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TARGETED microRNA PROFILING IN GASTRIC CANCER WITH CLINICAL ASSESSEMENT

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ABSTRACT

Although several microRNAs (miRNAs) have been associated with gastric cancer there is still the need for identification of stable and validated biomarkers. The purpose of this study was to determine the alterations of a specific set of miRNA levels in gastric adenocarcinoma tissues to identify and validate gastric cancer-specific miRNAs using paired normal and tumor samples in an independent patient cohort. Gastric adenocarcinoma and normal stomach tissue samples of 20 patients who underwent surgery for gastric cancer were studied. The miRNA expression profiling was performed for eight miRNAs in a total of 40 tissue samples using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Six out of these eight miRNAs, namely, miR-375-3p, hsa-miR-129-5p, miR-196a-5p, miR-376c-3p, miR-34c-5p and miR-767-5p, were significantly underexpressed in malignant tissues of our cohort. Furthermore, the expression of miR-662 although not significantly different between normal and tumor tissues, was inversely associated with age ($r = –0.440$, $p = 0.049$). The levels of miR-129-3p and miR34c-5p were correlated with an increase in the number of metastatic lymph nodes ($r = 0.470$, $p = 0.036$; $r = 0.510$, $p = 0.020$), while and miR-376c-3p levels were negatively associated with smoking ($p = 0.043$). In addition, we found that the variability of miRNA expression in cancerous tissues was lower than that in normal tissues. Alterations in miRNA expression in gastric adenocarcinoma tissues in comparison to healthy tissues of each individual serves for identification of consistent biomarkers that can be used for development of diagnostic tools for gastric cancer.

Keywords: Gastric adenocarcinoma tissues; Gastric cancer; Gastric cancer patients; Metastatic lymph nodes; microRNAs (miRNAs).

INTRODUCTION

Gastric cancer ranks fifth among the most common cancers and is the third top ranking factor in causing deaths related to cancer worldwide [1]. Patients with gastric cancer exhibit low life expectancy due to diagnosis at an advanced stage because of absence of specific clinical symptoms and late presentation of patients to the hospital. Over the years, treatments specific to gastric cancer have been designed using different classifications, which, more recently, have become based on the molecular properties of tumors [2,3].

Research has demonstrated that microRNAs (miRNAs) are associated with various diseases because of their oncogenic or tumor suppressor properties. microRNAs play a role in cancer formation and progression because of their involvement in angiogenesis, proliferation, invasion, cell cycle, and differentiation [4,5]. According to the "database of differentially expressed miRNAs in Human Cancers (dbDEMC)," as of June 2021, there are 3268 miRNAs identified for 40 different cancer types, of which 2584 are differentially expressed miRNAs seen in humans [6]. Gastric cancer ranks high among cancer types whose miRNAs have been identified, such as prostate cancer, breast cancer, esophageal cancer, lung cancer. Accordingly, 1621 upreg-ulated, 1725 downregulated (and some of which can be both downregulated and upregulated) miRNAs have been found to be associated with

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gastric cancer; and were significant in terms of diagnosis, prognosis, and treatment processes in studies conducted on tissue or bodily fluids [6].

The purpose of the present study was to determine the expression levels of a selected set of miRNAs in an independent cohort of adult patients aged >18 years, who were diagnosed with gastric adenocarcinoma with strict selection criteria. Accordingly, we have identified, *via* real-time quantitative polymerase chain reaction (qRT-PCR), the miRNA expression and then compared the clinicopathological characteristics of these miRNA markers that could potentially be used in early diagnosis and innovative treatments of gastric cancers.

MATERIALS AND METHODS

Ethics Approval and Informed Consent. Ethics committee approval was obtained from the Ethics Committee for the study with the decision numbered E-16-1152. The study protocol was explained to all the patients who participated in the study and a signed informed consent was obtained from all the subjects.

Inclusion Criteria. A total of 20 patients aged >18 years were included in this study. They were pathologically diagnosed with gastric adenocarcinomas (two were diagnosed as signet ring cell carcinoma, a subtype of adenocarcinoma), surgically treated and did not receive neoadjuvant chemotherapy or radiotherapy, and were not diagnosed with linitis plastica at the General Surgery Department, Ankara Numune Training and Research Hospital, Ankara, Turkey, between March 2018 and April 2019.

