Mir-608 Overexpression in Idiopathic Pulmonary Fibrosis (IPF) is Related to Acetylcholinesterase Single Nucleotide Polymorphism (SNP)

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Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is a chronic progressive disease that causes scarring of the lungs. The disease is associated with the Usual Interstitial Pneumonia (UIP) pattern, which was not yet fully recapitulated by an animal model. Therefore, the disease is considered 'human specific'. miRNA-608 is a primate specific miRNA with many potential targets, such CdC42 and Interlukin-6 (IL-6) that were previously implicated in IPF pathology.

Objective: To test miR-608 expression and its targets in IPF patient samples.

Methods: RNA was extracted from Formalin fixed paraffin embedded (FFPE) tissue sections (N=18). miRNA-608 expression and Cdc42 and IL-6 levels were analyzed by qPCR. Acetylcholinesterase (AChE) is another target of miRNA-608. Its' rs17228616 allele has a single-nucleotide polymorphism (SNP) causing weakened miR-608 interaction (C2098A). Thus, DNA was extracted from whole blood samples from 56 subjects with fibrosing interstitial lung disease (ILD) and this region was sequenced for assessment of rs17228616 allele polymorphism.

Results: MiR-608 is significantly overexpressed in IPF samples, in comparison with controls ($p<0.05$). Cdc42 and IL-6 levels were lower in the IPF patient samples compared with control samples ($p<0.001$ and $p<0.05$, respectively).

The frequency of the rs17228616 minor A-allele was 17/56 (30.4%) with all patients being heterozygous. This result is significant vs. the published Israeli cohort of healthy individuals, which reported 17% prevalence of this allele in healthy control volunteers ($p=0.01$, OR = 2.1, CI 95% [1.19-3.9]).

Conclusion: MiR-608 is overexpressed in IPF patients. While the exact mechanism remains to be discovered, it could potentially promote fibrotic disease.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic disease that causes scarring of the lungs. The disease is characterized by progressive worsening of dyspnoea and decline in lung function. Many patients with IPF have sub-clinical or clear co-morbid conditions including pulmonary hypertension, gastroesophageal reflux, obstructive sleep apnoea, obesity, emphysema, as well as depression and anxiety [1, 2].

The disease is limited to the lungs, and is associated with the histopathologic and/or radiologic pattern of Usual Interstitial Pneumonia (UIP). So far, no animal model fully recapitulated the UIP pattern [3] and the disease is considered 'human specific'.

miRNAs are a non-coding RNA sequences, of about 22 nucleotides long that have complementary sequences to particular regions of the mRNA, often in the 3'UTR, through which regulation occurs. Regulation generally proceeds via either mRNA degradation or inhibition, leading to gene silencing, with

the degree of inhibition highly depending on the degree of complementarity between the miRNA and its corresponding mRNA target.

Single-nucleotide polymorphisms (SNPs) are strongly associated with susceptibility to various diseases, including IPF [4–6]. SNPs within miRNA sequences may therefore change properties of the resulting inhibition by altering degree of complementarity [7, 8].

miRNA-608 (hsa-mir-608) is a long (25 nucleotides) primate specific miRNA. It has many potential targets, such as Rho GTPase CdC42 and Interlukin-6 (IL-6), bearing much experimental evidence [9, 10]. Both IL-6 and CdC42 have been shown to be involved in both anxiety and IPF [11]. Therefore, we tested miR-608 expression and its targets in IPF patient samples.

Materials And Methods

RNA purification and quantitative PCR (qPCR):

Total RNA (including miRNA) was extracted from Formalin fixed paraffin embedded (FFPE) tissue sections taken from 18 IPF patients and 8 Control samples, using the miRNeasy FFPE kit (Qaigen, USA) according to the manufacturer's instructions.

mRNA was converted to cDNA using the reverse transcription kit (Applied Biosystems, UK). Reactions were performed with SYBR Green PCR master mix (Applied Biosystems, UK). Primer sequences (purchased from Hylabs, Israel) are listed in Table 1. 18S served as the reference housekeeping gene. Primers were normalized by specific cDNA standard curves obtained from known amounts of cDNA.

