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Significance of Cyclooxgenase-2 gene polymorphism and related miRNAs in pulmonary arterial hypertension

Sinem Durmus [a], Ersan Atahan [b], Burcak Avci Kilickiran [c], Burak Onal [d], Ufuk Cakatay [a], Remise Gelisgen [a], Hafize Uzun [a,e,*]

[a] Department of Medical Biochemistry, Cerrahpasa Faculty of Medicine, Istanbul University-Cerrahpasa, Istanbul, Turkey
[b] Department of Chest Diseases, Cerrahpasa Faculty of Medicine, Istanbul University-Cerrahpasa, Istanbul, Turkey
[c] Department of Cardiology, Cerrahpasa Faculty of Medicine, Istanbul University-Cerrahpasa, Istanbul, Turkey
[d] Department of Medical Pharmacology, Medical Faculty, Biruni University, Istanbul, Turkey
[e] Department of Medical Biochemistry, Faculty of Medicine, İstanbul Atlas University, Istanbul, Turkey

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ABSTRACT

Background: Pulmonary arterial hypertension (PAH) is a rare disease with a poor prognosis. The suppression of cyclooxygenase-2 (COX-2) expression has been known to impair vascular function in endothelial cells; however, the epigenetic factors that cause this are largely obscure. Our aim in this study was to examine the polymorphisms in the gene for COX-2 (*PTGS2*) and related miRNAs regulating its level in a single-center cohort of patients with PAH.

Method: In this study, three SNPs and miRNAs (rs5275, rs689470, rs20417, miR-26b-5p, miR-146a-5p, and miR-101-5p) in the *PTGS2* were screened in PAH and controls by qPCR. In addition, the COX-2 level was determined by immunoassay to examine the effects of epigenetic factors on its expression levels.

Results: The non-dominant genotypes of rs20417 and rs5275 were found to be related to PAH (OR = 8.56, 95% CI = 3.39–21.63, $p < 0.0001$ and OR = 7.82, 95% CI = 3.30–18.53, $p < 0.0001$, respectively). We also observed a significant increase in the miR-26b-5p and miR-146a-5p levels in PAH patients (2.18 and 2.35-fold, respectively; for both, $p < 0.05$). In addition, it was found that SNPs influenced the COX-2, miR-26b-5p, and miR-146a-5p levels in PAH. A negative correlation was also found between COX-2 levels and miR-26b-5p and miR-146a-5p.

Conclusions: As conventional drug therapies may cause lower COX-2 levels, the development of new genetic or epigenetic biomarkers is crucially important for early diagnosis and prognosis. The presence of minor alleles for rs5275 and rs689470 might also be considered as a significant risk factor for developing PAH. Furthermore, locus-specific miRNAs, such as miR-26b-5p and miR-146a-5p, seem to play a critical role in the regulation of *PTGS2* expression.

1. Introduction

Pulmonary arterial hypertension (PAH) is a rare, progressive, and incurable cardiopulmonary disorder, which is characterized by the abnormal vascular remodeling process [1–3]. Increased vascular resistance and consequent vascular narrowing can be defined as the most prominent features of PAH. These vasculopathies cause right ventricular failure and may even result in death [4,5]. Although the exact vascular pathogenic mechanism is still obscure, causative factors such as hypoxia, inflammation or responses to drugs or toxins as well as genetic predisposition are considered as disease contributors [5].

Cyclooxygenase (COX, also known as Prostaglandin-endoperoxide synthase, *PTGS,* EC 1.14.99.1) isoforms (COX-1 and COX-2) transform arachidonic acid to various prostanoids, which are thought to be involved in the development of the vascular remodeling process [6]. The COX-2 isoform acts as a rate-limiting factor in prostaglandin biosynthesis. It has been reported that COX-2, which is constitutively expressed locally in the kidney, regulates blood flow and directs vascular homeostasis. In addition, it was concluded that COX-2 plays an important role in systemic vascular protection and its inhibition can give rise to cardiovascular side effects [7,8].

The *PTGS2* gene is located on Chromosome 1 (the minus

* Corresponding author at: Department of Medical Biochemistry, Faculty of Medicine, İstanbul Atlas University, Istanbul, Turkey.
E-mail address: hafize.uzun@atlas.edu.tr (H. Uzun).

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chromosomal strand) at q31.1 and comprises 10 exons and introns [9]. *PTGS2* gene regulation occurs at both the transcriptional and post-transcriptional levels. To date, more than 4000 single nucleotide polymorphisms (SNPs) have been identified in both coding and non-coding (5′-upstream, introns and 3′-untranslated) regions of *PTGS2* [10]. The widely studied SNP variant is rs20417 (-765G > C), which is located in the promoter region of *PTGS2*. The presence of the C variant has been associated with reduced COX-2 levels [11]. The rs5275 (8473 T > C) and rs689470 (+8365C > T) variants are located in the three prime untranslated region (3′-UTR) and are related to its expression level [12].

