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Evaluation of expression levels of microRNA processing elements in patients with sudden sensorineural hearing loss

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Abstract

Background MicroRNAs have a significant role in the function and development of the hearing system. Idiopathic sudden sensorineural hearing loss (SSNHL) is a complicated disorder with no long-established reason. Since microRNAs play imperative roles in every aspect of the neural system, their dysregulation may contribute to the onset of SSNHL. The current study aimed to assess the expression patterns of microRNA processing elements (DROSHA, DICER, and DGCR-8) as the vital factors in microRNA biology that can affect the expression levels of microRNA. This study assessed DROSHA, DICER, and DGCR-8 mRNA expression levels in the peripheral blood mononuclear cells (PBMC) of 50 patients with SSNHL and 50 matched controls. After the isolation of PBMC, total RNA was extracted, and the expression levels of DROSHA, DICER, and DGCR-8 genes were evaluated using quantitative real-time PCR.

Results The results illustrated significant up-regulation of DICER and DGCR-8 genes in SSNHL patients at the mRNA level. Furthermore, despite no significant change in DROSHA level, DICER and DGCR-8 were significantly correlated with SSNHL. However, there was no significant correlation between these gene expressions and the clinicopathological features of patients.

Conclusion This study verified for the first time that the DGCR_8 and DICER mRNA expression levels were significantly up-regulated in patients with SSNHL, proposing that microRNAs and their processing pathways play key roles in the progression and development of SSNHL.

Keywords MicroRNAs, DiGeorge syndrome critical region gene 8, Sudden hearing loss, DROSHA, Ribonuclease III, Dicer 1 ribonuclease III

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Background

Sudden sensorineural hearing loss (SSNHL), commonly known as sudden deafness, is almost always unilateral and usually affects adults aged 30–60 [1, 2]. Clinically, SSNHL is defined as losing more than 30 dB of sensorineural hearing through three constant test frequencies in three days [3]. The prevalence of SSNHL is between 5 and 20 cases per 100,000 individuals per year [4, 5]. SSNHL is usually described as an isolated event caused by viral infections, cochlear membrane ruptures, autoimmune diseases, and vascular impairment. Nevertheless, none of these elements can be definitively identified as the

primary cause of this condition [6, 7]. Furthermore, early detection and treatment significantly affect the prognosis of SSNHL. Due to obstacles in the determination of control groups and factors affecting patient prognosis, it is difficult to carry out clinical trial examinations for drugs that are subjected to SSNHL treatment. Therefore, there is a need to identify novel diagnostic and prognostic targets to determine SSNHL patients' prognosis and follow the effects of therapeutic agents (https://doi.org/10.3390/ijms21197248).

MicroRNAs (miRNAs) are short (~20–24 nucleotides in length) non-coding RNA that play an important regulatory role in regulating multiple signaling pathways in unicellular and multicellular eukaryotic organisms. MiRNAs post-transcriptionally regulate gene expression, participating in a wide array of biological processes through gene silencing [8]. Generally, miRNAs target genes via interacting with specific sequences located at the 3′ untranslated region (3′ UTR) of mRNAs, leading to mRNA degradation or translational repression. Nonetheless, miRNAs can interact with other regions in mRNA sequence in addition to 3′ UTR, such as 5′ UTR and coding sequence [9]. MicroRNA expression levels are regulated in two main stages. The first step is to adjust the transcriptional level of the DNA, which is done in the nucleus with the help of an enzyme polymerase II (the transcribed RNA is known as primary miRNA (pri-miRNA). The second step is the maturation of microRNAs (transition from PR-miRNAs to mature RNAs) (Friedlander, Lizano, et al.). Subsequently, RNAse III, Drosha, and DGCR8 (DiGeorge syndrome critical region gene 8) process pri-miRNA in the cell nucleus to transform it into pre-miRNA. Then, with the involvement of exportin-5, the synthesized pre-miRNA is transported to the cytoplasm, whereby another endonuclease III called Dicer processes the pre-miRNA into mature and functional miRNA [8–10]. Consequently, Dicer, Drosha, and DGCR8 play basic roles in regulating the processing and expression of miRNAs [10–15].

