ORIGINAL ARTICLE

Serum miRNA profile as a potential tool for non-invasive gastric cancer diagnosis in Mexican patients

Perfil de miARNs séricos como una potencial herramienta para el diagnóstico de cáncer gástrico no invasivo en pacientes mexicanos

Hugo Cuellar-Gomez[1], Mª Esther Ocharán-Hernández[1], Claudia C. Calzada-Mendoza[1], and David A. Comoto-Santacruz[2]*

[1]Department of Postgraduate Studies and Research, School of Medicine, Instituto Politécnico Nacional; [2]Department of Molecular Biology, Escuela Militar de Graduados de Sanidad. Mexico City, Mexico

Abstract

Introduction: *Gastric cancer (GC) is the third leading cause of cancer death and a major public health-care problem worldwide. At present, methods for plasma detection of cancer are limited. MicroRNAs (miRNAs) have recently been proposed as genetic regulators, which are deregulated in different types of cancer. The miRNAs are stable in serum/plasma and can be detected. Circulating miRNAs in plasma have been proposed as potential diagnostic biomarkers in GC.* **Materials and methods:** *After reviewing the relevant literature, the expression levels of seven miRNAs (miR-16, miR-21, miR-25, miR-26a, miR-92, miR-218, miR-223, and miR-451) were assessed by quantitative reverse transcription polymerase chain reaction using TaqMan microRNA Assays (Applied Biosystems) in plasma samples from GC patients (n = 80) and healthy controls (n = 80).* **Results:** *Our results demonstrated that the expression levels of miR-21 and miR-25 were significantly upregulated in GC patients compared to healthy controls with a Fold Change of 11.551 and 60.129, respectively, while miR-223 showed downregulation in GC patients compared to healthy controls with a Fold Change of –247.281. The absolute value of Fold Change > 2 was consider significant, p < 0.05.* **Conclusions:** *Our results indicated that miR-21, miR-25, and miR-223 in plasma samples can be served as a potential noninvasive tool in detection of GC.*

Key words: *Gastric cancer. MicroRNAs. Plasma. Biomarker. Diagnosis.*

Resumen

Introducción: *El cáncer gástrico (CG) es la tercera causa de muerte por cáncer y un importante problema de salud pública. Actualmente, los métodos para la detección de CG en plasma son limitados. Recientemente se han propuesto los microARNs (miARN) como reguladores genéticos en diferentes tipos de cáncer. Los miARN son estables en plasma, lo que permite una fácil detección, pudiendo usarse como biomarcadores en CG.* **Materiales y métodos:** *Los niveles de expresión de siete miARN seleccionados (miR-16, miR-21, miR-25, miR-26a, miR-92, miR218, miR-223, miR-451) fueron evaluados mediante qRT-PCR mediante análisis con microARN TaqMan (Applied Biosystems) en muestras de plasma de pacientes con CG (n = 80) y controles sanos (n = 80).* **Resultados:** *Se observo que los niveles de expresión de miR-21 y miR-25 estaban significativamente*

Correspondence:
*Mª Esther Ocharan-Hernández
Salvador Díaz Mirón esq.
Plan de San Luis S/N, Miguel Hidalgo
Casco de Santo Tomas
C.P. 11340, Ciudad de México, México
E-mail: estherocharan@gmail.com

Date of reception: 02-09-2020
Date of acceptance: 19-10-2020
DOI: 10.24875/CIRU.20000963

Cir Cir. 2021;89(6):748-754
Contents available at PubMed
www.cirugiaycirujanos.com

regulados al alza en los pacientes con CG en comparación con los controles con un Fold Change de 11.551 y 60.129 respectivamente, mientras que miR-223 mostró una regulación negativa con un cambio de -247,281. El valor absoluto de Fold Change >2 se consideró estadísticamente significativo, P <0,05. **Conclusiones:** *Nuestros resultados indicaron que miR 21, miR 25 y miR-223 en plasma pueden servir como una potencial herramienta no invasiva en la detección de CG.*

