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Evaluation of increased microRNA-21 in the serum of patients with cardia cancer

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ABSTRACT

MicroRNAs are a new group of regulatory RNAs that are about 22 nucleotides long. MicroRNAs are also involved in many human cancers and can actually act as oncogenes and suppressors. In this study, the relationship between miR-21 levels in the serum of sick and healthy individuals and the relationship between high concentrations of this microRNA and tumor staging and cancer progression was performed for the first time in Iran on gastric cardia cancer. This study was performed on 40 patients with gastric cardia cancer and 40 patients who were selected as a control group. The control group was selected from those who presented due to gastrointestinal disorders but were diagnosed as healthy or non-cancerous. Their serum miR-21 was measured using the Real-Time PCR technique. The relationship between the concentration of this microRNA in serum with tumor staging and cancer progression was evaluated. Findings showed that increasing the concentration of miR-21 in serum samples, serum miR-21 in patients was about 3.5 times that of controls ($P < 0.0001$), and serum miR-21 in patients was significantly related to staging tumor showed ($P = 0.01$), indicating a significant and direct relationship between miR-21 and tumor growth, which indicates the importance of this microRNA in the progression of gastric cancer. Given the role of miR-21 in the progression of gastric cardia cancer and its association with clinic-pathological factors, it can be introduced as a new diagnostic marker. It can also be used as a non-invasive method in the early diagnosis of gastric cancer patients. In this study, miR-21 has an oncogenic role that can be measured in patients' serum to assess the progression of gastric cancer.

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Introduction

Today, the incidence of cardia cancer has increased and the incidence of distal gastric cancers has decreased. The process of gastric cancer requires a slow and gradual progression from superficial gastritis, glandular atrophy to metaplasia, dysplasia, and finally, adenocarcinoma (1). This process of slow carcinoma production, which may take several decades, will provide an excellent opportunity for diagnosis and intervention to prevent further progression of carcinoma production and even attempt to reverse the carcinoma (2).

Cancer is the result of cells deviating from the regulatory, proliferative, and differentiating pathways (3, 4). The malignancy of cancer is caused by the lack of sensitivity to growth-inhibitory signals, avoidance of programmed cell death, unlimited proliferative potential, maintenance of angiogenesis and tissue invasion, and metastasis (5). MicroRNAs are a large subset of non-coding RNA 18-25 that are evolutionarily conserved (3). Numerous studies have shown that microRNAs play an important role in the onset and progression of cancer. MicroRNAs can be tumor inhibitors or oncogenes, depending on the type of mRNA they inhibit (6). The miR-21 stimulates, proliferates and invades cells and inhibits apoptosis. The interaction of microRNAs with target genes identifies their role in growth, programmed death, differentiation, and cell proliferation and confirms the direct function of microRNAs in cancer (7).

The structure of microRNAs and how they work show that many microRNAs are abnormally expressed in cancer specimens (8). In addition, functional differences between different types of tumors and different stages of cancer are associated with the expression of microRNAs. MicroRNA expression is associated with clinical and biological features of the tumor, such as tissue type, differentiation, invasion, and response to treatment (9). The use of microRNAs as diagnostic markers is possible through the

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examination of human serum or plasma so that cancerous microRNAs and tumor cells in serum or plasma can be identified without any invasive methods (6). The use of microRNAs closely related to malignant phenotypes as diagnostic markers is very helpful in diagnosing the disease in its early stages. Given the fact that most common methods for early-stage cancer screening are incapable of diagnosing the disease, identifying tumor microRNAs that are released into the bloodstream during the gradual progression of the disease is a key method for early detection of cancer (10). The oncogenic and anti-apoptotic role of miR-21 has been suggested in various studies by a group of scientists (9). The main purpose of this study was to evaluate the level of miR-21 in the serum of patients with gastric cardia.

