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Has-MiR-196a-2 is up-regulated and acts as an independent unfavorable prognostic factor in thyroid carcinoma

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Abstract. – OBJECTIVE: To identify the role of hsa-miR-196a-2 in thyroid cancer by bioinformatics analysis.

MATERIALS AND METHODS: The expression profiles of thyroid cancer was download from TCGA. The dysregulated microRNAs were obtained by edger R package. Then, the prognostic data were analyzed by K-M plot. The difference between different groups was analyzed by the t-test. At last, the biological processes of has-miR-196a-2 were obtained with GSEA.

RESULTS: In this study, we found that has-miR-196a-2 was upregulated in thyroid carcinoma by analyzing the TCGA database, which was inversely proportional to the prognosis of patients with thyroid carcinoma. Univariate and multivariate COX analysis showed that has-miR-196a-2 was an independent prognostic risk factor for thyroid carcinoma. Higher expressions of has-miR-196a-2 were found in patients with older age, advanced tumor stage, lymph node metastasis, and local infiltration through the t-test. We found that has-miR-196a-2 was enriched in adherent junction, focal adhesion, and actin cytoskeleton, which are closely related to the invasion and migration of the function pathway. Moreover, it is mainly enriched in tumor progression pathways, such as the PPAR pathway and WNT pathway.

CONCLUSIONS: Hsa-miR-196a-2 is overexpressed in thyroid tumors and is an independent prognostic risk factor for thyroid carcinoma.

Key Words:
Thyroid carcinoma, Hsa-MiR-196a-2, PPAR, WNT.

Introduction

Thyroid carcinoma is the most common malignancy of the endocrine system, accounting for 2% of systemic malignancy, which ranks the fourth in female malignancies worldwide[1]. In the past 30 years, the incidence of thyroid carcinoma has continued to rise in most parts of the world, except in Africa due to limited diagnosis techniques[2]. In 2012, there was about 298,000 newly diagnosed cases and 40,000 deaths of thyroid carcinoma worldwide. Although 37% of new cases of thyroid carcinoma were in Europe and the United States, the death cases occurred mainly in Asia. In China, new cases and death cases of thyroid carcinoma accounted for 15.6% compared with the 13.8% in the world[3]. In 1993, Lee et al[4] first found that miRNAlin-4 was binding to the 3'UTR of lin-4 mRNA when observing the development of *Caenorhabditis elegans*, thereby inhibiting protein expression of lin-4 and regulating the embryonic development of nematodes. MicroRNA is a class of endogenous noncoding small RNA that regulates gene expression, with the length of 16 to 29 nt (average 22 nt). So far, more than 1,000 microRNAs have been found in the human genome, which regulate about 30% of the genes encoding proteins in humans[5]. MicroRNAs are capable of regulating genes at the post-transcriptional level by completely or incompletely complementary pairing with the target mRNAs, thus leading to the mRNA degradation or translation inhibition[6]. Several studies[7,8] have shown that microRNAs can participate in this dynamic biological process by regulating different cellular functions, such as migration, invasion, differentiation, etc.

Researches have demonstrated that miR-221/222 regulates cell cycle distribution and inhibits cell proliferation of PTC cells by inhibiting the expression of cyclin-dependent kinase inhibitor 1B (P27Kip1)[9]. It has also been found that overexpression of miR-146 family regulated retinoic acid receptor beta (RAR-β) and promoted the proliferation of PTC cells[10]. MiR-145 acts as

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a tumor suppressor gene in the AKT3, it inhibits the proliferation and metastasis of thyroid carcinoma cells by regulating PI3K/AKT signaling pathway[11]. Another study[12] found that abnormally expressed miR-155 distinguishes benign and malignant thyroid nodules. Thus, miR-155 in peripheral blood circulation may serve as a target for the diagnosis of PTC. Therefore, the study of miRNA in the role of thyroid tumors is of significance.

In 2004, Yekta et al[13] reported for the first time that miR-196a was in the HOX family locus. MiR-196a contains two subtypes, miR-196a-1 and miR-196a-2, which are located on chromosome 17 and chromosome 12, respectively. In particular, miR-196a-1 is located between HOXB9 and HOXB13, and miR-196a-2 is located between HOXC10 and HOXC12. Multiple roles of miR-196a have been confirmed in a variety of tumors. For example, miR-196a inhibited proliferation and invasion of hepatocellular carcinoma cells by targeting FOXO1[14]. TGF-b1 promoted breast carcinoma invasion by degenerating miR-196a-3p[15]. The role of miR-196a in esophageal carcinoma[16], epithelial ovarian carcinoma[17], colorectal carcinoma[18], and bladder carcinoma[19] have also been reported. Unfortunately, there are no studies exploring the effect of miR-96 on thyroid carcinoma.