Collection of Tissue Samples. Samples collected from the cancerous stomach tissue of patients within the first half hour after the surgical removal of gastric tissue from the abdomen in the operating room along with those collected from normal stomach tissue of the same patients were rapidly frozen in liquid nitrogen. These samples were stored in a storage area at –80 °C until examination.

Selection of microRNAs. The miRCancer database (accessed February 18, 2019) [7], citing 5723 articles as references, were reviewed to identify relevant articles for the miRNAs we included in this study. According to the MiRCancer database, 323 miRNAs were associated with gastric cancer [7]. In addition, The National Center for Biotechnology Information [8] and the miRCancer databases were reviewed, and in total, eight miRNAs were identified, including seven that have previously been associated with gastric cancer in one or more articles, and one novel miRNA, not investigated in gastric cancer literature in detail. RNU6-1, recognized as the universal reference, was used as the reference miRNA in the study [9]. microRNAs used herein include hsa-mir-375-3p, hsa-mir-148a-3p, hsa-mir-196a-5p, hsa-mir-376c-3p, hsa-mir-129-5p, hsa-mir-34c-5p, the newly identified hsa-mir-767-5p, and the novel hsa-mir-662 (Table 1) [10-22].

Isolation of microRNAs and cDNA Synthesis. RNA isolation of the tissues to be used in the study was performed using miRNeasy mini kit (Cat. #217004; Qiagen Sciences Inc., Germantown, MD, USA) as described in the catalog. cDNA synthesis was performed using miScript kit II (Cat. #218161; Qiagen Sciences Inc.) according to the manufacturer's instructions.

microRNA Expression Detection and Analysis. In order to evaluate the miRNA expressions, cDNA samples were analyzed with qPCR. Qiagen miScript primer assays with miScript SYBR Green kit (Cat. #218073; Qiagen) and

Table 1. miRNAs used in the study and their properties.

miRNA ID	Accession Number; Sequence	Expression	Function	Refs.
Hsa-miR-375-3p	MIMAT0000728; UUUGUUCGUUCGGCUCGCGUGA	downregulation	apoptosis	[10-12]
Hsa-miR-148a-3p	MIMAT0000243; AAAGUUCUGAGACACUCCGACU	downregulation	D_X; metastasis; invasion; poor prognosis	[13]
Hsa-miR-196a-5p	MIMAT0000226; AAAGUUCUGAGACACUCCGACU	upregulation	poor prognosis; D_X	[14,15]
Hsa-miR-376c-3p	MIMAT0000720; AACAUAGAGGAAAUUCCACGU	downregulation; upregulation	apoptosis; cell proliferation	16,17]
Hsa-miR-129-5p	MIMAT0000242; CUUUUUGCGGUCUGGGCUUGC	downregulation	poor prognosis	[18,19]
Hsa-miR-34c-5p	MIMAT0000686; AGGCAGUGUAGUUAGCUGAUUGC	downregulation	apoptosis; cell-cycle	[20]
Hsa-miR-662	MIMAT0003325; UCCCACGUUGUGGCCCAGCAG	downregulation	unknown	[21]
Hsa-miR-767-5p	MIMAT0003882; UGCACCAUGGUUGUCUGAGCAUG	downregulation	cell proliferation; migration and invasion	[22]

ID: identity; Refs.: references.

Roche Multiwell Plate 96 (Cat. #4729692001; Hoffman La-Roche Ltd., Indianapolis, IN, USA) were used for this study. LightCycler 480 Instrument II (Roche Diagnostics International AG, Basel, Switzerland was used to determine the miRNA levels. Quantification of the miRNA expression values, and the threshold cycle (Ct) and melting temperature (Tm) determinations were performed using Light Cycler Software. In this study, eight miRNAs (Table 1), whose expression levels were normalized to those of RNU6-1 and their corresponding normal tissues using ΔCt and ΔΔCt methods [23].