Materials and methods

Patients

The statistical population in this study included patients referred to the hospital who were suspected of gastric cancer based on physical examinations and underwent gastric endoscopy and multiple biopsies of gastric lesions to confirm the disease. Thus, 40 patients with gastric cardia cancer were selected for this study. Also, 40 patients who were referred due to gastrointestinal disorders but were diagnosed as healthy or non-cancerous were also selected as the control group. Demographic information of patients was collected through face-to-face interviews and their medical records. All subjects participated in this study with informed written consent and this study did not impose any therapeutic limitations or loss of life on the subjects.

Preparation of blood serum

To prepare the serum, 5 ml of fasting blood was prepared from the subjects through a normal blood collection syringe in glass test tubes, and after half an hour of incubation, they were centrifuged at room temperature at a speed of 4000 rpm for 10 minutes. Serum samples were then collected in 1 ml aliquots in polypropylene tubes with special caps and stored at -80°C until further biochemical analysis. Serum RNA was extracted for liquid samples using miRVanaTM PARIS (Ambion) kit according to the manufacturer's instructions. To separate RNA from serum, 625µl of serum was mixed with a volume of 2-Mercaptoethanol solution at room temperature. Then, a solution of phenolic acid: chloroform (1250µl) was added to the mixture and vortexed for 60 seconds to mix well. The resulting mixture was centrifuged at room temperature for 100 minutes at a maximum speed of 10,000xg. Thus, the mixture was converted into two phases, aqueous (supernatant) and organic.

The aqueous phase (supernatant) was transferred to a new tube without disturbing the organic phase. To 300µl of the aqueous phase (supernatant), 375µl of ethanol (100% at room temperature) was added to a new tube and mixed well. A cartridge filter for each sample was placed inside the corresponding collecting tube and poured onto the filter cartridge with the help of a sample of the above-mentioned ethanol liquid mixture. The collecting tube was centrifuged with the cartridge for 30 seconds so that the mixture passed through the cartridge filter and was collected in the collecting tube. The collected liquid was discarded in the collecting tube. In the next step, 700µl of washing solution 1 was poured on the cartridge filter. The cartridge filter was then centrifuged with the collecting tube for 15 seconds so that the mixture passed through the cartridge filter and was collected in the collecting tube. Again, the collected liquid was discarded in the collecting tube. In the next step, 500µl of 100% ethanol solution was passed through this cartridge filter according to the previous step. This step was repeated for the second time. Then, once again, the cartridge filter was centrifuged for one minute with the collecting tube to separate all liquid residues from the filter. Next, the cartridge filter was placed inside another new collector tube. 100µl of nuclease-free water (preheated to 95°C) was poured onto the center of the cartridge filter. The cartridge filter was centrifuged with the new collecting tube for 30 seconds to separate RNA from the filter and collect it in the new tube.

Evaluate the efficiency and quality of isolated RNA

1.5µl of the RNA solution collected in the new tube was evaluated directly on a 1000A nanodrop spectrophotometer (NanoDrop, Wilmington, DE, USA) and its absorbance was measured at 260 and 280 nm. RNA concentration in each sample was estimated based on the absorbance at 60 nm. We also knew the purity of the isolated RNA, given that the adsorption ratio at 260 nm to the absorbance at 280 m

was approximately 1.8 to 2.1 for the samples. Electrophoresis on 1.2% agarose gel and ethidium bromide was used to determine the quality of RNA isolated.

Real-Time PCR reaction

Real-Time PCR operation was done using special primers (miScript Primer Assay, Qiagen), and miScript SYBR Green PCR Kit (Qiagen). First, 2x QuantiTect Universal SYBR Green PCR Master Mix solutions of 10x miScript Primer, 10xmiScript Primer Assay, nuclease-free water and synthesized template cDNA were brought to room temperature and each was mixed separately. To each of the plate wells of Real-Time PCR, 22.5µl of Master Mix reverse transcription mixture (including 12.5µl 2x QuantiTect SYBR Green PCR Master Mix, 2.5µl 10x miScript Universal Primer, 2.5µl 10x miScript Primer Assay, and 5µl of nuclease-free water) and 2.5µl of synthesized template cDNA for each sample were added. The plate was tightly covered by a layer of film. The plate was centrifuged at 1000 g at room temperature for one minute to expel air bubbles into the plate wells. Then the mentioned plate in Real-Time CFX96TM Real-Time PCR Detection (PCR), Bio-Rad, Hercules, CA, USA) (System) whose temperature program was previously set as follows. Initial activation of PCR was for 15 minutes at 95 ° C, which activates the enzyme HotStarTaq DNA polymerase.

