ORIGINAL ARTICLES

Mutations in the Gene Encoding Bone Morphogenetic Protein Receptor 2 in Patients With Idiopathic Pulmonary Arterial Hypertension

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OBJECTIVE: Pulmonary arterial hypertension (PAH) is a rare disease that can have a familial component. It has been shown that more than 50% of cases of familial PAH are associated with mutations in the gene encoding bone morphogenetic protein receptor 2 (BMPR2), which acts as a receptor for members of the transforming growth factor β, superfamily. Some studies in patients with idiopathic PAH have also shown varying percentages of mutations in this gene. The aim of this study was to determine the frequency of these mutations in a group of patients with idiopathic PAH.

PATIENTS AND METHODS: The study population included patients with idiopathic PAH who were seen during 2006 in our unit specialized in this entity. Patients were excluded if they had relatives who had been diagnosed with PAH or who had symptoms that led to suspicion of the disease. Diagnosis was obtained according to the protocol used in our unit. A hemodynamic study was carried out in all cases and patients were included if they had a mean pulmonary arterial pressure of greater than 25 mm Hg. DNA was extracted from peripheral leukocytes and amplified by polymerase chain reaction. Seventeen primer pairs were used for the 13 exons that make up the gene. Using the single strand conformational polymorphism (SSCP) technique we detected anomalous DNA fragments for subsequent sequencing.

RESULTS: The study included 8 patients (4 women). In 5 patients, no abnormalities were observed, whereas in the remaining 3, anomalous electrophoresis patterns were obtained in the SSCP and sequencing revealed mutations. In 1 case, 2 different electrophoresis patterns were observed by SSCP, but it was only possible to sequence 1 of them due to the low concentration of DNA obtained.

CONCLUSIONS: The presence of mutations in the gene encoding BMPR2 is not infrequent in patients with idiopathic PAH, suggesting that this family of growth factors may be important in the pathogenesis of the disease and could have therapeutic implications.

Key words: *Pulmonary arterial hypertension. BMPR2. Mutations.*

Mutaciones en el gen que codifica BMPR2 en pacientes con hipertensión arterial pulmonar esporádica

OBJETIVO: La hipertensión arterial pulmonar (HAP) es una enfermedad poco frecuente que puede tener un componente familiar. En este caso se ha comprobado que un porcentaje superior al 50% se asocia a mutaciones en el gen que codifica el receptor tipo 2 de las proteínas morfogenéticas del hueso (BMPR2), un receptor de la superfamilia del factor transformador del crecimiento beta. Algunos estudios en pacientes con HAP idiopática también muestran porcentajes variables de mutaciones en este gen. El objetivo de nuestro trabajo ha sido conocer la frecuencia de estas mutaciones en nuestros pacientes con HAP idiopática.

PACIENTES Y MÉTODOS: Los pacientes con HAP idiopática seguidos en nuestra unidad de HAP durante el año 2006 constituyeron la población de estudio. Se excluyó la existencia de familiares con diagnóstico de esta enfermedad o síntomas que pudieran hacer pensar en ella. El diagnóstico de HAP fue acorde con el protocolo utilizado en la unidad. En todos los casos se realizó estudio hemodinámico y se incluyó a pacientes con presión arterial pulmonar media mayor de 25 mmHg. Se efectuó extracción del ADN de los leucocitos periféricos y se amplificó mediante la técnica de reacción en cadena de la polimerasa. Se utilizaron 17 parejas de cebadores para los 13 exones que componen el gen. Mediante técnica de conformación de hebra simple y electroforesis posterior se detectaron los fragmentos anómalos, para posteriormente proceder a la secuenciación del gen mediante un lector automático.

RESULTADOS: Se estudió a 8 pacientes (4 mujeres). En 5 de ellos no se encontraron alteraciones, pero 3 mostraron patrones anómalos en la electroforesis y en la secuenciación se observaron mutaciones. En uno de los casos se presentaron 2 patrones anómalos en la electroforesis, pero sólo fue posible secuenciar uno de ellos por la baja concentración del ADN recogido.