On the other hand, miRNAs are small, non-coding RNAs that mainly regulate the expression of various genes, including the arachidonic acid pathway. The 3′-UTR regions contain binding sites for miRNAs. Moreover, *PTGS2* 3′ UTR contains several available miRNA binding sites [13]. miR-146a and miR-146b act as negative regulators of inflammatory *PTGS2* gene expression in a wide group of vascular cells, including monocytes, fibroblast, endothelium, airway smooth muscle, and epithelial cells [14]. It has been shown that miR-26b, miR-146a, and miR-101 inhibit the expression or induce degradation of COX-2 [14–18].

Conventional anti-inflammatory drug therapies such as Bosentan and Sildenafil affect *PTGS2* expression levels [19,20] Increased expression of some miRNAs and miRNA-related gene variations may also negatively affect the COX-2 levels. The development of robust genetic or epigenetic biomarkers is crucially important for early diagnosis and prognosis of PAH. The 3′ UTR segment of the *PTGS2* gene includes both miRNA binding and SNP regions. Therefore, we aimed to investigate the possible mechanistic relationship of *PTGS2* SNPs and related miRNA levels with an integrative approach in PAH.

2. Materials and methods

2.1. Study group

The study protocol was approved by the local Ethics Committee of the Cerrahpasa Faculty of Medicine, Istanbul University-Cerrahpasa (No: 224609, date:17/06/2016), and it was conducted in accordance with the Declaration of Helsinki. In total, 29 patients between the ages of 23 and 80 with PAH and corresponding 150 age and gender-matched healthy controls were included in the study. The anamnesis of the cases was taken, and general physical examinations were performed. Patients with mean pulmonary artery pressure higher than 3.33 kPa and right heart catheterization were diagnosed with pulmonary artery hypertension and classified as functional according to the functional classification criteria of World Health Organization (WHO). The exercise capacity of patients was evaluated with the six-minute walking test [21]. All patients with PAH had been previously diagnosed. Drugs that are effective on COX-2 levels, such as Bosentan and Sildenafil, were not administered to the patients with PAH in our study. Patients who suffered with any other chronic inflammatory conditions, such as inflammatory bowel disease, coronary artery disease, diabetes mellitus, obesity, malignancy, or a history of malignancy, were excluded from the study protocol. The healthy control group consisted of 150 individuals without any health complaints and medication. Patients and their respective controls who initially gave and later wished to withdraw their previous consent were also excluded from the study.

2.2. Sample collection

Venous blood samples were collected from patients with PAH and their healthy age-matched controls. Blood was drawn from all participants between 8 AM and 10 AM. To avoid any matrix interference, such as lipemia, we preferred morning fasting blood samples. Plain tubes with no additive-containing blood samples were centrifuged for 15 min at 5000 rpm and 4 °C to obtain serum samples. To perform the analysis of other analytes, such as COX-2 and miRNA, serum aliquots were frozen and stored at –80 °C until they were assayed. Venous whole blood samples were collected in K_3-EDTA tubes, and stored at −20 °C until DNA isolation.

2.3. Analytical assays

COX-2 was analyzed using a sandwich ELISA kit (Human COX-2 kit, Cat. No. E0780Hu, Bioassay Technology Laboratory, Shanghai, China) and the serum COX-2 levels of the samples were expressed as IU/L. The sensitivity (limit of quantification) of this commercial assay kit was 0.249 IU/L. The intra-assay coefficients of variability (precision within an assay; known concentrations were tested on one plate to assess the intra-assay precision by manufacturer) at 102.4 ± 3.66 IU/L was 3.6, as stated in the kit manufacturer's protocol. The concentration values for inter-assay precision (precision between assays; known concentrations were tested in separate tests by the manufacturer) were not provided in the kit protocol; however, the coefficients of variability were stated to be <10.

miRNAs were extracted from venous blood samples using an mirVana™ PARIS™ Kit (Life Technologies, Carlsbad, USA). The quantification of extracted RNA was realized by using the Qubit™ microRNA Assay Kit (Life Technologies Carlsbad, USA) with the Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, USA). In the first step, miRNA samples were transcribed into complementary DNA (cDNA) using the TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.). Subsequently, miRNA levels were analyzed with Taqman MicroRNA Assays (hsa-miR-26b-5p, hsa-miR-101-5p, hsa-miR-146a-5p, and RNU6B; Thermo Fisher Scientific Inc.) using the StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The relative levels of miR-26b-5p, miR-101-5p, and miR-146a-5p were calculated using the $2^{-\Delta\Delta CT}$ method. Using the $2^{-\Delta\Delta CT}$ method, data were presented as fold change (or fold difference, hereinafter referred to as fold difference) in miRNA levels, which were normalized to an endogenous reference gene and presented as relative to control [22]. RNU6B was used as endogenous control (housekeeping gene) for normalization. All calculations were made according to the following equation:

$$\Delta\Delta CT = \left(CT_{TargetmiRNA} - CT_{RNU6B}\right)_{Patient} - \left(CT_{TargetmiRNA} - CT_{RNU6B}\right)_{Control}$$

Genomic DNA was extracted from whole blood samples using the PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific, Inc.). The absorbance of the extracted DNA samples was measured at 260 nm wavelength. Furthermore, DNA purity was confirmed to be between 1.8 ± 0.2 OD values. The determination of polymorphic regions of *PTGS2* gene (rs689470, rs5275, and rs20417) was performed using an RT-PCR device and appropriate SNP assay (Taqman SNP Genotyping assays rs689470, rs5275, rs20417, Thermo Fisher Scientific, Inc.) according to the kit instructions. Each sample for all the miRNA analysis assays was assayed in triplicate. The CT value of each sample was calculated by averaging the three CT values.

