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ORIGINAL ARTICLE

# Genetic variants in a Polish population of patients with pulmonary arterial hypertension: sequencing of BMPR2, ALK1, and ENG genes

Barbara Uznańska-Loch[1], Kamil Wikło[1], Dominika Kulczycka-Wojdala[2], Bożena Szymańska[2], Łukasz Chrzanowski[1], Karina Wierzbowska-Drabik[1], Ewa Trzos[1], Jarosław D. Kasprzak[1], Małgorzata Kurpesa[1]

[1]Chair and Department of Cardiology, Medical University of Lodz, Bieganski Hospital, Lodz, Poland
[2]The Central Scientific Laboratory of Medical University in Lodz (CoreLab), Lodz, Poland

## Abstract

**Background:** Pulmonary arterial hypertension (PAH) is a rare disease with a very serious prognosis. It seems that mutations in genes related to transforming growth factor-$\beta$ signalling pathway are often related to the development of the disease. No study covers this problem in a Polish population.

**Aim:** To screen for genetic mutations in a Polish cohort of patients with pulmonary hypertension, especially with idiopathic PAH, treated in a single hospital in Poland.

**Methods:** DNA sequencing method was used. Samples from 50 patients with pulmonary hypertension were screened for mutations in type 2 bone morphogenetic protein receptor of the transforming growth factor-$\beta$ superfamily gene (BMPR2). Samples from 20 patients with idiopathic PAH (11 men, mean age 55 years) were also screened for mutations in activin A receptor-like type 1 gene (ALK1) and endoglin gene (ENG).

**Results:** No genetic variations were found for the BMPR2 gene. In all 20 samples from idiopathic pulmonary hypertension patients we found heterozygosity of single nucleotide polymorphism (SNP) rs 372023206 in ALK1 gene. Three samples from these patients showed variations of ENG gene: we found one sample with heterozygosity of SNP rs 200525684, one with heterozygosity of SNP rs 3739817, and one with both.

**Conclusions:** We detected benign polymorphisms or genetic variants of unknown importance. It is possible that the Polish population of PAH patients differs from the previously described populations of other countries in terms of the frequency and importance of mutations in BMPR2, ALK1 and ENG genes.

**Key words:** genetic mutations, pulmonary arterial hypertension, BMPR2 gene, ALK1 gene, ENG gene

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## INTRODUCTION

Pulmonary arterial hypertension (PAH) is a rare disease with a very serious prognosis, comparable to the prognosis in cancer. PAH is characterised by pre-capillary pulmonary hypertension in a haemodynamic examination (the mean pressure in the pulmonary artery ≥ 25 mmHg, pulmonary wedge pressure ≤ 15 mmHg, vascular pulmonary resistance > 3 Wood units) [1]. There are various forms of PAH (including sporadic and heritable) with a similar clinical picture and with pathomorphological changes in pulmonary circulation. It is estimated that about 50% of PAH cases are sporadic forms, where the cause remains unknown. However, there are no clear differences in the course of this disease in comparison with heritable PAH. It can be presumed that both forms involve de novo mutations of the same genes or genes that perform similar functions.

In patients with a family history of PAH, mutations can be identified in 75% of cases.

Most cases of heritable PAH are associated with heterozygous mutations in the BMPR2 gene, which denotes type

**Address for correspondence:**
Barbara Uznańska-Loch, MD, PhD, Chair and Department of Cardiology, Medical University of Lodz, Bieganski Hospital, ul. Kniaziewicza 1/5, 91–347 Łódź, Poland, e-mail: tyhe@op.pl

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Table 1. The scheme for genetic testing

| The sequenced genes | Idiopathic PAH | Eisenmenger's syndrome | Secondary PH |
|---|---|---|---|
| BMPR2 | Yes | Yes | Yes |
| ALK1 | Yes | Not done | Not done |
| ENG | Yes | Not done | Not done |

ALK1 — activin A receptor-like type 1 gene; BMPR2 — type 2 bone morphogenetic protein receptor; ENG — endoglin gene; PAH — pulmonary arterial hypertension; PH — pulmonary hypertension

2 bone morphogenetic protein receptor of the transforming growth factor-$\beta$ (TGF-$\beta$) cell signalling superfamily. In sporadic cases, mutations of this gene were found in between 11% and 40% of patients [2]. BMPR2 signalling influences many significant cellular processes, such as proliferation and apoptosis, differentiation, and migration.