Palabras clave: *Cáncer Gástrico. miARN. Plasma. Diagnostico. Biomarcador.*

Introduction

According to GLOBOCAN 2018 data, gastric cancer (GC) is the third leading cause of cancer deaths worldwide, only following lung and colorectal cancer in overall mortality. GC has the fifth highest incidence among cancers, with 5.7% of all new cases attributable to the disease[1]. In Mexico in 2012, cancer was the third cause of death after heart disease and diabetes mellitus[2]. In 2013, GC was the third leading cause of death from cancer in individuals 20 years of age or older. GC remains a public health problem in Mexico due to its high mortality and low survival rates and the significantly lower quality of life of patients with this condition[3]. The diagnostic methods are limited for early detection and no effective screening programs outside some Asian countries. Thus, early detection of GC is the key to prolong survival of patients and therefore health-care policy for GC should be focused on early detection and as well as novel treatment strategies[4]. To date, the commonly used diagnostic tools for detection of GC are endoscopy, computed tomography, magnetic resonance imaging, and Endoscopic Ultrasound and tumor markers (CA199, CEA, and CA724). However, these tools are valuable only in diagnosing late stage cancers[5]. Consequently, the high mortality rate in GC is partially associated with a lack of noninvasive tool for GC detection at early stages. Thus, there is an urgent search for new, preferentially non-invasive, and biomarkers to allow early detection of GC.

MicroRNAs (miRNAs) are non-coding RNAs of approximately 19-25 nucleotides, which suppress the translation of target genes by binding to their mRNAs. The miRNAs can post-transcriptionally regulate the expression of hundreds of their target genes, thereby controlling a wide range of biological functions, such as cellular proliferation[6], differentiation[7], and apoptosis[8]. Recent evidence indicates that miRNAs may function as tumor suppressors or oncogenes, and that alterations in miRNA expression may play a critical role in tumorigenesis and cancer progression, particularly in GC[9-12]. miRNAs have been found to be involved in known oncogenic pathways, including the phosphatidylinositide 3-kinase (PI3K)/Akt[13], the Ras/Raf/MEK/extracellular-signal-regulated kinase (ERK)[14], the slit family of guidance cues binds to Roundabout (Robo)[15], the Janus kinase/signal transducers and activators of transcription (JAK/STAT)[16], and the Wnt/β-catenin pathway[17], which are associated to genes targeted by miRNAs. PTE (phosphatase and tensin homolog) is one of the target genes of miRNA-21 that increases the proliferation and invasion of GC cells that belong to PI3K/Akt pathway[18]. A group of Chinese researchers demonstrated that miRNA29s could effectively inhibit protein expression/phosphorylation of Cdc42 and its downstream molecule PAK1, thereby influencing the Ras/Raf/MEK/ERK pathway[19]. Tie et al. have demonstrated that miRNA-218 coding genes are located in and transcribed together with Slit genes, which are Robo1 ligands, thus creating a negative feedback loop that regulates Slit/Robo1 signaling[20]. The previous research has shown that miRNA-375 may function as a tumor suppressor that potentially regulates GC cell proliferation by targeting the JAK2 oncogene in the JAK/STAT pathway[21]. Recent studies have revealed that many proteins, such as adenomatous polyposis coli and Axin, are involved in the regulation of the Wnt signaling pathway. Therefore, miR-27 may modulate Wnt signaling by interacting with adenomatous polyposis coli[22]. It is now well know that some plasma or serum miRNAs are quite stable and suitable for biomarker screening[23]. The stability of circulating miRNA in plasma along with aberrant expression of these small non-coding RNA in GC has clearly demonstrated in several studies which represents their potential applications in cancer diagnosis and prognosis[5,24,25].