CONCLUSIONES: La presencia de mutaciones en el gen que codifica BMPR2 no es infrecuente en pacientes con HAP idiopática, lo que probablemente indique un papel importante de esta familia de factores de crecimiento en la patogenia de la enfermedad y, por tanto, podría tener implicaciones terapéuticas.

Palabras clave: *Hipertensión arterial pulmonar. BMPR2. Mutaciones.*

Introduction

Pulmonary arterial hypertension (PAH) is an uncommon disease with an estimated incidence of around 1 to 2 cases per million individuals per year. Without treatment, the mean life expectancy is less than 3 years from diagnosis, although this varies in relation to functional class and exercise capacity.[1,2] PAH can occur in association with other disease such as AIDS, collagenosis, or portal hypertension, or as a result of drug treatments, or it can present without any apparent cause. The initial concept of vasoconstrictive disease has changed as aspects of the pathogenesis have been revealed, and it is now considered to be essentially a proliferative condition.[3] In 1954, Dresdale et al[4] recognized the possibility of a certain familial trend in some patients with PAH, and 30 years later it was demonstrated that this familial form exhibits autosomal dominant inheritance, although with reduced penetrance,[5] and that it does not differ clinically from idiopathic forms, except for a slightly earlier onset. In 2000, 2 groups simultaneously demonstrated that the anomaly which gives rise to the disease is situated in a gene on chromosome 2q33 encoding the type 2 receptor for bone morphogenetic proteins (BMPR2).[6,7] Since then, this gene has been the subject of intensive research.[8] Approximately 70% of patients with familial PAH carry a mutation in *BMPR2* and more than 140 such mutations have been described to date.[9] Studies have addressed the incidence of these mutations in idiopathic cases and significant variations have been found, with frequencies ranging from 10% to 25%. The largest study included a group of 99 patients in Germany.[10] Of those, 11 carried mutations. Some small studies have also been published on secondary PAH. *BMPR2* mutations were found in a group of patients with congenital heart disease (6% of the cases)[11] and again in cases of PAH associated with anorexigenic drugs[12]; however, no mutations have been found in patients with scleroderma[13] or AIDS.[14]

The aim of this study was to determine the incidence of described mutations in *BMPR2* in a group of patients with idiopathic PAH.

Patients and Methods

The study included patients with a diagnosis of idiopathic PAH seen in our unit in the first quarter of 2006. In all cases, diagnosis was established on the basis of initial clinical suspicion and an echocardiogram showing increased pulmonary arterial pressure, followed by a hemodynamic study with a vasodilator test (using prostacyclin in the first few patients and subsequently with nitric oxide) to confirm the initial diagnosis. A mean pulmonary arterial pressure greater than 25 mm Hg was considered abnormal. To exclude possible causes of secondary PAH, we applied a standard protocol that included the following: a detailed clinical history; laboratory workup, including antinuclear antibodies, liver enzymes, and thyroid hormones; complete coagulation studies; human immunodeficiency virus serology; computed tomography angiography; and lung function testing. A 6-minute walk test was done in all cases to assess exercise capacity. Patients who had been treated with drugs that have a known or probable association with increased pulmonary arterial pressure were excluded. Prior to enrollment, patients were questioned on the existence of first-degree or second-degree relatives who had been diagnosed with the disease or presented symptoms leading to clinical suspicion. In case of doubt, the patient was excluded from the study. All patients provided signed consent having been informed in detail of the aims of the study. The study was approved by the local ethics committee.

Extraction and Processing of Samples

All samples were obtained in our laboratory. A 10-mL sample of blood was collected with EDTA and the DNA extracted (DNA Isolation Mammalian Blood Kit, Roche, Basel, Switzerland). Samples were then stored at 4ºC prior to analysis.