In this investigation, we examined miR-196a-2 expression in thyroid carcinoma tissues by analyzing the carcinoma genome atlas (TCGA). The effect of miR-196a-2 on the prognosis of patients with thyroid carcinoma and its clinical value were also explored.

Materials and Methods

Data Collection

The microRNA expression data of breast carcinoma dataset were downloaded and pretreated from TCGA database (https://carcinomagenome.nih.gov/) using TCGAbiolinks R/Bioconductor package. The relative expression of miR-196a-2 was analyzed. Clinical data of patients with thyroid carcinoma were also downloaded for pathological correlation and prognostic analysis.

Correlation Analysis of Data Set Screening and Clinicopathological Parameters

Specimens of normal thyropathy and thyroid carcinoma expressing has-miR-196a-2, as well as clinicopathological parameters and prognostic data in accordance with the thyroid carcinoma tissues, were obtained by downloading data. Cases with unknown or incomplete clinicopathological parameters and prognostic follow-up data were excluded. Only cases with TCGA data sets containing clinical parameters and survival data were retained. Thyroid carcinoma tissues were divided into high and low expression group based on their has-miR-196a-2 expression.

Gene Enrichment Sets Analysis

The gene enrichment sets analysis (GSEA) 2.2.3 software was used for the analysis. Enrolled patients were divided into the high and the low expression group of has-miR-196a-2 according to the above-mentioned method based on the expression level of has-miR-196a-2. In this study, the c2.cp.kegg.v6.0.symbols.gmt data set was obtained from the Msig-DB database in GSEA website. Then, the enrichment analysis was carried out by the default weighted enrichment method, and the number of random combinations was set at 1000 times.

Statistical Analysis

Statistical analysis was performed with Statistical Product and Service Solutions (SSPS22.0, Armonk, NY, USA) software. The edger function was used to analyze the different miRNAs between normal and thyroid tissues. For the correlation analysis of clinicopathological parameters, χ^2-test and Fisher exact test were used in comparison among groups. Kaplan-Meier and Log-rank test were used for survival analysis. Univariate and multivariate analyses were performed using the COX regression model ($\alpha = 0.05$).

Results

Hsa-miR-196a-2 is Overexpressed in Thyroid Carcinoma

MiRNAs in the TCGA database were screened through the edger function for investigating has-miR-196a-2 expression in the normal and thyroid carcinoma tissues. Our results showed that has-miR-196a-2 expression in the thyroid carcinoma tissues was significantly higher that of the normal thyroid tissues (Figure 1A and 1B).

Hsa-miR-196a-2 is the Risk Factor for the Prognosis of Thyroid Carcinoma

Here, we investigated the relationship between has-miR-196a-2 and the prognosis of thyroid carcinoma. The expression level of miR-196a-2 was

Table I. Univariate and multivariate Cox regression analyses hsa-miR-196a-2 for OS of patients in study cohort.

	Univariate analysis			Multivariate analysis		
Variables	***p*-value**	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**
Age (>60 *vs.* ≤ 60)	0.568	1.114	0.268-1.162	0.667	1.375	0.561-1.945
Gender (Femal *vs.* Male)	0.818	1.154	0.341-3.902	0.612	1.411	0.373-5.336
Stage (I vs. II vs. III vs. IV)	0.046	3.976	2.671-5.029	0.037	3.829	2.238-4.889
Lymphatic metastasis (No *vs.* Yes)	0.037	3.228	2.337-4.430	0.043	5.960	2.204-7.507
Depth of invasion (T1 vs. T2 *vs.* T3 vs. T4)	0.013	2.673	1.841-4.066	0.025	2.854	1.846-4.064
Has-miR-196a-2 (High level *vs.* Low level)	0.006	5.006	3.737-7.745	0.008	5.111	3.724-7.706

found to be negatively proportional to the prognosis of patients with thyroid carcinoma by log-rank test (Figure 1C). Furthermore, factors influencing the survival of patients with thyroid carcinoma were analyzed by univariate and multivariate analysis. In the univariate analysis, we found that T stage, N stage, TNM stage, and has-miR-196a-2 expression could affect the prognosis of thyroid carcinoma ($p<0.05$), while age and sex did not affect the prognosis ($p>0.05$, Table I). The above factors were included in the COX regression model for multivariate analysis. Results showed that N stage, M stage, TNM stage, and has-miR-196a-2 were the independent prognostic factors of prognosis of breast carcinoma patients ($p<0.05$), whilst age and sex were not (Table I). Further analysis showed that the expression level of has-miR-196a-2 was significantly higher in the older age group (Figure 2A), T3 + T4 group (Figure 2B), lymph node metastasis group (Figure 2C), and Stage III/IV stage (Figure 2D) than that of the younger age group, T1 + T2 group, non-lymph node metastasis group and the Stage I/II group.