Statistical Analyses. Microsoft Excel 2010 (Microsoft, Albuuerque, NM, USA) was used to collect and group the patient data and to perform basic mathematical operations on these data. The Ct values of the miRNAs were evaluated using parametric paired *t*-tests in comparison of cancerous and normal tissues. Parametric *t*-test was also used to assess the relationship of demographic, clinical, and pathological results with miRNA expressions with categorical variables, and Pearson correlation score was used for continuous variables. The one-tailed F-test was used to test the difference in the variance of miRNA expression between tumor and normal samples in Microsoft Excel 2010, and those with p values of <0.05 were considered significant. Hierarchical cluster analysis was performed using the "Euclidean" distance and the "complete" linkage methods, while a heatmap was created using the log fold changes in miRNAs with age, smoking and the number of metastatic lymph nodes in the R platform with various R programing tools for plotting data (ggplots) package [24]. All the statistical analyses and graphic drawings for miRNAs and patient data were conducted using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) and Matlab 2017 (MathWorks Inc., Natick, MA, USA).

RESULTS

Evaluation of Patients' Demographic, Clinical, and Pathological Characteristics. In total, 17 (85.0%) study subjects were men, and three (5.0%) were women; the mean age was 64.9 ± 13.8 years. Moreover, nine patients were smokers and five used gastric acid-lowering drugs. Ten patients had cardiac tumors, while 10 had non cardiac tumors. Based on the evaluation of the histopathological examination report of the patients using the tumor size, lymph node, distant metastasis (TNM) staging system, 13 patients had T3, and six had T4 tumors, and only one had a T1 tumor. Based on the evaluation of the regional lymph nodes, three patients were classified as N0, while four, seven, and six patients belonged to N1, N2, and N3, respectively [TNM; American Joint Committee on Cancer (AJCC), 8th ed.] [25]. There were two patients who had signet ring cell carcinoma, the subtype of adenocarcinoma. We found that 18 patients had lymphovascular invasion, while 14 exhibited nerve invasions in the tumor. Additional clinical information has been made available in Table 2.

Table 2. Evaluation of the demographic, clinical and pathological features of the 20 patients.

Variables	*n*; mean ± SD; %
Age (years):	64.9±13.8
<60 years old	7; 50.00±8.75; 35.0%
>60 years old	13; 72.92±8.01; 65.0%
Gender:	
females	3; 15.0%
males	17; 85.0%
Helicobacater pylori	3; 15.0%
Smoking	9; 45.0%
PPI/H2RB	5; 20.0%
Family history	1; 5.0%
Blood group:	
A Rh (+)	8; 40.0%
O Rh (+)	5; 25.0%
B Rh (+)	4; 20.0%
A Rh (–)	1; 5.0%
O Rh (–)	1; 5.0%
AB Rh (+)	1; 5.0%
Preoperative:	
CEA (ng/mL)	12.94±21.75; normal: 0-5
CA19-9 (U/mL)	122.64±217.83; normal: 0-27
AFP (ng/mL)	2.84±1.60; normal: 0-7
Tumor location	
cardiac	10; 50.0%
non cardiac	10; 50.0%
Differentiation:	
poorly differentiated	7; 35.0%
mid differentiated	11; 55.0%
well differentiated	2; 10.0%
Borman classification:	
type 1	9; 45.0%
type 2	4; 20.0%
type 3	7; 35.0%
pT:	
pT1	1; 5.0%
pT2	0; 0.0%
pT3	13; 65.0%
pT4	6; 30.0%
pN:	
pN0	3; 15.0%
pN1	4; 20.0%
pN2	7; 35.0%
pN3	6; 30.0%
Lymphpvascular invasion	18; 90.0%
Nerve invasion	14; 60.0%
Stage:	
I	1; 5.0%
II	5; 25.0%
III	14; 60.0%

PPI/H2RB: proton pump inhibitor/histamine 2 receptor blocker; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; AFP: α-fetoprotein; pT: pathological t stage; pN: pathological n stage.

microRNA Expression Changes in the Tumor Tissues of Patients with Gastric Cancer. Table 3 presents the miRNA expression changes in cancerous and normal tissues of the patients, while Table 4 shows the mean changes observed in the miRNA expression levels in cancerous and normal tissues, separately, and the statistical significance of these changes. For the miRNAs hsa-miR-375-3p, hsa-miR-196a-5p, hsa-miR-376c-3p, hsa-miR-129-5p, hsa-miR-34c-5p and hsa-miR-767-5p, a significant decrease was noted in the cancerous tissues in comparison with the normal tissues. Overall, the miRNA expression levels were lower in cancerous tissues than in normal tissues (Figure 1). In addition, when each miRNA in the individual cancerous and normal tissue pair was examined, the expressions of miRNAs were significantly lower.

