



MicroRNA expression profiling provides novel insights into immune-related pathways involved in gastric cancer

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Abstract

Gastric cancer is one of the most common cancers, and an increasing number of studies have found that microRNAs (miRNAs) play essential roles in gastric cancer progression; however, the roles of specific miRNAs involved in the immune response to this disease remain unclear. We compared the miRNA expression in tissues from primary gastric cancer patients and healthy controls to find miRNAs dysregulated in gastric cancer and used bioinformatics tools to determine potential roles of these miRNAs in the immune system. We evaluated 25 primary gastric cancer tissues and five healthy gastric tissues. Quantitative real-time polymerase chain reaction was performed for a set of miRNAs, followed by the prediction of their target genes and functional enrichment analysis of these targets. Analysis of a microarray dataset showed that the miRNA miR-196a-5p was significantly upregulated, while miR-374a-5p and miR-375 were downregulated in gastric cancer patients. In addition, miR-374-5p was significantly downregulated in patients with metastasis compared with its expression levels in non-metastatic patients ($p=0.03$). Bioinformatics analysis suggested that the pathways regulated by these differentially expressed miRNAs were related to the immune response, cell adhesion, and cell migration. Most importantly, this study provides a new insight into the potential use of multiple miRNAs to find distinct pathways of immune regulation in gastric cancer.

Keywords Gastric cancer · MicroRNA · Microarray analysis · Immune system · Bioinformatics

Introduction

Stomach cancer is a public health problem worldwide, with an estimated 1,000,000 new cases and 783,000 deaths per year, thus accounting for 8.8% of the cancer deaths globally, with 70% of the new cases and deaths occurring in developing countries [1]. Brazil and, specifically, its Northeast region are high-incidence areas for this disease [1]. Thus, it is important to better understand the mechanism of gastric cancer (GC) progression, including proliferation, growth, migration, invasion, and apoptosis [2]. Numerous studies have demonstrated that non-coding RNAs play essential roles in GC initiation and progression [3, 4].

MicroRNAs (miRNAs, miRs) are small non-coding RNAs of 20–22 nucleotides in length, which are involved in crucial biological processes [5]. miRNAs regulate gene expression through binding to the 3' untranslated region (UTR) of target mRNAs, thus leading to mRNA cleavage or suppression of its translation [6]. Abnormal expression levels of miRNAs are a well-known and vital

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issue in cancer development [7] and have been shown to be involved in multiple steps in GC tumorigenesis [8, 9].

Recent evidence has indicated a promising role for miRNAs in the risk assessment, prevention, early diagnosis, and prognosis of GC [10, 11]. miRNA expression profiling is becoming an important tool to reveal different tumor types and gene activity in cancer [12, 13]. Owing to their involvement in the regulation of expression of many mRNAs, miRNAs can modulate various physiological and pathological responses [14], thus providing important insights into disease severity and prognosis. Studies of miRNAs have revealed a new layer of the regulation of gene expression that affects many biological systems, including the immune system [15]. More than 100 different miRNAs are expressed by cells of the immune system and have the potential to influence molecular pathways that control innate and adaptive immune responses [15, 16].

The present study aimed to identify differentially expressed miRNAs in GC, determine their associations with clinical variables, identify their putative target genes, and to assess their potential to influence the outcomes of immune responses, using a series of bioinformatics methods.

Materials and methods

Patients

The study included patients diagnosed with potentially curable GC at the Hospital do Cancer de Pernambuco from 2014 to 2017. The inclusion criteria of the study were histologically confirmed diffuse or intestinal-type gastric carcinoma, and all patients were newly diagnosed. None of the patients had received chemotherapy, radiotherapy, or immunotherapy before sampling. The exclusion criteria were past treatments of neoplasia or autoimmune disease. We prospectively collected information on the clinical characteristics of the patients. Cancer staging was performed according to the 7th edition of the American Joint Committee on Cancer tumor–node–metastasis (TNM) criteria [17].

