



# Correlation between the Expression Levels of Circulating miR-21 and miR-192 and Clinicopathological Features in Plasma of Patients with Gastric Cancer in Iran

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## Abstract

**Background:** Gastric cancer (GC) is the most prevalent malignancy worldwide and a common cause of death in Iran. Studies have proved that a variety of dysregulated microRNAs is involved in the development and progression of gastric cancer. The present study aimed to evaluate the expression levels of plasma circulating oncogenic miR-21 and miR-192 and their association with clinical phenotypes of patients with gastric cancer in the north of Iran.

**Methods:** Clinico-pathological analysis was conducted using a standard protocol and pathological tests. The expression levels of miR-21 and miR-192 were measured using quantitative reverse transcription-polymerase chain reaction in the plasma of twenty pre/post-operative gastric cancer patients and twenty healthy subjects. The receiver operating characteristic (ROC) curve of these microRNAs was analyzed to investigate their diagnosis properties.

**Results:** The study results indicated that plasma miR-21 expression was significantly associated with tumor stage and helicobacter pylori infection status ( $P=0.024$ ,  $P=0.0004$ , respectively). However, no association was observed between clinic-pathological characteristics and miR-192 expression. The results showed that the plasma levels of miR-21 ( $P=0.0001$ ) and miR-192 ( $P=0.0007$ ) were significantly higher in GC patients compared to those in healthy individuals. Furthermore, the ROC analyses yielded the mean  $\pm$ SD area under the curve (AUC) values of  $0.9525\pm 0.03$  ( $P<0.0001$ ) and  $0.5925\pm 0.09$  ( $P=0.316$ ) for miR-21 and miR-192, respectively. Pearson regression analysis showed that there was no significant correlation between the expression of miR-21 and miR-192 ( $P=0.1507$ ).

**Conclusion:** Based on the obtained results, the expression of the plasma level of miR-21 was significantly higher in gastric cancer patients compared to that in the healthy group. Furthermore, the higher levels of AUC in miR-21 indicated the potential role of miR-21 as a noninvasive biomarker for the prognosis of gastric cancer in the population of the north of Iran.

**Keywords:** Gastric cancer, miR-21, miR-192, plasma, Receiver operating characteristic analysis

## 1. Background

Gastric cancer (GC) is the fourth most prevalent malignancy and the second leading cause of mortality worldwide (1). Based on the latest update of the International Agency for Research on Cancer (IARC), the incidence rates of the disease in different geographical regions are distinctly varied (2). Although the mortality rates and incidence of GC have decreased in the past 70 years in Iran (3) it still is the most common cancer in males and the second malignancy-related death among females (4). Based on the plethora of evidence in the literature multiple factors involved in the etiology of gastric malignancy include genetic alterations (mutations, epigenetic changes, and genetic polymorphism), infection such as *Helicobacter pylori*, lifestyle (diet), and metabolic risk factors (serum cholesterol) (1).

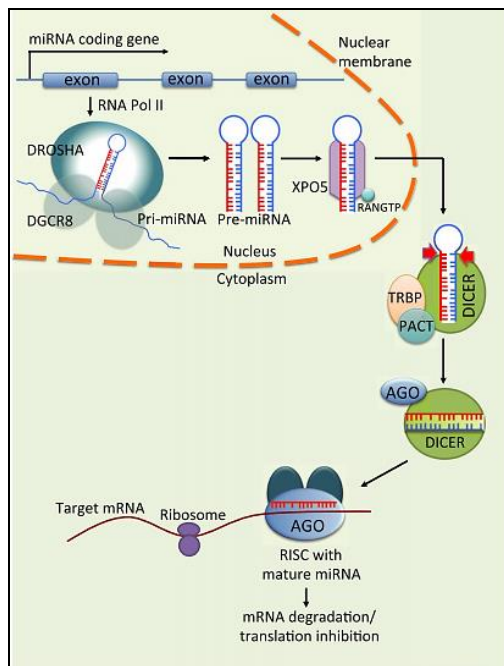
Molecular genetics studies proved that MicroRNAs (miRNAs) play diverse roles in the physiological and pathological state of GC (5, 6). miRNAs are a subset of non-coding RNAs with a length of ~22 nucleotides (6) that are transcribed but