Materials and methods

Subjects

A prospective study was carried out with a total of 80 gastric patients and 80 healthy adult volunteers. The patients included in the study had to have confirmation of the diagnosis by pathology and endoscopy. Sample collection was performed before any cancer

Table 1. Clinical variables of cases and controls

Variable	GC cases (n = 80)		Healty Controls (n = 80)		p value
Gender					
Male	55	68.80%	62	77.5%	> 0.05*
Female	25	31.30%	18	22.5%	
Age (years)					
< 60	24	30%	42	52.5%	> 0.05*
> 60	56	70%	38	47.5%	
TNM stage					
I-II	46	57.50%	-	-	-
III-IV	34	42.50%			
Borrman					
I-II	10	13.50%	-	-	-
III-IV	70	87.50%			

GC: Gastric Cancer, TNM: Tumor-node-metastasis, Borrman: Classification of advanced gastric cancer according to the appearance and growth state of the tumor. Student t-test. p < 0.05 were considered statistically significant*.

treatment including chemotherapy, radiotherapy, surgery, or comorbid malignancies from other organs, were excluded to no alter miRNAs behavior. The patients who had gastric tumors other than adenocarcinoma were excluded. Table 1 shows the clinical characteristics of GC patients and healthy controls. Hospital Central Militar Ethics Committee approved the written informed consent that was taken from all the participants.

Samples

Peripheral whole blood was collected in ethylenediaminetetraacetic acid tubes (3-5 mL/tube). Each blood sample was immediately centrifuged; up to obtain plasma aliquots (1 mL) which were stored at −80°C until further analysis.

Rna extraction

Total RNA isolation was performed from 200 μl of the thawed plasma using TRIzol™ Reagent (Life technologies) according to the manufacturer's instructions.

Rna quantification

The RNA (ng/μl) concentration was evaluated by spectrophotometry using NanoDrop 1000® equipment (Thermo Scientific). The reading was performed with an absorbance at 260 nm. Purity was determined by calculating the ratio A260/280 and A260/230.

Assessment of RNA integrity by agarose gel

To assess the integrity of the extracted RNA, electrophoresis was performed on 1% agarose gel (Ultrapura Agarose Invitrogen®) stained with ethidium bromide (Sigma®) using as buffer solution TBE 0.5X (Tris 54 g, boric acid 27.5 g, and 20 ml of ethylenediaminetetraacetic acid [EDTA] at 0.5M [pH 8.0]) subjected to a voltage of 75 mV for 45 min. The gel was photographed on a QUANTUM VILBER LOURMAT ultraviolet light transilluminator (312 nm) with the VISION CAPT program.

cDNA synthesis

cDNA synthesis was performed using the miScript II RT kit (Qiagen, Hilden, Germany) in the Veriti Thermal Cycler (Applied Biosystems), according to the manufacturer's instructions.

Quantification of miRNA by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The expression levels of miRNAs were obtained using the Qiagen methodology; qRT-PCR was performed in the Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) for each of the pool with the miRNAs related to GC selected from the already reported in the international literature. In addition, the Human miRNome miScript® miRNA PCR Array kit (Qiagen) was used according to the manufacturer's instructions.

Statistical analysis

miScript miRNA PCR Array Data Analysis (Qiagen) was used for statistical analyses. A global normalization was performed, which included the average cycle threshold of the 7 GC-miRNAs in the array plate, plus spike-in cel-miR-39 and endogenous small RNAs (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6). This is a more robust and preferred method for this type of study. Significance was assessed by the fold change ($2^{-\Delta\Delta Ct}$ method) of miRNAs between GC patients and healthy controls, also was compared with Student's *t*-test, the absolute value of fold change >2 was considered statistically significant with a $p < 0.05$.

Results

In total, 160 individuals containing 80 GC patients and 80 healthy controls were recruited in this study. No significant differences were observed between the GC patients and controls in the distribution of age and gender, cancer stage (TNM) showed heterogeneous distribution (Table 1). The expression levels of miRNAs in plasma were quantified by qRT-PCR using Spike-In Control (C. elegans miR-39) as a normalization control. The average ΔCt of cases and controls is shown in table 2. With an absolute value of fold change of more than 2 and a $p < 0.05$ as the cutoff, we found that the results of the present study demonstrated, that expression level of circulating miR-21 and miR-25 was significantly upregulated in GC patients than in healthy individuals with a Fold Change of 11.551 and 60.129, respectively ($p < 0.05$). The expression of miR-223 was downregulated in GC patients with a fold change of –247.281 ($p < 0.05$). The levels of the other miRNAs showed not significant expression (Fold change < 2, $p > 0.05$), table 3.