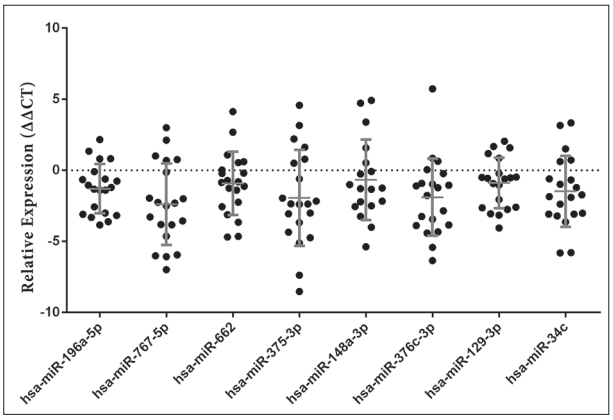

Figure 1. Expression changes of miRNAs in cancerous tissues.

The cancerous tissues also exhibited lower variability for hsa-miR-375-3p (p = 0.021), hsa-miR-196a-5p (p = 0.042), hsa-miR-376c-3p (p = 0.023), miR-34c-5p (p = 0.030) and hsa-miR-767-5p (p =.0.038). The narrower range we observed may indicate the presence of a stronger genome/transcriptome control (Figure 2).

Association of Tissue microRNA Expression Levels with the Available Patient Data. Based on the evaluation of the other known demographic, clinical, and histopathological characteristics of the patients, an optimal evaluation could not be made owing to the limited sample size and the lack of sufficient samples in the subgroups. No significant result was obtained for variables other than those for smoking, age, and the number of metastatic lymph nodes (Table 5 and discussion).

A hierarchical cluster analysis performed using the changes in the miRNA expressions and the heatmap cre-

Table 3. Exchange of miRNA expressions of cancerous and normal tissues of patients ($2^{-\Delta\Delta Ct}$).

Patient Code	Hsa-miR-375-3p	Hsa-miR-148a-3p	Hsa-miR-196a-5p	Hsa-miR-376c-3p	Hsa-miR-129-5p	Hsa-miR-34c-5p	Hsa-miR-662	Hsa-miR-767-5p
GCM-01	3.158	–5.382	0.803	–0.238	2.043	1.498	0.598	2.113
GCM-02	–1.958	1.573	–0.613	–1.103	–0.228	–1.213	–0.803	–3.278
GCM-03	2.198	–1.248	1.348	0.631	1.173	0.723	1.083	1.008
GCM-04	4.570	–2.495	0.810	5.725	1.670	3.340	2.680	3.000
GCM-05	1.618	4.908	2.158	0.843	1.573	3.158	–0.858	0.753
GCM-06	–2.393	–0.268	–0.758	–2.853	–3.058	–3.078	4.128	–4.633
GCM-07	–8.520	4.720	–0.095	–5.420	–0.610	–1.855	–1.255	–4.633
GCM-08[a]	–2.368	–2.553	–0.653	0.038	–0.498	0.618	–0.723	–1.958
GCM-09	–4.745	–0.085	–1.400	–4.415	–2.638	–1.905	–3.125	–5.945
GCM-10	–7.380	–3.240	–2.575	–4.335	–3.155	–5.810	–4.650	–6.015
GCM-11	–0.608	NA	–1.318	–1.763	0.852	–1.723	–2.243	–1.998
GCM-12	–4.353	NA	–3.178	–0.978	–2.053	–3.633	–3.653	–3.563
GCM-13	–3.673	–4.003	–3.613	–6.353	–4.053	–5.793	–4.688	–6.993
GCM-14	0.503	–1.293	–1.053	–1.048	–0.558	–0.608	0.543	0.688
GCM-15	0.788	–1.008	–1.208	–3.843	–0.533	–0.678	–0.203	–0.143
GCM-16	–0.3055	0.520	–3.315	–3.445	–2.595	–3.085	–0.225	–3.815
GCM-17	–2.360	–1.330	–3.095	–3.885	–2.750	–3.020	–2.560	–2.260
GCM-18	–3.005	–2.175	–1.395	–0.920	–1.040	–0.985	0.025	–2.495
GCM-19[a]	–2.168	3.388	–3.003	–1.228	–0.473	–2.388	–1.403	–3.838
GCM-20	–5.110	–2.210	–3.840	–3.250	–0.930	–3.205	–1.125	–2.310

GCM: gastric cancer miRNA; NA: not available.