not translated into proteins (7). It is believed that miRNAs have a unique ability to negatively regulate gene expression (8). Figure 1 presents a history of microRNAs biogenesis. Some microRNAs, such as miR-21 and miR-192 have oncogenic properties associated with various genetic pathways in GC (8, 9). Aberrant dysregulation of miRNAs has been observed in GC (5). Although different serum tumor markers, such as carcinoembryonic antigen and carbohydrate antigen 19-9 have been applied for the diagnosis of GC, these conventional tumor markers lack sufficient sensitivity and specificity to facilitate early detection of malignancies (10). Several studies have shown that some circulating miRNAs in GC patients were detectable in plasma. These miRNAs can be non-invasive blood-based biomarkers that were potentially used for the prognosis or diagnosis of GC patients in the future (10).

## 2. Objectives

The present study aimed to evaluate the correlation of clinicopathological features and the

expression pattern of oncogenic miRNAs (miR-21, miR-192) in patients with GC in the north of Iran.



**Figure 1.** Biogenesis of microRNAs. They are produced by RNA polymerase II enzyme from nuclear DNA to generate pri-miRNAs. This molecule is processed to pre-miRNAs and it is then transferred to the cytoplasm by Exportin-5. In the cytoplasm, it is cleaved by RNase III enzyme Dicer to produce miRNA duplexes. Following pre-miRNAs, the guide strands are selected by AGO enzyme for integration into the RISC (the RNA induced silencing complex) complex. This is the last stage of miRNAs maturation process. Mature miRNAs binds complementary to the 3'UTR of mRNAs resulting in denaturation or translational repression of target genes (Shea et al., 2016).

### 3. Methods

#### 3.1. Patients and samples

The study protocol was approved by the Committee of Human Experiments of Rasht Branch, Islamic Azad University, Rasht, Iran (IR.IAU.RASHT.REC.1399.010), and informed consent was obtained from the participants at the beginning of the study. Plasma samples were obtained from 20 patients with pre/post-operative GC as well as 20 healthy volunteers (control group) in Jam laboratory and Razi Hospital (Rasht, Guilan province, Iran). The participants' demographic, clinical, and pathological data were collected which included gender, age, smoking status, tumor size, tumor

location, tumor stage, helicobacter pylori infection status, and metastasis phenotype.

#### 3.2. RNA extraction and cDNA synthesis

Total RNAs were extracted from plasma using a BON-miR RNA extraction kit (Stem Cell Technology Research Center, Tehran, Iran) according to the manufacturer's protocol. All plasma samples were transferred to free EDTA tubes and incubated in RT for 30 minutes. The samples were centrifuged at 3000rpm for 15 minutes in the RT. The cell pellets were then lysed using a lysis buffer. Afterward, the suspension was incubated with chloroform at 12000 rpm. Subsequently, cold isopropanol was added and incubated overnight at -20°C. The precipitated samples were washed with ethanol and the precipitated miRNAs were diluted by DEPC water following isopropanol elimination. The cDNA synthesis was performed using BON-miR miRNA 1<sup>st</sup>-strand cDNA synthesis Kit (Stem Cell Technology Research Center, Tehran, Iran) according to the manufacturer's protocol. Activation of RNA Polyadenylation was performed using polyA polymerase at 37°C for 30 min. Afterward, polyadenylated RNA was mixed with 1µl of BON-RT adapter primer (10µM) and DEPC treated water and this mixture was incubated at 75°C for 5 min to remove secondary structures. Subsequently, RT enzyme, dNTP (10mM), and 5X buffer were added to the mixture. cDNA synthesis was carried out at 25°C for 10 min, 42°C for 60 min, and 70°C for 10 min.