Table 2. Average Ct of cases and controls

miRNAs	Average ΔCt	
	GC Group	Control Group
miR-16	34.08	35
miR-26a	31.13	30.87
miR-92a	28.15	29.14
miR-25	29.09	35
miR-21	31.47	35
miR-451	29.79	28.46
miR-223	30.91	22.96
Cel-miR-39 (Control miRNA)	29.37	29.37

GC: gastric cancer, miRNA: microRNA, Ct: cycle threshold.

Table 3. Over and under expression and fold change of genes

Genes over and under expression in GC patient's versus Control group			
	miRNAs	Fold Change (comparing to control group)	p value
Over Expression	miR-16	1.8921	-
	miR-92a	1.9810	-
	miR-25	60.1295	< 0.05*
	miR-21	11.5514	< 0.05*
Under Expression	miR-26a	–1.2010	-
	miR-451	–2.5162	-
	miR-223	–247.2812	< 0.05*

The absolute value of fold change>2 was considered statistically significant, p < 0.05*.

Discussion

The miRNAs have been identified at the extracellular level in many body fluids, including plasma, in which they are associated with proteins and lipoproteins, packaged within cellular structures (i.e., exosomes, microvesicles, or apoptotic bodies), giving them protection and stability against Ribonuclease (RNase), and allowing them to be detectable and quantifiable; serving as potential markers for various pathologies including cancer[10,25,26].

The expression levels of miRNAs in cancer have not showed the same expression rate, some of them have a tendency to over expression and others to under expression, due to the fact that some miRNAs act like proto-oncogenes, those that trigger tumor proliferation or like tumor suppressors which favor tumor development by gene inactivation[9]. Because of that some studies have focused on evaluating the relationship between GC and miRNAs expression. Tsujiura et al. (n = 69) reported for the 1st time the aberrant expression of circulating miRNAs (miR-17-5p, miR-21, miR-106a, miR-106b, and let-7a) in plasma in GC patients, the expression level of miR-17-5p, miR-21, miR106a, and miR-106b showed up-regulation while the only one that showed down-regulation was let-7a compared to healthy controls, demonstrating that can be found in plasma samples, and could be considers as tumor markers for this pathology[25].

Based on different expression profiles, the miRNAs can be associated with proliferation, progression, invasion, and prognosis in GC[11]. A systematic review study of human GC microRNA expression profiling realized by Sirjana et al. based on 200 studies. Were detected the expression levels of more than 350 miRNAs in GC patients, demonstrating that miR-21 was reported upregulated in 10 studies with a median Fold change of 4.05, followed by miR-25, miR-92, and miR223 upregulated in 8 studies with a Fold change of 2.55, 2.80, and 3.10, respectively, provide information on miRNAs with potential role as biomarkers in GC[27].

Table 4. The expression level of miRNAs in our study comparing to the previous miRNA studies and target genes

Sample	Study Result	Circulating miRNA	Fold Change	Reported result	Reported sample	Mechanism in GC	Target genes	Reference
Plasma	Upregulated	hsa-miR-16-5p	1.8921	Up-regulated	Plasma/Tissue	Proliferation and migration.	HGF/ c-Met	34
Plasma	Downregulated	hsa-miR-26a-5p	–1.2010	Down-regulated	Plasma/Tissue	Growth and metastasis	FGF9	35
Plasma	Upregulated	hsa-miR-92a-3p	1.9810	Up-regulated	Plasma/Tissue	Proliferation and invation.	FXR	36
Plasma	Upregulated	hsa-miR-25-3p	60.1295	Up-regulated	Plasma	Growth and motility	RECK	29
Plasma	Upregulated	hsa-miR-21-3p	11.5514	Up-regulated	Plasma	Growth and invation	PTEN, PDCD4	30
Plasma	Downregulated	hsa-miR-451a	–2.5162	Down-regulated	Plasma	Invation and, metastasis	MIF	37
Plasma	Downregulated	hsa-miR-223-5p	–247.2812	Down-regulated	Plasma	Proliferation, invation and migration	STMN1, ASCL2	32,33

HGF/c-Met: hepatocyte growth factor/c-Met receptor, FGD9: fibroblast growth factor 9, FXR: farnesoid X receptor, PDCD4: programmed cell death 4, PTEN: phosphatase and tensin homolog, MIF: the macrophage migration inhibitory factor, STMN1: Stathmin1, ASCL2: achaetescute homolog 2.