It was possible to connect nine other genes with PAH occurring in certain diseases. In hereditary haemorrhagic telangiectasia, patients with PAH most often had mutations of the activin A receptor-like type 1 gene (ACVRL1 or ALK1). Another gene in which mutations were shown is the endoglin (ENG) gene [3].

Products of these three genes are part of the TGF-$\beta$ signalling pathway.

Pulmonary arterial hypertension is more frequent in trisomy 21, but Down syndrome is often connected with congenital heart defects, which can lead to pulmonary hypertension on their own.

Genetic testing is time-consuming and expensive, and, for the time being, its results do not affect the treatment strategy. Therefore, currently genetic screening is not conducted on a large scale. So far it has not been performed in a Polish population of patients with pulmonary hypertension. In this study we present the results of DNA sequencing in 50 patients treated in a single cardiac clinic in central Poland.

## METHODS

Twenty patients with idiopathic PAH (IPAH), i.e. with PAH type 1.1 according to classification of the European Society of Cardiology (ESC), were included in the main study group.

Group 2 consisted of 10 patients with PAH, but with the aetiology of Eisenmenger's syndrome in the course of various congenital heart diseases, i.e. with pulmonary hypertension type 1.1.4 according to the ESC.

Group 3 consisted of 20 patients with pulmonary hypertension secondary to heart failure with impaired left ventricular ejection fraction (LVEF), i.e. with pulmonary hypertension type 2.1. This group served as a control group, in which the results were expected to be similar to those in the general population. Due to the lack of data regarding the frequency of BMPR2 gene mutations in the Polish population, we planned to compare possible abnormal findings from PAH patients with this control group.

The diagnoses were made in the reference centre by cardiologists with experience in treating pulmonary hypertension, in accordance with the ESC standards. All the patients gave informed written consent to undergo genetic testing. The study protocol was accepted by the local Ethics Committee.

Peripheral blood was collected on ethylenediamine tetraacetic acid (EDTA) anticoagulant and stored at –80°C.

To isolate genomic DNA, a GeneMatrix Quick Blood DNA Purification Kit (EURx, Gdańsk, Poland) was used, together with EURx silica membranes, in accordance with the manufacturer's protocol. DNA quality and concentration were evaluated spectrophotometrically by means of the Picodrop spectrophotometer (Picodrop Limited, Hinxton, United Kingdom). The isolated and purified DNA was stored at –20°C.

The scheme for genetic testing is presented in Table 1.

DNA sequencing was performed for protein-coding exons. Since primers encase larger fragments than exons, it was also possible to sequence parts of intron areas adjacent to exons.

### *Analysis of the BMPR2 gene*

All the exons coding for the BMPR2 gene were amplified in a polymerase chain reaction (PCR). The DNA isolated from the material under test underwent PCR, using 17 pairs of primers for the BMPR2 gene. The BMPR2 gene consists of 13 protein coding exons. Each of them was amplified by one pair of primers, except for exon 12. Owing to its length, this exon was amplified in five separate PCRs, which produced amplicons of approximately 350 base pairs (bp). The amplification was carried out by means of Promega GoTaq® G2 Flexi DNA polymerase in a GeneAmp PCR System 9700 thermocycler (Thermo Fisher Scientific, Waltham, MA, USA).