#### 3.3. miR-21 and miR-192 expression analysis by Q-RT-PCR

Quantitative expression of miR-21 and miR-192 was carried out using the ABI StepOnePlus™ instrument. The primer pairs were purchased from the Stem Cell Technology Research Center (Tehran, Iran). Primers and chromosomal locations of microRNAs are presented in Table 1. The PCR reaction was carried out in the final volume of 13µl using BON-miR High specificity miRNA QPCR kit (Stem Cell Technology Research Center, Tehran, Iran) containing 6.3µl of 2X miRNA QPCR master mix (CYBR green), 1µl of cDNA, 0.5µl of each primer (forward and universal reverse primer), and 4.7µl of distilled water. The PCR amplification was performed in a 40 cycle based on the following program: 95°C for 5s and 60°C for the 30s. The expression level of miR-21 and miR-192 was normalized by miR-93-5p (12). All reactions were performed at least in triplicate. Fold change of each microRNA was calculated by the equation  $2^{-\Delta\Delta Ct}$ .

**Table 1.** Primers sequence of miRNAs used in the study

MicroRNAs Symbole	Accession Number(miRBase)	Chromosome location	Primers (5'to 3')	Anealing/Extension temperature (°C)
miR-21	MI0000077	ch17q23.2	F.GCTTATCAGACTGATGTTG R.GCGAGCACAGAATTAATACGACTC	60
miR-192	MI0000234	ch11q13.1	F.TC TGA CTT ATG AAT TGA CAG C R.GCGAGCACAGAATTAATACGACTC	60
miR-93-5p	MI0000095	ch 7q22.1	F.TAAAGTGCTGTTCGTGC R.GCGAGCACAGAATTAATACGACTC	60

### 3.4. Receiver operating characteristic (ROC) curve analysis and microRNAs expression association

The ROC curve analysis was performed to evaluate the diagnostic value of miR-21 and miR-192 expression levels in gastric tumor and control samples. Furthermore, a regression analysis was performed to obtain the probability correlation analysis of microRNAs expression in GC.

### 3.5. Bioinformatics analysis of miRNAs-targets interactions

*In-silico* analysis was carried out to examine the probability of the interaction of miRNAs-targets in GS pathways. miRNA sequences were obtained from the miRBase database (<http://www.mirbase.org>) and the interaction process was carried out using online

RNA-Hybrid bioinformatics software (<http://www.bibiserv.cebitec.uni-bielefeld.de>). Interaction networks were built using Cytoscape software (version 3.7.1).

### 3.6. Statistical Analysis

Normality distribution of samples was tested using the Shapiro-Wilk test. The statistical analysis of miRNAs expression and clinic-pathology features in healthy individuals and those with GC was performed using the Mann-Whitney test. The ROC curve analysis was performed to evaluate the diagnostic value of miR-21 and miR-192 expression levels in the samples from plasma of GC patients using Prism software (version 8.0.2) based on RQ values. A P-value less than 0.05 was considered statistically significant. rest were nonsmokers. Based on TMN classification, 85% and 15% of patients were in stage III and stage IV. Moreover, 80% of patients (n=20) were positively in the metastasis stage. Analysis of tumor location revealed that both cardia and non-cardia GC were involved in the patient's pathogenicity. The cardia GC and non-cardia GC were diagnosed in 60% and 40% of patients, respectively. A brief history of clinical data is presented in Table 2.