The *BMPR2* gene comprises 13 exons. To obtain copies of all of them, polymerase chain reaction (PCR) was done with 17 pairs of primers (Table 1). Briefly, this technique requires the use of 2 primers, which are complementary oligonucleotides corresponding to the sequences located at either end of the DNA fragment of interest. In the presence of DNA polymerase, each of these primers initiates synthesis of the fragment complementary to the one it has bound, each in the opposite direction (forward and reverse). Amplification of the DNA fragment is achieved by repeated cycles of heat denaturation of double-stranded DNA, hybridization (annealing) of each of the primers to the corresponding strand, and DNA polymerization through the action of DNA polymerase. The amplification conditions were an annealling temperature of 55ºC in the presence of 1.5 nmol/L $MgCl_2$, except in the case of exon 2, for which an annealing temperature of 60ºC was used. The amplification products were visualized with ethidium bromide following electrophoresis in a 2% agarose gel. The fragments were analyzed by single-strand conformational polymorphism (SSCP). Briefly, when a double-stranded DNA fragment is heat denatured and then rapidly cooled, the strands remain separate. If the strands are then subjected to polyacrylamide gel electrophoresis, each strand renatures and adopts a specific conformation that affects its electrophoretic migration. A minimal difference in the sequence (a point mutation) will lead to a conformational change and therefore a different migration pattern. The effectiveness of this technique to detect mutations ranges from 75% to 98% according to the number of base pairs.[15] Point mutations affect a single codon, in other words, a base triplet. If they occur in a coding region of the gene, various things can happen: the new triplet can continue to code for the same amino acid, making it a silent mutation that would not alter the protein; it can code for a different amino acid, making it a missense mutation, in which the new amino acid may be chemically similar to the original and not alter the function of the protein or in which it may be sufficiently different to give rise to a structurally unstable protein or a protein with altered biological function; or the mutation can transform the triplet into a genetic stop codon, causing reading of the messenger RNA to be terminated prematurely and an incomplete, and therefore malfunctioning, protein to be released (a nonsense mutation). When the final outcome of the possible mutations is reduced synthesis of the protein, a situation referred to as haploinsufficiency occurs.

In the fragments in which abnormal electrophoretic mobility was detected compared with controls, the mutations were identified using the enzymatic Sanger sequencing method. This method involves the use of DNA polymerase to synthesize a strand of DNA from a previously denatured DNA template. Subsequent incubation with dideoxynucleotides (ddNTP) at 37ºC interrupts DNA synthesis due to the absence of a 3' hydroxyl group, which is necessary for elongation. The technique employs 4 different ddNTPs, leading to the production of a large number of differently sized fragments, which are then

TABLE 1
Primers Used for Amplification of DNA Fragments[a]

Exon	Forward	Reverse
1A	CCAGTCAAGGAAGAGGATTTGT	AGCAGGATGGTCCATGGTAG
1B	ATGAAAGCTCTGCAGCTAGGTC	GGACGCATGGCGAAGGGCAA
2	TGAAGTCATTCGGATAAGACAAAG	TTATTACCCAGGCTGGTCTCA
3	CCCCATGAAATGTCTTTGGTA	TGCAAATCTTTGGAGAAAGGA
4	CATTTCCTTTGATGCAAAAACA	AATAATCCAGTGGCATGGAAA
5	GTCTCCCAGAATTTGGCTTTC	TGCCTAGAATAGGCCTTGACA
6	GGTAGGAGCTTCATCAGCCATA	TACAGGCATAAGCCACCACA
7	CATGGAATCCTAGCCTATTTGC	AGCCCAGGAGTTTTACTCAGC
8	ATCTGAAGTGGCAGCATGTTT	CACCTGGCCAGTAGATGTTTT
9	TTCAGGAAGGGCATTTTATAGG	TGCATCCTGCTGCTAATAATGT
10	GCCTGAAGGGGATGAAAAA	GTTTGATTTGTGGCATTAGGC
11	TTTGAGCATGTTCCGTAATCC	TTCTTTGTTGGGTCTCAGTTTC
12A	TCAGAGGTGTTAAATTTGGAGAGA	GGTCTAGCTTGTTGGTTTCCA
12B	ACCACAAATGTTGCACAGTCA	ATGAGGTTCTGCTGCATTGAT
12C	GCAGCAAGCACAAATCAAACT	GAATTAGGCCTCTGTGCTCTTC
12D	CACAGTGTTAACTCCCATGCT	AGGTGTGAGCCACTGTGC
13	TCCTGAGACATTGGTTTGACC	TCTATTTAAAGCAAGTCTTTGTTGC