The PCR took place in 20 $\mu$L of reaction mixture. We used 50 ng of the matrix, 10 pmol of primers, 7.5 nmol of dNTP, MgCl2 at the concentration of 1.5 mM, and one unit of polymerase. Upon completion of the reaction, the resulting products were visualised by means of a Shimadzu (Kyoto, Japan) MultiNA microchip electrophoresis system. Before sequencing, the PCR products were purified using a EURx PCR/DNA Clean-Up Purification Kit (EURx, Gdańsk, Poland). The purified PCR products were sequenced using the Big Dye® Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). The same primers were

Table 2. Type 2 bone morphogenetic protein receptor (BMPR2) primers

| Primers | 5´→3´ | The length of the PCR product |
|---|---|---|
| BMPR2Ex1 for | GCATGAAAGCTCTGCAGCTA | 419 base pairs |
| BMPR2Ex1 rev | ATTTCCCTGGAAGGCATGG | |
| BMPR2Ex2 for | GTCATTCGGATAAGACAAAG | 335 bp |
| BMPR2Ex2 rev | TTTAACATACTCCCATGTCC | |
| BMPR2Ex3 for | TAGCTTACACGTACTCTCAC | 374 bp |
| BMPR2Ex3 rev | CCTGGCTTCAACCTTGAATG | |
| BMPR2Ex4 for | GGGTACAGCCTTTCTAAAGG | 335 bp |
| BMPR2Ex4 rev | GATACTATTGAGGCTGGGTG | |
| BMPR2Ex5 for | GCTGCTAATCTTTCTGCAGC | 273 bp |
| BMPR2Ex5 rev | GAATGAAGTCACTGTTCCAG | |
| BMPR2Ex6 for | CAGAGAGCTGTAGCATTCTG | 426 bp |
| BMPR2Ex6 rev | AAGTGATCCACCTGCCTTAG | |
| BMPR2Ex7 for | CGCATTTTTTCCTCTATA | 285 bp |
| BMPR2Ex7 rev | TGCATTTCATGCTGAATCTTTC | |
| BMPR2Ex8 for | GCAGAAAAATAATACTACTTCTATA | 319 bp |
| BMPR2Ex8 rev | GATGTTTTAATTAAATTATCATTTC | |
| BMPR2Ex9 for | AGAATATGCTACGTTCTCTC | 336 bp |
| BMPR2Ex9 rev | ACACTAGATAGCAATGAACTAAAGG | |
| BMPR2Ex10 for | GCCTGAAGGGGATGAAAAA | 388 bp |
| BMPR2Ex10 rev | GGCATTAGGCAACTCCAAAA | |
| BMPR2Ex11for | CCTCATGTGGTAAACTGAAAAGC | 436 bp |
| BMPR2Ex11 rev | TGCACTTGTACCAAACAAAAATG | |
| BMPR2Ex12_1 for | AAATTTGGAGAGACAGTTTGTCA | 391 bp |
| BMPR2Ex12_1 rev | TTCATCTGGGTATGGCATCTC | |
| BMPR2Ex12_2 for | AGCAAGCACAAGCTCGAATC | 393 bp |
| BMPR2Ex12_2 rev | AGTCCTGCTGTCCAGTTGCT | |
| BMPR2Ex12_3 for | TCTAGCTTGCTTTACCCACT | 351 bp |
| BMPR2Ex12_3 rev | AGCATGGGAGTTAACACTGT | |
| BMPR2Ex12_4 for | ACCTCATGTGGTGACAGTCA | 310 bp |
| BMPR2Ex 12_4 rev | ATTGGAATTAGTTCGGCCAC | |
| BMPR2Ex12_5 for | ATTCCAGTCCTGATGAGCAT | 343 bp |
| BMPR2Ex12_5 rev | AGTTATTTAAATGGCCCCAA | |
| BMPR2Ex13 for | TTACATCCCTTACCCGTTAT | 454 bp |
| BMPR2Ex13 rev | TTAAAGCAAGTCTTTGTTGC | |

PCR — polymerase chain reaction

used for the reaction and preamplification. The sequencing PCR was performed under the following conditions: 10 $\mu$L of the reaction mixture contained modified deoxynucleotides (the so-called dideoxy terminators), nucleotides, primers, water, and the DNA matrix. The samples were placed in the GeneAmp PCR System 9700 thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). The post-sequencing purification was performed using a BigDye XTerminator Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). The purified products of the sequencing reaction were separated in a 3130xl Genetic Analyzer capillary sequencer (Thermo Fisher Scientific, Waltham, MA, USA). The samples were analysed by means of a DNA Baser Sequence Assembler (Thermo Fisher Scientific, Waltham, MA, USA).