One of the factors that can significantly affect the maturation of miRNA is the deregulation of miRNA biogenesis-related elements. Dysregulated expression of these components occurs in various human conditions, including neurological diseases, malignancies, and immunologic disorders [16–18]. Subsequently, it has been theorized that the biogenesis pathway of miRNAs may participate in the development of SSNHL.

Therefore, in the current study, the changes in mRNA expression levels of the miRNA processing machinery system elements, including Drosha, Dicer, and DGCR8, were investigated in SSNHL patients to find out their diagnostic potential as biomarkers as well as their correlation with patients' clinical features.

Methods

Preparation of patient samples

This study was approved by the ethics committee at Tabriz University of Medical Sciences, Tabriz, Iran (Ethical Code: IR.TBZMED.REC.1398.808). Fifty patients diagnosed with SSNHL between August 2017 and June 2018 at Tabriz Medical Center by well-trained, specialized doctors were subjected to this study. The patients included in the study were recognized with the following symptoms: sensorineural hearing loss of less than 30 dB in more than three successive frequencies by pure tone audiogram with onset within 72 h and no sign of involvement of cranial nerves other than the eighth cranial nerve. The clinical features of SSHNL patients were recorded and are represented in Table 1. Fifty healthy volunteers with no clinical signs OR familial history of SSNHL or any other hearing disorders were gathered as the control group. The controls were checked by a pure-tone audiogram. The studies were approved by Tabriz University of Medical Sciences and the faculty of medicine following established guidelines.

Blood samples were obtained from all cases using sterile venipuncture into tubes filled with EDTA anticoagulant (0.5 M). Blood samples of SSHNL patients were taken in the acute phase of hearing loss. Using the Ficoll-Paque process (GE Healthcare Life Sciences), peripheral blood mononuclear cells (PBMC) were collected. Subsequently, the cream-colored inter-layer was washed using the Gibco Hanks Balanced Salt Solution (HBSS) to discard residual platelets, plasma, or other contaminants. PBMCs were pelleted at 2000 rpm at 10 °C for 10 min and stored at − 80 °C until mRNA was extracted [35].

Table 1 Demographic information of the patients

Variables	Groups		*P* value
	Case	Control	
Gender			
Male, *N* (%)	27 (54%)	27 (54%)	0.06
Female, *N* (%)	23 (46%)	23 (46%)	0.07
Age (years), mean (SD)	55.48 (2.3)	56.80 (2.6)	0.051
SSNHL laterality			
Unilateral SSNHL			
Right ear	24	–	–
Left ear	26	–	–
Bilateral SSNHL	0	–	–
Degree of hearing loss			
Mild	14	–	–
Moderate	23	–	–
Severe	8	–	–
Profound	6	–	–

RNA extraction and qRT-PCR

Quantitative real-time PCR (qRT-PCR) was employed to evaluate gene expression at mRNA levels in tissue samples. Then, using TRIzol reagent (Life Technologies), total RNA was isolated and subsequently incubated with DNase (AM1906 Ambion, Life Technologies) to remove any DNA contamination. The RNA quality and concentration were examined using optical density (OD) at 260 and 280 by a NanoDrop™ 2000c spectrophotometer (Thermo Scientific), and the samples with an OD260/OD280 ratio above 1.96 were subjected to RT-PCR. Then, 1000 ng of total RNA was converted to cDNA using the Takara cDNA Reverse Transcription Kit (PrimeScript cDNA synthesis kits, Takara, Japan) in a Bio-Rad T100 thermal cycler (Germany). Using the specifically designed primer pairs presented in Table 2 and TAKARA SYBR GREEN Premix (SYBR® Premix Ex Taq™, Japan), the expression levels of interested genes were evaluated in the Roche Light Cycler 480 real-time PCR instrument (Germany). The amplification conditions were set as follows: 95 °C for 3 min, followed by 45 cycles of 10 s denaturation at 95 °C, 30 s annealing at 59 °C, and 20 s extension at 72 °C for 20 s. GAPDH was used as an internal control to normalize target gene expression. The total volume was set at 20 µl, and each reaction was done in triplicate. The comparative 2-ΔΔCT method (Livak) was used to perform a relative quantification analysis between the groups.

