Circulating Micro RNA (106, 21) as Biomarker for *Helicobacter pylori* associated gastric cancer

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Abstract

Background: gastric cancer (GC) residue one of the major health burdens accounting for of all cancers and is the third leading cause of cancer-related deaths worldwide. Chronic inflammation due to Helicobacter pylori infection plays the role in triggering carcinogenesis. Gastric cancer managements must be quickly and the time of gastric cancer diagnosis is necessary for treatment, unfortunately, the existing circulating biomarker for gastric cancer diagnosis prognosis display low sensitivity and specificity, the gastric cancer diagnosis is based on only on the invasive procedures such as upper digestive endoscopy and diagnosis for the majority of patients is made at an advanced stage when only limited treatment options can be offered, therefore other the recent studies are suggests using miRNAs as gastric cancer biomarker. **Objective**: The study aims to evaluate miRNA-2l, miRNA-106 as a non invasive diagnostic biomarker for Helicobacter pylori associated gastritis and gastric cancer. **Patients and methods:** A case control study have been conducted and based on 3 group, The first group was included 20 patients with Helicobacter pylori infection associated gastric cancer, who were observation in Oncology Center at Al-Diwaniyah City, Second group was include 20 patients with Helicobacter pylori infection associated Gastritis who visited Endoscopy Department of Gastroenterology and Hepatology of Al-Diwanyiah Teaching hospital. Third group was include 40 healthy volunteers. Venipuncture used to collect samples of blood from these groups. The collection of three milliliters blood was payload out in plain non EDTA tube in order to clot. Further, centrifuges was utilized to separate the serum and then stored at -20oC which was further used to identify free miRNA-106 and miRNA-21 qPCR. **Results:** Current study reveals that miR-21 expression was significantly highest in patient with gastric cancer , and then patients with gastritis and then control group ($P < 0.001$), 6.28 (4.78) fold change versus 2.45 (1.79) fold change versus 1 (---) fold change In addition, the miR-106 expression was significantly highest in patients with gastric cancer and then patients with gastritis and control group ($P < 0.001$), 7.03 (4.81)fold change versus 3.02 (1.82)fold change versus 1 (---) fold change, respectively, the diagnostic value of both miR-21 and miR-106 was evaluate using receiver operator characteristic ROC curve analysis the cutoff value of miR-21 was > 4.32 fold change with an area under the curve (AUC) of 0.973, an accuracy of 97.3 %, a sensitivity of 100 % and a specificity of 95 %, Moreover, the cutoff value of miR-106 was > 4.84 fold change with an area under the curve (AUC) of 0.981, an accuracy of 98.1 %, a sensitivity of 100 % and a specificity of 96 %. **Conclusion**: We can obtain diagnostic biomarkers with high specificity and sensitivity, by using two miRNA miRNA-21 which show high sensitivity, and miRNA-106, which show high specificity, Perhaps this could complement each other in testing whilst increasing sensitivity when individually related to miRNA.

Keyword: Circulating Micro RNA, Biomarker, Helicobacter pylori, gastric cancer

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Introduction

The vast majority of gastric cancer worldwide is attributable to H. pylori, chronic inflammation due to H. pylori infection play a crucial role in causing carcinogeness according to a multi-stage cycle first described by Correa, H. pylori-driven inflammation triggers the development of gastric cancer leading to typical stages of mucosal alterations such as chronic gastritis, glandular atrophy, intestinal metaplasia, dysplasia before reaching the final stage of invasive gastric cancer [1]. Despite recent advances in diagnostic techniques and preoperative management, these methods, however, are costly, invasive, and can put too much risk on the patient. Improvement in GC's prompt diagnosis and care has a critical effect on the patients' optimum management and long-term survival [2], thus the establishment of novel strong specific biomarkers with sufficient sensitivity is an ideal strategy for improving the early detection of GC and the cure rates for patients. Many of these biomarkers are not specific for the early stages; however, some of possible non-invasive diagnostic biomarkers

for early-stage gastric cancer discovered circulating molecules genetic and epigenetic alterations [3]. Thus miRNAs present inside the bloodstream are called circulatory miRNAs. Therefore, these miRNAs were considered important biomarkers for early detection of cancer [4-5]. There are numerous reports that specify various miRNAs involved in GC tumerogenesis such as miR-106a [6] miR-106a can be followed in salid tumor, stool, and plasma/serum sample of patients with gastrointestinal tumor [7]. The new studies show that miR-106a levels are significantly correlated with the stage of tumor node metastasis (TNM), tumor size and differentiation, lymphatic and distant metastasis, and invasion [8]. The miR-21 is over expressed in various cancers and has been causally associated to cellular proliferation, apoptosis, and migration and it had been reported that miR-21 induces invasion, intravasation, and metastasis [9].