The BMPR2 primers are shown in Table 2.

### *Analysis of the ALK1 and ENG*

We analysed exons 2 to 10 of the ALK1 gene (since the first exon has not been shown to encode any protein) and 14 exons of the ENG gene. Amplification of the DNA of the protein-coding regions was performed using primers for the ALK1 and ENG genes, designed by means of Oligo Explorer

Table 3. Primers used for the polymerase chain reaction (PCR) of activin A receptor-like type 1 gene (ALK1)

| Primers | Sequence |
|---|---|
| ALK1_ForPCR0 | 5′ CTCTGTGATTTCCTCTGGGCA 3′ |
| ALK1_RevPCR0 | 5′ TACATTCTCCCCAGCT1TCTCAA 3′ |
| ALK1_ForPCR1 | 5′ TCAGACGAGAGGGACAGTAG 3′ |
| ALK1_RevPCR1 | 5′ GTAGCCAAAAACTCCCTCAC 3′ |
| ALK1_ForPCR2 | 5′ CTGGGTTTGGGTCTGGATTA 3′ |
| ALK1_RevPCR2 | 5′ CTGGGAAACATAGGGAGACC 3′ |
| ALK1_ForPCR3 | 5′ TCTCTGACCTAAGCCACATC 3′ |
| ALK1_RevPCR3 | 5′ GCTGATTCCCCTTTCCCTAC 3′ |
| ALK1_ForPCR4 | 5′ GGATAGAGGGTAGAAAAGGC 3′ |
| ALK1_RevPCR4 | 5′ CAGGGTTGAAAGAGGGAGTA 3′ |
| ALK1_ForPCR5 | 5′ GTCATCCTCTGTTCTCTCTC 3′ |
| ALK1_RevPCR5 | 5′ CTGTATTTTTGGCTGGGTGG 3′ |

Table 4. Primers used for the polymerase chain reaction (PCR) of endoglin gene (ENG)

| Primers | Sequence |
|---|---|
| ENGPCR1F | TGTGTCCACTTCTCCTGACC |
| ENGPCR1R | AAAGAGTCAGCCCAGTTTGC |
| ENGPCR2F | TAGTAGCGCTGTGACCTTGG |
| ENGPCR2R | TAACGAGGACTCAGCCACTG |
| ENGPCR3F | GGAGAGTGGAGTGGAAGCAT |
| ENGPCR3R | CATCAACCTGACTCCCACCT |
| ENGPCR4F | AGAAGGGGCTGATCTGACTG |
| ENGPCR4R | TTGTGGCATGTGAACTGTGG |
| ENGPCR5F | CCTGCCCCACCACTATCTTT |
| ENGPCR5R | AGAGGTTGGGAGTTTGGGTT |
| ENGPCR6F | ACCTATGCCCATACGTGAGG |
| ENGPCR6R | TCCATGTCATCCTGAGCCAG |
| ENGPCR7F | GAATGGCTGTGACTTGGGAC |
| ENGPCR7R | TGCTCCGGTCATACAGAAGG |
| ENGPCR8F | AACTCCACAGGGCCATGATG |
| ENGPCR8R | CATGTCCCTTCCTGCAAACC |
| ENGPCR9F | CCTCCCACAGCACAGATCTT |
| ENGPCR9R | GGCTCTCCATCTGCAAAGTG |
| ENGPCR10F | GGTTCAGAGAAGTCGAGGGT |
| ENGPCR10R | CTATGCCATGCTGCTGGTG |

1.2 software (Gene Link, Inc., New York, USA). The primers used are presented in Tables 3 and 4.