The dominant model is a genetic model that helps in estimating an OR, thereby clarifying a subject-level phenomenon. On the other hand, the allelic model evaluates the impact of individual alleles on PAH. All subjects mentioned in Fig. 3 were PAH patients, and patients were classified according to their genotype subgroups. In the dominant model, one group consisted of individuals carrying only the wild type allele (W), whereas the other group comprised individuals' minor (M) alleles (WM + MM).

2.4. Statistical analysis

All statistical analyses were performed with GraphPad Prism 9.0 for macOS (Version 9.3.1; GraphPad Software, LLC.) and p value <0.05 was considered to be statistically significant. The normal distribution of the variables was tested using visual (histograms and probability graphs) and analytic (Kolmogorov–Smirnov or Shapiro–Wilk tests) frameworks. The results for the continuous variables were expressed as means ±

standard deviations (median, Q1–Q3). The lower quartile (Q1) corresponds with the 25th percentile, whereas the upper quartile (Q3) corresponds with the 75th percentile. Categorical variables were expressed in terms of percentages or frequency, and they were compared using Pearson's χ^2 tests. The Hardy–Weinberg equilibrium of the genotype distribution among the controls was tested by a goodness-of-fit $\chi 2$ test. The statistical significance of the differences between means was determined by Student's *t*-test, Mann–Whitney *U* test, or analysis of variance (ANOVA), followed by post-hoc multiple comparisons using the Tukey's Honest Significant Difference (HSD) test. The sensitivity and specificity of the measured variables for biomarkers were examined using a receiver operating characteristic (ROC) curve analysis. Univariate and multivariate analysis were performed by using binary logistic regression (Enter method).

3. Results

In total, 29 diagnosed cases of PAH and 150 healthy control subjects were included in the study. The median age of the patients was 58 years (between 23 and 80 years), with a M:F ratio of 1.6:10. PAH patients and healthy control subjects appeared to be sufficiently matched in terms of age ($p = 0.221$) and gender ($p = 0.255$). The current results indicate the comparability of this case-control study design. mPAP, PASP, and PVR are also provided in Table 1 with median values (6.67 kPa, 9.87 kPa, and 0.91 MPa • s/m^3, respectively). Our study showed that the COX-2 levels were lower in PAH patients.

The genotypic and allelic distribution of the three selected SNPs of the *PTGS2* gene amongst PAH cases and controls are shown in Table 2. The observed genotype frequencies in patients were statistically consistent with the Hardy–Weinberg equilibrium expectation (Table 2). Our study had an 81.9 percent power gene variation distribution for rs20417 ($\chi 2 = 1.55$, $\alpha = 0.05$) and 88.8 percent power gene variation distribution for rs5275 ($\chi 2 = 3.78$, $\alpha = 0.05$) between the patients and controls.

Among the three SNPs, heterozygote genotypes of rs20417 (OR = 9.28, 95% CI = 3.62–21.63, $p < 0.0001$) and rs5275 (OR = 7.94, 95% CI = 3.25–19.40, $p < 0.0001$) were associated with a statistically significantly increased risk of PAH. In addition, a similar trend was observed in the combined genotypes of rs20417 (for GC/CC genotype: OR = 8.56, 95% CI = 3.39–21.63, $p < 0.0001$), rs5275 (for TC/CC genotype: OR = 7.82, 95% CI = 3.30–18.53, $p < 0.0001$) in the dominant model, and the minor alleles of rs20417 (OR = 5.90, 95% CI: 2.61–13.37, $p < 0.0001$) and rs5275 (OR = 5.13, 95% CI: 2.60–10.13, $p < 0.0001$). However, we did not find a similar relationship between rs689470 and the risk of PAH.

Table 1
Demographics, clinical characteristics, and COX-2 levels of the healthy controls and PAH.