Materials and Methods

Patient group and sample collection

This case control study comprised of 3 groups, 1st consisted of20 patients with H. pylori-associated Gastric Cancer who were observation in Oncology Hospital in Diwaniya in the period from March 2018to February 2019. Under the supervision of specialists, Lesions observed by endocytoscopy underwent endoscopic biopsy or endoscopic resection. Second group was 20 patients with H. pylori-associated Gastritis who visited Endoscopy Department of Gastroenterology and Hepatology of Al-diwanyia Teaching hospital. Individuals with gastritis were recruited consecutively from health checkup examinees that had undergone gastro copy and serologic analysis as part of a screening program for gastritis under the supervision of internal medicine specialists. While, 3rd group was include 40 healthy volunteers. Venipuncture used to collect samples from groups; 3 milliliter blood was collected in plain tubes without EDTA. Blood was permitted to clot in order to separate the serum through five minutes centrifugation at 13000rpm. The separated serum was then gathered in Eppendorf tubes and stored at -20 for further usage in miRNA-106 & miRNA-21 qPCR.

Total RNA extraction

The TRIzol® reagent kit (Bioneer, Korea) was used to extract the total RNA as per the instruction of the company, further, Nanodrop spectrophotometer (THERMO USA) was used to check the extracted genomic DNA. Thus, purity of DNA & concentration of DNA was estimated by reading the absorbance at 260/280nm.

STEM-LOOP RT-qPCR

The expression analysis of 106 miRNA & 21miRNA was quantified using stem loop RT-qPCR which was further normalized with GAPDH housekeeping genes in normal samples, blood patients & serum using the technique of Real-time PCR. This method was done according to the described method of [10].

Primers and probes

GAPDH gene Primers, probes

The Primer 3 Design Online & NCBI-Gene Bank database was used to design the GAPDH gene probes & primers. Macrogen Company, Korea provided all these primers which are given in the table below:

Gene	Sequence	
GAPDH primer	F	TCAGCCGCATCTTCTTTTGC
	R	TTAAAAGCAGCCCTGGTGAC
GAPDH probe	FAM- CCAGCCGAGCCACATCGCTC-TAMRA	

Micro RNA Primers, Probes

The Primer and probe for miRNA21 and miRNA 106a designed in this study by using [The Sanger Center miRNA database Registry to select miRNA sequence and using miRNA Primer Design Tool. These primer and probe were provided by (Macrogen Company, Korea) as following table:

Primer	Sequence	
hsa-miR-21 RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCAACA	
hsa-miR-21 primer	F	GTTTGGTAGCTTATCAGACTGA
	R	GTGCAGGGTCCGAGGT
hsa-miR-21 probe	FAM- TCAGTCTGATAAGCTA-MGB	
hsa-miR-106a RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGT ATTCGCACCAGAGCCAACACCAGA	
hsa-miR-106a primer	F	GTTTGAAAAGTGCTTACAGTGC
	R	GTGCAGGGTCCGAGGT
hsa-miR-106a probe	FAM- GCACTGTAAGCACTTTT -MGB	

Data analysis of qRT-PCR

The outcomes of q RT-PCR data for housekeeping gene and miRNA were examined with the assistance of relative quantification levels of gene expression (fold change).For this, the ΔCT method was preferred while utilizing the reference termed by (11) as following equations:

Ratio (reference/target) = 2CT (reference) – CT (target)

Results

Comparison of miR-21 and miR-106 fold change expression among patients with gastritis, patients with gastric carcinoma and control subjects