[a] GCM-08 and GCM-19 have signet ring cell carcinoma (a subgroup of gastric carcinoma).

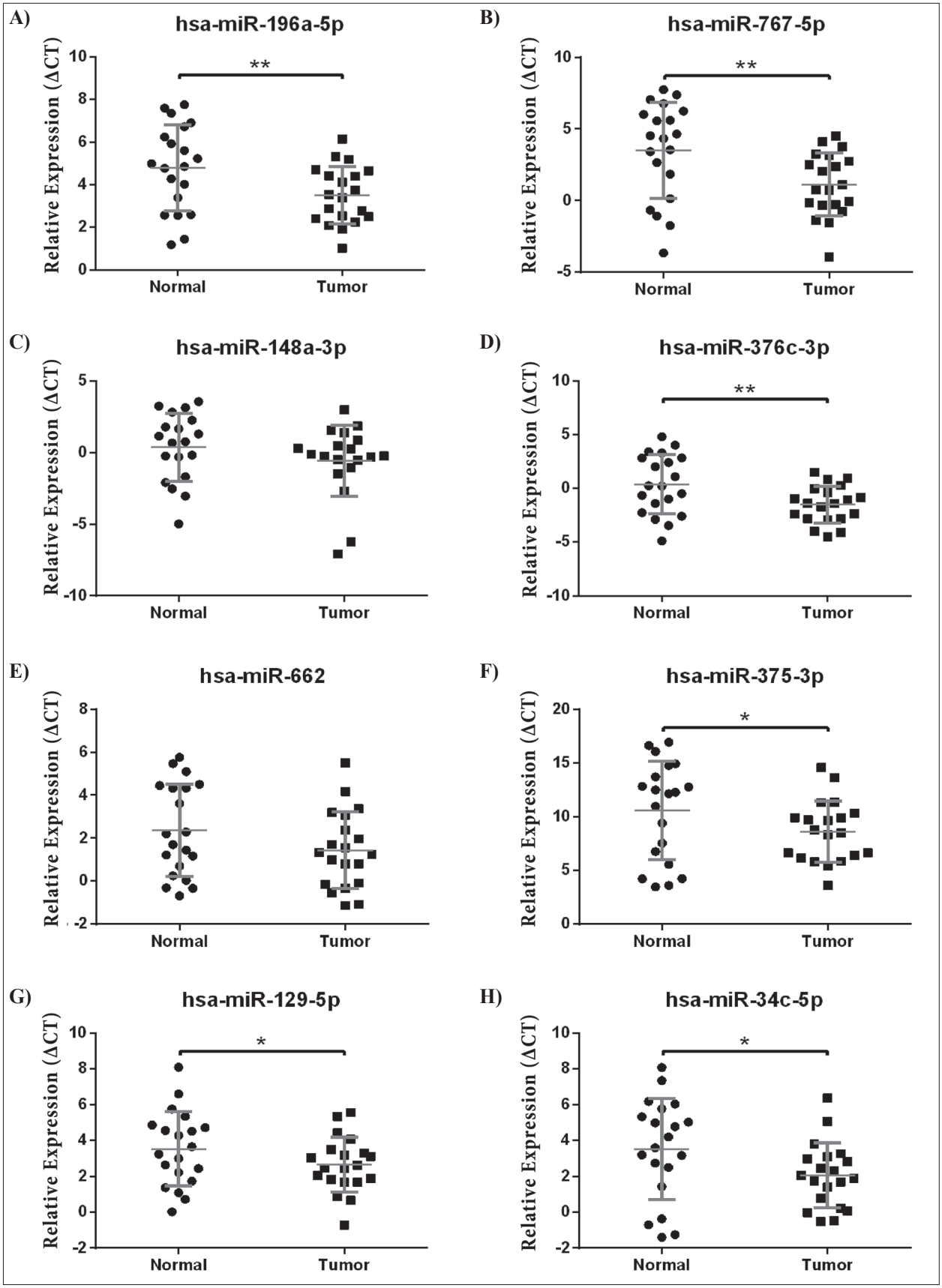

Figure 2. Relative expression changes of miRNAs in cancerous and normal tissues (*p <0,05, **p <0,01).

ated by the addition of annotations for smoking status, age and the number of metastatic lymph nodes, revealed distinct clusters of miRNAs and patients (Figure 3). For example, miR148a-3p had a relatively different expression pattern than other miRNAs, while miR-767 and miR-375-3p in one cluster and miR-196a-5p and miR-123-3p in the other, showed similar expression profiles. The miRNA expressions of patients with GCM-01, GCM-03, GCM-04 and GCM-06 code, were different from those of the other 16 patients, and exhibited a decreased expression of miR-148a-3p, unlike the other patients (Figure 3).