Table 1
List of primers

	Forward (5'-3')	Reverse (5'-3')
CDC42 variant 1 (NM_001791)	CTGTCAAGTATGTGTGGAGTGTTCTG	CTCTTCTTCGGTTCTGGAGGCT
CDC42 variant 2 (NM_044472)	TGCACTTACACAGAAAGGCC	CTTCTTCGGTTCTGGAGGCT
18S	AGGAATTGACGGAAGGGCAC	GGACATCTAAGGGCATCACA
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC

miRNA was converted into cDNA using the qScript microRNA cDNA synthesis kit (Quanta Biosciences, USA) and was then amplified using Perfecta Universal PCR primer kit (Quanta Bioscience, USA). qPCR was performed with Perfecta SYBR Green using specific primers for mir-has-608 (Quanta bioscience, USA). The reference genes (Snord 44 and RNV6) primers were supplied with the kit (sequence not available).

Human Blood Samples:

Whole blood samples were collected from 56 subjects with fibrosing interstitial lung disease (ILD). DNA was purified from these samples using QaiSymphony (Qaigen). Their demographic data, as well as disease progression and final diagnosis were also recorded.

Gene sequencing:

DNA was amplified using PCRBIO HS Taq master mix (PCR Biosystems) with specific primers for the AchE: Forward 5'-CGCTGGAGCTCCTACATGGT-3' and Reverse 5'-ATAGACTCGGCCCCGTGAT-3'. Products were purified using FastAP Thermosensitive Alkaline Phosphatase (Thermo-Fisher scientific). Then, sequencing was performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo-Fisher Scientific) according to manufacturer's instructions. The target sequence was analysed by 3130 Genetic analyser (Applied Biosystems) for determining A/C allele single nucleotide polymorphism (SNP) at rs17228616.

Statistical Analysis:

Statistical analysis was done using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). ANOVA was performed to compare differences between multiple cohorts. Paired Student's t-tests were employed to analyze differences between the two groups. An effect was considered significant when the P-value was < 0.05. All experiments were repeated at least three times.

Ethical approval:

This study was approved by the local Ethics Committee (MMC-18-18). Informed consent was obtained from all subjects.

Results

MiR-608 is overexpressed in lung tissue samples from IPF patients

The expression of miR-608 in was evaluated in IPF FFPE patient samples. Interestingly, a significant overexpression of miR-608 was found in IPF patient samples, in comparison with controls ($p<0.05$, Figure 1A). Using the miRDB search (http://mirdb.org/cgi-bin/search.cgi), 975 possible targets for miR-608 were identified. These results were then subjected to WEB-based Gene set analysis toolkit (WebGestalt, http://www.webgestalt.org). We analysed the overrepresentation analysis (ORA) for disease (OMIM) functional database. Interestingly, IPF was the most significantly over-represented disease, with an enrichment ratio of 78 (PULMONARY FIBROSIS, IDIOPATHIC, gene set 178500, FDR= 0.00025).

Cdc42 levels are significantly reduced in IPF patient samples

Cdc42 is an established target of miR-608 [9]. Moreover, it was recently implicated in lung fibrosis, as it was found that the loss of Cdc42 promotes the fibrotic process [11]. Thus, we measured the expression level of both Cdc42 mRNA variants (NM_001791 and NM_044472) in IPF patient samples, in comparison to controls. Both Cdc42 isotype levels in IPF patient samples were significantly lower compared with those of the control samples ($p<0.001$ and $p<0.001$, respectively, Figure 2A-B).

IL-6 levels are downregulated in IPF patient samples

IL-6 is well-known cytokine in IPF and a validated target of miR-608. In our recent work, we showed that the IL-6R protein level is reduced in tissue samples taken from IPF patient biopsies [12]. qPCR was performed to measure the expression of IL-6 in IPF patients compared with healthy individuals. In fact, IL-6 levels were lower in the IPF patient FFPE samples compared with controls ($p<0.05$, Figure 2C). This result is in key with the hypothesis stating an inhibitory relationship between miRNA-608 and its targets.

The C2098A substitution (minor rs17228616 allele) at the AChE sequence is more prevalent in IPF patients

It was previously shown that the miR-608 has differential affinity to the AchE sequence due to C to A change (C2098A) in its 3'-untranslated region (e.g. the minor 'A-allele' and 'C-allele', respectively), indicating weakened A-allele AChE–miR-608 interaction [9]. The impaired interaction of the A-allele AChE with miR-608 predicted both weakened AChE suppression and freed more miR-608 molecules to suppress other targets with tighter binding parameters, such as Cdc42 and IL6, as found in our results.