## 4. Results

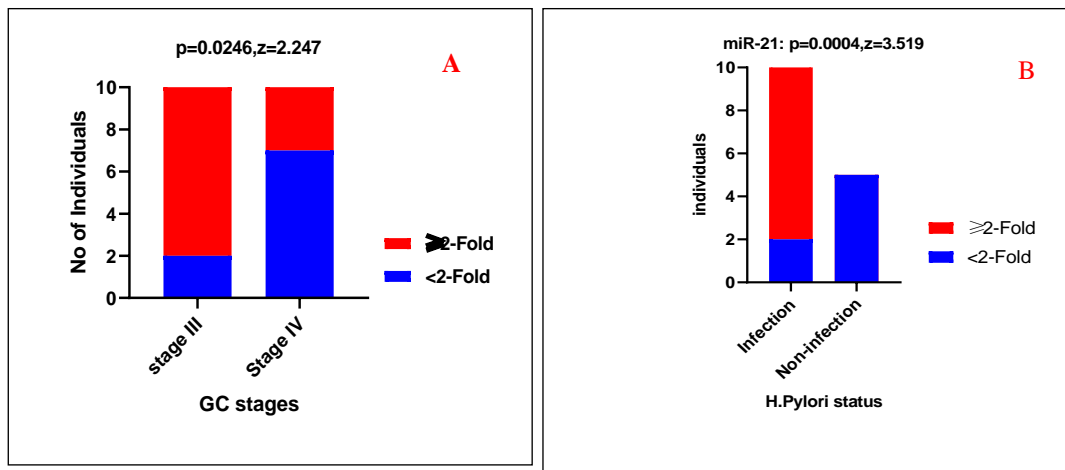
### 4.1. Clinico-pathological characteristics of the study participants

The participants in this study included twenty GC patients and twenty healthy individuals. The median age of participants was 65 years. It should be noted that 70% of patients (n=20) were smokers and the

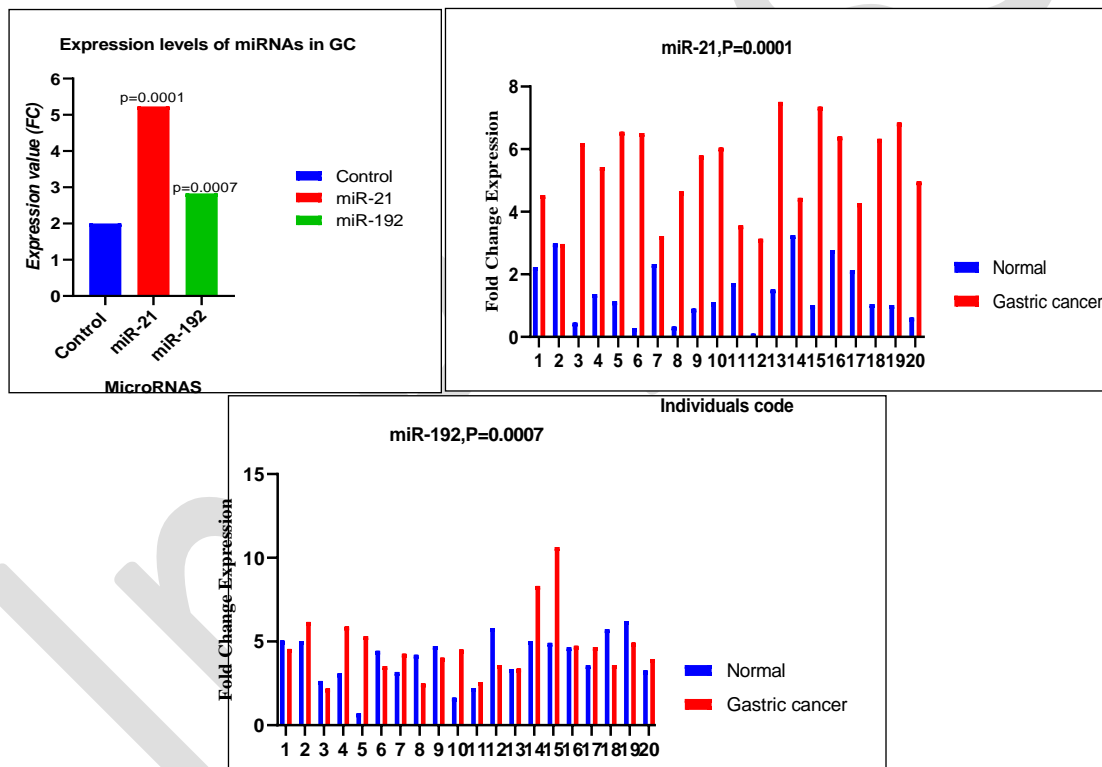
**Table 2.** Correlation of clinico-pathological features of GC patients with miR-21 and miR-192 expression