The periodic phase includes denaturation for 15 seconds at 95°C, annealing for 30 seconds at 55°C, elongation for 30 seconds at 72°C. This step was repeated 39 times.

Determining the baseline signal

By evaluating the linear view of the Real-Time PCR amplification curve, the location of 2 cycles before the first visible amplification signal was considered as the base signal.

Threshold cycle determination

By evaluating the logarithmic view of the Real-Time PCR amplification curve, the signal or threshold cycle was located above the background signal but in the lower half of the algorithmic range of the amplification diagram.

Melting curve analysis

At the end of replication, with the help of the melting curve analyzer, the replication product was performed to confirm the specificity and identity of the original reproduced product. For this analysis, the plate temperature was slowly increased from 65°C to 95°C, during which the fluorescence signal of the amplified product was continuously read by the device. The device then plots a curve or graph of fluorescence intensity against temperature.

All samples were dually assayed. The small androgenic nuclear RNA (RNU6B), which plays the role of the Housekeeping gene, was also assayed to normalize the accompanying assay of the samples at all stages (simultaneously with the dual assay of the samples on the same plate). This was done by purchasing a kit related to Human RNU6B ((RNU6-2) miScript Primer Assay, Qiagen). 10 samples of patients were prepared and their Real-Time PCR amplification product was analyzed. Their Ct diagram was plotted against their dilution. In addition, a negative control sample without cDNA (in pairs) was included in the plate along with the other samples, 10 healthy samples were mixed together and after vortexing, a pooled DNA sample was prepared. This recent pooled DNA sample was used as a calibrator.

Calculation of miRNA-21 gene expression in the studied samples

To normalize the miR-21 level in the samples, RNU6B RNA was used according to the $2^{-\Delta\Delta Ct}$ (Livak) method. The pooled DNA sample was used as a calibrator, which was measured along with other samples on the plate. First, the Ct of each sample was determined according to the method mentioned earlier. The ΔCt was then calculated. The value of ΔCt is obtained according to the following equation:

ΔCt = Ct (each sample) - Ct (RNU6B)

In the next step, the ΔΔCt value and the normalized value of each sample are calculated:

ΔΔCt = ΔCt (Serum sample of cancer patients or control) - ΔCt Calibrator serum sample)

The amount of normalized miRNA in each sample will be 2. The data of this study were analyzed after entering the computer with statistical software 16.SPSS vol. Statistical differences in miR-21 values

between patients and controls were tested by a two-tailed Mann-Whitney U test. Receiver-operating characteristic ROC analysis was performed to determine the diagnostic value of serum miR-21 in differentiating patients from controls. A value of p less than 0.05 was considered statistically significant.

Results and discussion

According to the results, the presence of two rRNA index bands (28S rRNA and 18S rRNA) on 2% agarose gel electrophoresis indicated the good quality of isolated RNA (Figure 1).

