JBUON 2017; 22(5): 1154-1159
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Analysis of expression profile of miRNA in stomach adenocarcinoma

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Summary

Purpose: *Stomach adenocarcinoma (STAD) has become the second main cause of cancer death around the world, and accumulated evidence has suggested that micro-RNAs (miRNAs) are likely to be served as indicator for diagnosis and prognosis in a variety of tumors. The purpose of this study was to discover the miRNAs acting as independent prognostic factors of STAD and to provide reference for the STAD diagnosis and prognosis.*

Methods: *In this study, we analyzed the clinical data collected from 393 STAD cases of the Cancer Genome Atlas (TCGA;USA) and the expression data of 1881 miRNAs to screen miRNAs that were associated with STAD via Cox regression univariate analysis and those miRNAs with independent effect on the prognosis of STAD via Cox regression multivariate analysis.*

Results: *We found differential expressions of 218 miRNAs in STAD tumors and normal tissues, and a key role of miR-7 in STAD. In Cox regression multivariate analysis, miR-378i (p=0.0010), miR-7-3 (p=0.01), miR-137 (p=0.03) and miR-372 (p=0.04) were independent prognostic factors, for which we performed Kaplan-Meier analysis, and the results showed that there was a statistically significant difference in comparison of the survival duration between the high-expression and low-expression of miR-7-3 groups, but no differences were identified between the groups of miR-378i (p=0.7), miR-137 (p=0.11) and miR-372 (p=0.13).*

Conclusion: *miR-7-3 is proved to be an independent prognostic indicator for STAD and this study confirmed that tumor-specific miRNA can predict the progression and prognosis of STAD.*

Key words: *gastric adenocarcinoma, miRNA, TCGA*

Introduction

STAD has become the second main cause of cancer death around the world, and its incidence in Asian countries including China and Japan is particularly high. Although a decrease in the incidence rate of STAD has been reported recently, there remain about 1,000,000 new cases of STAD and 850,000 STAD-associated deaths [1]. Such a high mortality rate is mainly caused by the delayed diagnosis of STAD in early stages, where no symptoms or no specific symptoms are reported, and, however, the survival rate is dependent on the stage of STAD [2]. Despite the progression in diagnosis and treatment methods of STAD, survival remains low [3]. Currently, patients who are diagnosed with early-stage STAD are about 25%, and the 5-year survival of STAD patients in China is only 20-25%, while this rate in Europe and USA is 26% [4,5].

miRNAs are small conservative non-coding RNAs that can regulate the genetic expression through inhibiting the mRNA translation or inducing degradation of mRNA [6]. miRNAs show a huge potential in serving as biomarkers of cancer for their tissue-specific expression pattern and the aberrant expression in tumor cells [7]. The expression of miRNAs in a tumor can be deregulated through multiple mechanisms, including transcriptional regulation, amplification, deletion,

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Received: 26/05/2017; Accepted: 05/06/2017

mutation and epigenetic gene silencing [8]. miRNAs exhibit a high specificity to different types of tissues, or different types of cells in those tissues, and many studies have detected the patterns of miRNAs in different carcinomas [9]. The biological function of miRNAs remains unclear yet, but analysis of miRNAs has shown that miRNAs have differential expression profiles in a variety of tumors in comparison with the normal tissues [7]. Thus, miRNAs are now been applied in diagnostic tests as biomarkers with high-tissue specificity, and these advancements have laid the foundation for the development of clinical diagnosis.

In this study, we extracted the data from the Cancer Genome Atlas (TCGA,USA), aiming to discover the miRNAs acting as independent prognostic factors of STAD, thereby providing reference for the STAD diagnosis and prognosis.

Methods

Collection of data on miRNAs in TCGA

According to the exclusion criteria, we obtained the sequencing data of STAD miRNA from the web of TCGA and the corresponding personal comprehensive information of 436 patients (Table 1). Exclusion criteria: a) Cases in which the patients were not diagnosed as STAD in the first histological examination; b) Cases with no integral data of tissue samples for analysis; c) Cases that were complicated with another malignant tumor in addition to STAD; d) Cases in which the survival duration of patients was no longer than 30 days. After screening, a total of 393 patients were enrolled in this study, and their data on gender, age, race, survival duration and pathological stages and TNM staging of the American Joint Committee on Cancer (AJCC) totally conformed to the publication guidance provided by TCGA. Since the data used in this study were obtained from the TCGA database, no approval of Ethics Committee was required.