We hypothesized that the presence of the minor A-allele could be higher in subjects with IPF. Thus, 56 patients were recruited and their DNA was sequenced for the A/C allele. Patient characteristics are listed in Table 2. 62.5% were male with the average age of 65.82±12.

Of these subjects, the frequency of the A-allele was 17/56 (30.4%) with all patients being heterozygous for the minor A-allele (Figure 3). This result is significant vs. the published Israeli cohort of healthy individuals, which reported 17% prevalence of this allele in healthy control volunteers ($p=0.01$, OR = 2.1, CI 95% [1.19-3.9]).

Of them, 64.3% were diagnosed with IPF and the rest with other types of ILD (e.g. NSIP, silicosis etc.). In addition, we followed up these patients' disease progression to determine whether they are rapidly progressing, as previously defined by the annual decline in FVC% [13-16]. We found that of the minor allele population, more patients presented with a rapid progressing disease (35.4% vs. 20%, $p=0.24$), yet this result did not reach significance.

When comparing between the A to the C allele groups' lung function tests at the time of diagnosis, a significantly reduced FVC at diagnosis for the A group was observed (Table 2, $p=0.02$).

Table 2:
Patient characteristics

Parameter	A-allele n=17	C-allele n=39	p-value
Age	62 ±14	62.4 ±11	0.9
Gender (%male)	12 (70%)	23 (58.9%)	0.41
Smoker	13 (76.4%)	20 (51.2%)	0.08
Rapidly progressing disease	6 (35.2%)	8 (20.5%)	0.24
FVC %	59 ±14.5	70 ± 19	0.05
DLCO %	46.2 ± 17	48.6 ± 17	0.66
BMI	27.6 ± 5	28.4 ± 4.5	0.61
IHD	5 (29.4%)	7 (17.9%)	0.34
CHF	1 (5.9%)	2 (5.1%)	0.9
Diabetes	7 (41.2%)	16 (41%)	1
Anxiety	1 (5.9%)	6 (15.4%)	0.3
Hypertension	6 (35.2%)	11 (28.2%)	0.64
Osteoporosis	4 (23.5%)	7 (17.9%)	0.63
Malignancy	1 (5.9%)	5 (12.8%)	0.44

Discussion

Numerous pathways were implicated and major progress has been made towards understanding IPF etiology [17]. However, as recently stated by McDonough et el, since the UIP pattern cannot be replicated in animal models, we have no information about what regulates progression of IPF in the human lung [18]. Thus, in this work we focused on the primate specific miRNA that was previously implicated in aging related diseases. We found that miR-608 was significantly upregulated in IPF tissue samples. In addition, we found that the minor rs17228616 allele was more abundant in IPF patients than in the general population.

miR-608 is located on human chromosome 10q24.31. Although not afermentioned in IPF [19], current studies in tumors indicate that miR-608 affects cell proliferation, invasion, migration and apoptosis [20, 21]. Although the expression level of miR-608 was found to be downregulated in several types of cancer [8, 22-25], these studies did not take into account the SNPs that can significantly affect miRNA stability and function [7].

The major SNP of miR-608 mentioned in cancer is the rs4919510 variant G allele. This SNP was suggested to affect the expression of mature miR-608, as well as that of the proinflammatory cytokines TNF-α, IL-6, and IL-1β. Nevertheless, there are conflicting results for the association between the presence of miR-608 rs4919510 and susceptibility to tumors. [8, 26-28]. Although IPF and lung cancer are sometimes seen in the same patients [29], since we observed an upregulation in the miR-608, rs4919510 was not studied and our focus was shifted to other directions.

To date, limited targets of miR-608 have been confirmed by *in-vitro* studies performed in mostly tumor cell lines [30]. A recent study by Wu et al, suggested that Cdc42 is an important post-transcriptional regulator and may play a significant role in the process of inflammation .Although IPF is not considered to be an inflammatory disease per se, pro-inflammatory factors, such as IL-6, TNF-alpha and IFN-y were shown to contribute to disease progression [12, 31].

Several studies reported that symptoms of depression and anxiety are common in patients with IPF. Such studies indicated prevalence of depression ranges from 24.3–49.2%, while that of anxiety reaching 60% in patients with IPF and other ILDs [32-36]. Although a causality of anxiety or depression could be expected, it is possible to assume a genetic predisposition as well.