All samples were obtained from surgical specimens of patients with GC, and all patients provided written informed consent for the use of these tissues for research purposes. We obtained 25 GC tissues and also collected five normal gastric biopsy samples from the antral part of the stomach of control subjects who were referred for upper gastrointestinal endoscopy due to symptoms of dyspepsia and had no previous history of malignancy. The study protocol was reviewed and approved by the Ethics Research Committees of Hospital do Câncer de Pernambuco (approval number. CAAE 28932814.2.0000.5205).

miRNA expression analysis by TaqMan® low-density array

For this analysis, we used 23 gastric tumor tissues and five healthy gastric tissues as controls. Two patients were excluded because of inadequate samples. miRNA expression profiles were determined using a TaqMan low-density array (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Quantitative real-time polymerase chain reaction (PCR) was performed using a 7900 HT Fast real-time PCR system (Applied Biosystems), and relative miRNA quantification was performed using the $2^{-\Delta\Delta C_t}$ method [18]. Data analyses were performed by comparing the tumor and control groups. A pool of the normal samples was used for calibration, and hsa-miR-10b, which showed the most stable expression in the sample set, was used as a reference. Calculations were performed using the R software (<https://cran.r-project.org/>), and only differentially expressed miRNAs, with a false discovery rate (FDR) ≤ 0.01 , were considered for further analysis.

Differences in miRNA expression according to clinical variables

The most differentially expressed miRNAs between tumor and healthy tissues were selected based on fold changes (FCs; the ratio of median relative expression in the tumor group to that in the control group > 10.0) and further investigated for their association with clinical variables. Power analysis for the study design was conducted using G*Power 3 [19] based on the means and standard deviations of relative miRNA levels, as reported by Baggish et al. [20]. Power calculations were also performed on several physiological measures from the current study to ensure that the sample sizes were adequate for detecting between-group differences in these variables. A sample size of $N = 30$ was required to detect between-group differences in the mean miRNA levels by the Mann–Whitney test (power of 80%; $\alpha = 0.05$). miRNA expression was considered to be significantly different between the two groups if the FDR-adjusted p -value was < 0.05 , and FC of expression was > 10 in either direction. In the patient group, the relative expression of each miRNA was analyzed according to the histological grade (G1/G2 vs. G3), lymph node involvement (negative vs. positive), tumor location (proximal vs. distal), metastasis (positive vs. negative), and Lauren classification (diffuse vs. intestinal). Considering that variables did not show a normal distribution (using the Kolmogorov–Smirnov test), the Mann–Whitney U -test was performed using GraphPad Prism version 7.00 for

Windows (GraphPad Software, La Jolla California USA). For all statistical tests, p -values were two-tailed, and p was set to 0.05.

Target prediction and functional annotation

Potential mRNA targets of the miRNAs selected for analysis according to clinical variables were predicted using miR-Walk v2 [21, 22], with its algorithm and three other tools (RNA22, miRanda, and TargetScan) integrated into the system. We only used 3'-UTRs of mRNAs for target prediction and considered interactions with the miRNA seed region (nucleotides 2–8). Only targets predicted with at least three tools were considered for further analysis. Functional annotation was conducted separately for targets of each selected miRNA differentially regulated in GC. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) v. 6.8 [23] was used for pathway enrichment assessment (Kyoto Encyclopedia of Genes and Genomes pathways).

Results

The clinical and pathological characteristics of GC patients are summarized in Table 1.

miRNA expression in gastric cancer and healthy gastric tissues

miRNA expression patterns significantly differed between GC and healthy gastric tissues (Fig. 1 and Table 2). FC filters included the requirements that the miRNA expression was more significant than or equal to two-fold of the control level for upregulated miRNAs and less than two-fold of the control level for downregulated miRNAs.

Using an arbitrary FC cutoff of > 10 and a p -value cutoff of < 0.01 , among the differentially expressed miRNAs, one (miR-196b-5p) was found to be significantly upregulated, and two (miR-374-5p and miR-375) were found to be significantly downregulated in GC tissues versus normal gastric tissues (Table 2).

Analysis of miR-196b-5p, miR-374-5p, and miR-375 according to clinical parameters

miRNA expression analysis, according to clinical parameters, revealed that miR-374-5p was significantly downregulated in GC patients with metastasis compared with its expression in the no-metastasis group ($p = 0.03$).

