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Original Article (Pages: 14907-14913)

Evaluation of miR-301b and miR-302b Expressions in the Serum of Cystic Fibrosis Patients and their Association with Clinical Scoring System

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Abstract

Background: Cystic fibrosis (CF) is an autosomal recessive disorder caused by a mutation in CF transmembrane conductance regulator gene (CFTR). Clinical manifestations of the disease and their severity have considerable variations in patients having similar mutations in CFTR gene. This can be due to different polymorphisms, epigenetic changes and microRNAs (miRNAs) as gene modifiers. Considering the proven roles of miR-301b and miR-302b on infection and inflammation, expression of these miRNAs might change in CF patients.

Methods: In this study, 30 CF patients (homozygous for ΔF508 mutation) and 30 healthy individuals were participated and their demographic data were recorded. The whole RNA was extracted from serum samples and cDNA was synthesized. Using Real-Time PCR, expression levels of miR-301b and miR-302b were measured between the patient and normal groups. Patient classification was carried out based on Shwachman-Kulczycki score, and expression levels of these miRNAs were determined in these classifications. All statistical analyses were performed using IBM SPSS software, version 21.

Results: Statistical analyses of qRT-PCR results showed a significant increase in serum levels of miR-301b and miR-302b expression (p-Values of 0.02 and 0.03; fold changes of 3.73 and 1.95, respectively) in CF patients compared to healthy controls. A significant increase ($p<0.05$) in miR-301b expression level was observed in severe, moderate and mild groups, while miR-302b expression level was increased in CF patients of severe and moderate groups according to Shwachman-Kulczycki score.

Conclusion: Expression levels of miR-301b and miR-302b are different based on the clinical scoring system. This data suggests that expressions of these two miRNAs are influenced by infection and inflammation of CF patients. Further studies can lead to the development of innovative treatment strategies.

Key Words: Cystic fibrosis, MicroRNA, miR-301b, miR-302b, Shwachman-Kulczycki score.

*Please cite this article as: Jalalimanesh N, Ghanavi J, Hassanzad M, Farnia P, Sayedi J, Akbar Velayati A. Evaluation of miR-301b and miR-302b Expressions in the Serum of Cystic Fibrosis Patients and their Association with Clinical Scoring System. Int J Pediatr 2021; 9 (11): 14907-14913. DOI: **10.22038/IJP. 2021.60598.4683**

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Received date: Sep.26,2021; Accepted date:Oct.20,2021

1- INTRODUCTION

Cystic Fibrosis (CF) is a lethal autosomal recessive disease caused by mutations in the related CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) gene. CFTR codes a transmembrane conductance regulator protein, a chloride channel which is expressed in various cell types including epithelial cells and macrophages (1). Accordingly, any dysfunction in this protein can lead to many lung disorders like inflammation, pulmonary fibrosis and remodeling of the airways (2). This protein directly affects hemostasis and indirectly regulates the functions of some other ion channels like sodium channel, and the transfer of some molecules such as glutathione (3, 4).

The CFTR gene is located at 7q31.2 and there are about 2000 reports of pathogenic and non-pathogenic variants for this gene, among which the single nucleotide alterations and deletions are the most common types of mutations (5). Although environmental factors have been shown to be directly associated with CF (6), studies have revealed that genetic factors regulating gene expression play a critical role in CF manifestations (7). Expression regulating factors like microRNAs (miRNAs) post-transcriptionally affect the expression of CFTR protein (8). Recently, numerous studies have investigated microRNAs which are a group of non-coding RNAs with short length that function in post-transcriptional regulation. They act through binding to a regulatory region in 3`UTR of mRNA, named miRNA recognition elements (MREs), and accordingly suppress the expression and degradation of mRNAs (9, 10). There are many reports of miRNA alterations in CF patients, reporting both overexpression and downregulation of different miRNAs (11). The first studies on the role of miRNAs in this disease were carried out on bronchial brushing samples, by comparing the results from CF patients and healthy controls. miRNAs can act through many mechanisms in pathogenesis of CF such as disruption of innate immunity and consequently inflammation, deficiency in bacterial infection clearance and macrophage activity, development of lung fibrosis, disruption in ion conductance, reactions of the cell toward accumulation of misfolded or unfolded CFTR protein (UPR: unfolded protein response), etc. (12). Although many studies have reported the role of miRNAs in the development of CF, there is still a long way to understand its whole mechanism and to recognize all miRNAs involved in pathogenesis of this disease. Two newly identified miRNAs including miR-301b and miR-302b are known to play roles in many biological processes such as immunity responses, inflammation, cell division and differentiation (13, 14). It has been shown that miR-301 inhibits PI3K-Akt pathway, and downregulation of this miRNA is also witnessed in different types of cancers (15). miR-302 has been shown to be a pluripotency activator through activating specific genes including SOX2, OCT4, and NANOG (16). Inflammation is a fundamental burden in cystic fibrosis, and inflammatory response mediated by neutrophils is the main causative agent in this disorder where IL-8 plays a crucial role (17). Similarly, miR-155, miR-93 and miR-17 target IL-8 transcript, and downregulation of these miRNAs along with the increase of IL-8, leads to inflammation in CF patients (7, 17). miR-301 is also shown to activate NFKB and STAT3 pathways to enhance pro-inflammatory responses (18).

