MiRNA biomarkers and potential critical genes associated with the prognosis of gastric cancer

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Research Article

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Abstract

Background Dysregulated expression of miRNAs in gastric cancer (GC) is associated with tumor progression. MiRNA markers are important for the prognosis and therapeutic targeting of GC patients.

Methods To detect differentially expressed miRNAs in GC from the TCGA database and predict their target genes. We downloaded RNA sequencing (RNA-seq), miRNA-seq and clinical data of GC from TCGA. Differential expression analysis of RNA-seq and miRNA-seq data was performed by R 3.6.1. MiRNAs associated with prognosis were evaluated with the Cox model, and differentially expressed miRNAs were assessed by Kaplan–Meier curve analysis. Risk factors were identified in the Cox model. Target genes of differentially expressed miRNAs were searched in three databases. GO enrichment and KEGG pathway analyses were used to evaluate the biological functions of these target genes.

Results Five miRNAs (hsa-miR-135b-3p, hsa-miR-143-5p, hsa-miR-196b-3p, hsa-miR-942-3p, hsa-miR-9-3p) were related to survival. Eight target genes (AKAP12, AR, DZIP1, PCDHA11, PCDHA12, PI15, SH3BGRL and TMEM108) were closely correlated with patient overall survival (OS).

Conclusion Differentially expressed miRNAs and their target genes have an important influence on the diagnosis and prognosis of GC and may be used as tumor biomarkers in further studies and as potential therapeutic targets.

Background

GC is a malignant tumor that ranks as the fifth most common cancer and the third leading cause of death from cancer globally [1]. The 5-year survival rate of GC is generally 25-30% or lower, even in developed countries [2]. Although the 5-year survival rate has improved due to increased diagnostic activity, early stage at diagnosis, radical surgery, and reduction in postoperative mortality, a large proportion of GC patients are diagnosed at an advanced stage or with tumor metastasis or local invasion, which affects their survival, particularly in less developed countries. Fortunately, the 5-year survival rate of early GC is as high as 90% [3]. Therefore, biomarkers for the early diagnosis and high treatment efficacy of GC are key to improving GC survival. The identification of microRNAs (miRNAs) and their target genes that are associated with the occurrence and development of GC will help characterize the molecular biology mechanisms of GC and contribute to exploring effective treatments.

MiRNAs are endogenous noncoding small single-stranded RNAs composed of approximately 22 nucleotides and play key roles in the regulation of gene expression [4]. MiRNAs incorporated within the RNA-induced silencing complex (RISC) guide target messenger RNA (mRNA) degradation or translational inhibition via base pairing with target mRNAs [5,6]. The dysregulation of miRNA expression is closely related to the development, invasion, migration and treatment response of various tumors [7,8,9]. In GC studies, several miRNA biomarkers with upregulated or downregulated expression involved in signaling pathways have been reported to induce cell proliferation, invasion, metastasis and angiogenesis by binding to target mRNAs and are potential diagnostic and prognostic indicators [10]. However, there are

still many undiscovered miRNAs that can predict overall survival (OS) or molecular markers of immunotherapy response in GC patients. In this study, dysfunctional miRNA microenvironments were identified, and useful biomarkers for miRNA therapy were established by using RNA sequencing (RNA-seq) and miRNA sequencing (miRNA-seq) data of GC from The Cancer Genome Atlas (TCGA).

Materials And Methods

RNA-seq and miRNAs-seq data collection

RNA-seq data and miRNA-seq data were obtained from TCGA, which represents a comprehensive multi-omics approach to studying multiple cancers. Finally, RNA-seq data of samples from 407 (32 normal and 375 tumor) patients and miRNA-seq data of samples from 491 (45 normal and 446 tumor) patients were selected for analysis. The collected clinical data included age, gender, grade, stage, TNM classification, survival status and the number of survival days (Table 1).

