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Bone morphogenetic protein receptor 2 in patients with idiopathic portal hypertension

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Abstract

In idiopathic portal hypertension (IPH) typical vascular lesions are present in the branches of the portal vein or in the perisinusoidal area of the liver. Similar histological alterations have been reported in the pulmonary vasculature of patients with idiopathic pulmonary artery hypertension (IPAH). As IPAH is associated with mutations of the bone morphogenetic protein receptor 2 (*BMPR2*) gene, the aim of this study was to investigate whether this association might also be found in patients with IPH. Twenty-three samples belonging to 21 unrelated caucasian patients with IPH followed in the hepatic haemodynamic laboratory of the Hospital Clinic in Barcelona were included in the study. All patients were studied for the entire open reading frame and splice site of the *BMPR2* gene by direct sequencing and multiple ligation probe amplification (MLPA) in order to detect large deletions/duplications. None of the 23 patients had pulmonary artery hypertension. Four patients presented one single nucleotide polymorphism (SNP) in intron 5, four patients had a SNP in exon 12 and a SNP in exon 1 was found in two cases. Two patients had both intron 5 and exon 12 polymorphisms. All SNPs were previously described. Except for these three SNPs, neither mutations nor rearrangements have been identified in the *BMPR2* gene in this population. We did not detect mutations or rearrangements in the coding region of the *BMPR2* gene in our patients with IPH. These findings suggest that, in contrast to IPAH, mutations in *BMPR2* are not involved in the pathogenesis of IPH.

Keywords: hepatoportal sclerosis • non-cirrhotic portal hypertension • bone morphogenetic proteins • pulmonary artery hypertension • HIV • *BMPR2* gene

Introduction

Idiopathic portal hypertension is a progressively debilitating and life-threatening disease of unknown etiology characterized by the absence of cirrhosis or portal vein obstruction [1].

Typical lesions are generally vascular and are present in the portal vein, its branches or in the perisinusoidal area of the liver. Essentially, there is a marked sub-endothelial thickening of the large and medium-sized branches of the portal vein, with obliteration of small portal venules, microthrombi incorporated into the vessel wall and preisinusoidal fibrosis [2, 3].

The mechanisms causing these lesions remain largely unknown. Prothrombotic disorders are considered important causal features [4, 5], but also infections [6], trace metals and chemicals [7] and immunological factors [8, 9] have been proposed. Furthermore, genetic mutations may play a role in the pathogenesis of IPH [10, 11]. Familial aggregation has been described, raising the question about the existence of one or more genes at the origin of this disorder [11].

Interestingly, the pathological alterations observed in the smallest vessels of the lung of patients with IPAH, *i.e.* intimal

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proliferation with perivascular fibrosis and muscular hypertrophy of the media [12] are very similar to those found in the liver in IPH [5]. In addition, some patients with IPH also present clinical features of IPAH [13].

Bone morphogenetic protein receptor 2 (*BMPR2*) gene, which encodes a membrane receptor of the transforming growth factor beta (TGF-β) superfamily, has been mapped on chromosome 2q33 (locus PPH1) and the sequence of this 4-kb gene is composed by 13 exons and encodes a 1038–amino acid protein [14]. Mutations in *BMPR2* account for 7–25% of the IPAH forms and for up to 80% of the familial forms of pulmonary arterial hypertension [15–17]. Large rearrangements account for 12% in familial forms of pulmonary arterial hypertension and 5% in sporadic cases [18]. In a Spanish study, these proportions resulted significantly lower (11% and 25%, respectively) [19]. This difference could be attributable to population heterogeneity or to a clinical selection or failure to detect mutations by the technology used (single-strand conformation polymorphism, SSCP, analysis).

A total of 144 distinct mutations in the *BMPR2* gene have been so far described in 210 patients with pulmonary arterial hypertension [20]. Approximately 70% of the *BMPR2* mutations underlying pulmonary artery hypertension are predicted to lead to premature truncation of the *BMPR2* transcript and are likely to be lost by the process of nonsense-mediated decay. All the currently known mutations cause a loss of receptor function.