Statistical analysis

GraphPad Prism (version 6.0) was employed to perform all statistical analyses. Examination of all mRNA information on interest gene CT rates was performed and standardized to the geometric mean of GAPDH. All data are shown as the mean ± standard error (SE). *P* values less than 0.05 were considered statistically significant. We used the Kolmogorov–Smirnov test for normality analysis and the unpaired *t*-test and *F* test for comparison between groups.

Results

Clinical features of SSNHL patients

A total of 50 patients diagnosed with SSNHL in the ENT and Audiology Department of the Imam Reza Hospital, Tabriz University of Medical Sciences, Tabriz, Iran, between August 2017 and June 2018 were included in the current study. In our study, 23 (46%) patients were females and 27 (54%) were males. All patients ($n=50$; 100%) were unilateral cases, including 26 (62%) right-side unilateral cases and 24 (48%) left-side unilateral cases. The range of patients' ages was between 23 and 69 years, with a median of 54.23 years (Table 1).

Drosha, Dicer, and DGCR8 expression levels in SSNHL patients

To investigate the effect of dysregulation in miRNA processing machinery system elements, including Drosha, Dicer, and DGCR8 during SSNHL development, their expression levels were quantified in 50 SSNHL patients compared to 50 healthy cases. Prior to statistical analysis, raw qPCR data for the mRNA expression of target genes were normalized to the internal control of GAPDH as the reference gene. According to our results, Dicer and DGCR-8 expression levels were considerably higher in the blood samples of SSNHL patients ($P=0.02$) compared to normal samples (Table 3). However, the upregulation of Drosha in patients with SSNLH was not significant (Fig. 1). There is no significant relationship between target gene expression and the clinical features of our patients.

Drosha, Dicer, and DGCR8 as potent biomarkers for SSNHL

Our results indicate that the expression levels of DGCR-8 and Dicer, according to their AUC values equaling 0.67

Table 3 Target gene expression in SSNHL patients

Gene	Fold change	*P* value
DGCR-8	1.53	0.0089
DICERDICERDICERDICER	1.43	0.0037
DROSHA	1.03	0.1831

Table 2 Primer sequences used in quantification real-time PCR

Primer name	Left primer	Right primer
mRNA expression primers		
DROSHA	CATGTCACAGAATGTCGTTCCA	GGGTGAAGCAGCCTCAGATTT
DICER	TTAACCTTTTGGTGTTTGATGAGTGT	GGACATGATGGACAA TTTTCACA
DGCR-8	GCAAGATGCACCCACAAAGA	TTGAGGACACGCTGCATGTAC
GAPDH	CGAGATCCCTCCAAAATCAA	TTCACACCCATGACGAACAT