Demographical/ Pathological data	miR-21 Expression			miR-192 Expression		
	<2-Fold (N=20)	≥2-Fold (N=20)	P-Value	<2-Fold (N=20)	≥2-Fold (N=20)	P-Value
<b>Age (Year)</b>						
≥65=8	3	5	0.848	6	2	0.999
≤ 65=12	4	8		9	3	
<b>Gender</b>						
Male=12	4	8	0.456	6	6	0.58
Female=8	4	4		3	5	
<b>Smoking</b>						
Smokers=14	6	8	0.768	8	6	0.768
Non=6	3	3		3	3	
<b>Site of tumor</b>						
Upper Stomach (Cardia)=12	6	6	0.219	6	6	0.76
Middle Stomach (Body)=5	3	2		2	3	
Whole Stomach (Antrum)=3	0	3		2	1	
<b>Tumor size</b>						
< 4cm= 12	5	7	0.443	6	6	0.99
4-8 cm =8	2	6		4	4	
<b>Tumor Stage</b>						
Stage III=10	2	8	0.024*	2	8	0.91
Stage IV=10	7	3		2	8	
<b>Gross appearance</b>						
Superficial type=10	4	6	0.422	5	5	0.859
Borrmann, I,II type=8	4	4		3	5	
Borrmann III,IV type=2	1	1		1	1	
<b>Lymph node metastasis</b>						
Positive=16	2	14	0.093	8	8	0.99
Negative=4	2	2		2	2	
<b>H. Pylori status</b>						
Infection=15	2	13	0.0004*	7	8	0.79
Non-infection=5	5	0		2	3	

\* Pathological significant changes presented as star



**Figure 2.** Correlation of Clinico-pathological features of GC patients and expression pattern of oncogenic miR-21 in plasma of GC patients. Data analysis revealed a significant association between A) tumor stages (P=0.246) and B) H.Pylori status (P=0.0004) with miR-21 expression in patients



**Figure 3.** Relative expression of A) miR-21, B) miR-192 in patients and healthy individuals; and C) mean of their expression in two groups. miRNAs expression was normalized to miR-93-5p as endogenous internal control. The normalized expression value (FC) was 2

**4.2. Quantitative expression of miR-21 and miR-192**

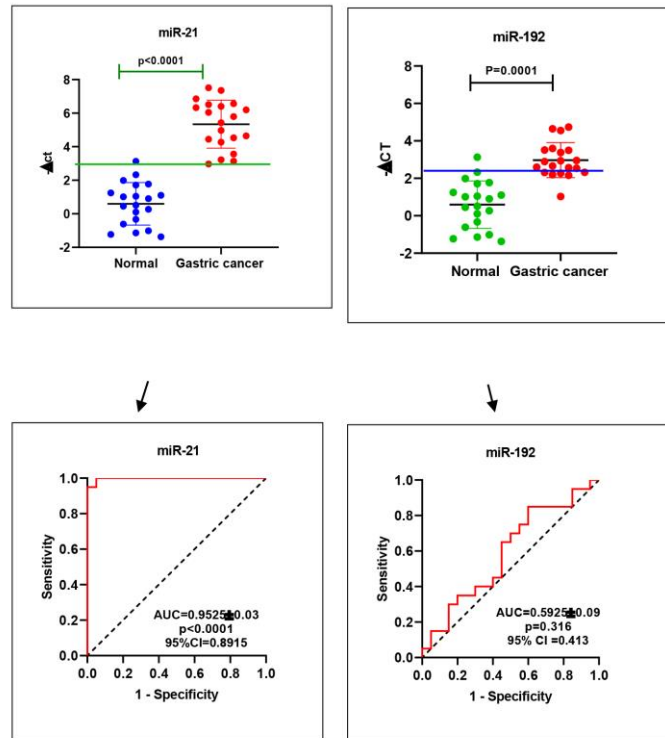
The study analysis showed significant overexpression of miR-21 and miR-192 oncogenes (P<0.0001 and P=0.0007, respectively) in plasma of

GC patients compared to that of the healthy individuals, with an average of 5.2 and 2.83-fold change, respectively (cut-off was set to 2- fold). The results are presented in Figure 3 and Table 3.