Processing the miRNAs data

Through analysis of miRNA expression data of 393 STAD patients, we detected a total of 1881 miRNAs in their tissue samples, which were further assayed using the Illumina HiSeq platform and the Illumina Genome Analyzer platform. To screen the miRNAs with differentiated expression, the screening criterion was set as log fold change >2 and the relative $p<0.05$. In addition, the miRNAs with upregulated and downregulated expression in the tissue samples of STAD patients were also screened, and the miRNA expression was subjected to the standardized processing.

Statistics

Log 2 transformation of miRNA expression was carried out, and the univariate Cox proportional hazard regression model was used to screen the miRNAs and clinical features that were associated with the survival of STAD patients. Thereafter, multivariate Cox proportional hazard regression model was utilized to screen the independent prognostic factors of STAD. In addition, we also used the Kaplan-Meier analysis for the independent prognostic capacity of miRNA in STAD, in which the expressions of miRNAs were classified into high and low expression with the median of expression as the critical point. Data were processed using the survival package of R software.

miRBase was used to predict the target gene of independent prognostic miRNA of STAD, and after the repeated target genes were screened and excluded, we adopted the Database for Annotation, Visualization and Integrated Discovery (DAVID) to perform Gene Ontolo-

Table 1. Clinical characteristics of stomach adenocarcinoma

Characteristics	*Data (TCGA) n (%)*
Gender	
Male	259 (69.50)
Female	134 (34.09)
Age (years)	
<65	170 (43.25)
≥65	217 (55.21)
NA	6 (1.52)
Pathologic stage	
I	51 (12.97)
II	122 (31.04)
III	168 (47.74)
IV	38 (9.66)
NA	14 (3.56)
Pathologic T stage	
T1	19 (4.83)
T2	86 (21.88)
T3	182 (46.31)
T4	101 (25.69)
NA	5 (1.21)
Pathologic N stage	
N0	116 (29.51)
N1	110 (27.98)
N2	78 (19.84)
N3	79 (20.10)
Nx	10 (2.54)
Pathologic M stage	
M0	353 (89.82)
M1	26 (6.61)
Mx	14 (3.56)
Race	
White	244 (62.08)
Asian	82 (20.86)
Black or African American	12 (3.05)
NA	55 (13.99)
Ethnicity	
Not Hispanic or Latino	282 (71.75)
Hispanic or Latino	5 (1.27)
NA	106 (26.97)

NA: not available

gy (GO) enrichment analysis (p<0.05), in which the data were processed using ggplot 2 package of R software (Figure 2) to figure out 5 functions in closest association with the target genes. Cytoscape and kobas were also used to perform the enrichment analysis on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of target genes to identify 5 pathways in closest association with the target genes.

Results

In this study, we collected the data of 393 STAD patients for analysis, including not only the data of expressions of 1881 miRNAs, but also the patient clinical features (Table 1), such as age, race, survival status, survival duration, gender, pathological types and TNM staging. Besides, we selected miRNAs with upregulated and downregulated expressions in STAD, in which the miRNAs with the most significant upregulated expressions included hsa-miR-21, hsa-miR-196a-1, hsa-miR-196a-2, hsa-miR-135b, hsa-miR-146b, hsa-miR-196b, hsa-miR-501, hsa-miR-183,hsa-miR-18a and hsa-miR-500a (Table 2), while those with the most significant downregulated expressions included hsa-miR-139, hsa-miR-6510, hsa-miR-365a, hsa-miR-365b, hsa-miR-145, hsa-miR-133a-2, hsa-miR-133a-1, hsa-miR-1-1 and hsa-miR-1-2 (Table 2).

Moreover, the univariate Cox proportional hazard regression model (Table 3), showed that pathological features, such as pathologic stage (p=1.10E-06), pathological N stage (p=1.28E-05), pathological T stage (p=0.0004), pathological M stage (p=0.0011242) and age (p=0.04), were all closely correlated with the survival of STAD patients. On the other hand, in 219 miRNAs with