To find a miRNA that could have a significant expression level in early cancer stages, Zhu et al., designed a four-phase study, where the expression levels of miR-16, miR-25, miR-92a, miR-451, and miR-486-5p were evaluated in GC patients and healthy controls (n = 160 vs. n = 160), showed consistently elevated levels in plasma and were identified to be potential markers for GC with area under the receiver operating characteristic curves ranging from 0.850 to 0.925 and 0.694 to 0.790[24].

Recently, Wang et al.[28] assessed the diagnostic performance of circulating miRNAs for the detection of gastrointestinal cancer in a meta-analysis including 21 GC studies. Most of the GC studies were of Asian ethnicity, and the most frequent miRNAs found in plasma or serum was miR-106b and miR-21. In Caucasian patients with GC, they described miR-203, miR-146b-5p, miR-192, and miR-200c as potential biomarkers in plasma. However, many of these biomarkers have been tested in very restricted parameters and are highly influenced by ethnic and environmental factors, thus making it even more difficult to find specific biomarkers for GC.

Based on the foregoing, we decided to choose the most frequent and relevant miRNAs found in the literature and assess in Mexican population to in observer their behavior. In our study, a panel of 7 miRNAs was screened, 4 were found upregulated (miR-16, miR-92a, miR-25, and miR-21), and 3 downregulated (miR-26a, miR-451a, and miR-223) as shown in table 3.

We observed that miR-25 and miR-21 showed the highest expression levels with a fold change of 60.195 and 11.551 among the other miRNAs, which is correlated with the literature[24]. Both miRNAs are associated with the development, growth and size of tumor, targeting RECK gene (tumor suppressor) in case of miR-25[29], and targeting PTEN gene (tumor suppressor) for miR-21[30], allowing to be expressed from the initial stages of the disease, and permitting early detection.

The miRNAs that showed downregulation in our study were miR-26a-5p, miR-451a, miR-223-5p. The miR-223 was significantly down-regulated respect to the others with a Fold Change of -247.28, contrary to our study, some authors have reported that this miRNA is over-expressed in GC targeting EPB41L3 gene (tumor suppressor), which is associated to tumor invasion and metastasis related to advance cancer stages[31]. However, Kang et al. observed a low miR-223 expression level in GC cell lines and an inverse relationship between miR-223 and STMN1 protein expression, permitting upregulation of STMN1 in gastric adenocarcinoma, and the expression was correlated with poor disease-specific survival in diffuse type GC[32]. Zou et al. demonstrated that ASCL2 gene was able to downregulate the expression level of miR-223, contribute to epithelial-mesenchymal transition and

promote gastric tumor metastasis[33]. This could explain the highest under-expression observed in our study.

The miR- 26a-5p and miR-451a showed under-expression, while miR-16, and miR-92a showed over-expression but not significant association was found between GC patients y and healthy controls (Fold change < 2 and p > 0.05). Table 4[34-37] shows a comparation between our study and previous miRNA studies and target genes.

Conclusion

In the present work, we were able to identify plasma miRNA from patients with GC. We demonstrated that expression levels of circulating miR-21, miR-25, and miR-223 in GC patients' sample were higher than those in noncancerous samples. Some of those miRNAs are associated to early cancer stages, a correlation could not be made because GC cohort was heterogeneous, containing early, and advances stages. However, the combination of several miRNAs reached higher specificity and sensitivity than single miRNA. Altogether, this data suggests its potential application as a minimally invasive tool in GC detection. Despite the standard protocol of sample treatment and the suitable internal controls should be further established to make the detection comparable before the miRNA biomarkers being fully used in clinical practice.

Conflicts of interest

The authors declare that they have no conflicts of interest

Funding

The authors declare that there was no funding used for this research project.

Acknowledgments

H Cuellar-Gomez gratefully acknowledges the scholarship from CONACyT to pursue his postgraduate studies.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

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