Our work was inspired by a group of researchers who studied miR-608 in the context of anxiety [9]. They investigated the interaction of miR-608 with AChE (major rs17228616) and the resulting changes, which give rise to a higher ratio of suppression by miR-608 of its other targets, including CdC42 and IL-6, as shown in our results. They showed that young, healthy volunteers with the minor rs17228616 allele showed elevated blood pressure and reduced cortisol, predicting risk of aging-related diseases, such as IPF. Our cohort of patients with fibrosing ILDs, mostly IPF, was shown to include significantly higher prevalence of minor A-allele in comparison to the healthy cohort presented by this group. Since both populations were from Israel, we can also assume similar genetic backgrounds. These results require further investigation in a large cohort to determine the polymorphism in this patient population.

In conclusion, although the number of patients was limited, a significant effect was reached. We found that miR-608 is overexpressed in IPF patients and that this patient population includes 30 percent of a specific SNP in AChE that was previously implicated as relevant to aging related diseases. These findings require further research in a large study cohort.

Declarations

Ethics approval and consent to participate:

The study was approved by the Ethics Committee of Meir Medical Center. Signed informed consent was obtained from all patients.

Consent for publication:

Not applicable

Availability of data and material:

Any data can be supplemented on demand.

Competing interests:

all authors declare no conflict of interest and have consented for publication.

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Authors' contributions:

GES drafted the manuscript, designed the experiments and analyzed the results, IK performed the experiments and analyzed the results, OW revised it critically for important intellectual content, while DS and LIS contributed to conception and design, drafting the manuscript for important intellectual content and revised the final version.

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Figures

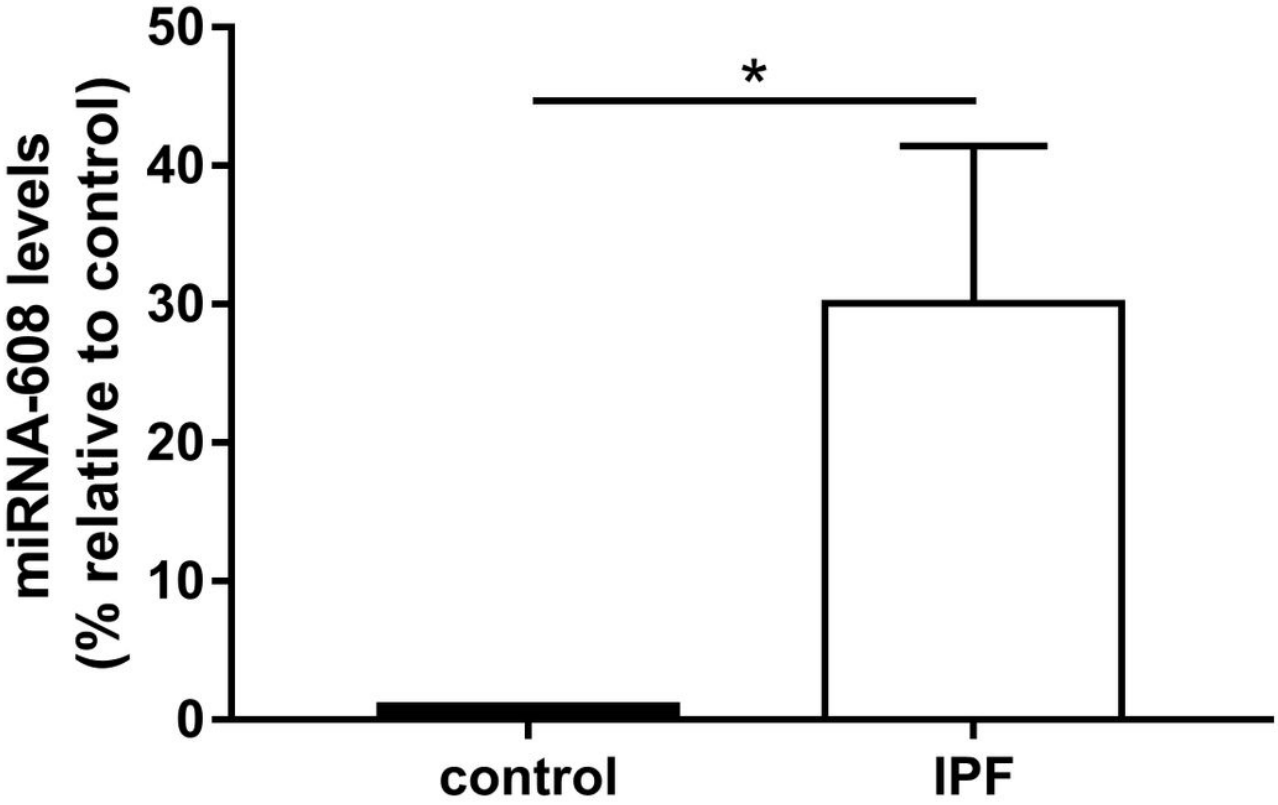

Figure 1

miR-608 is overexpressed in lung tissue samples from IPF patients RNA was extracted from IPF and non-IPF (control) FFPE samples. miRNA-608 levels were evaluated by qPCR. *** p≤0.001, Student's paired t-test (n>3).