Table 2. The top ten up and down-regulated miRNAs

miRNA	*logFC*	*logCPM*	*p value*	*FDR*
Up				
hsa-miR-21	1.895352725	17.92267236	4.98E-49	3.92E-46
hsa-miR-196a-1	4.57300425	6.474830007	6.57E-33	9.41E-31
hsa-miR-196a-2	4.614126184	6.635284735	3.20E-32	4.20E-30
hsa-miR-135b	3.193577279	5.781172728	1.41E-28	1.48E-26
hsa-miR-146b	1.854802944	9.416321501	4.08E-28	4.01E-26
hsa-miR-196b	4.479127275	8.890973666	8.89E-26	6.66E-24
hsa-miR-501	1.575517396	5.721397732	3.65E-23	1.98E-21
hsa-miR-183	1.955122805	12.03159787	3.92E-18	1.55E-16
hsa-miR-500a	1.255445517	9.059697334	6.62E-18	2.54E-16
hsa-miR-18a	2.027514649	5.754601169	1.15E-17	4.32E-16
Down				
hsa-miR-139	-2.276107095	6.068183569	1.08E-54	1.71E-51
hsa-miR-6510	-4.228159939	1.896768895	1.91E-44	1.00E-41
hsa-miR-365a	-1.508479067	6.058189126	1.04E-43	3.47E-41
hsa-miR-365b	-1.507622785	6.057114965	1.10E-43	3.47E-41
hsa-miR-145	-2.398892054	12.53632707	1.51E-41	3.96E-39
hsa-miR-133a-2	-3.203947706	6.478947181	2.14E-39	4.81E-37
hsa-miR-133a-1	-3.164947895	6.608185169	3.36E-39	6.60E-37
hsa-miR-1-1	-3.010607062	6.107021956	4.46E-35	7.80E-33
hsa-miR-1-2	-3.000724166	6.192474163	7.37E-35	1.16E-32
hsa-miR-5683	-2.932903623	2.533973897	1.23E-31	1.49E-29

FC: fold change, CPM: count per million, FDR: false discovery rate

Table 3. Univariate Cox proportional hazards regression

Factors	*coef*	*exp (coef)*	*se (coef)*	*z*	*Pr (>|z|)*
Pathologic stage	0.498688676	1.646560679	0.102347447	4.872507231	1.10E-06
Pathologic N stage	1.046217687	2.846863002	0.239801331	4.36285187	1.28E-05
Pathologic T stage	3.882414632	48.541283	1.097881661	3.53627788	0.000405808
hsa-miR-328	1.040722892	2.831262973	0.304219832	3.420956769	0.000624013
Pathologic M stage	0.888415132	2.431273349	0.272733721	3.257445132	0.0011242
Age	0.015188749	1.015304685	0.007488907	2.028166334	0.042543275
hsa-miR-125a	2.08548965	8.048531478	0.658097387	3.168968138	0.001529812
hsa-miR-7-3	-0.438918701	0.644733193	0.142350973	-3.083355821	0.002046803

differential expressions, there were 36 miRNAs in close correlation with the survival of STAD patients, in which hsa-miR-328 (p=0.0006), hsa-miR-125a (p=0.001) and hsa-miR-7-3 (p=0.002) were the most closely correlated with the survival of STAD patients (Table 3). The results of multivariate Cox proportional hazard regression model are listed in Table 4 and showed that hsa-miR-378i (p=0.0010), hsa-miR-7-3 (p=0.01), hsa-miR-137 (p=0.038) and hsa-miR-372 (p=0.04) were independent prognostic factors of STAD, for which we further utilized Kaplan-Meier survival analysis (Figures 1,2,3 and 4). The results indicated that there was a statistically significant difference in comparison of the survival duration between the hsa-miR-7-3 high expression and low expression groups, but no statistically significant differences were identified in comparisons between the hsa-miR-378i, hsa-miR-372 and hsa-miR-137 high and low expression groups.

Furthermore, we adopted the miRBase to predict the target genes of those four independent

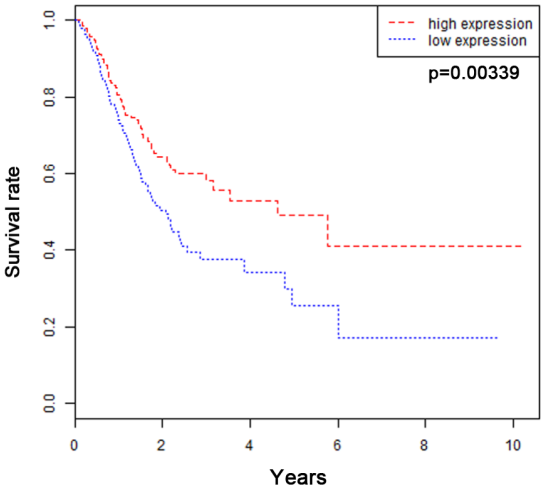

Figure 1. Kaplan-Meier survival curves of hsa-miR-7-3. The expression level of hsa-miR-7-3 was independent prognostic factor of STAD.