Table 1 Clinical and pathological characteristics of gastric cancer patients

Variables	$N = 25$
Age (years), median (IQR 25th–75th)	64 (34–85)
Sex	
Male	15 (60%)
Female	10 (40%)
Lymph node involvement	
Negative	7 (35%)
Positive	13 (65%)
Distant metastasis	
Negative	20 (80%)
Positive	5 (20%)
TNM ^a	
I/II	8 (32%)
III/IV	17 (68%)
Grade	
Differentiated (G1/G2)	7 (28%)
Undifferentiated (G3)	18 (72%)
Lauren's criteria	
Intestinal	7 (28%)
Diffuse	14 (56%)
Indeterminate	4 (16%)

Values are presented as absolute numbers and percentages or median (IQR)

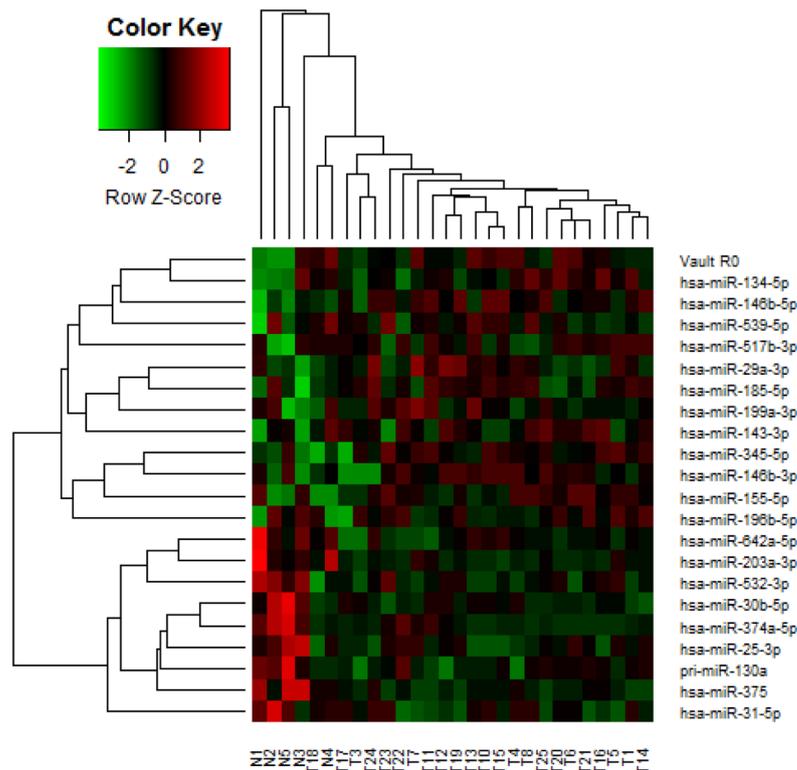
IQR interquartile range, TNM tumor–node–metastasis

^aClassification according to the American Joint Committee on Cancer, 7th edition [17]

miR-375 was significantly upregulated in the histological G3 group compared with its expression in G1 and G2 tissues ($p = 0.05$). No statistical differences were found in the expression of miR-196b-5p, miR-374-5p, and miR-375 depending on the lymph node involvement, tumor location, and Lauren classification. Besides, miR-196b-5p was not associated with metastasis or histological grade parameters (Table 3).

Analysis of miR-196b-5p, miR-374-5p, and miR-375 associations with biological pathways by functional enrichment

The pathways for miR-375 (Fig. 2a), miR-196b-5p (Fig. 2b), and miR-374-5p (Fig. 2c), identified by microarray analysis, included the gap junction, mRNA surveillance pathway, gonadotropin-releasing hormone signaling pathway, proteoglycans in cancer, Ras signaling pathway, pathways in cancer, pancreatic and colorectal cancer, and tumor necrosis factor (TNF) signaling pathway. Further details of the pathways associated with miR-196b-5p, miR-374-5p, and miR-375 are shown in Fig. 2.



Note: False Discovery Rate (corrected P -value) ≤ 0.01 . In green, down-regulated miRNAs; in red, up-regulated miRNAs. N – normal tissue; T – gastric tumor.