Groups Variable	Control (n = 150)	Patient group (PAH) (n = 29)	p values
Age (years)	51.25 ± 15.03 (50.00)	54.45 ± 14.72 (58.00; 46.00–65.00)	0.221
Gender Male/Female ratio (%)	35/115 (30%)	4/25 (16%)	0.255
mPAP (kPa)	N/A	6.88 ± 2.73 (6.67; 4.67–9.00)	N/A
PASP (kPa)	N/A	10.56 ± 3.87 (9.87; 7.33–14.27)	N/A
PVR (MPa • s/m^3)	N/A	7.92 ± 2.90 (0.91; 0.47–1.41)	N/A
COX-2 (IU/L)	56.97 ± 31.57 (51.48; 36.50–69.45)	14.21 ± 4.06 (15.65; 10.39–17.07)	<0.001

Values are provided as mean ± S.D. (median; Q1–Q3) and ratio (%). **mPAP:** Mean pulmonary artery pressure; **PASP:** Pulmonary artery systolic pressure; **PVR:** Pulmonary vascular resistance; **COX:** Cyclooxygenase; **N.A.:** Not applicable; $p < 0.05$ are considered as significant. The lower quartile (Q1) corresponds with the 25th percentile while the upper quartile (Q3) corresponds with the 75th percentile.

In comparison with the controls, a significant alteration was observed in two miRNAs in PAH patients, namely miR-26b-5p and miR-146a-5p. The fold difference using RNU6B revealed the upregulation of hsa-miR-26b-5p (fold difference: 2.18; $p < 0.05$) and hsa-miR-146a-5p (fold difference: 2.35; $p < 0.05$), whereas hsa-miR-101-5p (fold difference: 1.13; p greater than 0.05) deregulation was not significant (Fig. 1).

To investigate the possibility that these miRNAs and the COX-2 level may serve as new and potential biomarkers for PAH, ROC analysis was performed. The sensitivity can be defined as the proportion of patients with PAH, and the specificity of the proportion of healthy controls for ROC analysis. As shown in Fig. 2A and B, the AUC values for miR-26b-5p and miR-146a-5p were 0.6608 (95% CI = 0.5404–0.7812; $p = 0.0126$) and 0.6588 (95% CI = 0.5396–0.7779; $p = 0.0138$), respectively. In Fig. 2C, the AUC for the COX-2 level was 0.9932 (95% CI = 0.9804–1.000; $p < 0.0001$), whereas the AUC measured for miR-101-5p was 0.5507 and not significant. Therefore, we concluded that miR-101-5p might not separate the differences between PAH and controls (Fig. 2D). Our current result indicates that COX-2 level might be an efficient diagnostic biomarker for PAH. The gender factor was found to be insignificant for both univariate and multivariate analyses (Table 3). According to the multivariate analysis, the R^2 value was found to be 0.898 (around 90 percent). This result advocates the gender-independent diagnostic value of our studied biomarkers.

Our results showed that COX-2 activities were significantly increased in PAH patients. To examine the impact of SNPs on the *PTGS2* gene and miRNAs targeting this gene in terms of this increase, miRNA level changes and COX-2 levels were examined based on the dominant model in rs20417 and rs5275 (Fig. 3). In comparison with PAH patients with wild type genotype (GG), miR-26b-5p (fold difference: 3.20; $p = 0.0048$) and miR-146a-5p (fold difference: 3.98; $p = 0.0065$) were significantly upregulated in PAH patients with GC/CC combined genotype (Fig. 3A). When COX-2 activities were compared in the same subgroup, the COX-2 levels were found to be significantly lower in the GC/CC combined genotype group (Fig. 3A, $p < 0.0001$). Similarly, when PAH patients with rs5275 SNP wild type genotype (TT) were compared with PAH patients with the variant allele (TC + CC), miR-26b-5p (fold change: 4.08, $p < 0.0001$) and miR-146a-5p (fold difference: 4.25, $p = 0.0019$) were upregulated (Fig. 3B), and COX-2 levels were found to be significantly lower (Fig. 3B, $p < 0.0001$). In the control group, no significant difference was found between the participants with the wild type of genotype and the WM/MM genotype in terms of miRNA and COX-2 levels. Our results strongly suggest that the decrease in COX-2 activities in PAH patients may be attributed to both miRNA upregulation and the impact of variant alleles.

In addition, a significant moderate negative correlation was found between COX-2 levels and miR-26b-5p and miR-146a-5p in the PAH group ($r = -0.676$, $p < 0.001$ and $r = -0.753$, $p < 0.01$, respectively).

4. Discussion

Several genetic and epigenetic factors are known as considerable risk factors for the development of PAH [23]. Our studied *PTGS2* gene promoter region contains TATA box and binding sites for the transcription factor such as NF-κB, NF-IL6/C/EBP, and the CRE binding protein [24]. *PTGS2* also includes AU rich elements (AREs) in the 3′UTR region, which controls the post-transcriptional regulation through the degradation rate of its mRNA and the translation rate of COX-2 [25]. It has been known that the 3′ UTR region of the *PTGS2* gene includes the miRNA binding region and the polymorphic variants. Thus, we aimed to investigate the possible relationship of miRNAs with the risk of developing PAH and COX-2 levels and its role as a diagnostic or prognostic biomarker. The genetic variants we examined in patients with PAH have not been previously studied. As the variants are located in the miRNA binding site,

Table 2
Genotype and allele distribution of *PTGS2 polymorphism*.