Current study reveals that the miR-21 expression was significantly highest in patient with gastric cancer , and then patients with gastritis and then control group (P < 0.001), 6.28 (4.78) fold change versus 2.45 (1.79) fold change versus 1 (---) fold change In addition, the miR-106 expression was significantly highest in patients with gastric cancer and then patients with gastritis and control group (P < 0.001), 7.03 (4.81)fold change versus 3.02 (1.82)fold change versus 1 (---) fold change, respectively, the diagnostic value of both miR-21 and miR-106 was evaluate using receiver operator characteristic ROC curve analysis . the cutoff value of miR-21 was > 4.32 fold change with an area under the curve (AUC) of 0.973, an accuracy of 97.3 %, a sensitivity of 100 % and a specificity of 95 %, Moreover, the cutoff value of miR-106 was > 4.84 fold change with an area under the curve (AUC) of 0.981, an accuracy of 98.1 %, a sensitivity of 100 % and a specificity of 96 %,as shown in table 1 and figure 2.

Table 1: Comparison of miR-21 and miR-106-fold change expression among patients with gastritis, patients with gastric carcinoma and control subjects

miR fold change	Statistic	Control *n* = 40	Gastritis *n* = 20	Gastric cancer *n* = 20	*P1*	*P2*	*P3*
miR-21	Median (IQR)	1(---)	2.45 (1.79)	6.28 (4.78)	<0.001 € HS	<0.001 € HS	<0.001 € HS
	Range	1-1"	1.95 -9.00	4.44 -23.43			
miR-106	Median (IQR)	1(---)	3.02 (1.82)	7.03 (4.81)	<0.001 € HS	<0.001 € HS	<0.001 € HS
	Range	1-1"	2.72 -9.52	5.23 -24.63			

n: number of cases; IQR: inter-quartile range; €: Mann Whitney U test; HS: Highly significant at $P \leq 0.01$; (---): Inter-quartile range cannot be calculated because fold change is constant in control group

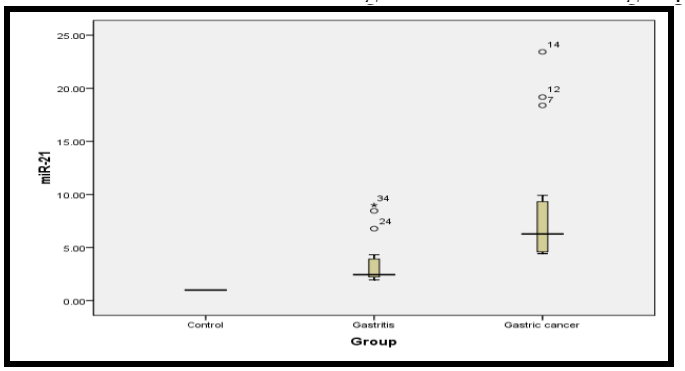

Figure (1): Box plot showing comparison of miR-21 fold change expression among patients with gastritis, patients with gastric carcinoma and control subjects

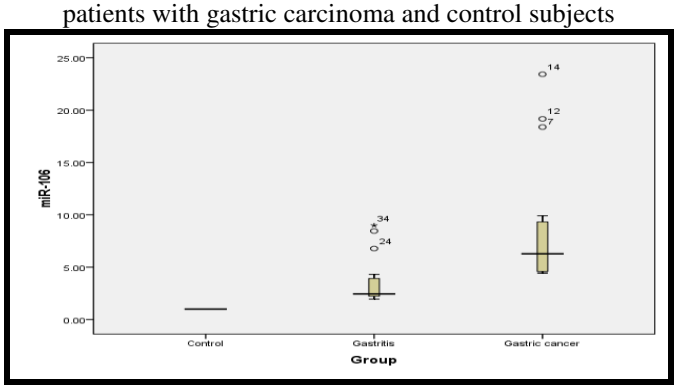

Figure 2: Box plot showing comparison of miR-106 fold change expression among patients with gastritis, patients with gastric carcinoma and control subjects