Fig. 1 Heatmap summarizing the expression patterns of 22 miRNA loci that were differentially expressed in gastric cancer compared to healthy tissue

Relation of miRNAs differentially expressed in gastric cancer to cancer hallmarks and mediators of tumor immune escape

The target prediction analysis revealed 10,435 putative target genes for the positively regulated miRNAs in the tumor, and 10,278 putative targets for the negatively regulated miRNAs. From these data, the statistically significant biological pathways related to the two groups of target genes were identified by functional annotation, revealing several pathways associated with hallmarks of cancer and the immune system relevant to cancer development. We found miRNA target genes TNFRSF1B and TRAF1 in important immune pathways, including hsa04668:TNF signaling pathway, related to aberrant miRNA expression. The gene PDCD1 (PD-1), a well-reported gene in tumor escape process, was identified in the enriched pathway hsa04660:T-cell receptor signaling pathway and hsa04514:cell adhesion molecules (CAMs). The gene CD274 (PDL-1) was revealed in a pathway linked to cancer hallmarks, hsa04514:CAMs. The CD40LG (CD40L) gene was observed in several biological pathways related to tumor-regulated miRNAs (with increased expression in the

tumor relative to the control group). The CD40L-involving biological pathways were found to be linked to the immune system, as well as the hsa04660:T-cell receptor signaling pathway, hsa04514:CAMs, and hsa04060:Cytokine-cytokine receptor interaction. Further details of the pathways associated with the positively and negatively regulated miRNAs are shown in Fig. 3 (The ten most significantly enriched pathways).

Discussion

In the present study, we established a carefully designed procedure to identify a miRNA profile of GC patients. Aberrant miRNA expression was reported in various solid tumors, including breast, lung, pancreas, and colon cancer [24, 25]. miRNAs can act as tumor suppressors and as oncogenes [26]. The relationships between miRNAs and tumors have thus become the focus of cancer studies [27].

Our study revealed that the miR-196b-5p expression was higher in GC tissues than in normal tissues. To date, there have been a few studies on miR-196b-5p in GC.

Table 2 MicroRNAs with altered expression in gastric cancer tissues (FDR ≤ 0.01)

miRNA kit	Expression fold change (tumor/control)	FDR <i>t</i> test	miRNA name
hsa-miR-374-000563	- 16.83	0.010592087	hsa-miR-374a-5p
hsa-miR-375-000564	- 14.39	0.010179736	hsa-miR-375
hsa-miR-203-000507	- 7.05	0.004724326	hsa-miR-203a-3p
hsa-miR-30b-000602	- 5.74	0.012233919	hsa-miR-30b-5p
hsa-miR-31-002279	- 4.45	0.004128206	hsa-miR-31-5p
hsa-miR-642-001592	- 4.23	0.004724326	hsa-miR-642a-5p
hsa-miR-25-000403	- 3.86	0.004128206	hsa-miR-25-3p
hsa-miR-130a-000454	- 3.14	0.014106465	hsa-miR-130a-5p, hsa-miR-130a-3p
hsa-miR-532-3p-002355	- 2.97	0.010665846	hsa-miR-532-3p
hsa-miR-199a-3p-002304	2.42	0.004724326	hsa-miR-199a-3p
hsa-miR-539-001286	2.72	0.012233919	hsa-miR-539-5p
hsa-miR-345-002186	2.74	0.004128206	hsa-miR-345-5p
hsa-miR-29a-002112	3.03	0.004128206	hsa-miR-29a-3p
hsa-miR-143-002249	3.23	0.004897369	hsa-miR-143-3p
hsa-miR-155-002623	3.39	0.004128206	hsa-miR-155-5p
mmu-miR-134-001186	3.67	0.004897369	hsa-miR-134-5p
hsa-miR-146b-001097	4.12	0.004897369	hsa-miR-146b-5p
hsa-miR-185-002271	4.28	0.004128206	hsa-miR-185-5p
hsa-miR-886-5p-002193	4.69	0.01164442	Vault RNA
hsa-miR-146b-3p-002361	6.60	0.004128206	hsa-miR-146b-3p
hsa-miR-517b-001152	7.23	0.004128206	hsa-miR-517b-3p
hsa-miR-196b-002215	184.65	0.014802397	hsa-miR-196b-5p