Table 4. Statistical comparisons between cancerous and normal tissues in patients with gastric cancer.

miRNA ID	Average Change	95% CI	*p* Value
Hsa-miR-375-3p	–1.943	–3.521/–0.365	0.018[a]
Hsa-miR-148a-3p	–0.676	–2.085/0.731	0.324
Hsa-miR-196a-5p	–1.299	–2.115/–0.483	0.003[b]
Hsa-miR-376c-3p	–1.891	–3.160/–0.623	0.006[b]
Hsa-miR-129-5p	–0.892	–1.726/–0.059	0.037[a]
Hsa-miR-34c-5p	–1.482	–2.650/–0.314	0.016[a]
Hsa-miR-662	–0.922	–1.964/0.118	0.079
Hsa-miR-767-5p	–2.387	–3.735/–1.040	0.001[b]

ID: identity; 95% CI: 95% confidence interval.
[a] A *p* value of <0.05 was significant.
[b] A *p* value of <0.01 was significant.

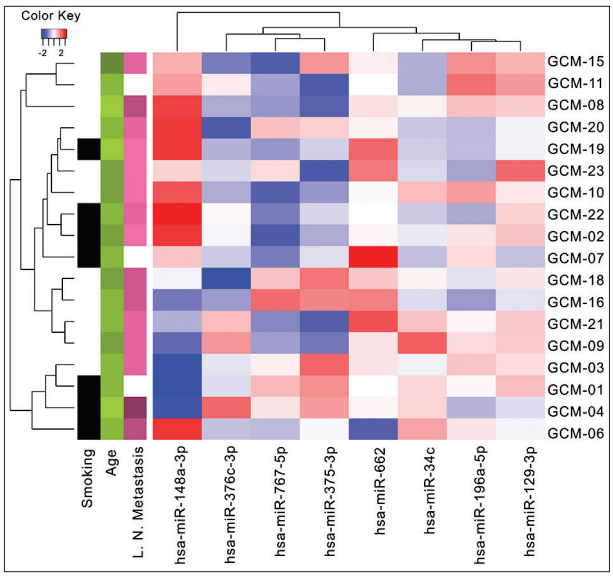

Figure 3. Hierarchical clustering and heatmap of miRNAs. Hierarchical cluster using "Euclidean" distance and "complete" linkage. (L.N.: Lymph node).

Table 5. Comparison of patient age, metastatic lymph node number and smoking with miRNA expressions.

miRNA ID	Age; *r* Value	Age; *p* Value	Metastatic Lymph Node; *r* Value	Metastatic Lymph Node; *p* Value	Smoking *p* Value
Hsa-miR-375-3p	–0.160	0.512	0.350	0.135	0.102
Hsa-miR-148a-3p	–0.320	0.196	0.290	0.240	0.245
Hsa-miR-196a-5p	–0.330	0.159	0.430	0.056	0.412
Hsa-miR-376c-3p	–0.420	0.063	0.410	0.075	0.043[a]
Hsa-miR-129-5p	–0.320	0.173	0.470	0.036[a]	0.150
Hsa-miR-34c-5p	–0.380	0.102	0.510	0.020[a]	0.274
Hsa-miR-662	–0.440	0.049[a]	0.210	0.370	0.195
Hsa-miR-767-5p	–0.330	0.156	0.400	0.077	0.344

ID: identity; 95% CI: 95% confidence interval.
[a] A *p* value of 0.05 was significant.