In this study, we quantified the expression of both miR-301b and miR-302b in CF patients, compared to healthy controls. Next, patient classification was carried out based on Shwachman-Kulczycki score, and expression levels of these miRNAs were determined in these classifications.

2- MATERIALS AND METHODS

In this study, 30 patients with diagnosed cystic fibrosis (homozygous for ΔF508 mutation), as well as 30 normal controls with no evidence of lung disorder were participated, ranging from 5 to 27 years of age. Written informed consent was signed by the subjects before the sampling procedure and the patients were diagnosed using clinical symptoms, chloride sweat test and genetic analysis for ΔF508 mutation. The study was approved by our Institutional Review Board of National Research Institute of Tuberculosis and Lung Disease (NRITLD). Demographic data including sex, age, nutrition and clinical symptoms were collected and the Shwachman-Kulczycki score was calculated. Based on Shwachman-Kulczycki score, patients were divided into 5 groups including severe (<40), moderate (41-55), mild (56-70), good (71-85), excellent (86-100). 4 mL of blood samples were collected from each case and the whole RNA was extracted using an RNA extraction kit (CinnaGen, Iran). The quantity and quality of extracted RNA was investigated using electrophoresis and NanoDrop spectrophotometer (Biotek, USA). The cDNA synthesis was carried out using the related kit (Zistroyesh, Iran) with primers for RT Stem-loop HK (U6) as housekeeping gene and RT Stem-loop miRNA (miR-301b & 302b) specific primers (19). The expression levels of microRNAs were studied using Real-Time PCR Rotor-Gene Q (QIAGEN, USA) and SYBR Premix Ex Taq II (Takara, Japan). The real-time PCR experiments were performed in triplicate and the results were expressed as mean ± SD. All the statistical analyses were carried out using IBM SPSS software. 21.

3- RESULTS

The samples were separated based on patient`s data, and disease severity was evaluated by Shwachman-Kulczycki score. The results of the grouping of patients based on the Shwachman-Kulczycki score are illustrated in **Fig. 1**. Total patients consisted of 24% severe, 40% moderate, 22% mild, 14% good and 0% excellent cases.

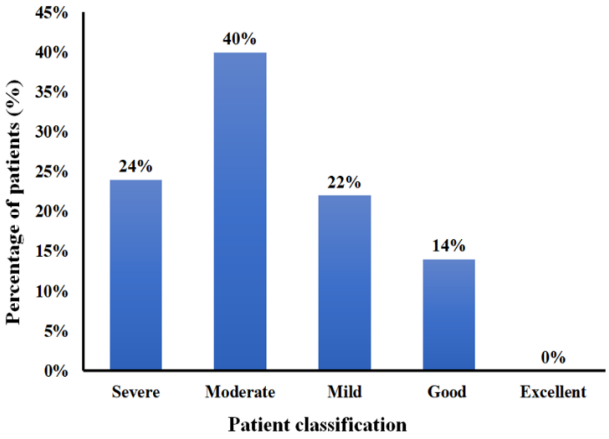

Fig. 1: Patients groups based on Shwachman-Kulczycki score

The expression levels of miR-301b and miR-302b were quantified in CF patients and healthy control groups. Age and gender were not determinant factors for miRNA expression in the studied cases. The expression level of miR-301b in CF patients was significantly upregulated 3.73 times greater than that of the control group (p-Value= 0.02). Relative expression of miR-301b in serum indicates significant alteration (**Fig. 2**).