The diagnostic value of both miR-21 and miR-106 was evaluated using “receiver operator characteristic" ROC curve analysis and the results were shown in figures 3 and 4 and table 2. The cutoff value of miR-21 was > 4.32 fold change with an area under the curve (AUC) of 0.973, an accuracy of 97.3 %, a sensitivity of 100 % and a

specificity of 95 %, as shown in figure 3 and table 2. Moreover, the cutoff value of miR-106 was > 4.84 fold change with an area under the curve (AUC) of 0.981, an accuracy of 98.1 %, a sensitivity of 100 % and a specificity of 96 %as shown in figure 4 and table 2. Consistent with previous findings about the expression levels of miR-21, as GC related miRNAs, in GC plasma samples compared to healthy controls. According to their data, the plasma expression level of miR-21 (P < 0.0001) and was higher in GC patients than in healthy controls. Furthermore, ROC curve for miRNAs of interest showed circulating miR-21 with an AUC of 0.893 (95% CI: 0.755–1.000; P < 0.0001), can be served as a potential biomarker for GC diagnosis.

Table 2: Characteristics of ROC curve

Characteristic	miR-21	miR-106
Cutoff	> 4.32	> 4.84
AUC	0.973	0.981
95 % CI	0.909 to 0.996	0.918 to 0.993
Accuracy	97.3	98.1
P	<0.001 HS	<0.001 HS
Sensitivity	100.0	100.0
Specificity	95.0	96.0

AUC: area under curve; CI: Confidence interval; HS: Highly significant at $P \leq 0.01$

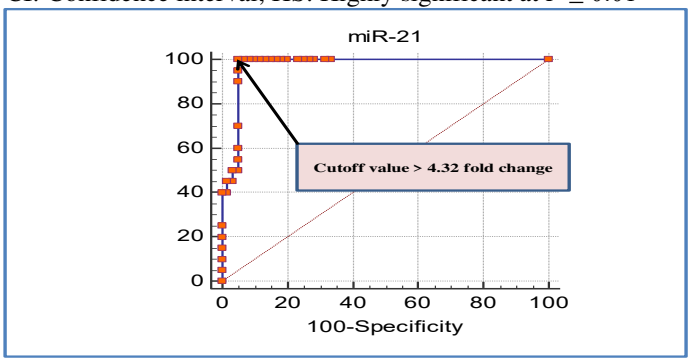

Figure 3: Receiver operator characteristic (ROC) curve to find the best miR-21 cutoff value that can predict a diagnosis of gastric cancer with highest accuracy level

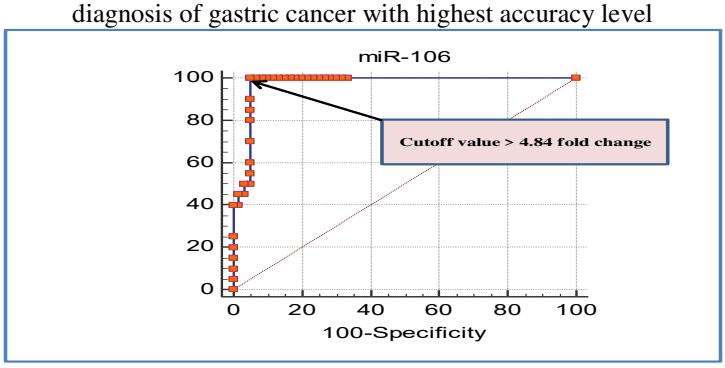

Figure 4: Receiver operator characteristic (ROC) curve to find the best miR-21 cutoff value that can predict a diagnosis of gastric cancer with highest accuracy level

Discussion

Due to the complicated mechanism of oncogenesis and the therapeutic difficulties of gastric cancer, the development of more responsive and reliable methods for early detection as well as prognosis is important. Even though there are some medical examination procedures, such as needle biopsy and endoscopy can provide accurate diagnosis results, researchers still make every effort to lower the cost and develop non-invasive tests for reduce patient's burden. Checking urinary miRNA expression level is a desired way for being a non-invasive test and for routine cancer monitoring [8]. Previous research indicated that miR-106b-25 and miR-21 are oncogenic and have an abnormal up - regulation in gastric cancer [12-13]. MiR-106b-25 targets include cycline-dependent kinase inhibitor (CDKN1A; p21CIP1 / WAF1), E2F1, and proapoptotic factor Bim leading to reduced gastric cell response to TGF-b, [14-15] and miR-21 targets and inhibits tropomyosin 1 (alpha) (TPM1), programmed cell death 4 (neoplastic transformation inhibitor) (PDCD4), and Phosphatase and Tensin homologue (PTEN) and other tumor-related genes [16-18] It was recorded that in 69 patients with gastric cancer, the plasma concentrations of miRNAs, miR-17-5p, miR-21, miR-106a and miR-106b were significantly