Recent studies suggest that *BMPR2*-related IPAH is due to the failure of BMPR2 opposing a competing TGF-β signalling function, whose activation causes an increase in the TGF-β activity that has been shown to promote fibrogenesis, intimal hyperplasia and smooth-muscle growth [21, 22]. This hypothesis implies that the fundamental mechanism of *BMPR2*-related pulmonary artery hypertension is an imbalance of growth signalling caused by a reduction in the braking function of BMPR2.

As the pathophysiology of IPH remains largely unknown and IPH and IPAH share similar histopathological vascular lesions, we hypothesized that mutations in the *BMPR2* gene may be found in patients with IPH. Consequently, the aim of this study was to assess the prevalence of mutations in *BMPR2* in patients with IPH.

Materials and methods

Patients

Diagnosis of IPH was based on the following criteria: (1) presence of unequivocal signs of portal hypertension (gastroesophageal varices, ascites, splenomegaly and/or presence of portosystemic collaterals), (2) absence of cirrhosis or advanced fibrosis or of other additional causes of chronic liver diseases causing portal hypertension, at liver biopsy (performed in all patients), (3) absence of hepatic or portal vein thrombosis at imaging studies performed at diagnosis, (4) absence of toxic exposure to arsenic, vinyl chloride or copper sulphate (clinical history). All liver biopsy specimens were re-evaluated for the purpose of the study by an experienced pathologist (M.B.). These criteria were selected based on two reference papers from Japan and Europe [23, 24].

Patients with IPH followed-up at the Liver Unit of our Hospital, who have given written informed consent to obtain a blood sample for genetic studies, were considered eligible for the study. To avoid genetic noise related to inherited genetic traits and to have a homogeneous ancestry population we selected only caucasian patients for the study. Because of the exploratory nature of this study and the elevated costs, the study was limited to the initial consecutive 23 patients.

The protocol was approved by the Institutional Review Board of Hospital Clinic in Barcelona. Clinical, epidemiological, laboratory and imaging features were recorded in a pre-designed case report form.

BMPR2 gene molecular studies

Aliquots of whole blood were stored at −80°C. DNA extraction from whole blood was performed using an automatic MagnaPure system (Roche Diagnostics, Madrid, Spain) according to the manufacturer instructions.

BMPR2 exons and their associated boundary regions were amplified by PCR with previously reported primers [15, 19]. PCR products were sequenced using the Big-Dye Terminator Chemistry Kit v3.1, run on an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analysed using ABI PRISM GeneMapper software version 3.0. All sequences were compared to NCBI RefSeq NM_001204.6. The standard nomenclature recommended by HGVS (www.hgvs.org/mutnomen) was used to number nucleotides and name mutations.

Frequencies of *BMPR2* SNPs were compared to those previously described in the HapMap database (http://hapmap.ncbi.nlm.nih.gov/index.html.en).

Multiple ligation probe amplification (MLPA) reaction

Multiple ligation probe amplification analysis was performed with Salsa P093-B2 HHT (MRC-Holland, Amsterdam, the Netherlands) as described previously by Madrigal *et al.* [25]. Samples were loaded onto an ABI3130 Genetic Analyzer and results were visualized using the Gene Mapper program and analysed with the SEQUENCE Pilot-module MLPA® program (JSI Medical Systems GmbH, Kippenheim, Germany).

Quantitative polymerase chain reaction (qPCR)

DNA copy number of two *BMPR2* genes was determined by qPCR using SYBR Green on an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems). Primers were designed for exons 4, 9, 11 and 12 (4F-CAGCCTTTCTAAAGGGCAGTC, 4R-CCAAAGCATAAGGCAACTATC; 9F-AGAATATGCTACGTTCTCTC, 9R-CCTGGGAAGAGGTCTGTACATC; 11F-CAGGCAGTGAGGTCACTCAA; 11R-TGATAGATGCCACACCCCTTA; 12F-GTGTGCCAAAAATTGGTCCT; 12R-TTGTGCTTGCTGTCGTTCAT). Each reaction was performed in triplicate. Amounts of DNA in each amplification were determined by comparing the results to a standard curve produced by real-time PCR of serial dilutions (*e.g.* undiluted, 1:4, 1:16 and 1:64) of a known amount of DNA.