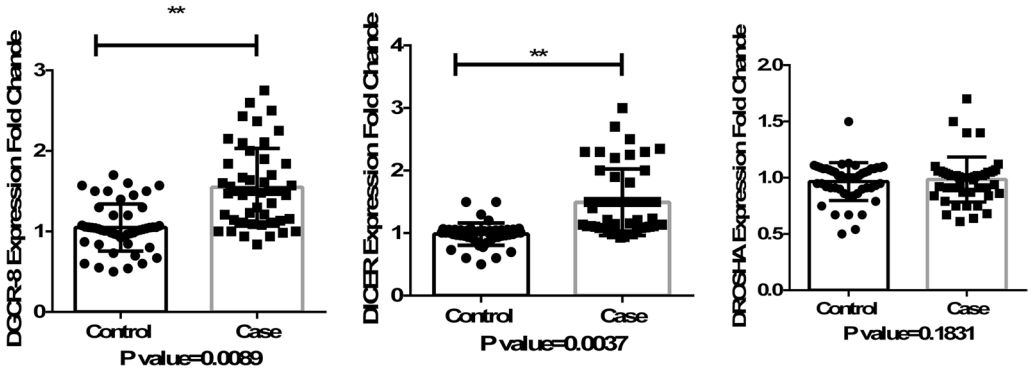

Fig. 1 Expression of Dicer, DROSHA, DGCR-8

($P=0.039$) and 0.73 ($P=0.001$), respectively, could be potential biomarkers for SSNHL (Fig. 2).

Discussion

SSNHL has been reported to be correlated with several clinical conditions, and its etiopathogenesis remains uncertain [19]. The doubt of sensible etiologies has stimulated continuous research to find the most considerable pathological description. Furthermore, it has encouraged renewed interest in the inclining genetic pathways that might play a substantial role in initiating or continuing SSNLH. The lack of a clear etiology in most cases of SSNHL has motivated research into determining an underlying etiology [20–22].

Furthermore, prior studies have reported the relevance of the deregulation of various miRNAs and their processing components in a vast category of diseases [23, 24].

Accordingly, Drosha, Dicer, and DGCR8 exhibited different expression patterns through the development of various types of disease. Also, the abnormal expression patterns of these genes are linked with dysregulated expression of miRNAs, which in turn lead to the initiation and advancement of neural-related diseases [25–27].

However, there is no obvious evidence to indicate that, besides the involvement of these genes in miRNA processing, their deregulated expression directly affects miRNAs as well [28].

It has been shown that several predictive factors are meaningfully related to the clinical features of SSNHL. Some of the best-recognized SSNHL-associated prognostic factors include audiogram patterns, initial hearing loss level, presence or absence of vertigo, age, and tinnitus [29, 30]. Additionally, for a better understanding of SSNHL, it is necessary to investigate molecular pathways to find out the reasons for the occurrence of SSNHL. In this study, we evaluated the expression levels of genes participating in miRNA biogenesis and their correlation with the clinical features of SSNHL patients. Kim et al. demonstrated that the expression level of Dicer and Drosha at the mRNA level was not correlated with the clinical parameters [31]. Also, our study showed no significant correlation between the expression of these genes and the clinicopathological features of patients, which is consistent with their findings.

Dicer is one of the RNA-induced Silencing Complex [RISC] components that catalyze the formation of mature miRNAs from their precursors in the miRNA

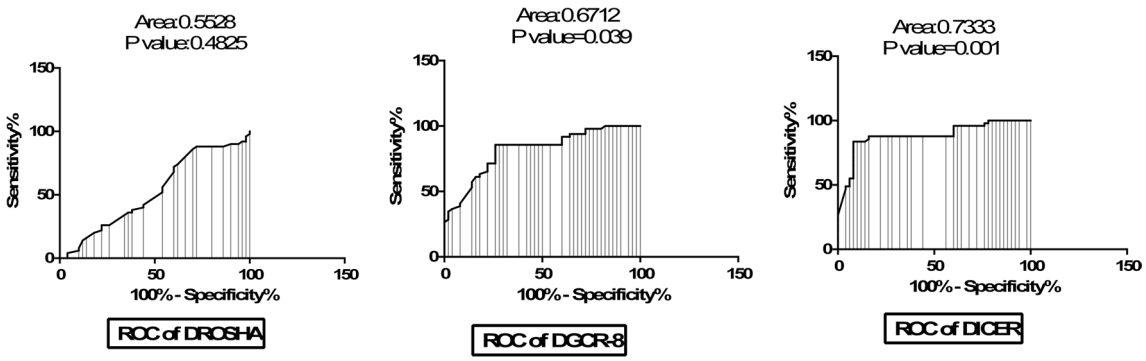

Fig. 2 ROC curve analysis of Dicer, DROSHA, DGCR-8

biogenesis process [32]. Previous findings indicated that the miRNA machinery components dysregulations (Dicer/Drosha) are involved in several human disorders. The expression levels of Dicer and Drosha at the mRNA level were reduced in endometrial and ovarian cancers [33, 34].