Expression of has-miR-196a-2 in Thyroid Carcinoma and its Clinicopathological Correlation

We found that the expression level of miR-196a-2 was positively correlated with age, T stage, TNM stage, and N stage of patients with thyroid carcinoma ($p<0.05$) by χ^2-test, but it was not correlated with sex (Table II).

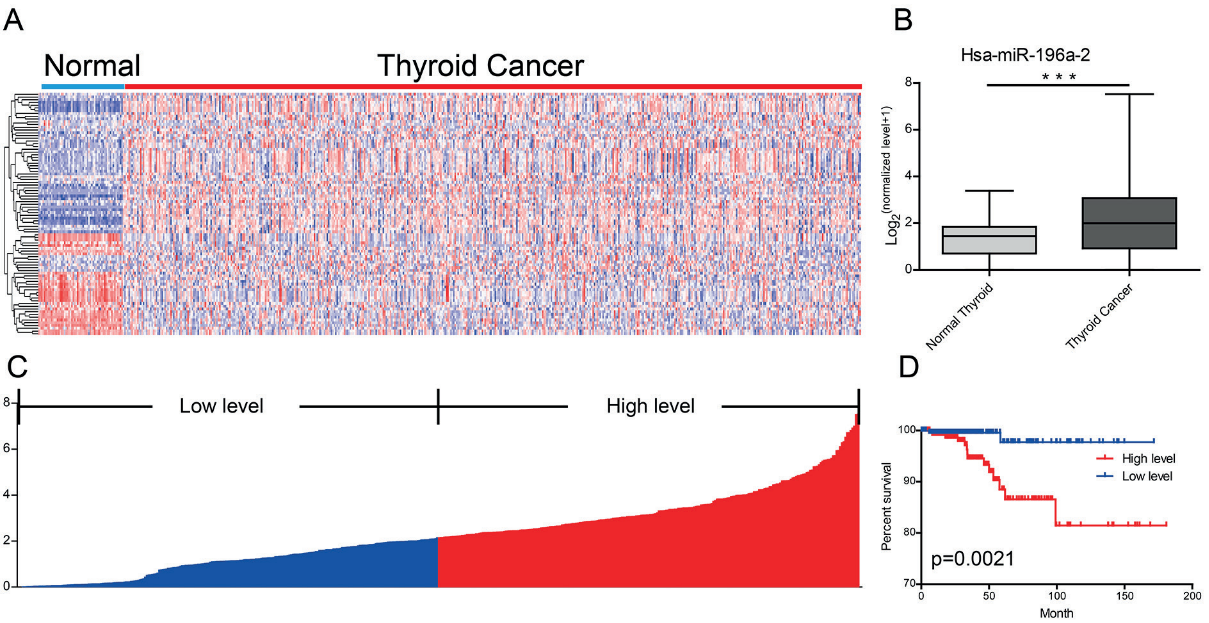

Figure 1. Hsa-miR-192a-2 is up-regulated in thyroid cancer in TCGA. ***A,*** The dysregulated microRNAs in thyroid cancer. ***B,*** Hsa-miR-192a-2 is up-regulated in thyroid cancer in TCGA. ***C,*** The patients were divided into two group according to the media expression level of hsa-miR-196a-2. ***D,*** The expression level of hsa-miR-196a-2 is conversely correlated with patients' outcome.