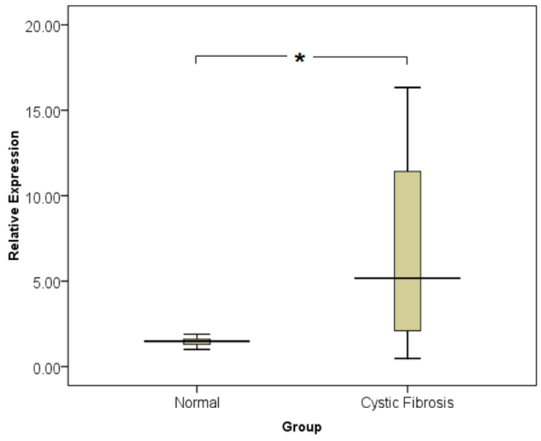

Fig. 2: Comparison of miR-301b relative expression levels in CF patient and control groups

The expression level of miR-302b was significantly increased in CF patients in comparison to the healthy controls (*p*-Value= 0.03). Relative expression of miR-302b in serum of CF patients was slightly downregulated (**Fig. 3**).

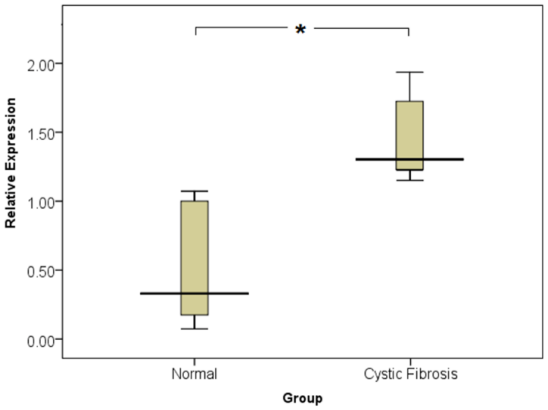

Fig. 3: Comparison of miR-302b relative expression levels in CF patient and control groups

Then, the expression level of miR-301b was determined according to patient classifications based on Shwachman-Kulczycki score. A significant increase in miR-301b expression level was witnessed in severe, moderate and mild groups (p<0.05) (**Figure.4**).

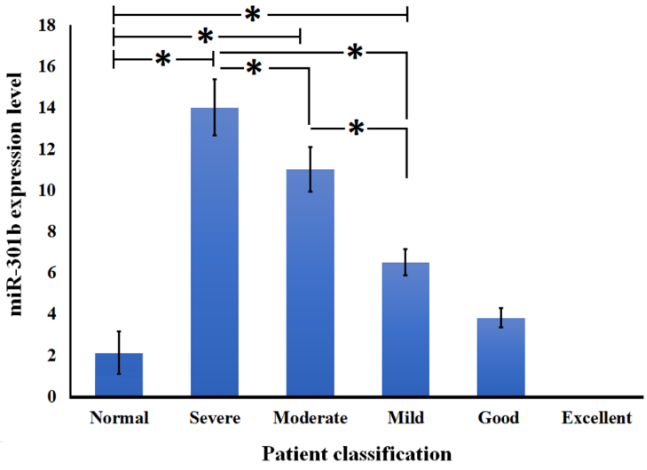

Fig.4: Correlation of miR-301b expression level in different CF patient groups based on Shwachman-Kulczycki score

In addition, miR-302b expression level was increased in CF patients in the severe group (p<0.05), according to the Shwachman-Kulczycki score (**Fig. 5**).

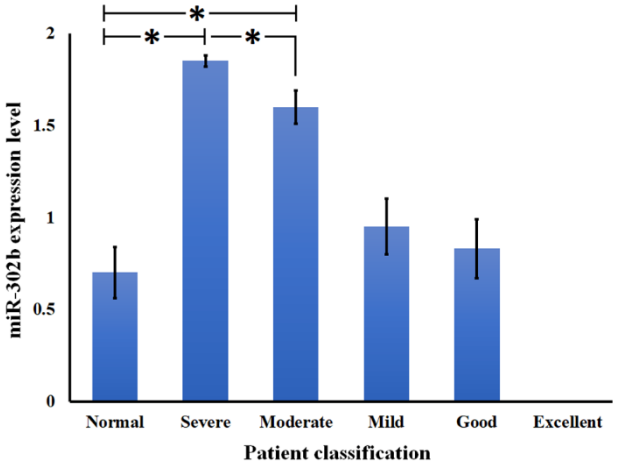

Fig.5: Correlation of miR-302b expression level in different CF patient groups based on Shwachman-Kulczycki score