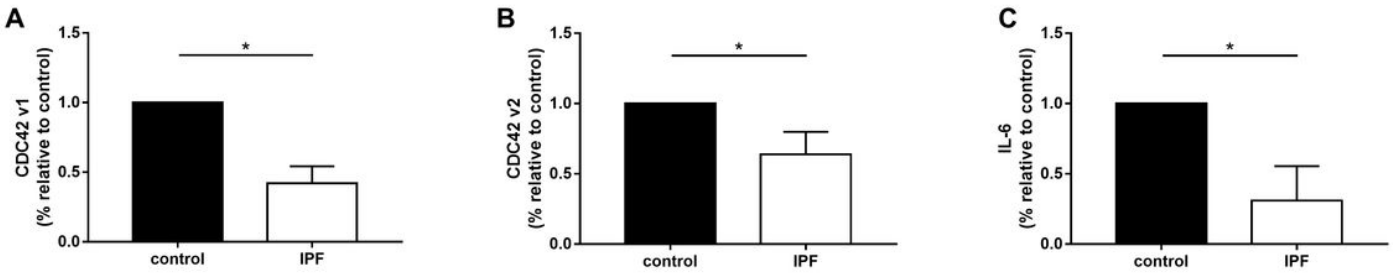

Figure 2

miR-608 target levels are significantly reduced in IPF patient samples RNA was extracted from IPF and non-IPF (control) FFPE samples. The two variants of Cdc42 (A-B) and IL-6 (C) mRNA levels were evaluated by qPCR. * p≤0.5, Student's paired t-test (n>3).

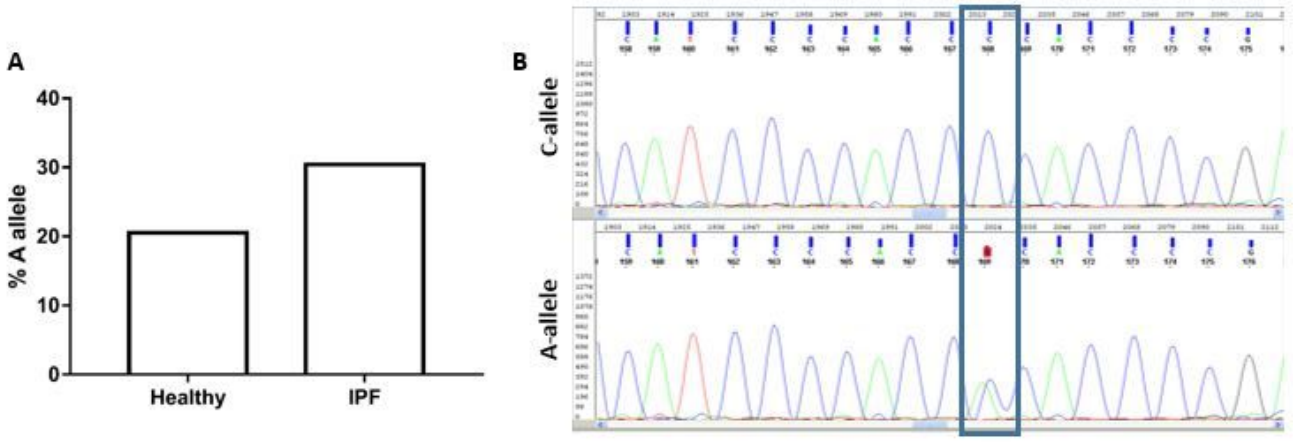

Figure 3

The C2098A substitution (minor rs17228616 allele) at the AChE sequence is more prevalent in IPF patients DNA was extracted from 56 subjects with progressive fibrosing ILD. AChE miR-609 target sequence was analysed for determining A/C allele rs17228616 SNP (A). B is a representative image of sequencing output showing C (top panel) and A heterozygous allele (bottom panel).