Table II. Correlation between hsa-miR-196a-2 expression and clinicopathological characteristics of thyroid cancer.

	has-mir-196a-2		
Clinical Parameter	**Low expression**	**High expression**	***p*-value**
Age			
≤ 60	224	190	3.55E-04
> 60	41	75	
Gender			
Female	198	184	0.1753
Male	67	81	
Stage			
I+II	194	157	6.47E-04
III+IV	70	107	
Lymphatic metastasis			
No	140	91	7.60E-06
Yes	100	149	
Depth of invasion			
T1+T2	185	135	8.46E-06
T3+T4	79	129	

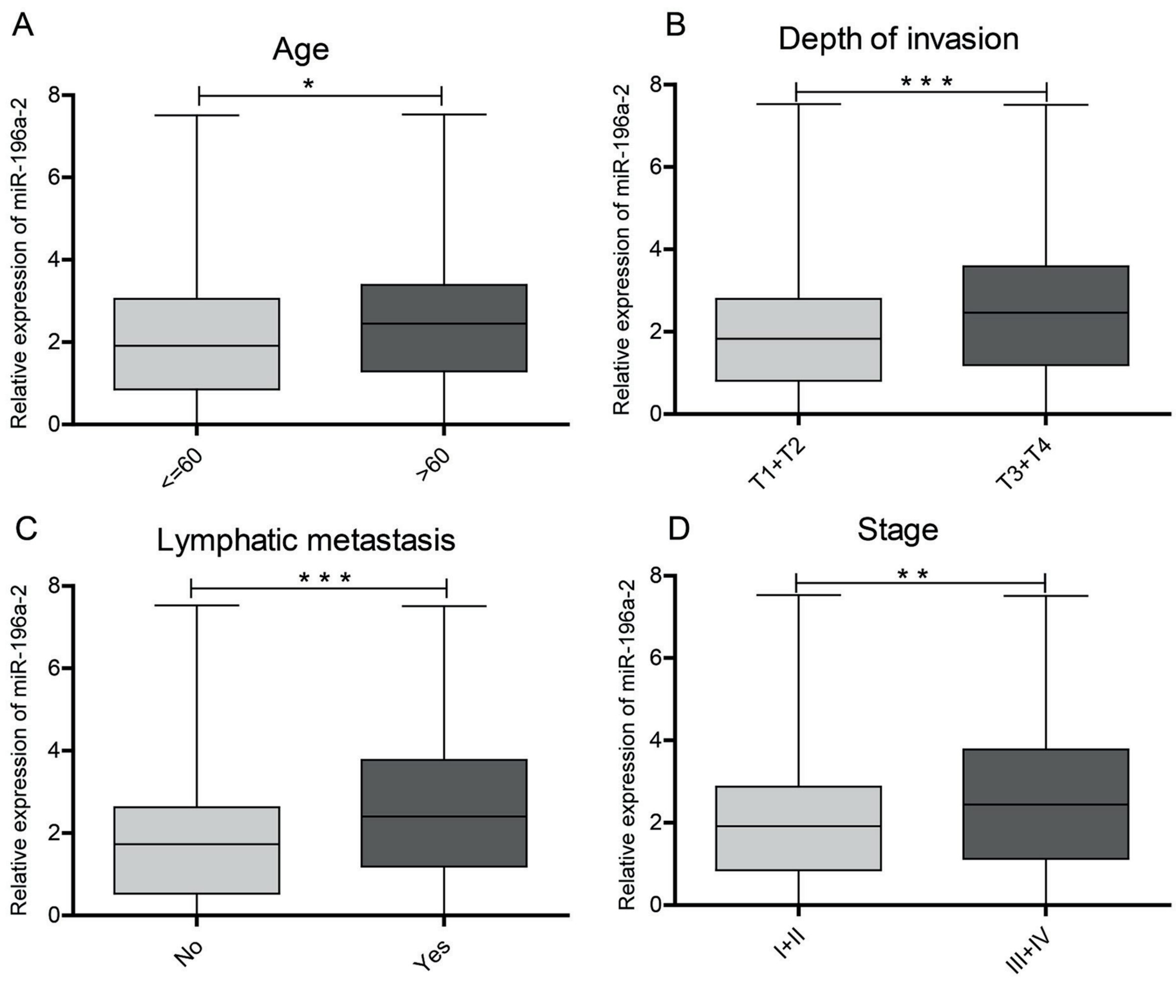

Figure 2. The expression level of hsa-miR-196a-2 in different group. ***A,*** Hsa-miR-196a-2 is up-regulated in patients with ages greater than 60. ***B,*** Hsa-miR-196a-2 is up-regulated in deeper invasive group. ***C,*** Hsa-miR-196a-2 is up-regulated in deeper lymphatic metastasis group. ***D,*** Hsa-miR-196a-2 is up-regulated in advanced stage group.