Table 1
The characteristics of gastric cancer from
TCGA database

Variable	Number of samples
Age(years)	
≤65/≥65/NA	185/253/5
Gender	
Male/Female	285/158
Grade	
G1/G2/G3/NA	12/159/263/9
Stage	
I/II/III/IV/NA	59/130/183/44/27
T	
T1/T2/T3/T4/NA	23/93/198/118/10
N	
N0/N1/N2/N3/NA	132/119/85/88/19
M	
M0/M1/NA	391/30/22
Survival(days)	42/396/5
≤30/≥30/NA	

Establishment and validation of miRNA-related prognostic model

The mRNA and miRNA expression profiles of GC were normalized by using edgeR (R package). A false discovery rate (FDR) < 0.05 and $|\log_2FC| \geq 1$ were used to define differentially expressed mRNAs and miRNAs between normal stomach samples and GC samples. All miRNAs were assigned to a training group or a testing group by using the caret package. A prognostic model was constructed by using data from the training group, and the risk score formula for this prognostic model was as follows: (expression of miRNA1 $\times$ coefficient of miRNA1) + (expression of miRNA2 $\times$ coefficient of miRNA2) + $\dots$ + (expression of miRNA n $\times$ coefficient of miRNA n). Univariate Cox regression analysis was used to identify miRNAs associated with prognosis that included survival status and survival time (patients who died within 30 postoperative days were excluded). The significant miRNAs detected in the univariate Cox regression analysis and clinical factors were used in the multivariate Cox regression analysis. MiRNA-related survival curves and risk-related survival curves were plotted by the survival package. A time-dependent receiver operating characteristic (ROC) curve was plotted by the survivalROC package to predict the accuracy of the prognostic model for GC. A survival diagram was constructed according to survival time and risk score. Prognostic factors among age, sex, grade, stage, T stage, M stage, N stage, and risk score were identified.

Gene function analysis

Candidate target genes of prognostic miRNAs were obtained from 3 databases: miRDB, TargetScan and miRTarBase, which were plotted by the VennDiagram package. The potential link between miRNAs and target genes was explored by using Cytoscape 3.7.1. Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed by the R package.

Results

Construction of miRNA-related prognostic model

A total of 7531 differentially expressed mRNAs (4395 upregulated and 3136 downregulated) and 267 differentially expressed miRNAs (185 upregulated and 82 downregulated) were selected from the TCGA database. The heatmap of the top 20 upregulated and downregulated genes and the volcano plot of differentially expressed mRNAs are presented in Figure 1a-b. The heatmap of the top 20 upregulated and downregulated miRNAs and the volcano plot of differentially expressed miRNAs are presented in Figure 1c-d. Overall, 15 miRNAs were identified to be significant prognostic factors in the univariate Cox regression analysis (Table S1), and 5 miRNAs were identified to be significant prognostic factors in the multivariate Cox regression analysis (Table S2). Finally, these five differentially expressed miRNAs (hsa-miR-135b-3p, hsa-miR-143-5p, hsa-miR-196b-3p, hsa-miR-942-3p, hsa-miR-9-3p) were selected as independent prognostic factors for GC patients in the training group. The risk score formula for our prognostic model was: risk score = (expression value of hsa-miR-9-3p $\times$ 0.147) + (expression value of hsa-miR-196b-3p $\times$ 0.134) - (expression value of hsa-miR-135b-3p $\times$ 0.148) - (expression value of hsa-

miR-196b-3p $\times$ 0.307) - (expression value of hsa-miR-942-3p $\times$ 0.178). In addition, the risk score of each patient in our study datasets was calculated. The datasets were then assigned to high-risk and low-risk groups by using the median risk score as the cutoff value in the training group, testing group and all patients.

Survival results and examination

As showed in Fig. 2a-e, low expression of hsa-miR-942-3p ($p = 2.478e - 02$), hsa-miR-196b-3p ($p = 9.559e - 03$), and hsa-miR-135b-3p ($p = 8.348e - 03$) and high expression of hsa-miR-143-5p ($p = 2.14e - 02$) and hsa-miR-9-3p ($p = 1.119e - 02$) predicted poor OS. For the training group, testing group, and all patients, poorer survival outcomes were showed in the high-risk group than in the low-risk group ($p = 1.417e - 04$, $p = 2.131e - 02$ and $p = 1.436e - 05$, Fig. 2f-h). An area under the ROC curve (AUC) of 0.719 in the training group, 0.660 in the testing group, and 0.689 in all patients indicated moderate specificity and sensitivity of this prognostic model (Fig. 2i-k). The mortality rate of patients increased as the risk score increased, as shown in the survival state plots (Fig. 2l-n). The 5-miRNA-based signature (HR = 1.971, 95% CI 1.557–2.494, $p < 0.001$), age (HR = 1.027, 95% CI 1.010–1.045, $p = 0.002$), and gender (HR = 1.510, 95% CI 1.02–2.218, $p = 0.036$) were confirmed as independent prognostic factors for OS (Fig. 3).