The PCR for the ALK1 gene with PCR0 and PCR4 primers and for the ENG gene took place in 20 $\mu$L of reaction mixture. We used 50 ng of the matrix, 10 pmol of primers, 7.5 nmol of dNTP, MgCl2 at the concentration of 1.5 mM, and one unit of polymerase. Additionally, the PCR for the ALK1 gene with PCR1 and PCR3 primers contained the PCR enhancer (5xGC Additive; DNA Gdańsk); in the PCR with PCR2 primers we used MgCl2 at the concentration of 1.0 mM and the PCR enhancer 5xGC Additive. The amplification took place in the GeneAmp PCR System 9700 thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). Upon completion of the reaction, the resulting products were visualised using a Shimadzu MultiNA microchip electrophoresis system. The purification of the PCR product was carried out by means of the PCR/DNA Clean-Up Purification Kit (EURx, Gdańsk, Poland). The purified PCR products were sequenced using the appropriate for-primers (in one direction). The second thread was sequenced selectively in order to confirm a positive result or to exclude artefacts. The sequencing PCR was carried out in 10 $\mu$L by means of the Big Dye® Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). The primers used for ALK1 are presented in Table 5 [4], and the primers used in ENG sequencing are presented in Table 6 [5].

The reaction took place in a GeneAmp PCR System 9700 thermocycler. The post-sequencing purification was performed by means of the BigDye XTerminator Purification Kit. The purified products of the sequencing reaction were separated in a 3130xl Genetic Analyzer capillary sequencer (Thermo Fisher Scientific, Waltham, MA, USA). The samples were analysed using the DNA Baser Sequence Assembler.

Table 5. Primers used for activin A receptor-like type 1 gene (ALK1) sequencing

| Primers | Sequence |
|---|---|
| ALK1_2For | 5′ CTCTGTGATTTCCTCTGGGCA 3′ |
| ALK1_2Rev | 5′ TACATTCTCCCCAGCT1TCTCAA 3′ |
| ALK1_3For | 5′ AGCTGGGACCACAGTGGCTGA 3′ |
| ALK1_3Rev | 5′ GGAGGGCAGGGGCCAAGAAGAT 3′ |
| ALK1_4For | 5′ AGCTGACCTAGTGGAAGCTGA 3′ |
| ALK1_4Rev | 5′ CTGATTCTGCAGTTCCTATCTG 3′ |
| ALK1_5For | 5′ AGGAGCTTGCAGTGACCCAGCA 3′ |
| ALK1_5Rev | 5′ ATGAGAGCCCTTGGTCCTCATCCA 3′ |
| ALK1_6For | 5′ AGGCAGCGCAGCATCAAGAT 3′ |
| ALK1_6Rev | 5′ AAACTTGAGCCCTGAGTGCAG 3′ |
| ALK1_7For | 5′ TGACGACTCCAGCCTCCCTTAG 3′ |
| ALK1_7Rev | 5′ CAAGCTCCGCCCACCTGTGAA 3′ |
| ALK1_8For | 5′ AGGTTTGGGAGAGGGGCAGGAGT 3′ |
| ALK1_8Rev | 5′ GGCTCCACAGGCTGATTCCCCTT 3′ |
| ALK1_9For | 5′ TCCTCTGGGTGGTATTGGGCCTC 3′ |
| ALK1_9Rev | 5′ CAGAAATCCCAGCCATGAGCCAC 3′ |
| ALK1_10For | 5′ TCTCCTCTGCACCTCTCTCCCAA 3′ |
| ALK1_10Rev | 5′ CTGCAGGCAGAAAGGAATCAGGTGCT 3′ |