**Table 3.** miRNAs biological role, their targets in Gastric cancer and dysregulation of microRNAs involved in Iranian GC. The data presented in CI=95%

Name	Biological role in GC	mRNAs Targets	Expression Pattern in GC	References	Dysregulated pattern in GC-Patients		
					Fold	P-Value	CI 95%
<b>miR-21</b>	Oncogenic(OncomiR), Cell proliferation, metastasis,	RECK,TIMP3,MMP2, MMP9,SERPIN1, TMP1,FASLG,BTG2,	Upregulated	(14-16)	5.2	0.0001	1.223 - 2.564

	malignant transformation, invasion	<i>PTEN,PDCD4,AKT,NF-BK,EGR2,SMAD7</i>					
<b>miR-192</b>	Oncogenic(OncomiR), Cell proliferation, invasion	<i>ERCC1,ERCC2,BIM</i>	Upregulated	(17-19)	2.83	0.0007	1.102 - 2.008
<b>miR-93</b>	Housekeeping gene	-	-	(14)	2	-	-



**Figure 4.** ROC curve analysis. The figure shows the comparison of two microRNAs and their specific AUC based on their  $\Delta Ct$  distribution. miR-21 with  $0.95 \pm 0.03$  AUC (optimal cut off value = Youden index = 3.1) compared with miR-192,  $AUC = 0.5925 \pm 0.09$  (optimal cutoff value = Youden index = 2.1) play as a potential biomarker for GC diagnosis patients in Iranian population. The curve was analyzed using graph pad prism version 8.0.2

#### 4.3. Correlation of clinicopathological feature and miRNAs expression

Histopathological data revealed a significant association between miR-21 expression in tumor stage ( $P = 0.024$ ) and *H. pylori* status ( $P = 0.0004$ ) in gastric cancer patients (Figure 2). This clinicopathological association was not observed in miR-192 expression status. The analysis revealed that tumor stage ( $P = 0.024$ ) and *H. pylori* infection status ( $P = 0.0004$ ) were statistically different in patients with over-expressed miR-21 ( $F \geq 2$ ) compared to those with low miR-21 ( $F < 2$ ). However, there was no significant difference in other clinic-pathological features. In addition, no significant difference was observed in clinicopathological features with

dysregulation of miR-192 in plasma of GC patients ( $P > 0.05$ , Table 2).

#### 4.4. ROC curve analysis and miRNAs expression association evaluation

Diagnostic evaluation of the ROC curve based on the distribution of  $\Delta Ct$  of miR-21 and miR-192 in GC

patients revealed that the mean of area under the curve (AUC) for miR-21 and miR-192 was estimated at  $0.9525 \pm 0.03$  ( $P < 0.0001$ , 95% CI = 0.89) and  $0.5925 \pm 0.09$  ( $P = 0.316$ , 95% CI = 0.41), respectively. The ROC analysis showed that miR-21 had the highest AUC value compared to miR-192, indicating that miR-21 had a strong potential diagnosis value for GC detection in the Iranian population (Figure 4). Moreover, Pearson regression analysis (Figure 5) showed that there was no correlation between the expression levels of miR-21 and miR-192 ( $y = 0.5113 + 3.83$ ,  $R^2 = 0.1113$ ,  $P = 0.1507$ ).

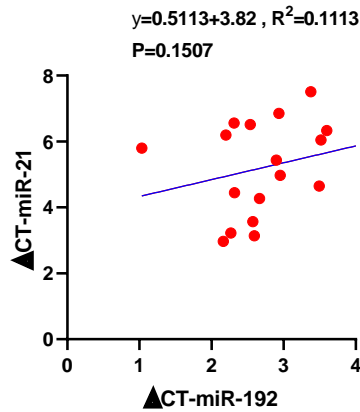
#### 4.5. In-silico analysis

*In-Silico* pathway and gene enrichment analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG) showed genetic interactions between miR-21, miR-192, and their genetic targets involved in GC. Data analysis revealed that these microRNAs regulate genes involved in the crucial signaling pathways, such as Apoptosis, AKT signaling pathway, tumorigenesis, and proliferation pathways. The potential target genes of these microRNAs were graphically presented as a