MiRNA-target gene analysis

The predicted target genes of the five miRNAs from three databases (red represents miRDB, purple represents TargetScan, and green represents miRTarBase) are shown in Venn diagram (Fig. 4a-e). Figure 4f showed that all five miRNAs had predicted target mRNAs (red indicates upregulated and green indicates downregulated). GO enrichment and KEGG pathway analyses were used to evaluate the biological functions of these target mRNAs. The GO enrichment results revealed that the biological processes (BP) were enriched in limbic system development, notch signaling pathway, and response to glucose. The cell components (CC) were mainly enriched in apical part of cell, transporter complex, and plasma membrane protein complex, while the molecular functions (MF) were mainly enriched in RNA polymerase II proximal promoter sequence – specific DNA binding, DNA – binding transcription activator activity, RNA polymerase II – specific, proximal promoter sequence – specific DNA binding, and ion channel activity. KEGG pathway analysis revealed that these target genes were mainly enriched in the cAMP signaling pathway, neuroactive ligand – receptor interaction, MAPK signaling pathway, and TGF – beta signaling pathway (Fig. 5).

Target mRNAs associated with survival outcomes

In the analysis of the impact of target mRNAs on patient survival, eight mRNAs ($p < 0.01$), AKAP12 ($p = 1.29e - 03$), AR ($p = 9.578e - 03$), DZIP1 ($p = 5.605e - 03$), PCDHA11 ($p = 1.625e - 03$), PCDHA12 ($p = 6.907e - 04$), PI15 ($p = 9.468e - 03$), SH3BGRL ($p = 5.428e - 03$), and TMEM108 ($p = 6.22e - 03$), were revealed to be significantly related to patient survival (Fig. 6). Construction of the protein-protein interaction (PPI) network revealed 129 potential critical genes (Fig. 7a), and the top 15 critical genes were identified by using Cytoscape 3.7.1 (Fig. 7b).

Discussion

MiRNAs are located in non-protein-coding regions or in the introns of protein-coding transcripts and are classified into two categories based on their regulatory functions in cancers: tumor suppressor miRNAs and oncogenic miRNAs (oncomiRs) [11]. The downregulation of tumor suppressor miRNAs or upregulation of oncomiRs can promote the development of tumors [12]. In GC studies, a variety of abnormally expressed miRNAs were discovered to contribute to the proliferation, radioresistance, chemoresistance and targeted therapy sensitivity of cancers [13–15]. In addition, miRNAs that are stable in serum and easy to detect represent a noninvasive, convenient screening method and could be used as indicators for early screening of GC and evaluating clinical efficacy [16]. Therefore, understanding the basic molecular mechanisms of miRNA regulation contributes to the development of effective GC treatments. In this study, we established an effective prognostic index based on miRNAs that have a strong prognostic value, and the miRNAs expressed in GC patients identified from TCGA may be potential biomarkers for therapeutic intervention.

In this study, five differentially expressed miRNAs (hsa-miR-135b-3p, hsa-miR-143-5p, hsa-miR-196b-3p, hsa-miR-942-3p, hsa-miR-9-3p) were selected as independent prognostic factors for GC patients in the training datasets. Wu et al. [17] revealed that miR-143 expression was aberrantly downregulated in GC cell lines (AGS). STAT3 was identified as a potential target of miRNA-143 in AGS cells by using TargetScan analysis and the dual luciferase assay, and miRNA-143 overexpression resulted in considerable downregulation of STAT3 expression, which eventually caused the inhibition of cell proliferation, migration and invasion of AGS cells. The same results have also been found in studies of cell proliferation and migration in prostate cancer and cervical cancer [18, 19]. Zhang et al. [20] found that miR-143 expression in advanced nasopharyngeal carcinoma patients was downregulated after concurrent chemoradiotherapy, suggesting that low expression of miR-143 indicated better OS. Zhou et al. [21] discovered that the miR-135b level in the plasma of GC patients was significantly higher than that in healthy individuals, and high expression of miR-135b predicted malignant transformation and poor prognosis of GC. Han et al. [22] observed that the expression of miR-135b could be upregulated by IL-1 signaling in GC cells and organoids, which promoted the invasiveness and stem-cell features of GC cells in culture by reducing FOXN3 and RECK mRNA levels. Bai et al. [23] found that miR-135b promotes the proliferation and migration of GC cells by negatively regulating TGFBR2 expression, indicative of its role as an oncomiR. In hepatocellular carcinoma patients, overexpression of miR-942 was associated with shorter OS [24, 25]. Nevertheless, Du et al. [26] found that the expression of miR-942-5p was markedly reduced in ovarian cancer tissues and cells compared with control tissues and cells. Stenholm et al. [27] discovered that miR-196 was significantly associated with OS in advanced GC. MiR-196 has been found to be upregulated in various cancers and to promote cancer cell proliferation, migration, invasion and chemosensitivity [28–30]. In conclusion, the miRNAs mentioned above may be potential biomarkers for the treatment of GC and need to be verified by clinical studies.