Table 6. Primers used for endoglin gene (ENG) sequencing

| Primers | Sequence |
|---|---|
| ENGex1F | ACTGGACACAGGATAAGGCCCAGC |
| ENGex2F | CACCTTATTCTCACCTGGCCTCTT |
| ENGex3F | GGGTGGCACAACCTATACAAAT |
| ENGex4F | CTACATGGGATAGAGAGGGCAC |
| ENGex5F | TGAGGGAAGGGACTGAGGTGC |
| ENGex6F | GGCCTGTCCGCTTCAGTGTT |
| ENGex7F | CCCCCTGTTCTGCCTCTCTC |
| ENGex8F | ACACAGTGATCACACAGTGACCA |
| ENGex9F | CTCCTGATGGTGCCCCTCTCTTC |
| ENGex10F | CTGCAGGGGCTCAGAACACA |
| ENGex11F | ATTGACCAAGTCTCCCTCCC |
| ENGex12F | GGTGGGGTGAAGAGCAGCTG |
| ENGex13F | GAGTAAACCTGGAAGCCGC |
| ENGex14F | CCAGCACAACAGGGTAGGGGAT |

## RESULTS

### *Characteristics of patients from whom DNA samples were collected*

The main group with IPAH comprised 20 people, including 11 men. The mean age was 55 (range 22–82) years. Mean LVEF was 55.2%. All patients had undisputed evidence of PAH confirmed by right heart catheterisation. At the time of inclusion into the study group, the patients had already been under treatment for a minimum of two months, according to the National Health Fund medication programme.

Group 2 comprised 10 people, including six men, with Eisenmenger's syndrome. The mean age was 42 (range 26–68) years. Mean LVEF was 56.44%. At the time of inclusion into the study group, the patients had already been under specific treatment for a minimum of one month. Table 7 presents the characteristics of the congenital heart defects that caused the appearance of PAH in these patients.

Group 3 comprised 20 people, including 17 men, with pulmonary hypertension secondary to heart failure due to left ventricular dysfunction. The mean age was 61 (range 31–75) years. In 18 patients, left ventricular dysfunction was caused by coronary artery disease and in two patients by dilated cardiomyopathy. Mean LVEF was 29.45%. In three patients LVEF was between 40% and 44%, which classified them into the category of heart failure with mid-range ejection fraction; 17 patients had LVEF ranging between 15% and 36%, which means they were placed in the category of heart failure with reduced ejection fraction, according to the latest ESC guidelines [6].

### *Results of DNA sequencing*

**The BMPR2 gene.** Comparing the results of DNA sequencing for the BMPR2 gene with the reference sequence showed no differences. In all the 50 samples tested, the investigated sequence corresponded to the reference sequence.

**The ALK1 gene.** Comparing the results of DNA sequencing for the ALK1 gene with the reference sequence showed the same difference in all the 20 samples tested. There was heterozygosity within the tenth exon — a variant classified as single nucleotide polymorphism (SNP) rs 372023206. This polymorphism consists in changing guanine to adenine, which results in the GTG to GTA codon change; both versions encoding valine.

**The ENG gene.** Twenty samples were analysed.

In two samples (number 4 and number 12) we discovered heterozygous polymorphism SNP rs 3739817 within the eighth exon. This polymorphism consists in the ACC to ACT codon change; both versions encoding threonine.

In two samples (number 12 and number 17) we discovered heterozygous polymorphism SNP rs 200525684 in position 9:127824773, in the intron area adjacent to the seventh exon. This polymorphism consists in changing the

Table 7. Patients with Eisenmenger's syndrome — pulmonary arterial hypertension secondary to congenital heart defect (group 2)

| Sample | Sex | Age | Congenital heart defect |
|---|---|---|---|
| 1 | Male | 47 | Ostium secundum atrial septal defect |
| 2 | Female | 68 | Ventricular septal defect |
| 3 | Male | 35 | Common atrioventricular canal (trisomy of the 21st chromosome) |
| 4 | Male | 41 | Ventricular septal defect |
| 5 | Male | 27 | Ostium secundum atrial septal defect, patent ductus arteriosus |
| 6 | Female | 26 | Mitral atresia, hypoplastic left ventricle, double ventricular septal defect, atrial septal defect, patent ductus arteriosus |
| 7 | Female | 35 | Subaortic ventricular septal defect, overriding aorta, subvalvular right ventricular outflow tract obstruction |
| 8 | Male | 27 | Common atrioventricular canal (trisomy of the 21st chromosome) |
| 9 | Female | 55 | Ventricular septal defect |
| 10 | Male | 60 | Ostium secundum atrial septal defect |