Passon et al. demonstrated that the expression levels of DGCR8 and AGO2 at the mRNA level were positively associated with each other in SSNHL patients and controls, proposing that Dicer and Drosha genes share common mechanisms that led to their deregulation in SSNHL [35]. Kim et al. showed that the expression level of Dicer is decreased in SSHNL patients, but the expression of Drosha was not changed in the patients which indicated that miR processing at the nuclear level was not affected [31]. Our study did not show any significant changes in Dicer expression in SSHNL patients, which differs from Kim et al.'s findings which may related to difference in race, ethnicity, and geography of two studies that affect the gene expression.

Jafari et al. showed that Drosha, DGCR8, and Dicer expression was up-regulated in multiple sclerosis (MS) compared with healthy controls, which indicated the essential role of Drosha, DGCR8, and Dicer dysregulation as major components of the miRNA machinery system in the pathogenesis of MS [36].

Also, our study showed that the expression levels of Dicer and DGCR8 were significantly up-regulated in the PBMC of SSHL patients, which is in line with Jafari et al.'s finding. Therefore, Dicer and DGCR8 could be considered promising diagnostic targets for SSNHL. However, considering the ROC curve analysis and AUC values, this hypothesis should be validated with a larger sample size. On the other hand, no significant relationship was observed between the expression levels of target genes and patients' clinical characteristics.

Previous work has verified that suppressing the genes involved in the miRNA machinery system led to overall reduced expression levels of miRNAs in different cell types [37]. Subsequently, the idea that Drosha, Dicer, and DGCR8 up-regulation could increase miRNA production seems reasonable. Nevertheless, despite the overexpression and overactivity of miRNA biogenesis enzymes, some miRNAs might be down-regulated through different biological and pathological processes. This could be considered the consequences of the activity of some regulatory mechanisms participating in the stability of miRNA, including the addition of untemplated nucleotidyl to the 3′ end of miRNA, miRNA methylation, the conversion of adenosine to inosine, and cleavage at the terminal loop of miRNA [38].

Conclusion

In summary, Dicer and DGCR8 expression levels, as the most pivotal enzymes involved in the miRNA processing system, were significantly up-regulated in SSNHL compared with normal samples, suggesting their involvement in SSNHL pathogenesis. Conversely, no significant correlation was seen between the expression profile of these genes and the clinical and pathological features of the patients. However, more studies are needed to confirm these findings and further illustrate the exact function of the miRNA processing system in SSNHL development.

Abbreviations

DGCR8	DiGeorge syndrome critical region gene 8
HBSS	Hanks Balanced Salt Solution
miRNA	MicroRNA
OD	Optical density
PBMC	Peripheral blood mononuclear cell
pre-miRNAs	Precursor miRNAs
pri-miRNA	Primary miRNAs
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
SE	Standard error
SSNHL	Sensorineural hearing loss
UTR	Untranslated region

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Author contributions

YJ-M contributed to study concept and design, and acquisition of data study supervision. DS contributed to study concept and design, and acquisition of data drafting/revising the manuscript for content. MA contributed to acquisition of data drafting. SR-S contributed to drafting/revising the manuscript for content. VH did all of the experiments, contributed to acquisition of data, and reviewing and editing the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Data and materials will be available if needed.

Declarations

Ethics approval and consent to participate

The present study was ethically approved by ethical committee of Tabriz University of Medical Sciences, Tabriz, Iran (ethical Code: Ethical Code: IR.TBZMED.REC.1398.808), and all participants signed the consent form for participation in the study.

Consent for publication

All of the authors declared their consent for publication of the manuscript in this journal.

Competing interests

The authors declare no conflicting interest.

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