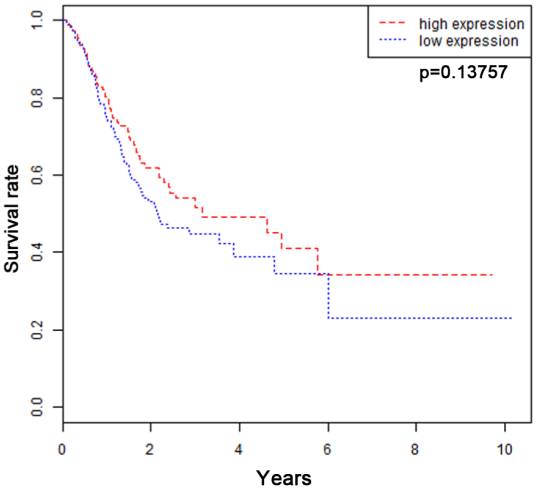

Figure 3. Kaplan-Meier survival curves of hsa-miR-372. The expression level of hsa-miR-372 was independent prognostic factor of STAD.

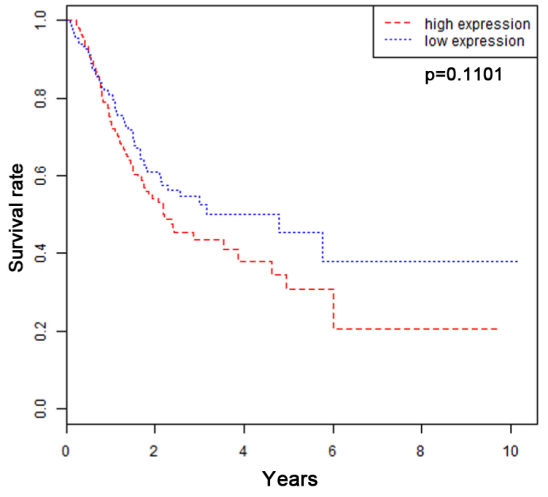

Figure 2. Kaplan-Meier survival curves of hsa-miR-137. The expression level of hsa-miR-137 was independent prognostic factor of STAD.

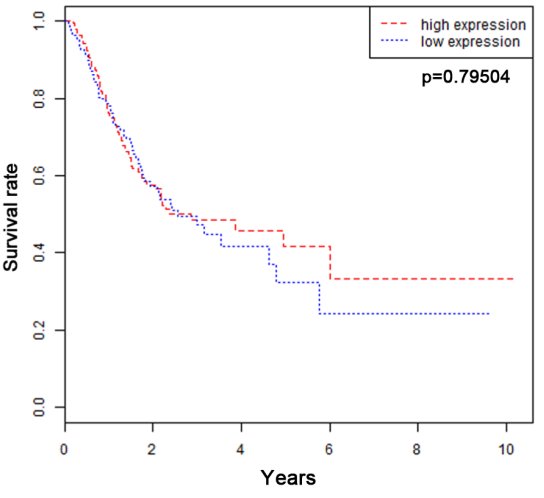

Figure 4. Kaplan-Meier survival curves of hsa-miR-378i. The expression level of hsa-miR-378i was independent prognostic factor of STAD.

Table 4. Multivariate Cox proportional hazards regression

Factors	*coef*	*exp (coef)*	*se (coef)*	*z*	*Pr (>|z|)*
Age	0.038173074	1.038911026	0.009759069	3.911548594	9.17E-05
hsa-miR-378i	-0.951531073	0.386149348	0.36793647	-2.586128725	0.009706067
hsa-miR-7-3	-0.464839511	0.628235922	0.188244879	-2.469334168	0.013536474
Pathologic M stage	0.935616467	2.548784215	0.423173787	2.210950905	0.027039237
hsa-miR-137	0.284291703	1.328820495	0.137177573	2.07243573	0.038224824
hsa-miR-372	-0.357082281	0.699714922	0.174893847	-2.041708659	0.041180439

prognostic miRNAs, and then DAVID was applied to perform the GO enrichment analysis for all target genes (Table 5). The three most significant functions were regulation of gene expression, regulation of macromolecule biosynthetic process and regulation of cellular macromolecule biosynthetic process. And finally, Cytoscape and kobas were used to conduct the KEGG pathway analysis for target genes, in which oxidative phosphorylation was identified as the most correlated pathway (Table 6).