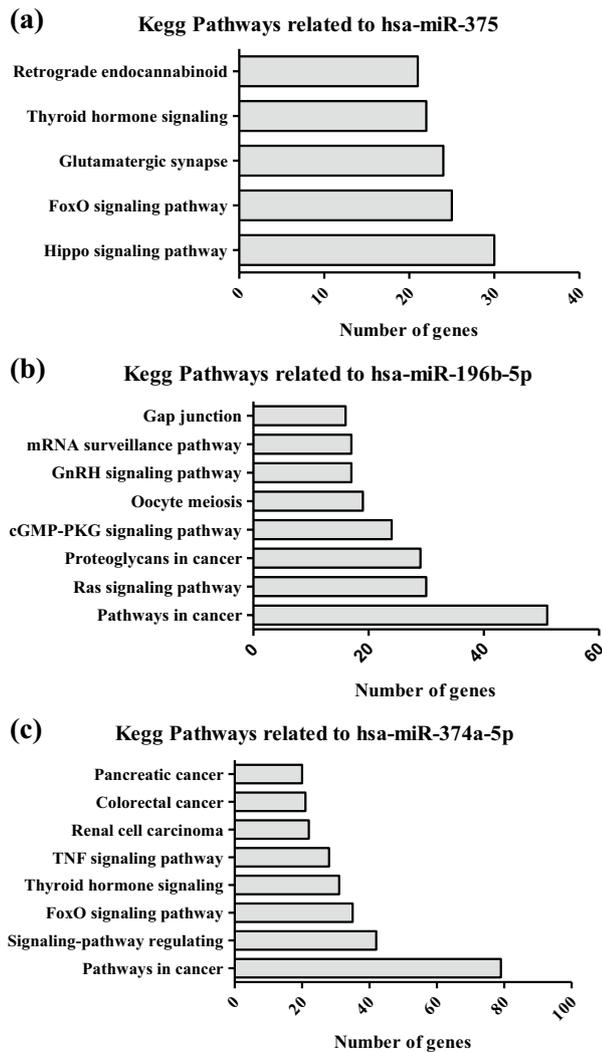
Table 3 Correlation between microRNA expression and clinical characteristics of gastric cancer patients

Variables	miR-196b-5p		miR-374-5p		miR-375	
	Mean fold change	<i>p</i> -Value	Mean fold change	<i>p</i> -Value	Mean fold change	<i>p</i> -Value
Histological grade						
Differentiated (G1/G2)	555.5	0.40	0.10	0.73	0.39	0.05*
Undifferentiated (G3)	184.2		0.16		0.66	
Lymph node involvement						
Negative	256.6	0.23	0.14	0.64	0.07	0.13
Positive	54.1		0.15		0.41	
Metastasis						
Negative	156.4	0.70	0.16	0.03*	0.25	0.72
Positive	36.6		0.03		0.09	
Lauren's criteria						
Intestinal	493.2	0.64	0.09	0.38	0.46	0.09
Diffuse	313.1		0.17		0.12	

**p* ≤ 0.05

miRNAs are abnormally expressed in tumor tissues and body fluids of GC patients. Because detection of miRNAs in the plasma, serum, urine, and gastric juice can be used in the diagnosis, several studies have evaluated the diagnostic performance of miRNAs in body fluids in GC patients, and the results showed that many miRNAs

had certain value for GC diagnosis, recurrence monitoring, and/or clinical staging. Tsai et al. [28] have reported that the lack of promoter methylation in miR-196b may explain its overexpression in the majority of GCs, which can be used as a tumor biomarker. Lee et al. [29] have reported that miR-196b-5p is upregulated in a Korean GC



Note: Statistically significant pathways (corrected P -value ≤ 0.05).

Fig. 2 Analysis of pathways related to miRNAs identified through microarray analysis: **a** miR-375, **b** miR-196b-5p, and **c** miR-374a-5p

population. However, Liu et al. [30] have reported that miR-196b-5p is downregulated in a GC cell line. This result contradicts to our data and those of Lee et al. [29]; however, the study [30] was conducted on a GC cell line rather than on primary tumor tissues, which suggests that the tumor microenvironment and lifestyles may affect the regulation of miR-196b-5p and indicates that in vitro studies, using a tumoral lineage rather than a primary tumor for miRNA analysis, may not be adequate.

miR-375 is frequently downregulated in various tumor types, being a tumor suppressor and inhibiting malignant characteristics of cancer cells, and it is associated with the proliferation, apoptosis, invasion, and metastasis [31]. Our results showed differences in the expression of miR-375 between histological types of GC.