Table 8. Patients with idiopathic pulmonary arterial hypertension (group 1)

| Sample | Sex | Age | DNA concentration [ng/μL] | BMPR2 | ALK1 | ENG |
|---|---|---|---|---|---|---|
| 1 | Female | 28 | 68.4 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 2 | Male | 32 | 53.4 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 3 | Male | 31 | 57 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 4 | Male | 56 | 51.7 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Heterozygosity of SNP rs 3739817 |
| 5 | Male | 82 | 43.7 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 6 | Female | 40 | 24.2 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 7 | Male | 22 | 37.1 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 8 | Female | 44 | 76 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 9 | Female | 57 | 25.7 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 10 | Female | 61 | 36.9 | Like reference sequence | heterozygosity of SNP rs 372023206 | Like reference sequence |
| 11 | Male | 78 | 39.9 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 12 | Male | 63 | 55.2 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Heterozygosity of SNP rs 200525684 and SNP rs 3739817 |
| 13 | Male | 36 | 46.3 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 14 | Male | 63 | 27.2 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 15 | Female | 69 | 41.7 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 16 | Female | 82 | 44.4 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 17 | Female | 79 | 40.8 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Heterozygosity of SNP rs 200525684 |
| 18 | Female | 63 | 57.8 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 19 | Male | 51 | 49.1 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |
| 20 | Male | 60 | 46 | Like reference sequence | Heterozygosity of SNP rs 372023206 | Like reference sequence |

SNP — single nucleotide polymorphism; other abbreviations — see Table 1

nucleotide thymine to cytosine. In the reference database this polymorphism was described for a complementary strand as a change from adenine (A) to guanine (G).

Results of the analysis of the BMPR2, ALK1, and ENG genes in the samples from patients with IPAH are presented in Table 8.

## DISCUSSION

To the best of our knowledge, this is the first such large, systematically performed genetic study of pulmonary hypertension patients in a Polish population, in which the sequencing of genome DNA was used.

The main study group in our study included 20 patients with IPAH. Although we had no data on the heritable nature of the disease in the family history, we expected to find at least a few sporadic pathogenic mutations. The results of the genetic analysis are surprising. In the DNA samples from the IPAH patients we did not find a single unequivocally pathogenic mutation. None of the following mutation types were found: nonsense, missense, frameshift, or deletion.

In the case of the BMPR2 gene, the analysis involving the IPAH group and additionally involving a group of patients with Eisenmenger's syndrome and a group of patients with left ventricular dysfunction and secondary pulmonary hypertension did not show any differences between the DNA from the samples and the reference sequence from the database. Our results are different from the literature. The latter mentions 668 variants underlying PAH, which places the BMPR2 gene as the most important gene in the pathogenesis of heritable PAH, and in many cases treated earlier as idiopathic [7]. As mentioned before, in the case of sporadic PAH the frequency of the mutation of the BMPR2 gene is estimated at between 11% and 40%, whereas in hereditary PAH mutations are discovered in as many as 75% to 80% of patients. We have not found a Polish study that would cover this problem. In a study by German researchers, mutation of the BMPR2 gene was discovered in 11 out of 99 patients, and the authors emphasised that this was a smaller percentage than described for North-European or Japanese populations [8].

In 2007 a Polish study including 22 patients with IPAH, 13 patients with Eisenmenger's syndrome, and 10 healthy subjects was published [9]. However, the methodology used in that study was different. The researchers isolated RNA from the leukocytes of peripheral blood and evaluated the expression levels of TGF-$\beta$1 and its receptor genes. In that study, TGF-$\beta$1 expression was similar in the IPAH group and in the controls, but lower in patients with Eisenmenger's syndrome. Expression of the two types of receptors for the TGF-$\beta$1 was higher in IPAH patients, whereas in Eisenmenger's syndrome patients, expression of all the three types of receptors was more intense. Therefore, the authors suggested that cytokines play a significant role in the pathogenesis of pulmonary hypertension.