SNP	*Genotype or Allele*	*Control (n:150) n/freq.*	*PAH (n:29) n/freq.*	OR (95% CI)	z	p
Upstream variant						
PTGS2:2KB rs20417 (−765G > C)	GG *(WW)*	137/0.913	16/0.552	1.00 (Reference)	–	–
	GC *(WM)*	12/0.080	13/0.448	9.28 (3.62 – 23.75)	4.64	p < 0.0001
	CC *(MM)*	1/0.007	0/-	2.78 (0.11 – 71.01)	0.62	0.537
	GC + CC *(WM + MM)*	13/0.087	13/0.448	8.56 (3.39 – 21.63)	4.54	p < 0.0001
	G *(W)*	286/0.953	45/0.776	1.00 (Reference)	–	–
	C *(M)*	14/0.047	13/0.224	5.90 (2.61 – 13.37)	4.26	p < 0.0001
Downstream variants						
PTGS2: 3 Prime UTR variant rs5275 (+8473 T > C)	TT *(WW)*	127/0.847	12/0.414	1.00 (Reference)	–	–
	TC *(WM)*	20/0.133	15/0.517	7.94 (3.25 – 19.40)	4.54	p < 0.0001
	CC *(MM)*	3/0.020	2/0.069	7.06 (1.07 – 46.45)	2.03	0.042
	TC + CC *(WM + MM)*	23/0.153	17/0.586	7.82 (3.30 – 18.53)	4.68	p < 0.0001
	T *(W)*	274/0.913	39/0.672	1.00 (Reference)	–	–
	C *(M)*	26/0.087	19/0.328	5.13 (2.60 – 10.13)	4.72	p < 0.0001
PTGS2: 3 Prime UTR variant, rs689470 (+8365C > T)	CC *(WW)*	115/0.767	27/0.931	1.00 (Reference)	–	–
	CT *(WM)*	31/0.207	1/0.034	0.14 (0.02 – 1.05)	1.91	0.056
	TT *(MM)*	4/0.027	1/0.034	1.06 (0.11 – 9.91)	0.055	0.956
	CT + TT *(WM + MM)*	35/0.233	2/0.069	0.24 (0.06 – 1.07)	1.87	0.062
	C *(W)*	261/0.87	55/0.948	1.00 (Reference)	–	–
	T *(M)*	39/0.13	3/0.052	0.37 (0.11 – 1.22)	1.63	0.103

freq.: frequency; **M:** minor allele; **W:** wild type allele; **OR:** Odds ratio. The observed genotype frequencies in patients were statistically consistent with the Hardy–Weinberg equilibrium expectation (for rs20417, $\chi 2 = 2.42$; df = 1, p = 0.12; for rs5275, $\chi 2 = 0.879$; df = 1, p = 0.349, and for rs689470, $\chi 2 = 2.42$; df = 1, p = 0.12), and in controls (for rs20417, $\chi 2 = 1.53$; df = 1, p = 0.22; for rs5275, $\chi 2 = 3.73$; df = 1, p = 0.054, and for rs689470, $\chi 2 = 1.12$; df = 1, p = 0.29).

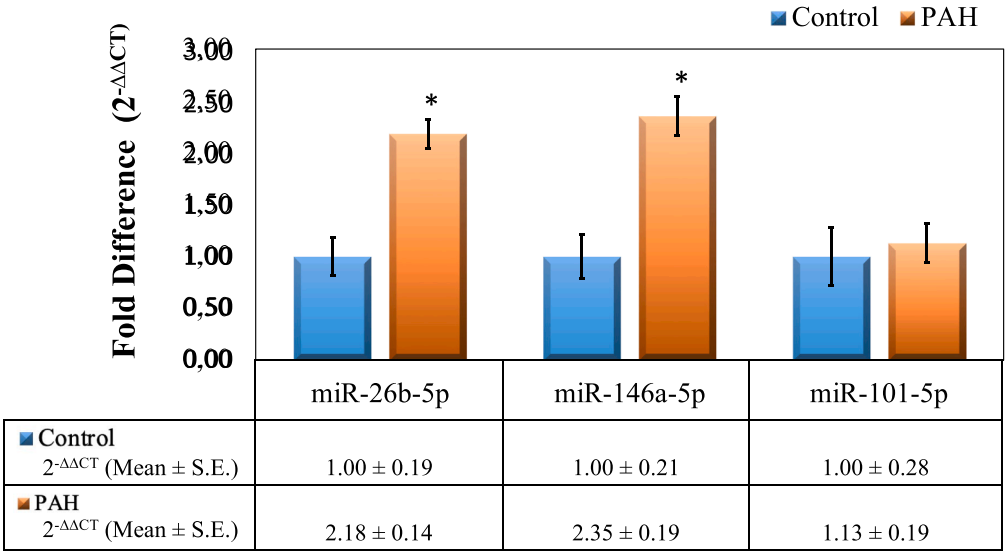

Fig. 1. Normalized relative levels of miRNAs in PAH patients.

we currently propose that these variants contribute to the development of PAH by affecting the expression levels of COX-2. Therefore, one SNP located in the promoter region (rs20417) and two SNPs located in the 3′UTR region (rs5275 and rs689470) were assayed in our study. The non-dominant genotypes of rs20417 (CT + GG) and rs5275 (CC + TC) were found to be related to PAH. In addition, we concluded that the presence of minor alleles in patients with PAH exhibit lower COX-2 activities in a gender-independent manner.