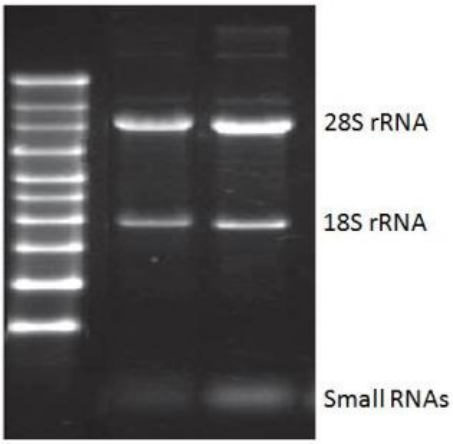

Figure 1. The presence of two rRNA index bands (28S rRNA and 18S rRNA) on 2% agarose gel electrophoresis

By evaluating the specific and exclusive peak of miRNA-21 as well as the peak dimer of the primers, we confirmed the specificity of Real-Time PCR amplification (Figure 2).

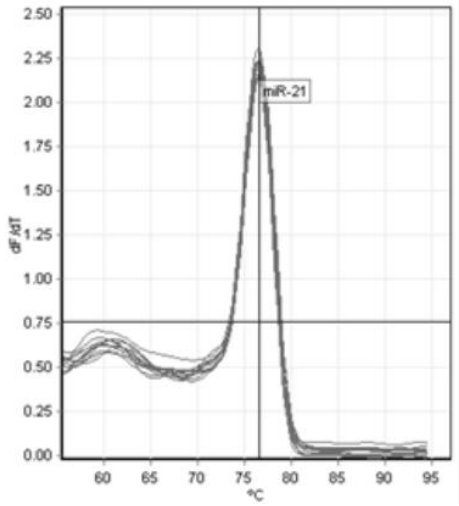

Figure 2. Melting curve analysis for miR-21

The results of clinical and demographic characteristics for both patient and control groups are shown in table 1.

Table 1. Demographic and clinical characteristics of Patient group and control group

Variable		Patient group (n = 40)	Control Group (n =40)	P-value
Gender	male	21	20	0.82
	Female	19	19	0.82
Age (year)		57.35 ± 10.13	57 ± 10.34	0.85
Smoking		20%	15%	0.56
Tumor staging	I	9	-	
	II	7	-	
	III	7	-	
	IV	17	-	

MiR-21 levels in the serum of patients and controls are shown in Figure 3. The serum level of miR-21 in these patients was about 3.5 times that of controls (P = 0.0001).

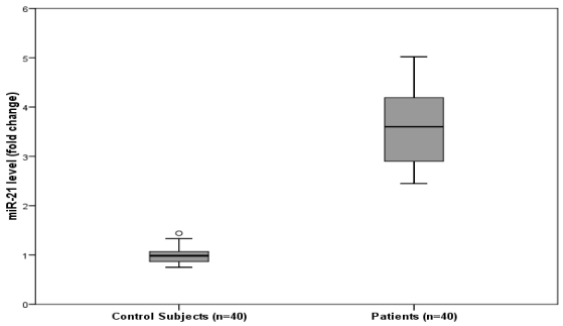

Figure 3. The serum level of miR-21 in the control group and patient group

According to Figure 4, the level of miR-21 in patients's serum showed a significant relationship with tumor staging (P = 0.01).

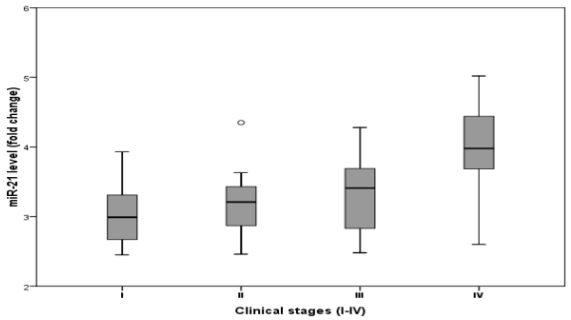

Figure 4. The level of miR-21 based on clinical tumor stage

Receiver-operator characteristic (ROC) curve analysis showed that the serum miR-21 value could be well differentiated between patients and controls

(Figure 5). By analyzing the curve and preparing the cut-off value for this biomarker, it was found that the serum miR-21 value above 1.47 times higher than healthy individuals (calibrators), can provide patients with 77% specificity and 79% sensitivity to recognizing healthy people. Based on the results, it was found that miR-21 has a significant relationship with tumor staging, and measuring the amount of this microRNA in serum is a good criterion for differentiating patients from healthy individuals.