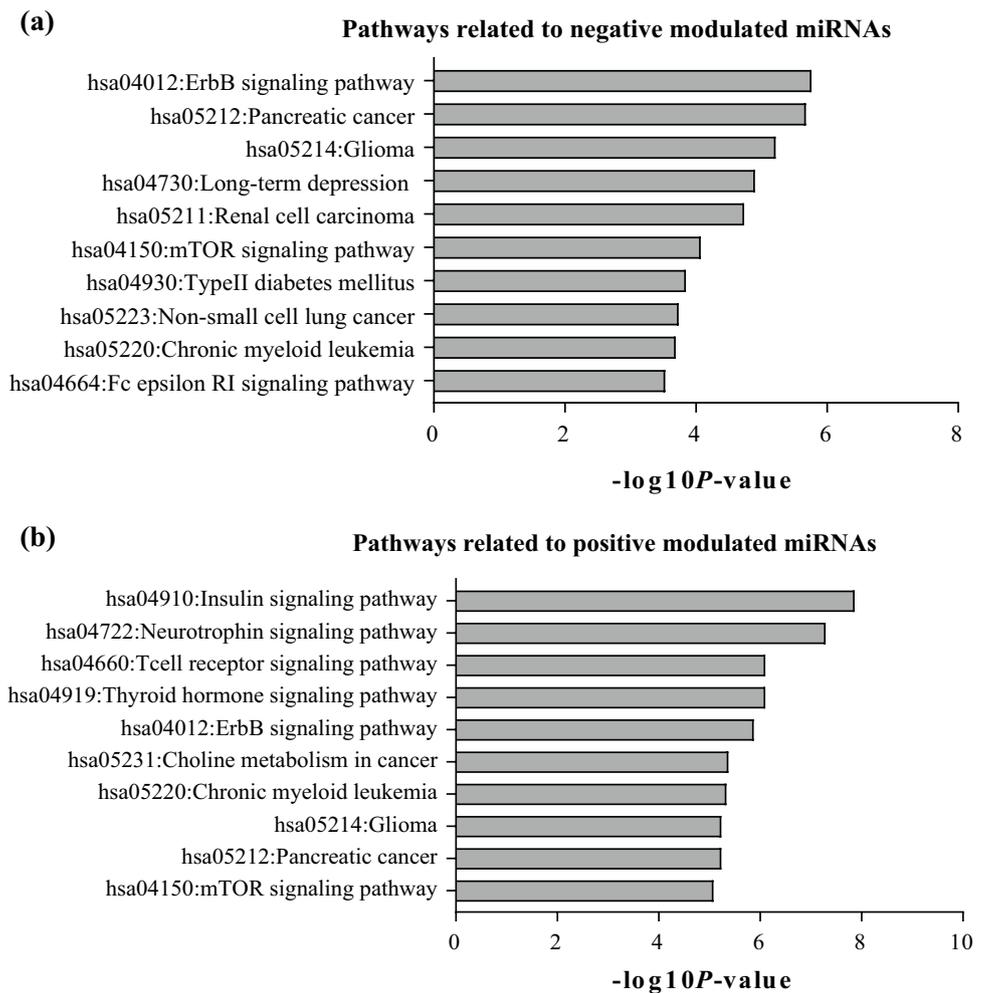
In this study, the expression of miR-374a-5p was significantly lower in gastric adenocarcinoma tissues than in normal tissues. Xie et al. [32] have demonstrated that miR-374a-5p is involved in tumor metastasis and invasion. Our results suggest that miR-374a-5p could be a useful biomarker for the risk of GC metastasis. Wu et al. [33] have found that miR-374a inhibited the cell proliferation and invasion of lung adenocarcinoma, at least partially, through the repression of transforming growth factor alpha (*TGFA*) gene expression. This newly identified miR-374a-mediated *TGFA* gene silencing may lead to a better understanding of the molecular mechanisms of GC progression.

Recently, emerging bioinformatics methods have accelerated the identification of the mechanisms involved in GC development at the molecular level. miRNAs have been reported to regulate the activity of ~30% of all protein-coding genes in the human genome [34]. We predicted, using bioinformatics resources, possible genetic pathways involving the three miRNAs mentioned above, among which both miR-196b-5p and miR-374a-5p were associated with the regulation of cancer pathways. The interactions between dysregulated miRNAs and their potential target genes are complex and can be influenced by various factors, such as pathology, hypoxia, and infection [35].

It has also become clear that miRNAs play multiple roles in the negative regulation of numerous immune checkpoints. A network of miRNAs, directly and indirectly, controls the expression of immune checkpoint receptors, and several miRNAs can target multiple checkpoint molecules, mimicking the therapeutic effect of a combined immune checkpoint blockade [36]. Standard treatment options for patients with advanced GC show limited efficacy and are associated with some toxicity, which necessitates the development of more effective therapies for improving treatment outcomes. Immunotherapy involving immune checkpoint inhibitors has emerged as a new treatment option, and shortly, miRNAs may become essential components of immune checkpoint therapy [37].