The three miRNAs (miR-26b-5p, miR-101-5p, and miR-146a-5p), which we examined in our study, comprise miRNAs that target the 3′UTR region of the *PTGS2* gene. We found that miR-26b-5p and miR-146a-5p were 2.18-fold and 2.35-fold upregulated in PAH patients, respectively. Consequently, we concluded that upregulated miRNAs may contribute to PAH development by suppressing COX-2 levels. In the current study, the negative correlation finding between miR-26b-5p and miR-146a-5p and COX-2 levels supports our established hypothesis. As PAH is a rare disease, one of general limitations for the clinical studies is the presence of a restricted number of patients. This issue would be hopefully resolved with multi-center studies. Similarly, in a study conducted with nasopharyngeal carcinoma epithelial cells treated with deferoxamine, it was shown that miR-26b suppressed the expression of the protein as a result of its direct association with COX-2 mRNA [17]. It was also found that miR-26b suppresses the expression of COX-2 in breast cancer cells [18]. Consistent with our findings, the findings of the

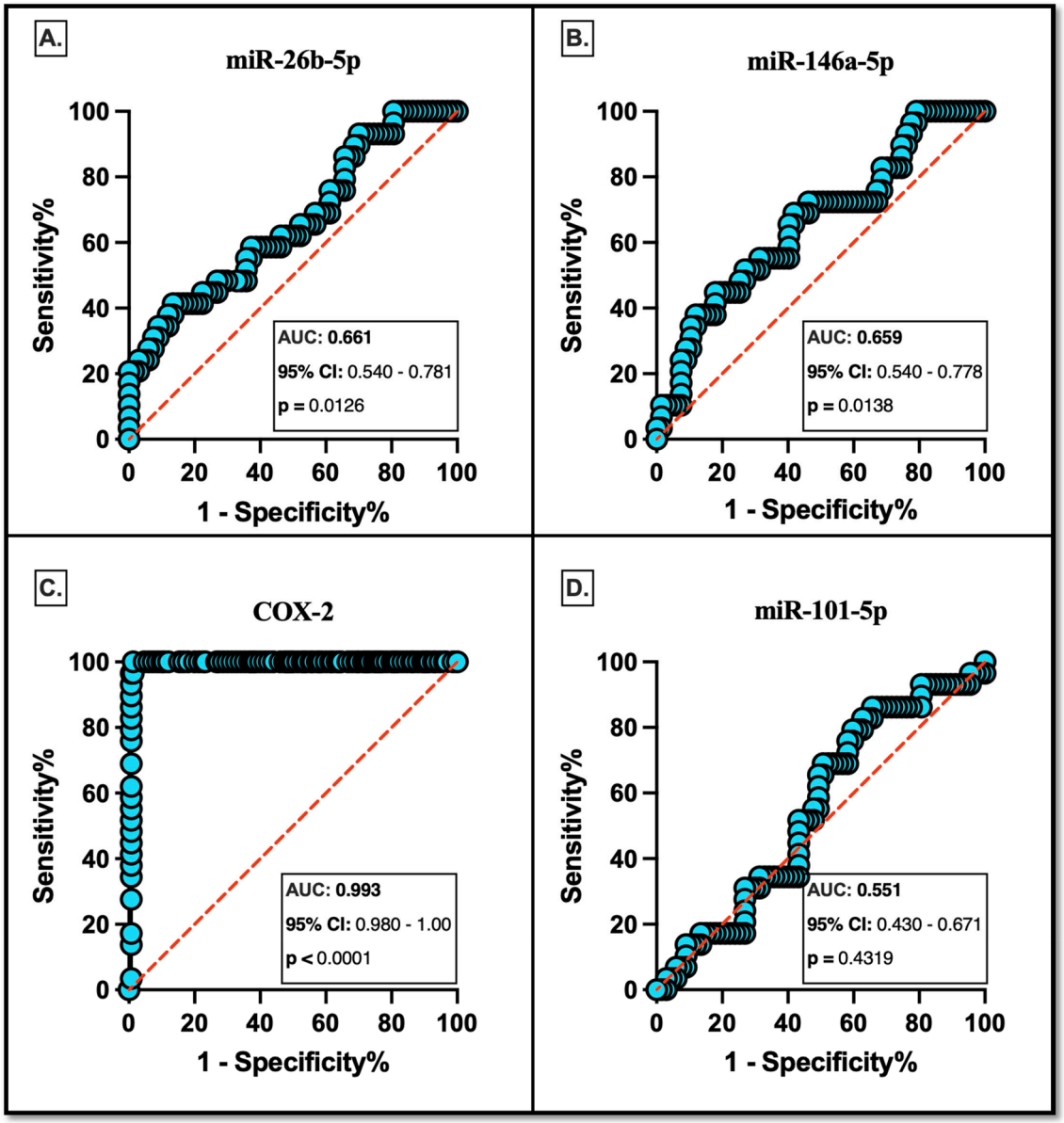

Fig. 2. Comparisons of the sensitivity and specificity of the diagnosis by miR-26b-5p, miR-146a-5p, miR-101-5p, and COX-2 in PAH patients. The ROC curve of **A.** miR-26b-5p; **B.** miR-146a-5p; **C.** COX-2; **D.** miR-101-5p.

Table 3
Univariate and multivariate regression analysis.

	Univariate			Multivariate analysis (Enter Method) $R^2 = 0.898$		
	p	OR	95% CI for OR	p	OR	95% CI for OR
Gender	0.261	0.526	0.171–1.613	0.071	0.060	0.003–1.269
miR-26b-5p	0.010	1.016	1.004–1.029	0.175	0.980	0.953–1.009
miR-146a-5p	0.091	1.003	1.00–1.005	0.250	1.006	0.996–1.015
miR-101-5p	0.683	1.000	1.000–1.00	0.934	1.000	1.000–1.000
COX-2	<0.001	0.551	0.403–0.753	0.002	0.608	0.446–0.829

OR: Odds ratio; **CI:** Confidence interval.

study conducted by Kaneto et al. showed how miR-26 is upregulated in patients with pulmonary hypertension [26].

miR-146a and miR-146b act as negative regulators of inflammatory gene expression in many diverse cells, including monocytes, fibroblast, endothelium, airway smooth muscle, and epithelial cells. In a study with human airway smooth muscle, it was reported that miR-146a mimics diminished expressions of COX-2 and IL-1β in asthma cells. Additionally, the miR-146a inhibitor increases the expression of COX-2 and IL-1β in these groups of cells [14]. In a study conducted on chronic obstructive pulmonary disease (COPD), miR-146a expression was reported to be a trigger by cytokines in the fibroblasts obtained from patients, and it was reported that miR-146a causes COX-2 degradation [27]. In another similar study conducted in the recent years, miR-146a was shown to be upregulated in children with PAH [28].

miR-101 is known as a tumor suppressor miRNA and its suppressive impact on cell proliferation and cell migration in many cancer types, such as prostate, breast, and liver, has been demonstrated in previous studies [29–31]. In an *in vitro* study with gastric cancer cells, it was found that mir-101 inhibits *EXH2*, *COX-2*, *Mcl-1*, and *Fos* gene