DISCUSSION

Currently, an increasing number of studies are being conducted on the biogenesis of gastric cancers, and effective diagnostic methods and treatments are investigated, helping understand the disease progression at the molecular level [25]. The miRNAs are relatively novel molecules with their poorly understood regulatory mechanisms and molecular interactions and can provide novel opportunities for gastric cancer diagnosis and prognosis [27,28]. Current knowledge states that miRNAs play active roles in cancer biogenesis with tumor-suppressive or oncogenic activities *via* modulating targets involved in carcinogenesis and tumor progression [2,3]. The prognoses and treatment responses for cancers differ, based on demographics, including the geography and ethnicity, as well as molecular characteristics. Several studies that exist in the literature for gastric cancer use paraffin-embedded formalin fixed tissue [29], and thus, our study using an independent cohort of freshly frozen tumor-normal pairs adds to the existing literature, using gastric cancer paired miRNA biomarkers obtained from cancer-adjacent normal fresh tissue collection.

We found that miR-375-3p, miR-129-5p and miR-34c-5p decreased significantly in gastric cancer, while miR196a-5p, miR376c-3p and miR-767-5p exhibited the most significant downregulation of expression. Our findings could provide good candidates for further testing as biomarkers in different gastric cancer cohorts. The present study also showed that the expression levels of miRNAs varied more in the adjacent normal tissue in comparison to paired tumors, supporting the notion that miRNA expression could be regulated in a stronger manner in cancerous tissue in accord with the literature [30].

Although the sample size limited the scale of our clin-icopathological assessments, we found associations with age and the number of metastatic lymph nodes. It was observed that lower miR-662 expression levels were significantly associated with increased age, and the expressions of miR-129-3p and miR-34c-5p correlated with the number of metastatic lymph nodes in the gastric cancer tissues. Indeed, previous studies have identified a correlation between miRNA levels and age [31] and invasion into lymph nodes [32]. Our findings provide novel miRNA candidates associated with these selected clinical parameters in gastric cancers.

One of the miRNAs we identified as being downregulated, miR-375-3p (2q35), first found in pancreatic β cells playing a role in pancreatic organogenesis and β cell function [30], has been shown to be decreased in laryngeal, esophageal, gastric, hepatocellular cancers and pancreatic ductal adenocarcinoma, while increasing in hepatitis B virus (HBV)-positive hepatocellular cancer, as well as lung and breast cancers [10-11,33-35]. The miR-375 targets JAK2, a cytokine signaling pathway molecule, leading to cell proliferation in gastric cancer [11]. The first article showing the relationship between miR-375 and gastric cancer indicated that it has a direct effect on PDK-1 and 14-3-3 ζ(YWHAZ) and the caspase-dependent apoptotic pathway, leading to cancer development *via* DNA methylation and histone deacetylation [10]; miR-375 also modulates JAK2/STAT3 signaling pathway [36]. Accordingly, miR-375 is a tumor suppressor miRNA whose downreg-ulation can be important for determining the degree of cancer progression. Other mechanisms are arising for the actions of miR-375 *via* its correlation with increased levels of Recepteur d'Origine Nantais (RON), a tyrosine kinase receptor [37]; miR-375 targets SLC7A11 directly and triggers ferroptosis [38]. These studies also indicate that modulation of target gene expression by miR-375 may also be considered for developing novel treatments for gastric cancer [33]. In the present study, we support the tumor suppressive role of miR-375 in our independent and paired cohort, in a manner consistent with the literature [10-12].

Previous studies have also shown that miR-148a-3p is downregulated in gastric cancer tissues with a diagnostic significance. miR-148a-3p has been reported as having an association with TNM staging and lymph node metastasis [39]. A previous study using tumor-normal pairs from gastric cancer patients has identified miR-148-3p as a downregulated biomarker [40]. Although the miR-148a-3p value exhibited a trend for downregulation in our study, there was no statistically significant result ($p = 0.325$) due to observed variability among patients. Potentially, this shows that the molecular subtype composition within and between patient cohorts could affect the significance of this miRNA in gastric cancer diagnosis. In our study, miR-148a-3p level was relatively lower in patients encoded with GCM-01, GCM-03, GCM-04 and GCM-06, who might represent different molecular characteristics from other patients and warrants further study.

On the other hand, miR-196a-5p is known to be effective in gastric cancer diagnosis/prognosis in the literature and shown to achieve this *via* its overexpression [14,41]. miR-196a is in a genomic region where the homeobox (*HOX*) genes are located and exerts an effect on these genes, along with high mobility group A2 (*HMGA2*), annexin A1 (*ANXA1*) and keratin 5 (*KRT5*) [42]. It has also been reported to be upregulated in other cancers, such as breast, esophageal and colorectal cancers, and leukemia [42]. In the present study, the level of miR-196a appears to be significantly downregulated as in acute lymphoblastic leukemia (ALL) and melanoma. Previous gastric studies of miR-196a were conducted in Far Eastern countries, such as China and Japan. This difference in expression in the present study may have resulted from geography, ethnicity as well as cohort specific molecular subtype composition, however, more studies are needed to confirm direction of this miRNA in gastric cancer using larger cohorts.