Functional Gene Sets Enrichment Analysis of miR-196a-2

GSEA analysis suggested that the main function of has-miR-196a-2-2 was enriched in the regulation of adherent junction (Figure 3A), focal adhesion (Figure 3B), and actin cytoskeleton (Figure 3C). Besides, it was mainly enriched in PPAR pathway (Figure 3E) and WNT pathway (Figure 3F).

Discussion

The thyroid malignancy is the most common thyroid carcinoma, a very small number of which may be malignant lymphoma and metastases. In addition to medullary carcinoma, the vast majority of thyroid carcinoma originated in follicular epithelial cells. The incidence of thyroid carcinoma was related to region, ethnicity, and sex. The incidence of thyroid carcinoma in the United States was high. It has been reported that the annual incidence of thyroid carcinoma in the United States increased by about 2.4 times from 1973 to 2002, and it is still increasing year by year[20]. In China, the incidence of thyroid carcinoma was lower which was (0.8-0.9/10 million in men and 2.0-2.2/10 million in women[21)]. Most often the first symptom of thyroid carcinoma is a nodule in the thyroid region of the neck, which is easy to produce oppressive symptoms, such as hoarseness, difficulty in breathing and swallowing, local tenderness and other oppressive symptoms. When the jugular vein is under compression, there may be ipsilateral venous engorgement and facial edema, especially in the goiter with unilateral vocal cord paralysis, which is one of the characteristics of thyroid carcinoma[22].

MiR-196a is proved to be capable of regulating somatic cell differentiation. Qiu et al[23] injected miR-196a precursors into embryos by microinjection techniques in the early differentiation of *Xenopus laevis*, which induced ocular deformity in *Xenopus laevis*. In 2004, Mansfield et al[24]