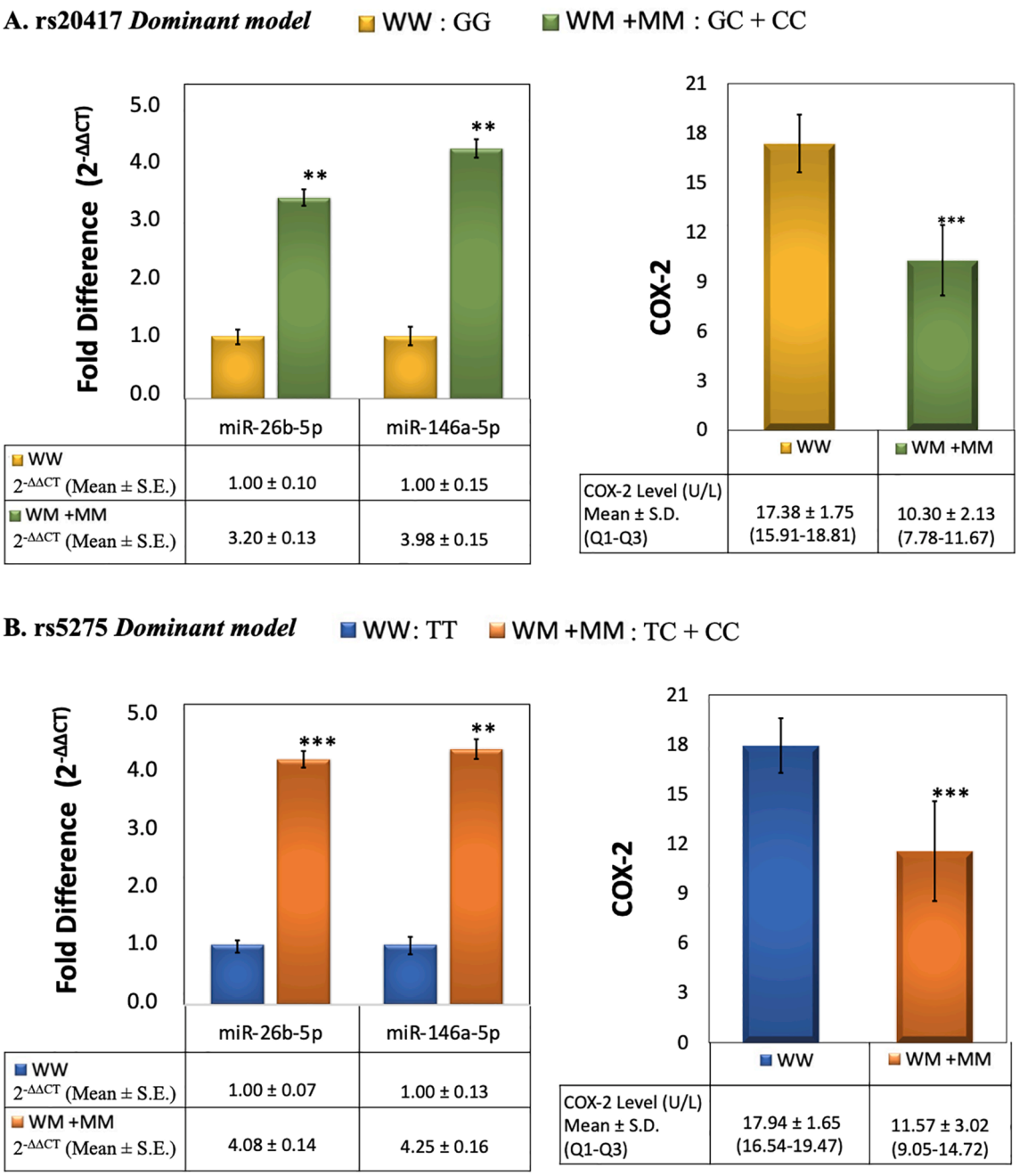

Fig. 3. Normalized relative levels of miR-26b-5p, miR-146a-5p, and COX-2 activities in PAH patients' subgroup according to **A.** Rs20417, **B.** rs5275.

expressions. Consequently, it is thought that miR-101 is able to inhibit the proliferation, migration and invasion of cancer cells [16]. However, in our study, no variation was determined at the miR-101 level.

Selective COX-2 inhibitors and all non-steroidal anti-inflammatory drugs inhibit COX-2 at the therapeutic dose level [32]. Although the patients in our study did not use this group of drugs, drugs, such as Bosentan and Sildenafil, that are used to treat PAH may also cause COX-2 inhibition [19,20]. Therefore, even though COX-2 seems to be an effective biomarker as a result of ROC analysis, we believed that these diminished COX-2 levels may be related to drug-induced effects; therefore, COX-2 may not be used as a reliable marker. In addition to this hypothesis, we thought that the decrease in COX-2 levels may not only be related to conventionally used PAH drugs but also the presence of *PTGS2* minor variants (for rs20417 and rs5275) and the suppressive effect of miR-26b-5p and miR-146a-5p.

Compared to PAH patients with the wild-type genotype, PAH patients with non-dominant genotypes of rs20417 (CT + GG) and rs5275 (CC + TC) exhibited higher levels of both miR-26b-5p and miR-146a-5p as well as diminished COX-2 levels. These findings strengthen our hypothesis that both SNPs and miRNAs are involved in COX-2 activation. According to the results of ROC analysis, we concluded that miR-26b-5p and miR-146a-5p may be promising biomarkers, even though they are not perfect. Considering the deleterious cardiovascular effects of COX-2 inhibitors, we thought that the presence of both minor alleles and miRNA levels may be