Discussion

In recent years, most STAD patients exhibit obvious symptoms in early-stage disease in clinical practice, but the diagnosis of STAD has always been made in advanced stages, contributing to the poor prognosis and low survival rate [12]. A miRNA is a small molecule that exerts its functions through mediating the expression of target genes, and has become a hotspot in the current tumor research [13-16]. The aim of this study was to identify independent prognostic factors of STAD to provide the basis for diagnosis and evaluation of prognosis of STAD. In this study, we used the data collection of TCGA, in combination with the analytic methods of bioinformatics, and confirmed that miRNA-7 is highly expressed in STAD-affected tissues, and higher expressions of miRNA-7 are found in tissues with advanced TNM stage and poorly differentiated tissues in STAD. Furthermore, we assessed the effect of miRNA-7 on the evaluation of prognosis of STAD patients, and the results showed that patients with high miRNA-7 expression had shorter survival duration. The results of this study suggested that STAD patients with high expression of miRNA-7 usually have poor prognosis.

Surgical resection is the preferable choice in the treatment of STAD, and the possibility for cure is increased for patients who are subjected to radical resection as soon as possible. But STAD is usually discovered at an advanced stage, resulting in low survival rates. In this study, we found that miR-7-3 may be an independent prognostic factor for STAD development. miR-7-3 is the miRNA precursor of miR-7 family, and miR-7 plays an important role in many tumors according to various authors. Liu et al. [17] confirmed that miR-7 can inhibit cell growth and induce cell apoptosis in cervical cancer. Existing evidence indicates that the downregulated expression of miR-7 is correlated with the epithelial-mesenchymal transition and metastasis [18]. Skalsky, Cullen and Kefas [19,20] reported that the expression of miR-7 is downregulated in glioblastoma, significantly inhibits the expression of epithelial growth factor receptor (EGFR) and suppresses independently the protein kinase B pathway through specifically acting on the upstream modulators [19,20]. A study reported that, compared with the normal tissues, the expression of miR-7 is upregulated in renal carcinoma cells, and the downregulation of miR-7 inhibits the proliferation of renal carcinoma cells and induces apoptosis, suggesting that miR-7 is characterized as oncogene in renal carcinoma cells [21]. These studies indicate that expression of the same RNA is differently expressed in various and diverse tumor tissues, and miR-7-3 in this study is identified as independent prognostic factor of STAD, which could serve as relevant reference for clinical treatment and survival prognosis of STAD, thereby providing the basis for therapies in the future.

GO enrichment analysis and KEGG analysis were also performed in this study. The significant results of GO enrichment analysis included

Table 5. Analysis of GO enrichment

GO-ID	*p value*	*Corrected p value*	*Description*
50794	1.05E-30	9.04E-27	regulation of gene expression
50789	4.84E-29	2.09E-25	regulation of macromolecule biosynthetic process
65007	1.28E-27	3.67E-24	regulation of cellular macromolecule biosynthetic
5622	4.06E-23	8.75E-20	regulation of nucleic acid-templated transcription
5488	6.83E-23	1.18E-19	regulation of transcription, DNA-templated

Table 6. Analysis of KEGG enrichment

Term	*Database*	*ID*	*p value*	*Corrected p value*
Oxidative phosphorylation	KEGG PATHWAY	hsa00190	1.68E-06	7.28E-05
Parkinson's disease	KEGG PATHWAY	hsa05012	2.30E-06	7.28E-05
Non-alcoholic fatty liver disease (NAFLD)	KEGG PATHWAY	hsa04932	3.07E-06	7.28E-05
Alzheimer's disease	KEGG PATHWAY	hsa05010	5.10E-06	9.04E-05
Huntington's disease	KEGG PATHWAY	hsa05016	9.81E-06	0.000139316

the regulation of gene expression, regulation of macromolecule biosynthetic process and regulation of cellular macromolecule biosynthetic process, suggesting that these genes in association with GO enrichment analysis may be conducive to the onset of STAD. KEGG analysis showed that oxidative phosphorylation may play an important role through involvement in the onset of STAD. In addition, McCully suggested that the oxidative phosphorylation is associated with the pathogenesis of atherosclerosis [22].

In this study, the data that were extracted from TCGA database (Home - The Cancer Genome Atlas - Cancer Genome) were relatively simple, and we did not carry out any experiments; however, the data could not totally indicate the expressions of miRNAs. Thus, in future studies, analysis and discussion should be carried out in combination with the immunohistochemistry methods. In addition, the severely imbalanced gender ratio, and excessive unknown data of race and ethnicity led to the lack of differences among different races in this study.

In conclusion, we confirmed that miR-7-3 is an independent prognostic factor of STAD, and may play an important role through those pathways that are involved in STAD.

Conflict of interests

The authors declare no confict of interests.

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