The potential target mRNAs of these miRNAs were predicted by using three free online databases, and the functions of these target mRNAs, including MF, CC and BP, were analyzed. KEGG pathway analysis

revealed that these target genes were mainly enriched in the cAMP signaling pathway, neuroactive ligand – receptor interaction, MAPK signaling pathway, and TGF – beta signaling pathway. cAMP, known as the "second messenger" of hormonal action, is an important factor involved in regulating substance metabolism and biological function in cells, and it delivers messages carried by hormones, neurotransmitters or other extracellular cues into cells and triggers a cascade of biochemical reactions [31]. Zhu et al. [32] discovered that DARPP-32, a phosphoprotein regulated by cAMP, plays a critical role in the activation of STAT3 and contributes to gastric tumorigenesis. Moreover, cAMP signaling activation highly enhances tissue angiogenesis, promoting the development of tumors [33]. The MAPK signaling pathway can promote the proliferation, invasion and migration of GC cells [34]. G3BP1, which is highly expressed in GC, activates the TGF- β /Smad signaling pathway to promote GC cell proliferation, migration and invasion [35]. Suppression of the TGF- β /EMT signaling pathway resulted in the increased invasiveness of gastric tumors [36]. Abnormal methylation may be an early and necessary step in the development of GC, [37] and Kim et al. [38] discovered that gastrointestinal hormone receptor genes in the neuroactive ligand-receptor interaction pathway are mainly hypermethylated in GC. Therefore, these target mRNAs involved in GC signaling pathways could be potential biomarkers for GC gene therapy. Ultimately, eight target mRNAs (AKAP12, AR, DZIP1, PCDHA11, PCDHA12, PI15, SH3BGRL, TMEM108) were significantly correlated with OS.

Conclusions

In summary, we established prognostic miRNA markers and potential therapeutic target mRNAs for GC from the TCGA database. However, our detection of biomarkers in GC is limited and requires further exploration.

Abbreviations

GC: Gastric cancer

TCGA: The cancer genome atlas

ROC: Receiver operating characteristic

AUC: Area under the curve

GO: Gene ontology

KEGG: Kyoto encyclopedia of genes and genomes

PPI: Protein-protein interaction

STRING: Search Tool for the Retrieval of Interacting Genes

BP: Biological process

CC: Cellular component

MF: Molecular function

log2FC: Log2-fold change

FDR: False discovery rate

OS: Overall survival

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: *The datasets analysed during the current study are publicly available in the TCGA* [<https://portal.gdc.cancer.gov/>].

Competing interests: The authors declare that they have no conflict of interest.

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Authors' contributions

WZ, JZ and ZC were responsible for the overall study design. ZC, SD, HX and ZB performed the data analysis. ZC and HX drafted the paper. All authors read and approved the final manuscript.

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Figures

Figure 1

The heatmap of the top 20 upregulated and downregulated genes or miRNAs and the volcano plot of differentially expressed genes or miRNAs. a,b mRNA; c,d miRNAs.

Figure 2

Overall survival of a-e miRNAs; f Training group; g Testing group; h all patients. ROC curves of i Training group; j Testing group; k All patients. Survival status plot in l Training group; m Testing group; n All patients.

Figure 3

Univariate and multivariate Cox analysis to identify the risk factors. a Univariate; b Multivariate.

Figure 4

The number of predicted target genes for five miRNAs from miRDB, TargetScan and miRTarBase databases. f The potential link between miRNAs and target genes was explored by using Cytoscape.

Figure 7

a The protein-protein interaction (PPI) network. b Top 10 critical genes (red and orange squares).

Supplementary Files

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