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ORIGINAL RESEARCH

Expression Level of miR-34a in Tumor Tissue from Patients with Esophageal Squamous Cell Carcinoma

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Abstract

Background miR-34a has been shown to be involved in P53 regulation. In this study, we aimed to evaluate the expression level of miR-34a in esophageal cancer and compare it with that of the normal marginal tissues.

Methods Tumor and marginal tissues were obtained from 50 patients with esophageal cancer. After RNA extraction, expression level of miR-34a was determined using SYBR green master mix and real-time quantitative PCR.

Results It was observed that there was a downregulation of miR-34a in tumoral tissue of esophageal patients in comparison to normal marginal tissues. Moreover, the expression level of miR-34a was correlated with clinicopathological specifications of the patients.

Conclusions miR-34a may be involved in the pathogenesis and development of esophageal cancer and have the potential to be used as a diagnostic and therapeutic marker in esophageal cancer.

Keywords Esophageal cancer · miR-34a · Gene expression

Introduction

Esophageal cancer is one of the most common cancers and is the sixth cause of cancer-related deaths worldwide [1]. Two common types of esophageal cancer are esophageal squamous cell carcinoma (ESCC) and adenocarcinoma that are pathologically and ethnically different from each other. The ECSS form is more prevalent than adenocarcinoma. Like other cancers, early diagnosis of ESCC could reduce mortality rate, and identification of biological markers like DNA alterations and gene expression levels is a potential tool to reach this goal [2–6]. Although, mutations of P53 are the most frequent mutation in ESCC, some oncogenes like cyclin D and KRAS are also prevalently mutated in ESCC [7–11].

MicroRNAs (miRNAs) are a class of non-coding RNAs that are about 22 nucleotides in length and do their functions in cells by targeting other mRNAs and, therefore, controlling their expression levels [12–16]. miR-34a belongs to the miR-34 cluster and is involved in regulation of P53 pathway; other members of this cluster are miR-34b and miR-34c [17–19]. Studies indicate that miR-34a targets the mRNA of genes such as E2F3, c-MYC, Bcl2, and MET, which play important roles in controlling the cell cycle [20]. Recently, studies have demonstrated a downregulation of mir-34a in ESCC and its correlation with some clinical features of patients [21].

In this study, it is aimed for the first time, to the best of our knowledge, to investigate the mir34a expression level in ESCC and evaluate the correlation of miR-34a expression level with the clinical outcomes of the patients with Iranian Azari ethnicity.

Patients and Methods

Study Subjects

Fifty esophageal cancer and their marginal tumor-free tissues were collected from Iranian patients of the Azari race, northwest of Iran. All samples were obtained from patients referred to Imam Reza Hospital of Tabriz University of Medical Sciences during 2011–2015. In this study, we used marginal tissue as a control group and

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patients were confirmed histologically as esophageal squamous cell carcinoma. Through sample gathering, we eliminate patients who had undergone chemotherapy and radiation therapy. All samples were collected through surgery and then transferred into RNase inhibitor solution (Qiagen, Cat No./ID: 76104) and stored at − 80 °C until RNA extraction. Clinicopathological manifestations of the patients are summarized in Table 1. The local Ethical Committee of Tabriz University of Medical Sciences approved the study protocol and written informed consent was endorsed through all subjects.

RNA Isolation

In order to extract total RNA from tumoral and marginal tissues, Tripure isolation reagent (Roche, Cat No. 11667165001) was applied according to the manufacturer's protocol. Yield and purity of RNA were determined using a NanoDrop spectrophotometer at 260/280 nm (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA). Moreover, for quality, we examined the samples by gel electrophoresis on 1% agarose. Afterwards, RNA samples were stored at – 80 °C until performing the cDNA synthesis.

cDNA Syntheses and Real-time PCR

In this study, two-step real-time PCR was used in order to quantitatively measure the miR-34a expression level. At the first step, the Universal cDNA Synthesis Kit (Exiqon Cat No. 40023301) was used. After that, quantitative real-time PCR was conducted by Exlent SYBR Green Master Mix (Exiqon, Cat No. 400203421) and miR-34a specific primer set (Exiqon, Cat No. 400204481). For normalizing the expression level of the target gene, the expression level of U6, as the housekeeping gene, was used. At the end, the average of the duplicated C_t values was calculated and relative expression level of miR-34a was determined by the comparative C_t method, as explained by Pfaffl [22].

Table 1 Clinicopathological properties of patients with esophageal cancer

Characteristic	Classification	Value
Age	> 55	19
	< 55	31
Sex	Female	22
	Male	28
Lymph node metastases	Yes	17
	No	33
Differentiation	Good	19
	Moderate	22
	Poor	9
Tumor site	Up site	16
	Mid site	23
	Low site	11
Tumor grade	t1–2	31
	t3–4a	19

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 6 (GraphPad Software Inc. San Diego, CA, USA). Kolmogorov-Smirnov's normality test was applied for evaluating normality of data. Two-sample *t* test was conducted to compare target gene expression level between CRC tissues and their paired marginal tissues. Pearson's correlation test was performed for evaluating the correlation between expression of target genes and patient's clinical parameters. All results were expressed as mean ± standard deviation (SD). Statistical significance level for all *P* value was less than 0.05.