[a]Once the 2 DNA strands have been separated by increasing the temperature, these primers are needed to synthesize the complementary strands of interest. The primers bind to the corresponding regions in each of the strands and, following cooling to 55ºC in the presence of DNA polymerase, synthesis of the new strand is initiated from the point at which the primer is bound. As DNA is double stranded, 2 primers are required, one directing synthesis in a forward direction and the other in a reverse direction.

separated by polyacrylamide gel electrophoresis. Each ddNTP is labeled with a different fluorochrome and sequencing is carried out with an automatic sequencer that uses a laser reader to determine the order of the signals from the 5' and 3' ends (Abi Prism 310, Applied Biosystems, Foster City, California, USA).

The study was descriptive and qualitative, and as such, no statistical analysis was performed.

Results

Eight patients (4 women; mean age at onset of symptoms, 63 years) met the inclusion criteria. All were treated with bosentan at a dose of 125 mg every 12 hours. Five patients had normal SSCP patterns for all of the fragments analyzed. Mutations were identified in the 3 remaining patients (38%), the characteristics of whom are shown in Table 2. Table 3 shows the characteristics and site of the mutations. In all cases they were missense mutations, in other words, base changes that gave rise to a codon encoding a chemically different amino acid and thereby giving rise to a protein in which function was altered. In case 2, different SSCP patterns were found for exons 6 and 12D (Figures 1 and 2). Sequencing revealed a guanine-to-thymine substitution leading to a substitution of alanine 269 with a valine residue. In case 6, a cytosine-to-thymine substitution in exon 1B led to replacement of threonine 10 with a tryptophan residue. Two changes were found in case 8, one in exon 3 and the other in exon 12A. The first corresponded to a thymine-to-cytosine substitution in a noncoding region 54 base pairs from the start of the exon (Figure 3). In this case, various computer programs were used to confirm that it did not involve a change in the splice site at the intron-exon junction, and the results were negative. A change in the conformation of exon 12A was observed in SSCP (splitting of the lower band). However, the low concentration of DNA obtained was insufficient for sequencing.

TABLE 2
Characteristics of the 3 Patients With Mutations in BMPR2

Case	Sex	Age at Onset of Symptoms, y	Mean PAP, mm Hg	FC	Treatment
2	M	62	60	III	Bosentan
6	F	72	36	II	Bosentan
8	M	65	40	II	Bosentan

Abbreviations: F, female; FC, functional class; M, male; PAP, pulmonary arterial pressure.

TABLE 3
Mutations Identified in *BMPR2*

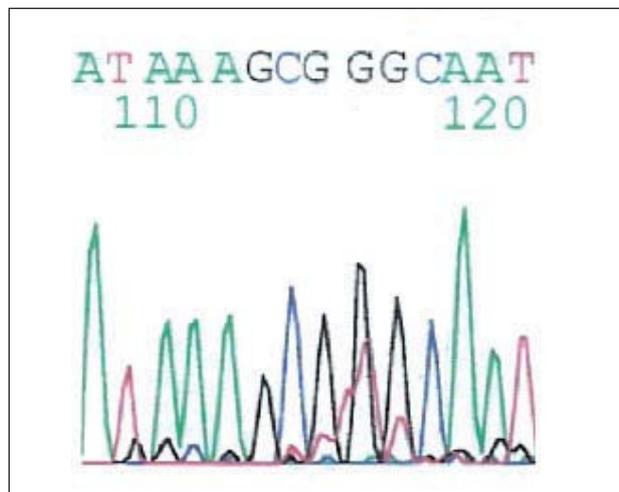| Case	Exon	Mutation	Change (Position)
2	6	806 G→T	Alanine → valine (268)
6	1B	28 C→T	Threonine → tryptophan (10)
8	3	247-54 T→C	(?)