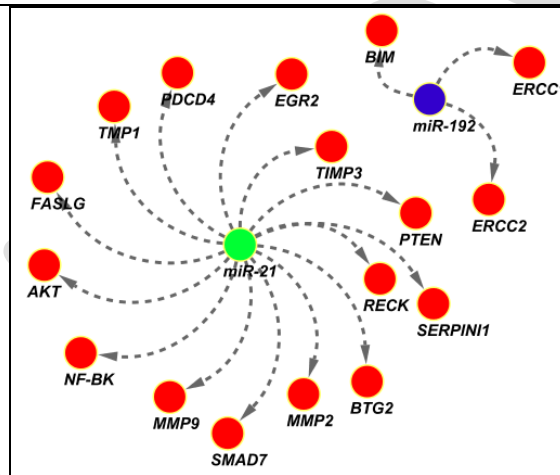


network in Figure 6. The network was built using Cytoscape software (version 3.7.1). A genetic pathway

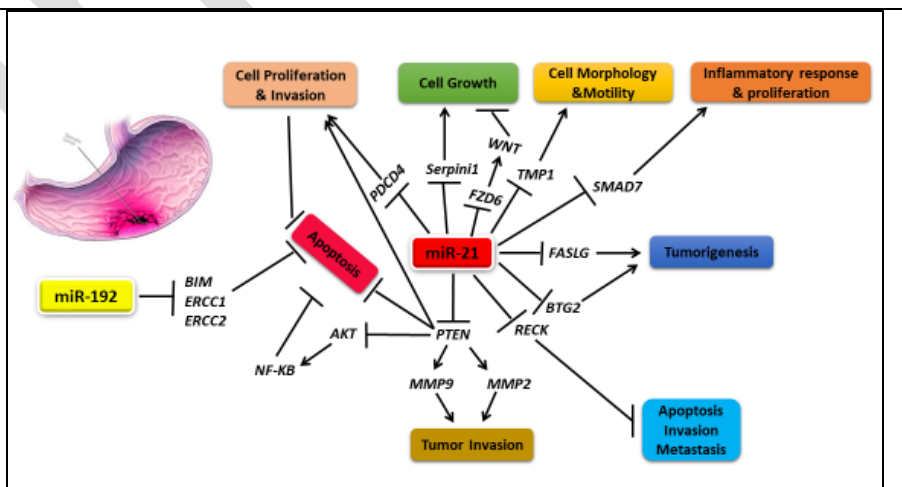
of dysregulated microRNAs is presented in Figure 7.



**Figure 5.** Pearson regression analysis of miR-21 and miR-192 expression based on  $\Delta Ct$  values. There was no significant correlation between the expression of two miRNAs ( $P=0.1507$ )



**Figure 6.** A Schematic network of *In-silico*-pathway analysis show the relationship between miRNAs and their target genes in Gastric cancer



**Figure 7.** Genetics pathway of dysregulated miR-21, miR-192 and their genetics action in Gastric cancer. Pathway analysis show that miRNAs target different genes in various pathways that are crucial in tumor progression

## 5. Discussion

Despite remarkable progress in the diagnosis and treatment of GC cases, the rate of mortality due to this cancer is still high. Many studies have demonstrated the dysregulation of miRNAs in gastric tumorigenesis and the fundamental role of these negative regulators in tumor progression (5, 9, 12, 16). Some miRNAs, such as miR-21 can in the future serve as a non-invasive biomarker for GC detection (17). The use of quantitative RT-PCR showed significant overexpression of miR-21 and miR-192 in 20 GC patients compared to controls.