RNA extraction and cDNA synthesis

Total RNA was extracted from peripheral blood using the PAXGene Blood RNA Kit following their basic protocol (QIAGEN, Germany). RNA

concentration and purity were measured with a spectrophotometer (NanoDrop™ Spectrophotometer ND-100; Thermo Fisher Scientific, Madrid, Spain). Finally, cDNA was synthesized with 200 ng of the extracted RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions.

Real-time quantitative PCR (RT-qPCR) and data analysis

Real-time quantitative PCR experiments were carried out in an ABI7300 Real-Time PCR System (Applied Biosystems). No cDNA was added to negative control reactions. Beta-glucoronidase (GUS-β) was used as a housekeeping gene RT-qPCR and analysis was performed as previously described [26].

mRNA expression analysis

PCR was performed using cDNA to amplified exons 4, 11 and 12 of *BMPR2* gene. Primers used were the ones previously described at the quantitative real-time PCR method.

Results

Study population

Clinical, demographic characteristics and laboratory findings of the 23 patients with IPH (21 unrelated) included in the study are shown in Table 1. Five patients belonged to three unrelated families (two brothers, a father and a son both included in the study and a woman whose sister was not included in the study). Three patients presented features associated with immunological diseases. The first one was diagnosed with rheumatoid arthritis, the second suffered from anti-phospholipid syndrome and the third one was a patient with positive anti-transglutaminase antibodies, but with a normal duodenal biopsy.

Table 1 Clinical, demographic characteristics and laboratory findings of the 23 patients with IPH included in the study

	Median (range) or *n* (%)
Number of patients	23
Age at diagnosis of IPH	28 (9–86)
Gender (male) (%)	14 (61%)
HCV infection	0 (0%)
HBV infection	1 (4%)*
HIV infection	4 (17%)
Signs of portal hypertension	
Presence of oesophageal or gastric varices	23 (100%)
Ascites	5 (22%)
Splenomegaly	17 (74%)
Laboratory data†	
Leucocytes (G/L)	4.7 (1.2–8.9)
Haemoglobin (g/L)	128.8 (100–167)
Platelet count (G/L)	112 (27–308)
Prothrombin time (%)	75 (40–100)
Creatinin (mg/dl)	0.87 (0.60–1.28)
AST (IU/l)	39 (17–95)
ALT (IU/l)	38 (12–95)
GGT (IU/l)	81 (7–423)
Total bilirubin (mg/dl)	1.45 (0.40–7.8)
Sodium (mEq/l)	140 (135–145)
Albumin (g/l)	42 (32–51)

IPH: idiopathic portal hypertension. *HBV past infection. †Data at the time of blood draw for genetic analysis.

Mutation analysis of the BMPR2 gene

We did not detect any causative mutations in the *BMPR2* gene in the 21 unrelated patients with IPH included in the study. However, in 12 patients SNPs were detected. Four patients presented one SNP in intron 5 (rs7575056), four patients had a SNP in exon 12 (rs1061157) and two patients had a SNP in exon 1 (5′-UTR-301G>A). Two additional patients had both polymorphisms at intron 5 and exon 12. No other SNPs were identified.

All SNPs that we found have been previously described in the HapMap database (http://hapmap.ncbi.nlm.nih.gov/index.html.en). The prevalence of these SNPs in our patients was similar to the prevalence described in the healthy caucasian population.

Multiple ligation probe amplification

Screening of *BMPR2* by using the MLPA technique revealed five patients with possible duplications of *BMPR2* gene: two patients presented a duplication of exon 4, one patient of exon 9 and two other patients of exons 11 and 12.