Figure 1. Case 2, exon 6: guanine-to-thymine substitution.
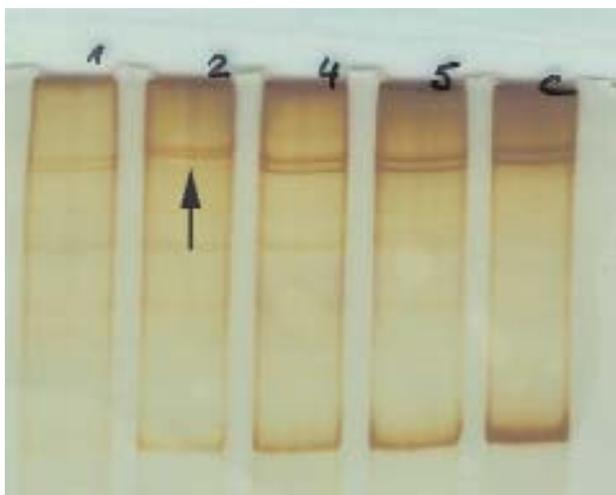
Figure 2. Polyacrylamide gel electrophoresis. Exon 12D: a change (arrow) is observed in the migration pattern of sample 2 compared with the control and the other samples analyzed.
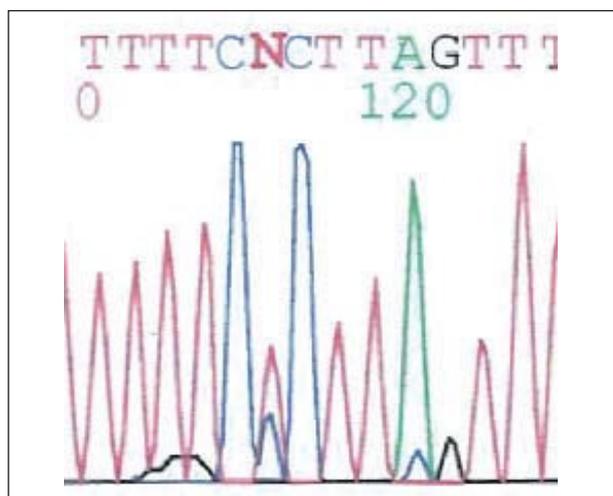

Figure 3. Case 8, exon 3: thymine-to-cytosine substitution 54 base pairs from the beginning of the exon. Given that the mutation is located in a noncoding region it is impossible to determine its effect.

Discussion

In our study, we found 3 cases of mutations in the *BMPR2* gene from a total of 8 patients with idiopathic PAH. Clearly, the size of the sample does not allow conclusions to be drawn regarding the prevalence of such mutations in this disease; however, it is apparent that they are not rare and may therefore play some role in the development of the disease. The methods used, involving PCR amplification followed by SSCP analysis, proved to have a high sensitivity for detection of most variations in the sequence of a DNA strand. The size of the fragment to be analyzed affects the results somewhat, given that, if it is large, a small change in the conformation may not affect electrophoretic mobility, whereas small fragments can adopt different conformations for the same nucleotide sequence. It has recently been reported that another type of genetic alteration may occur that is not detectable by sequencing,[16] meaning that the percentage of patients with idiopathic PAH who carry mutations in *BMPR2* may be higher than reported to date. More than 140 mutations in *BMPR2* have been identified in patients with PAH. All experimental studies suggest a mechanism involving haploinsufficiency—that is, reduced synthesis of the protein to levels that are insufficient to sustain normal function—as the cause of the disease.[17]