Result

It was observed that there was a significant downregulation of miR-34a in tumor tissues of ESCC patients in compression with marginal matched normal tissues ($P < 0.0001$, Fig. 1). Lymph node metastases and differentiation were two pathological features which had significant relations with miR-34a expression level ($P < 0.05$). Furthermore, in cases without lymph node metastases, miR-34a expression level was significantly higher than cases with metastasis. Alternately, we detected high level of miR-34a expression level in cells with good differentiation in compression to poorly differentiated cells. However, age, sex, tumor site, and tumor grade of the esophageal cancer patients were not significantly related to mir-34a expression level (Table 2).

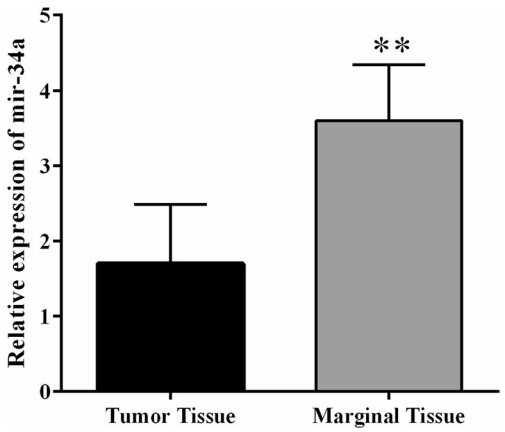

Fig. 1 Expression level of mir-34a in esophageal cancer tissue in comparison to marginal tissue (**$P < 0.0001$)

Table 2 Correlation between clinicopathological characteristics of patients with transcript level of miR-34a

Characteristic	*P* value
Age	0.1248
Sex	0.6585
Lymph node metastases	0.0115
Differentiation	0.0041
Tumor site	0.0987
Tumor grade	0.1254

Discussion

miRNAs are involved in most of the cell activities like proliferation, migration, and apoptosis and, therefore, can be a tumor suppressor gene or oncogene. In addition, it has been shown that abnormal expression of miRNAs is related to cancer progression and development. Furthermore, recent data approved that miRNAs have the potential to be applied as a diagnostic biomarker or therapeutic tool in cancers, especially in ESCC [23, 24]. The miR-34a family consists of three members including 34a, 34b, and 34c, which play roles in P53 pathway. Expression of these miRNAs is triggered by P53 itself as a result of DNA damage [20]. miR-34a as one of the important actors in this pathway, along with other microRNAs, regulates its target proteins' expression level after transcription by targeting their mRNAs. Bcl2 and HMGA2 are involved in apoptosis and cell proliferation that are most known targets of mir-34a; hence, this miRNA participates in such cell process. Recent studies have shown downregulation of miR-34a in cancer cells and its induced mimicry expression in vitro demonstrated it as a tumor suppressor in various type of cancer [12, 25]. Previous studies approved that altered expression level of multiple genes are involved in esophageal carcinogenesis and development [26–30]. On the other hand, dysregulation of miR-34a occurs in various types of human cancers and, in some cases, is related to clinical features of the patients [12, 31, 32].

In vitro investigations revealed miR-34a overexpression in ESCC cell lines, which positively correlated with increased apoptosis rate and decreased clonogenic formation. Furthermore, miR-34a upregulation inhibited invasion and migration of ESCC cell lines through suppressing the transcription of matrix metalloprotease (MMP)-2 and MMP-9. In ESCC cell lines, Yin Yang-1 (YY1) was a direct target of miR-34a. By activating YY1 expression, miR-34a exerted its suppressive effects on invasion and migration [33]. A significant downregulation of miR-34a in tumoral tissues of ESCC patients [21]. Moreover, correlation of expression level of miR-34a and clinical features of patients with ESCC has been observed [21]. In the present study, we found significant downregulation of miR-34a in tumoral tissues of ESCC patients compared with marginal tissues. Additionally, lymph node status, TNM stage, and differentiation level were the three features which had correlation with miR-34a expression level in Iranian Azari ESCC population.

In spite of vast survey of miRNAs in ESCC, validation of them as potential biomarkers of diagnosis, prognosis, or response to treatment needs more investigations, regarding the different tumor phenotypes and the mechanisms of genetic varieties are different. Additionally, such studies are beneficial considering the analysis of different populations, since the geoepidemiological factors influence these regulatory mechanisms, namely epigenetic regulatory mechanisms. These epigenetic mechanisms relate the environmental factors to the final fate of gene transcription and protein translation.

Conclusion

Our results reveal that there is significant decrease in expression of miR-34a in ESCC tumor tissues in our population, which propose its potential as biomarker for ESCC. Hopefully, further research into miRNAs will provide new strategies for gene-based therapy and miRNA-drug development for esophageal cancer. This needs further investigations considering other members of this cluster in esophageal cancer and other cancers.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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