Quantitative PCR only confirmed three of these duplications in two patients (exon 4) and one patient (exons 11 and 12). However, these duplications do not alter the normal expression of the gene, regarding RNA studies.

Discussion

Mutations in *BMPR2* gene have been involved in the pathogenesis of IPAH [27]. Based on the observation that IPAH and IPH share common vascular alterations, our aim was to investigate whether IPH also present mutations in the BMPR2 gene.

In contrast to our initial hypothesis, in this study we did not find any possible causative mutations in the *BMPR2* gene in patients with IPH. In addition to this finding, several other results that have emerged from this study, *i.e.* the confirmation of known SNPs and DNA duplications, as well as an unchanged mRNA expression, clearly argue against a possible role of *BMPR2* in the pathogenesis of IPH. Structural alterations such as deletions or duplications affecting the *BMPR2* gene have been described as a cause of familial pulmonary hypertension. In our samples, however, although at DNA level we have found some duplications, we could not confirm the alteration at mRNA and cDNA levels.

Previous studies in IPAH included small number of patients (less than 50) and the prevalence of *BMPR2* mutations was up to 26% [28]. One of the largest series included 126 patients with IPAH and the prevalence was 21% [18]. Also an increased occurrence of BMPR2 duplications has been reported in IPAH [29].

With the lack of evidence of any *BMPR2* mutation in this study and bearing in mind the low prevalence of IPH in western countries [5, 30], the number of patients in this study should be considered sufficient to reasonably believe that mutations in this gene may not be involved in the pathogenesis of IPH. Similarly, studies in familial PAH including a highly variable number of patients showed a prevalence of *BMPR2* mutations of up to 82% [31]. Consequently, more than one of our patients should have had the *BMPR2* mutation, reinforcing the finding that *BMPR2* gene mutations are not involved in the pathogenesis of familial forms of IPH.

Our study, however, showed that in 12 patients (52%) with IPH three different SNPs were detectable. The three SNPs identified in our patients, in intron 5, exon 12 and exon 1, have been previously described in a normal population with the same prevalence as in the population of our study. In addition, the possibility that *BMPR2* mutations occur in a subpopulation of patients with IPH also remains potentially open, because the sample size in this study (21 unrelated patients) was relatively small. However, due to the low prevalence of this disease in western countries [5, 30], the number of patients in this study should be considered sufficient to reasonably exclude this hypothesis.

It is currently recognized that up to 30% of hereditary and 80% of idiopathic cases of pulmonary artery hypertension do not have mutations identified in *BMPR2* despite comprehensive testing. Hence, it is likely that mutations at one or more other loci contribute to the pathogenesis of IPAH, as demonstrated by the association of IPAH with hereditary haemorrhagic telangiectasia and mutations in activin-like kinase type 1 and endoglin [32]. Similarly, familial or IPH may be associated with mutations in genes other than *BMPR2*.

An association of IPH with immunological abnormalities has been described [8, 9]. In this study we identified three patients in whom an autoimmune disease was known. However, due to an important diagnostic heterogeneity, we recognize that it is not possible to draw any conclusions from this study in terms of relationship between autoimmunity and the development of IPH.

Idiopathic portal hypertension has also been associated with HIV infection [33] and recent data by Caldwell *et al.* [34] indicate that HIV may repress BMPR2 transcription in macrophages. In the present study we focused on possible genetic alterations in the *BMPR2* gene and we did not assess the level of expression of BMPR2. Considering this methodological difference, whether the expression of BMPR2 was decreased in HIV patients in this study remains unknown. This intriguing hypothesis deserves further investigation.

In conclusion, the data of this study suggest that, in contrast to IPAH, IPH is highly unlikely that mutations in the *BMPR2* gene were involved with pathogenesis of IPH. Idiopathic portal hypertension may be related to other mutations in the TGF-β superfamily and further investigation is needed to understand the pathogenesis of this condition.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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