4- DISCUSSION

MicroRNAs are part of the regulatory network in innate immune response, functioning as the first line of the immunity. Activation of the innate defense pathways leads to changes in miRNA expressions which may ultimately result in regulation of the inflammation (20). Chronic inflammation in CF patients leads to damages to the lung tissue which can eventually result in respiratory failure. Although the inflammation is usually initiated by an infection, researches have indicated that in some cases, evidence of inflammation is detected in the lungs before any signs of infection in the CF infants and toddlers (21). The decrease in the function of ionic channels can cause dryness in the airway and disruption in mucus clearance which will finally lead to chronic respiratory inflammation (11). Chronic inflammation can trigger

destruction of lung tissue through fibrosis and result in pulmonary ulcer (5); therefore, the increase in inflammation can have a substantial importance in exacerbation of disease.

In this study, two miRNAs including miR-301b and miR-302b were investigated which have been shown to be involved in the signaling pathway of inflammation and infection control. miR-301b has impact on alterations in expression levels of the anti-inflammatory and pro-inflammatory cytokines (13), and miR-302b is shown to be effective on production of IL1β, IL-6, IL-8 and TNF-α (14, 22); the effects of these inflammatory cytokines are well confirmed in the inflammation of CF patients (23, 24). The present study was conducted to evaluate the expression levels of these miRNAs in 30 CF patients compared to 30 normal controls in an Iranian population. In addition, the patients were divided into five groups of disease severity according to the Shwachman-Kulczycki score, and miRNA expression levels were subsequently determined in these classifications.

Pseudomonas aeruginosa is the most prominent bacterial pathogen which is prevalently associated with immunocompromised and CF patients. Lipopolysaccharide (LPS) produced by this pathogen chiefly contributes to the virulence and has a main role in both innate and acquired immune responses against infection (25). Previous studies have indicated that *Pseudomonas aeruginosa* infection can induce expression of miR-301b through TLR4/MYD88/NFKB pathway. This microRNA regulates IL-4 and TGF-B1 through inhibiting the function of c-myb, and leads to inhibition of the anti-inflammatory cytokines, and hence results in excessive inflammation and impaired host defense. miR-301b can inhibit neutrophils initial recruitment against bacterial infection (13). The expression of this microRNA significantly increases in inflamed mucosa and serum samples of the patients with inflammatory bowel disease (IBD) (26). These findings are in agreement with the results of the present study, in that an increase in miR-301b expression level was observed in CF patients compared to healthy controls, and this increase was more perceived in patients with lower Shwachman-Kulczycki score.

Similar studies have shown the role of miR-302b in inflammation and infection. It has been demonstrated that miR-302b can be a proper candidate for the treatment of gout disease. This miRNA can reduce IL1β during the inflammation through targeting Caspase-1 and NFKB pathway (14). Upon infection by *Pseudomonas aeruginosa*, miR-302b is induced by Toll-like receptor 2 and 4 (TLR2 and TLR4) via ERK-p38-NF-kB signaling pathways (27). LPS-induced treatment of the cells which were transfected with miR-302b displayed a significant increase in expression levels of the inflammatory cytokines (IL1β, IL-6, IL-8 and TNF-α) (22). In the present study, miRNA-302b expression level was increased in CF patients compared to healthy controls and this increase was more perceived in patients of severe and moderate groups based on Shwachman-Kulczycki score.

As mentioned above, most of the studies have shown the role of miR-301b and 302b in infection and inflammation. On the other hand, there are some other miRNAs which are related to inflammation in CF patients. For instance, miRNA-25-3p, which is reported to be increased in CF cases, has been shown to repress TGF-β and the expression of its receptor, which is an inhibitor for NFKB/IL-8 pathway (28, 29). Our study demonstrated that miR-301b and 302b expression levels were increased in CF patients. Due to *Pseudomonas* infection which is the predominant infection in CF,

and the inflammation which leads to deterioration of disease, the increase in expression of these two miRNAs in CF patients compared to healthy controls as well as their higher expression in patients with lower Shwachman-Kulczycki score is considerable. This might need further investigations.

5- CONCLUSION

The expression levels of miR-301b and miR-302b varied along with the severity of CF disease. More investigations are recommended to understand the certain mechanism of action for these miRNAs and further studies can lead to the development of treatment strategies by suppressing the expression of these two mRNAs. The findings of this study can be applied to a wider range of patients to obtain more applicable results.

6- CONFLICT OF INTEREST: None.

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