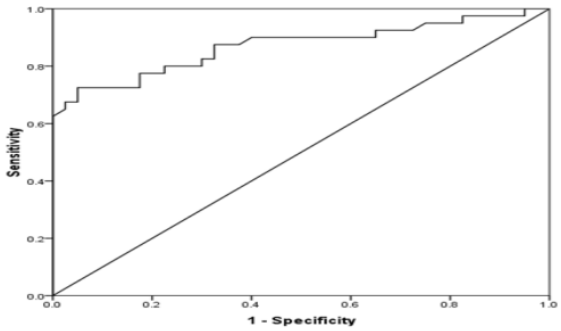

Figure 5. Rock curve for serum miR-21 levels to differentiate patients from controls; the area under the rocking curve for this analysis was 0.86 (CI: 0.73-0.96, 95%)

Gastric cancer is the most common cancer in men and the third most common cancer in women after breast and colon cancer (11). Given the importance of the issue and late diagnosis of the disease, our effort is based on the principle that we can provide methods for early and timely diagnosis of gastric cancer (12). In patients with gastric cancer, increased miR-21 expression in the blood may act as a diagnostic marker (6). Increasing this microRNA also reduces the expression of tumor-inhibiting proteins and increases the expression of oncogene proteins. Given these roles, miR-21 could be a good option for investigating cancer and metastasis (13). Due to the increased expression of miR-21 in cancerous tissues, it can be used for early detection and prevention of metastasis, and because this microRNA affects tumor cells and has oncogenic and anti-apoptotic properties, it disrupts the apoptotic pathway, which is beneficial for the survival of cancer cells (12).

The study on the anti-apoptotic role of this biomarker remains somewhat unclear (11). However, according to the above information, research on it can be a perspective in the early stages of diagnosis and treatment of cancer (14). Other studies have also used flow cytometry to examine the expression level of regulatory miRNAs in different types of cancer and have used them to classify different types of cancer (10). Clinical studies have shown that the expression of miR-21 in gastric cancer tumor tissue is significantly higher compared to healthy peripheral tissue. Increased miR-21 levels in gastric cancer tumor tissue have been shown to be strongly associated with disease invasiveness, tumor size, and staging. MiR-21 is a well-known microRNA that has been studied extensively (15).

This microRNA has an oncogenic role and by turning off a series of important genes in the cell, it leads the cell to become cancerous. There are traces of this micro RNA in various cancers (16). In this study, which was performed on 40 patients with cardia gastric cancer and 40 healthy individuals were selected as a control group, we found a direct relationship between increased expression of miR-21 and tumor staging. The results of this study also showed that in patients, serum miR-21 level showed a significant relationship with tumor staging. Zheng *et al*. (17) also found a positive association between this microRNA and tumor size and also clarified that patients with high miR-21 expression also have larger tumors. In the present study, the study of increasing serum miR-21 levels helps to differentiate between patients and controls, which consistent with the results obtained by Quan *et al*. (18). However, the difference between our works with the mentioned study was that our study was on blood serum, but they investigated the expression of miR-21 in tumor tissue.

MR-21 is directly related to tumor size, tumor staging, and cancer progression but is unable to predict disease prognosis. Based on this evidence, it can be concluded that miR-21 is produced by gastric cancer tumor tissue, and its serum level is significantly higher in patients than in controls, thus differentiating between the two groups. It also helps, and the higher the expression of 21-miR in the serum, the greater the relationship with tumor size, tumor staging, and the severity of disease invasion. As a result, measuring serum miR-21 levels can be used as a non-invasive method in the early diagnosis of gastric cancer patients. In fact, the expression of this microRNA in serum was considered as a diagnostic marker.

Acknowledgments

Not applicable.

Interest conflict

The authors declare that they have no conflict of interest.

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