promising biomarkers in PAH. In addition, other COX-2 downregulation-related miRNAs, such as miR-143, miR-30a, miR-1297, miR-216a, miR-16, and miR-144 [33], need to be assayed for further PAH studies in order to elucidate the mechanism of COX-2 inhibition and their diagnostic or prognostic biomarker effectiveness.

As conventional drug therapies may cause lower COX-2 levels, the development of new genetic or epigenetic biomarkers is crucially important for early diagnosis and prognosis. Increased expression of

some miRNAs and miRNA-related gene variations may also affect COX-2 levels. Our study originally indicates the presence of minor alleles for rs5275 and rs20417 as an important risk factor for the development of PAH. Our current findings need to be re-evaluated with different perspectives in multi-centered cohort studies. In addition, locus-specific miRNAs, such as miR-26b-5p and miR-146a-5p, seem to play a critical role in the regulation of *PTGS2* expression. ROC analysis and logistic regression analysis indicate that COX-2 could be considered as a highly valuable biomarker for PAH. We also concluded that miR-26b-5p and miR-146a-5p was associated with the *PTGS2* gene expression, and it may be a novel target for new therapeutics. It is a widely known fact that selective COX-2 inhibitors and all non-steroidal anti-inflammatory drugs inhibit COX-2 with the therapeutic dose. Therefore, we concluded that COX-2 levels might be partially used as a diagnostic biomarker in PAH patients. On the other hand, the clarification of medication-independent factors in terms of both COX-2 and our newly suggested biomarkers, such as miR-26b-5p and miR-146a-5p, needs to be further studied in future projects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2022.06.001.

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