We found several target genes, such as *TNFRSF1B*, *TRAF1*, *PDCD1* (*PD-1*), *CD274* (*PDL-1*), and *CD40L*, which are involved in immunological pathways and are significantly affected by the miRNA in GC. TNF- α and its receptors, TNFRSF1A and TNFRSF1B, belong to the TNF/TNFR superfamily (TNFRSF), and interactions of these genes regulate the inflammation and increase the invasive capacity and metastatic potential of tumor cells. TNF- α induces a cascade of other inflammatory cytokines and chemokines and has been considered one of the pivotal mediators of the immune system. TNF- α plays a therapeutic role in destroying tumor blood vessels and inducing the apoptosis and necrosis of tumor cells [38]. However, when chronically produced, with persistent inflammation in the tumor microenvironment, TNF- α can act as a tumor

Fig. 3 The ten most significant biological pathways related to positively and negatively modulated miRNAs identified through microarray analysis



promoter by promoting DNA damage, enhancing proangiogenic functions, and increasing the expression of matrix metalloproteinases and endothelial adhesion molecules [39]. Hamano et al. [40] showed that TNF, in concert with interleukin (IL)-2, preferentially upregulated the mRNA and surface expression of TNFR2, 4-1BB, and OX40 on regulatory T cells (Tregs) [40]. Thus, TNF amplifies its stimulatory effect on Tregs by inducing TNFRSF members [40]. Our previous study has demonstrated a higher expression of OX40 in T cells of GC patients [41]. These findings indicate that miRNAs may be important regulatory molecules in T cells in GC.

The interaction between PD-1 and PD-L1 has been demonstrated to negatively regulate T-cell activation and function, leading to the inhibition of the immune response in cancer patients [42]. However, little is known about the operation of the PD-1/PD-L1 pathway in gastric adenocarcinoma patients. Chiu et al. [43] characterized the PD-1 expression profile in gastric adenocarcinoma patients and found a significant increase in PD-1 expression in peripheral blood and gastric tissue, which could promote the apoptosis

of CD3⁺ T cells. Our findings corroborated the results of this previous study and provided new insights into possible immune targets in GC.

We identified miRNAs with significantly up- or down-regulated expression in GC, potentially influencing *CD40L* gene expression. These findings indicate that these miRNAs may downregulate *CD40L* mRNA and repress its protein translation. CD40L is a pivotal molecule in adaptive immune function. In its absence, T-dependent humoral immunity is debilitated, and several critical T-cell functions are compromised, including the activation of antigen-presenting cells (APCs) [44]. In vitro stimulation with CD40L has been demonstrated to enhance the efficiency of dendritic cells (DCs) as APCs for the antitumoral response [45], and CD40 stimulation can be significantly enhanced by an additional stimulus.

There are a few limitations to this study, including a relatively small number of patients. Thus, further large-scale research is required to elucidate the exact roles of miRNAs in GC. However, our study provides basic data on miRNAs in GC, which should facilitate further research on GC

and clarify the functional roles and clinical significance of miRNAs.

Collectively, our results can drive new research on the mechanism of immune evasion by tumors and their ability to attenuate immune responses. Furthermore, our data demonstrated that miRNAs could make two distinct impacts, on gastric adenocarcinoma cells and cells of the immune system, seemingly affecting a crucial part of the antitumor response. The screened genes and pathways provided new insights into molecular mechanisms underlying GC occurrence and progression, holding promise to serve as potential therapeutic targets.

Conclusions

Our study revealed, via analysis of a microarray dataset, that three miRNAs (miR-196a-5p, miR-374a-5p, and miR-375) were significantly up- or downregulated in GC patients. Further studies, involving larger cohorts, are needed to validate the clinical application of these miRNAs. Most significantly, this study provides a new insight into the potential use of multiple miRNAs to find distinct pathways of immune regulation in GC. Further understanding of the immunopathogenesis of gastric adenocarcinoma will be critically important for the development of effective therapeutic strategies against the disease. Our future research will focus on exploring immune checkpoint receptors in various populations of cells of the immune system and their relation to GC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest (including financial and non-financial interests).

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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