Under proper conditions proteins of some types of BMP — which are ligands of BMPR2 and the above-mentioned TGF-$\beta$1 — inhibit the proliferation of smooth muscle cells. There have been reports, however, of altered responses to TGF-$\beta$1 and to BMP-2, BMP-4, and BMP-7 in smooth muscle cells isolated from the pulmonary arteries of patients with primary pulmonary hypertension [10]. The results obtained in the material from six patients with primary pulmonary hypertension were different from those in the material from five patients with pulmonary hypertension secondary to the congenital heart defect, and from nine control samples. In PAH patients, a resistance to the antiproliferative effect of the TGF-$\beta$1 and BMP proteins was demonstrated, which the authors connected with gene mutations, a dysfunction of the BMPR2 protein, or dysfunction of the closely linked signalling pathways. The mutations of BMPR2 gene were, however, detected only in one out of the five samples from a patient with the sporadic form of PAH.

In the case of the ALK1 gene, heterozygous SNP rs 372023206 was observed in all the samples in the IPAH group. A change of the nitrogen base of the third nucleotide from the codon, in this case a change from guanine (G) to adenine (A), did not change the encoded amino acid. The reference sequence and the SNP rs 372023206 variant both encode valine, so the change of the sequence does not affect the structure of the protein. Logically speaking, such a change cannot be considered a pathogenicity. Also, in the clinical database the significance of the variant is described as unknown.

In the case of the ENG gene, differences between the sample DNA and the reference sequence were detected in three samples. In two samples we detected heterozygous polymorphism SNP rs 3739817. The reference sequence and the SNP rs 3739817 variant both encode threonine, therefore also in this case the change of the sequence has no influence on the structure of the protein. This polymorphism is described as mild or probably mild. Additionally, a single nucleotide variation in an intron was discovered in two samples. The clinical significance of this change is not known.

A study by Pousada et al. [5] examined 29 patients with IPAH (including nine men) — in seven of them a mutation of the ENG gene was found. Another group included 28 patients with associated PAH, and here mutations were also fairly frequent: in the course of connective tissue disease (mutation in four out of 19 patients), of portopulmonary hypertension (mutation in three out of five patients) and of a HIV infection (in two out of four patients). Those researchers analysed also 55 control samples taken from healthy subjects and did not find any mutations of the ENG gene. Moreover, the authors identified five patients in the PAH group, who had mutations also of other genes: two patients had mutations of the BMPR2 gene, classified as pathogenic, one patient had a different mutation of the BMPR2 gene, and two other patients had mutations of the ALK1 gene. In comparison to our results, such a large number of mutations discovered in their study is surprising. Additionally, the authors observed that the presence of an ENG mutation was associated with the appearance of symptoms of the disease eight years earlier than in patients without this mutation. In another study, the team of Girerd et al. [11] suggested a link between the presence of an ALK1 mutation and the PAH symptoms appearing earlier, in comparison with patients carrying a BMPR2 mutation and those without it.

Of course, the sequencing method we used has its limitations. The present study concerned only the protein coding regions, i.e. regions undergoing translation. We could not exclude the presence of mutations in introns or promoter regions. Owing to equipment limitations (length of the capillaries, polymer) in the CoreLab, we could use the capillary sequencer to divide short sections of only about 500 bp. We could not use other methods of searching for possible mutations, such as multiplex ligation-dependent probe amplification — the method used for detecting large mutations — or next-generation sequencing — the method which enables full sequencing, including also non-coding segments, where mutations are often found.

For our analysis we have chosen three genes in which mutations connected with/linked to pulmonary hypertension seem to occur most frequently. It must be added, however, that pathogenic mutations were detected in many other genes, which we did not study in this project.

The results may have also been influenced by the single-centre design of the study.

To sum up, in our study the material obtained from 20 patients with IPAH revealed only unidentified, and in most cases probably mild, polymorphisms. It is possible that the importance of mutations of individual genes and the role of pathogenic mechanisms in the Polish population of PAH patients are different than in the previously described populations of other countries. Our results should encourage further research in this area.

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