higher than in 30 healthy controls indicating the greatest AUC value for miR-106b by ROC analysis. The serum miR-106b results in the present study were similar to those of previous study investigating miRNAs expression in the gastric mucosa. In the high-risk group's gastric mucosa, the oncogenic miRNAs (miR-106b-93-25 cluster, miR-21, miR-194, and miR-196) levels were significantly higher compared with controls independent of H. pylori eradication [19]. As a significant oncomi R, MiR-21 induced several related cell signaling pathways including cell growth, tumor formation, angiogenesis and metastasis [20]. MiR-21 involves in abnormal cancer related processes by targeting of several genes such as SATB1, TIAM1, PDCD4, PTEN, APAF1, TIMP3, TGF-β, and PLAG1 [21]. Several articles have recently revealed the potential of miRs for early detection and prognosis in several malignancies, such as lung cancer [22], colorectal cancer and early breast cancer [23]. The GC miRNA expression profile was analyzed using different methodologies and some miRNA [24]. Notably, circuiting miR-21, miR -20a, miR -221, miR -378 and miR -421 have been reported to be potential biomarkers for tumor prognosis and diagnosis. In previous study for GC, the AUC value of miR-21 in serum was 0.912, with the sensitivity of 88.4% and the specificity of 79.6% [25]. Concentrating on the miR 106b~25, our study showed that AUC, sensitivity and specificity of miR 106b were higher than miR 421, miR 20a, miR 221 and miR 378. In a large-scale analysis, the AUC of miRNA106b was the greatest compared with the other miRs (AUC = 0.721), which was in line with our study. In CAI's research, the AUC using miR-106b was 0.7733 [26]. MiRs had the advantage of high room temperature stability, low degradation in multiple freezing thawing processes and high specificity of tissue distribution [27]. Thus, quantification of miRs can involve stabilizing miRs sample and shortening testing time to protect the samples from degradation. In present study Samples were immediately processed for miR extraction after blood collection to minimize miR degradation, and stored at -80 ° C before PCR analysis. The expression of some miRs is specific to tissues or biological stages, and their level can be easily detected by various methods. Data collection has shown that miR 106b~25 cluster performs oncogenic roles in cancers. Many cancers have documented up-regulation of this cluster, including multiple myeloma, esophageal squamous cell carcinoma, hepatocellular carcinoma, pancreatic cancer. [28-29]. The trigger targets of this oncogenic process are E2F1 , TGF-β [29], while other target just like Retinoblastom protein (RB) gene (28), and Phosphatase and Tensin homolog deleted on chromosome (PTEN) have been demonstrated in mechanism of miR 106b~25. All of these targets as an intrinsic factor of gastric carcinogenesis play a crucial role. Researchers have shown in previous studies that the expression of miR 106b and miR 25 in GC cell lines was significantly higher [30], tumor tissues and plasma form GC patients [31]. Although new biomarkers, further research is still needed to verify if these miRs have advanced sensitivity and specificity over conventional tumor markers. In this research, we assessed the optimal cut-off point of for miR 106, and miR- 21 diagnosis GC by ROC curve. All of the miRs were performed well in malignancy diagnosis as compared with traditional tumor biomarkers. Diagnostic efficacy of miR 106b and miR21 were significantly higher than CA724, CA242 and CA199. To boost GC's early diagnosis, early GC should be sub-analyzed more rigorously. In addition, multicenter and larger-scale studies were required to verify the accuracy of the clinical diagnosis in early GC [32].

Conclusion

We can obtain diagnostic biomarkers with high specificity and sensitivity, by using two miRNA miRNA-21 which show high sensitivity, and miRNA-106, which show high specificity, Perhaps this could complement each other in testing whilst increasing sensitivity when individually related to miRNA.

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