The literature also presents contradictory findings about miR-376c-3p, although only a few studies have investigated this miRNA [16,17]. Our findings supported that miR-376c was highly significantly downregulated in cancerous tissue, while there was a significant relationship between miR-376c-3p and smoking ($p = 0.043$).

miR-129-3p, another miRNA known to be downregulated in gastric cancer, exerts its effect on cell proliferation by acting through its target cyclin-dependent kinase 6 (*CDK6*) [18]. In the present study, we confirmed the decrease in the miR-129 level in the paired cancerous tissue as compared to that in the normal tissue, consistent with previous reports. In addition, we found that the number of metastatic lymph nodes exhibited a significant correlation with the miR-129 level. Previous studies identified an upregulated, metastasis associated long noncoding RNA target (*AC130710*) as one of the targets of miR-129-5p

in gastric cancers [19] as well as significant association between miR-129 levels and clinical parameters such as tumor size and lymph node metastasis, which support our findings.

miR-34c-5p has been frequently studied in the literature as it plays an important role in cancer biogenesis with its effect on DNA methylation and shows downregulation in gastric cancer [6]. In the present study, we found that miR-34c was significantly associated with both the number of metastatic lymph nodes and the lower expression in cancerous tissue, consistent with previous reports [20].

miR-662 is best known for its role in the immune response against viruses and cancerous tissues by affecting the *NLRC5* gene [43]. miR-662 upregulation could also be a factor responsible for distant metastasis in lung cancer [44]. To the best of our knowledge, no previous studies have investigated the relationship between miR-662 and gastric cancer. In the present study, the miR-662 in cancerous tissue showed downregulation with a p value only approaching significance due to variance observed among patients (p = 0.079). Future studies need to be performed using larger patient cohorts to help understand the source of this variability of expression among subtypes of gastric cancers.

Few studies have investigated miR-767-5p in the literature; upregulation of miR-767-5p through hypomethylation is thought to play a role in tumor development [45] as in melanoma [46]. There is no study other than that recently published by Geng *et al.* [22], demonstrating an association between gastric cancer and low levels of miR-767-5p. In our paired cohort, we also showed that miR-767, was highly significantly downregulated in cancerous tissues (p = 0.001). Although recent studies show that overexpression of miR-767-5p is associated with cell proliferation in multiple myeloma, hepatocellular carcinoma and prostate cancer [47-50], the findings in this study and Geng *et al.* [22], point to lower miR-767-5p in gastric cancer when compared with paired normal tissue as in glioma [51] and more recently in gastric cancer [22]. These implicate that the same miRNA may function in the opposite directions in different cancers and future studies are needed to understand the mechanisms at work. Moreover, the demonstration of the similarity between the expression of miR-375-3p, which is known to be downregulated based on the literature, [10-12] and miR-767-5p in patients supports this statistical significance.

In conclusion, to the best of our knowledge, this is the second report of miR-767 and its downregulation, while the first analysis on miR-662 expression levels in gastric cancer tissues. More importantly, our results implicate for the first time, differential expression of two miRNAs, namely, miR-662 and miR-376c-3p, respectively, with age and smoking in gastric cancer. We also associate the increased number of metastatic lymph nodes with the downregulation of gastric cancer specific miRNAs.

Our study showed that miR-375, miR-196a, miR-376c, miR-129, miR-34c and miR-767, all downregulated, may be used as biomarkers in development of diagnostic tools for gastric cancer. Moreover, miRNAs in cancerous tissues are likely to be expressed in a narrower range, and thus, could be under a stronger control of genomic/transcriptomic elements based on previous research. This could also be due to presence of higher cellular heterogeneity in the normal adjacent tissues of gastric cancers. The present study may lead to the understanding of the biogenesis of gastric cancer *via* finding targets of the miRNAs studied and to the discovery of novel molecular targets for treatment. However, further genetic, and functional studies on this subject with larger sample sizes are needed.

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