI 7300 real-time PCR machine (Applied Biosystems). We performed cDNA synthesis from miRNA using the reverse transcription system kit (Zist Royesh, Iran) with miR-specific primer loop primer [18]. The U6 small nuclear RNA was measured as an internal normalization control using the 2^{-dct} method [19]. The reactions were incubated at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. To statistically analyze the qRT-PCR results and based on the normality of data distribution in case and control groups, Student's t and Mann-Whitney U were tested in SPSS 16.0 statistical software. We accepted the difference with a probability value of less than 0.05 as statistically significant.

RESULTS

The demographic and clinical characteristics of the studied groups are compared in Table 1. There was no statistically significant difference between case and control groups regarding sex and age.

As shown in Figure 1, the expression of miRNA-217 was different in the serum of patients with achalasia and controls, but this difference was not significant (p-value = 0.572). Besides, the results demonstrated that the serum expression of miRNA-143-3p was not significantly different between the patients with achalasia and the control group (p-value = 0.366).

Figure 1 Relative expression of hsa-miR-217 and hsa-miR-143-3p in the serum of the patients with achalasia compared to the controls by the quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Relative expression was calculated using 2^{-dct} formula. Significant differences (p-value) have been shown in each graph.

DISCUSSION

The study of miRNA in achalasia has been performed in limited studies [20, 21]. But as far as we know, this is the first study to examine circulating miRNA as biomarkers in achalasia. As previously mentioned, serum MiRNAs are used as minimally invasive biomarkers to

diagnose and evaluate the prognosis of various pathological conditions. According to our previous study, two miRNAs, hsa-miR-217 and hsa-miR-143-3p, were evaluated in serum for their potential diagnostic biomarker for achalasia.

Based on NGS data, we have previously shown that has-mir-143-3p expression was increased in the esophageal tissue of achalasia patients [17], but in the present study, we could not find significant upregulation of miRNA 143 in the serum of achalasia patients by qRT-PCR. Contrary to our study, some studies have shown that miR-143 was up-regulated in the serum of patients with hepatocellular carcinoma and could be a potential circulating biomarker [22-24] . In another study conducted by Yu Han et al., sepsis was associated with increased levels of serum miR-143 [19].

Although in the previous study we showed that the expression of mir-217 was significantly reduced in the esophageal tissue in patients with achalasia, the results of qRT-PCR on serum of patients were not significant. Previous studies demonstrated that the serum mir-217 expression was downregulated in some disease, including pancreatic ductal adenocarcinoma, interstitial pneumonia, and membranous nephropathy [25-27]. These findings are contrary to the results of our study.

Although miRNAs are useful biological tools for the diagnosis and prognosis approach of diseases, accurate measurement of circulating miRNAs, including serum, is challenging. Various technical factors affect the circulating miRNAs measurement, which, if not addressed, could be affecting the results [28]. Precise measurement of the quality and concentration of circulating miRNAs in biological fluids posed many challenges due to their small amount, short length, and high GC concentration [12].

Numerous technical aspects and pre-analytical differences in sample collection and preparation may affect the outcome. To improve comparability between studies and the development of biomarker finding methods, various issues should be considered, including standardization and management of clinical sample preparation protocol and RNA isolation, use of normalization methods, and application of analytical standards [29].

Conclusion

We could not confirm our hypothesis about serum hsa-miR-217 and hsa-miR-143 as a non-invasive biomarker for achalasia. We suggest additional studies with more sample size and more accuracy to important technical aspects to reduce the limitations of this type of study.

Compliance with Ethical Standards

This study was approved by the Ethics Committee of Golestan University of Medical Sciences (Ethics Code= 31078693122415). Declaration of Helsinki in 1995 (as revised in Edinburgh 2000) was considered throughout the designing of the study protocol and its implementation. Participation in this study was optional, and all participants expressed their satisfaction by written informed consent, and participants' anonymity was preserved. The analysis and aggregation of information have not been individualized and the privacy of the participants has been considered.

Conflict of Interest

The authors declare that they have no competing interests.

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Table 1 Clinical data for 16 Achalasia patients and 17 controls

Characteristic	Patients †	Controls †
Mean Age (SD‡), year	39.75 (1.07)	44.23 (7.2)
Male/Female No. (% male)	5/11 (31.2)	7/10 (41.2)
Achalasia subtype¶	n (%)	
Type 1	5 (31.2)	
Type 2	11 (68.8)	
Type 3	0 (0)	
Mean duration (months) of symptoms (SD)	25.81 (1.74)	
Baseline symptoms	n (%)	
Dysphagia	8 (50)	
Chest pain	6 (37.5)	
Regurgitation	2 (12.5)	

† Unless otherwise indicated data are expressed as number (percentage) of patients. Percentages have been rounded and might not total 100.

‡ SD: Standard Deviation

¶Achalasia subtype: Type 1 (classic) with minimal contractility in the esophageal body, type 2 with intermittent periods of panesophageal pressurization, and type 3(spastic) with premature or spastic distal esophageal contractions[30]