Regarding clinicopathological characteristics, expression of plasma miR-21 was closely associated with tumor stages (III and IV;  $P=0.024$ ) and H.pylori infection status ( $P=0.0004$ ). It has been reported that patients with positive serologic tests for H. pylori have a higher risk of the development of GC (18). Previous study results revealed that tumor stages had a close association with GC tumorigenesis (19). In a study conducted by Wu et al., no correlation was found between miR-21 expression level and clinicopathological characteristics in the serum and peripheral blood mononuclear cell (PBMCs) of GC patients in 2010. Liu et al. reported that in 20 pairs of paraffin-embedded specimens of GC patients no correlation was observed between miR-21 and such factors as age, differentiation degree, histological type, lymph node metastasis, and H. pylori infection (20). The differences in miRNAs levels in normal and pathological conditions can be regarded as a promising biomarker for the diagnosis of GC patients. Based on the obtained results, circulating miR-21 can be introduced as a miR biomarker in stages III and IV of GC in cases with H. pylori infection in the north of Iran.

In the present study, ROC analysis showed a significant mean AUC of  $0.95\pm 0.03$  in miR-21 ( $P<0.0001$ ) that can be used as a diagnostic marker for GC patients. These findings were in line with those obtained in other regions of Iran and other countries (10, 21, 22). miR-21 is overexpressed in various cancers, such as breast cancer (23), lung cancer (24), colon cancer (25), and GC (19). Overexpression of miR-21 can change the biological process in the cell including proliferation (SMAD7, SERPIN1), apoptosis (RECK), invasion, (PTEN, RECK), and tumorigenesis (FASLG, BTG2) via regulating important target genes (8, 18).

There was no association between clinicopathological characteristics and expression of oncogenic miR-192 in the present study. The results of the study performed by Xu and Fan revealed that overexpression of miR-192 was related to the clinical features of GC, such as lymph node metastasis (26). Therefore, miR-192 can not be considered as a biomarker in GC patients in the study population.

A previous study showed that miR-192 has a dual

function as oncomiR and tumor-suppressive miR in gastric GC. The results of the study conducted by Chiang et al. revealed that miR-192 was downregulated in GC patients compared to that in the individuals in the control group (27). However, Jin et al., showed that miR-192 has been significantly overexpressed in primary GC (28). Studies have shown that miR-192 inhibits the apoptosis process by targeting the main target genes including *BIM*, *ERCC1*, and *ERCC2* in GC (14, 16, 29). Although miR-192 has not previously been described as a predictive factor in GC, it has been detected in other malignancies as possible predictors (30). In a research study conducted by Odental et al., miR-192 was introduced as an indicator of a major histopathological response in esophageal cancer (31). However, based on the results of the present study, miR-192 was significantly overexpressed ( $P=0.0007$ ) and the mean AUC analysis was not significant ( $0.5925\pm 0.09$ ,  $P=0.316$ ). Moreover, the miR-192 overexpression pattern was not associated with AUC; therefore, it cannot be considered as a prognostic biomarker in GC patients in the north of Iran.

## 6. Conclusion

Quantitative analysis showed significant overexpression of circulating miR-21 and circulating miR-192 ( $P<0.001$ ) in the plasma of GC patients compared to healthy individuals. A significant correlation was observed between miR-21 expression and tumor stages III and IV and H. pylori. The higher levels of AUC in the circulating miR-21 indicated the potential role of circulating miR-21 as a noninvasive biomarker for the prognosis of GC in the north of Iran.

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## Footnotes

**Conflicts of Interest:** The authors declare that there is no conflict of interest regarding the publication of the present study.

## Authors' Contribution:

F. A. R. and N. R. contributed in the conceptualization and design of the article. F. A. R., N. R. and H. S. S. contributed analyzed and interpreted the data. F. A. R., and N. R. conducted the statistical analysis. F. A. R. and N. R. wrote and revised the manuscript. N. R. gave the final approval for this article. All authors are accountable for all aspects of the work.

**Ethical Approval:** The study protocol was approved

by the Committee of Human Experiments of Rasht Branch, Islamic Azad University, Rasht, Iran (IR.IAU.RASHT.REC.1399.010). Informed consent was obtained from the participants at the beginning of the study.

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