BMPR2 probably plays an important role in the development of PAH. The demonstration of mutations in the gene may represent the most significant advance in the understanding of the pathogenesis of this disease. The receptor binds ligands (the proteins with which it interacts) belonging to the bone morphogenetic protein (BMP) family of growth factors. Signal transduction requires an interaction between 2 receptors, BMPR2 and BMPR1, in which BMPR1 is phosphorylated.[18] This conformational change activates cytoplasmic proteins known as Smads, which act as transcription factors. They are responsible for signal transduction in all members of the transforming growth factor β (TGF-β) family. By regulating gene expression, they lead to the synthesis of other proteins that interrupt cell proliferation and favor apoptosis.[19] Interestingly, BMPs also display pleiotropic effects. This means that they will have different effects according to the microenvironment (presence of one or another class of cytokines), cell type, or ligand involved. For instance, in contrast to their effects in the pulmonary arteries, BMPs appear to inhibit apoptosis of cardiac myocytes.[20] An additional step in determining the various interactions between growth factors in the pulmonary vessels has been the observation in patients with sporadic PAH of increased levels of angiopoietin-1 —a potent stimulator of proliferation of arterial smooth muscle cells—in association with a sharp reduction in the expression of BMPR1, which, as mentioned, is essential for BMP signaling.[21] Angiopoietin inhibits synthesis of BMPR1 and, therefore, also inhibits the pathway involving BMPR2. This once again highlights the importance of this family of growth factors in the pathogenesis of PAH, irrespective of the trigger, and of course, facilitates the design of specific targeted treatments. It would be of interest to know whether the signals activated by BMPR2 affect endothelial cells. The group led by Stewart in Toronto, Canada, addressed this question and found that there was a notable increase in apoptosis (almost 3-fold) in these cells following inhibition of BMPR2 expression by more than 50% through transfection with small interfering RNA. In other words, the effect of this signal in the pulmonary arterioles appears to differ between smooth muscle cells on the one hand and endothelial cells on the other. The mutations in *BMPR2*, by acting on the endothelial cells, would favor cell turnover via an increase in apoptosis, and this would subsequently give rise to resistant cells with greater proliferative potential. Some vascular growth factors, such as vascular endothelial growth factor, have been shown to prevent the appearance of PAH, possibly by reducing endothelial apoptosis.[23] Inhibition of this factor in association with hypoxia gives rise to severe PAH.[24]

Although it has been observed in some cases that the endothelial cells of plexiform lesions exhibited monoclonal expansion,[25] it could not be demonstrated that BMPR2 initiated this process.[26]

Of course, not all cases of PAH can be attributed to mutations in genes encoding members of the TGF-β superfamily. There is significant variation in the reported percentages of patients with idiopathic PAH who carry mutations in *BMPR2*. In the first study of *BMPR2* mutations in this context, 13 of 50 unrelated British, French, and American patients (26%) had mutations.[27] That study included 150 healthy control subjects, none of whom were found to carry mutations. In another study undertaken in 30 Japanese patients, 12 (40%) were found to have mutations,[28] a rate that is very similar to the one observed in our study. A study undertaken in a genetically homogeneous population in Finland found 3 cases in a total of 26 patients analyzed (12%).[29] A study carried out in a German population included a larger number of patients, a total of 99, of whom 11 had mutations.[10] As mentioned, it has been reported that mutations can occur through gene rearrangement that would not be detected by sequencing of coding regions.[16] A European study undertaken in 126 patients with both idiopathic and familial PAH, including some Spanish patients, used multiplex ligation, which allows rapid analysis of complete genes; the authors found 6 mutations in addition to the 20 that had been identified using sequencing methods.[30] This would correspond to an increase from 16% to 21% of cases carrying mutations. That study also showed conclusively that haploinsufficiency (reduced protein synthesis, in this case of BMPR2) is the principal mechanism through which these mutations predispose to the appearance of PAH. Analysis of lung tissue has shown that patients with idiopathic PAH have reduced expression of BMPR2, but when there is a mutation in the gene, this reduction is much more marked.[31] In cases of secondary PAH, the reduction, although significant compared with controls, was much less.

Our study represents a small contribution to the increasing evidence supporting the importance of the BMP family in the pathogenesis of PAH. Given the sample size, the percentage of patients with mutations in *BMPR2* is not representative in itself of the situation in all cases of sporadic PAH, but the finding of 3 cases out of 8 after careful selection of patients to avoid confounding factors does appear to indicate an appreciable prevalence of such mutations. Our patients belonged to the same geographic region, Galicia, and all of the known family of 2 of the patients also came from that region. This could indicate a possible source of bias, as found in the Japanese case series,[28] which reported a similar percentage of patients with mutations as in our study. Although it is difficult to determine exact figures, the number of patients in Galicia with PAH, with an incidence of 1 to 2 new cases per million inhabitants per year, does not appear to be higher than that calculated for nearby countries. We also did not detect families with PAH in our area of influence.

In conclusion, mutations in *BMPR2* are not infrequent in patients with idiopathic PAH. This is suggestive of a genetic basis in some of these patients and supports a role for members of the TGF-β superfamily in the pathogenesis of the disease.

Acknowledgments

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