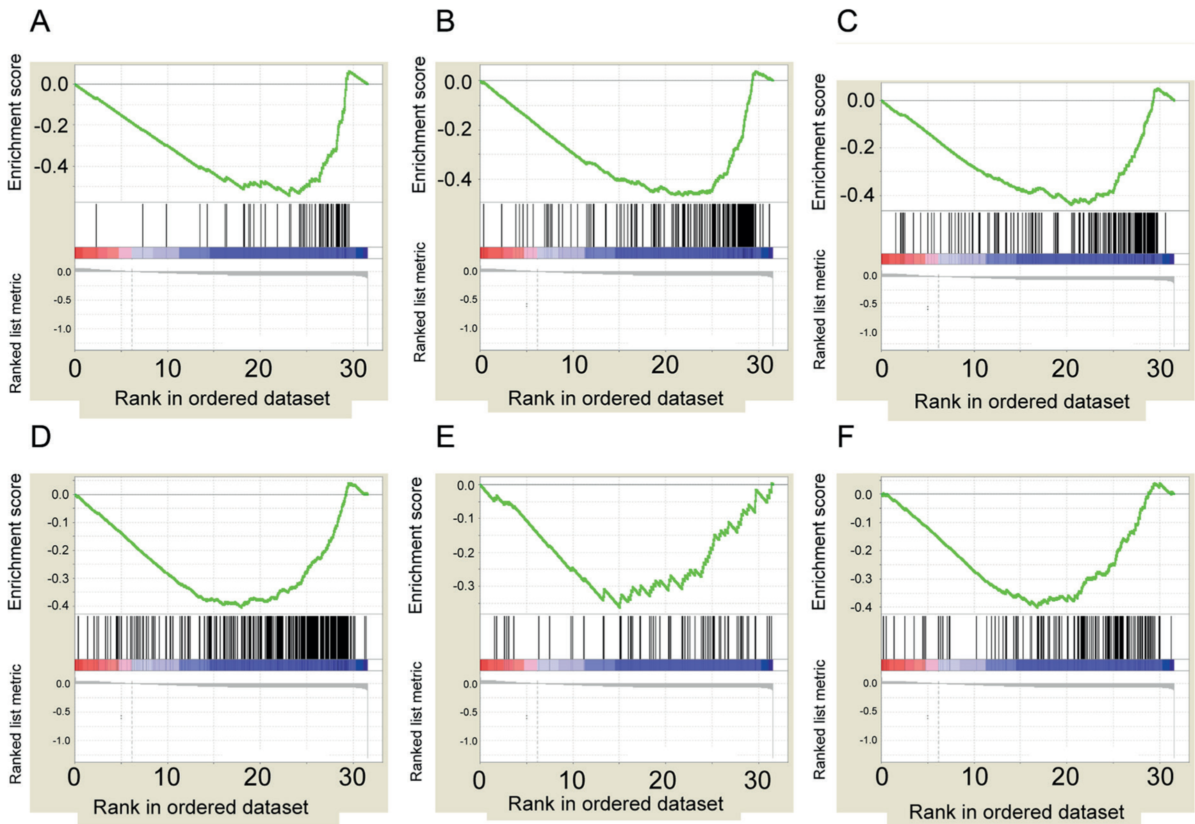

Figure 3. The biological process that hsa-miR-196a-2 in thyroid cancer. ***A,*** Hsa-miR-196a-2 could regulate adherence junction. ***B,*** Hsa-miR-196a-2 could regulate focal adhesion. ***C,*** Hsa-miR-196a-2 could regulate the actin cytoskeleton. ***D, E,*** and ***F,*** Hsa-miR-196a-2 could regulate PPAR pathway (***E***) and WNT pathway (***F,***).

found that miR-196a could participate in mouse embryogenesis by negative regulation of HOXB8 gene. Kim et al[25] found that overexpressing miR-196 inhibited the proliferation of human adipose tissue-derived mesenchymal stem cells (hASCs) and inhibited osteogenic differentiation. Further experiments showed that, after overexpression of miR-196a, both protein and mRNA levels of HOXC8 were decreased. Sun et al[26] detected miR-196a expressions in 36 pairs of gastric cancer tissues and adjacent normal tissues, as well as three gastric cancer cell lines and normal gastric cell lines, by Real-time quantitative PCR (qRT-PCR); the results found that miR-196a-2 was significantly up-regulated in the cancer tissues and cancer cell lines. Moreover, miR-196a-2 expression was positively correlated with tumor size and clinical stage. Kaplan-Meier survival analysis showed that miR-196a-2 expression was negatively correlated with the survival of patients with thyroid carcinoma. Further, *in vitro* experiments confirmed that miR-196a could negatively regulate the target gene p27kip1 thus inhibiting the proliferation of gastric cancer cells. Schianski et al[27] found that miR-196a was significantly overexpressed in colorectal cancer tissues and cell lines, which promoted invasion and migration of colon cancer cells, as well as increased the cell resistance to platinum drugs via AKT signaling pathway. Huang et al[28] found that the miR-196a was up-regulated in the pancreatic cancer cell line PANC-1 using miRNA microarray and qRT-PCR. Luciferase reporter assay confirmed that it bound to mRNA 3'UTR region in NFKBIA. *In vitro* experiments also confirmed that miR-196 could negatively regulate target gene NFKBIA to inhibit the proliferation and migration of PANC-1 and block the cell cycle in G0/G1 phase. MiR-196 decreased expressions of cell-related proteins, Cyclin D1 and CDK4/6. Meanwhile, molecular markers related to epithelial-mesenchymal transition (EMT), including E-cadherin, N-cadherin, and Vimentin, were also differentially expressed. Liu et al[29] demonstrated that miR-196a inhibited apoptosis, promoted proliferation, invasion, and migration of PANC-1 cells by negative regulation of growth inhibitory factor 5 (ING5). In addition, miR-196a played an important role in promoting cancer in breast cancer[30], lung cancer[31], esophageal cancer[32], and laryngeal cancer[33], but its role in thyroid cancer has not reported.

In this study, we found that has-miR-196a-2 was overexpressed in thyroid carcinoma, which was inversely proportional to the prognosis of patients through analyzing the TCGA database. The higher the has-miR-196a-2 expression, the worse the prognosis of thyroid carcinoma. Further, we found that has-miR-196a-2 was an independent prognostic risk factor for thyroid carcinoma by single factor and multivariate COX analysis. Higher expressions of has-miR-196a-2 were observed in patients with older age, advanced tumor patients, lymph node metastasis, and local infiltration. By correlation analysis, a significant increase of has-miR-196a-2 expression was found in patients with older age, advanced tumors, lymph node metastases, and locally deep infiltrates. Also, we found that has-miR-196a-2 was enriched in adherent junction[34], focal adhesion[35], and actin cytoskeleton[36], which was closely related to invasion and migration pathways. It was also mainly enriched in PPAR[37] and WNT[38] pathway, which were closely related to tumor progression.

Taken together, the clinical significance of has-miR-196a-2 in thyroid tumors and its possible influence on influencing cytological functions were investigated by analyzing the TCGA database, which provides the basis for the treatment of thyroid tumors.

Conclusions

We observed that Hsa-miR-196a-2 was overexpressed in thyroid tumors and is an independent prognostic risk factor for thyroid carcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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