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Penetrance of Pulmonary Arterial Hypertension is modulated by the expression of normal *BMPR2* allele

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Abstract

Familial pulmonary arterial hypertension (FPAH) is a progressive, fatal disease caused by mutations in the *bone morphogenetic protein receptor type 2* gene (*BMPR2*). FPAH is inherited as an autosomal dominant trait and shows incomplete penetrance in that many with *BMPR2* mutations do not develop FPAH suggesting a role for, as yet unidentified, modifier genes in disease penetrance. We hypothesized that variable level of expression of the wild type (WT) *BMPR2* allele could act as a modifier and influence penetrance of FPAH. WT *BMPR2* levels were determined by real-time PCR analysis in lymphoblastoid (LB) cell lines derived from normal controls and individuals with FPAH. The FPAH kindreds analyzed carried mutations that result in the activation of nonsense mediated decay (NMD) pathway, which leads to the degradation of the mutant RNA thus ensuring that only the WT *BMPR2* transcripts will be detected in the real-time assay. Our data show that WT and mutant *BMPR2* levels can be reproducibly measured in patient derived LB cell lines and that unaffected mutation carrier derived LB cell lines have higher levels of WT *BMPR2* transcripts than FPAH patient derived LB cell lines ($p \leq 0.005$). Our findings suggest that the levels of expression of WT *BMPR2* allele transcripts is important in the pathogenesis of FPAH caused by NMD^+ mutations. Furthermore, our study illustrates a novel application of lymphoblastoid cell lines in the study of PAH, especially important because the affected site, i.e. lung is not available for unaffected mutation carriers.

Keywords

BMPR2; Penetrance; Pulmonary arterial hypertension; Modifier; FPAH; PPH; NMD

Introduction

Pulmonary arterial hypertension (PAH) is a progressive, fatal disease characterized by vascular remodeling of the small pulmonary arteries, which results in increased vascular resistance and subsequent right heart failure (Chan and Loscalzo, 2008; Pietra, et al., 2004). Familial PAH (FPAH) has an autosomal dominant mode of inheritance with reduced penetrance, variable age of onset and a 2.4:1 (female to male) sex ratio (Newman, et al., 2001). Mutations in the *BMPR2* gene (MIM# 600799), a member of the transforming growth factor (TGF-β) super family, are found in the majority of cases of FPAH and constitute the largest known risk for the development of FPAH (Cogan, et al., 2006; Deng, et al., 2000; Lane, et al., 2000). While both haploinsufficient (HI) and dominant negative

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(DN) mechanisms of disease have been proposed (Machado, et al., 2001; Rudarakanchana, et al., 2002), reduced penetrance and variable age at diagnosis suggest there are additional modifiers of disease expression (Newman, et al., 2008).

RNA studies have shown that some *BMPR2* mutations produce stable transcripts while others contain premature termination codons (PTC) and are rapidly degraded through the nonsense mediated decay (NMD) pathway (Cogan, et al., 2006). NMD is an mRNA surveillance system that degrades transcripts containing PTCs to prevent translation of unnecessary or harmful transcripts (Gonzalez, et al., 2001; Maquat, 2004). We have recently demonstrated that FPAH patients with *BMPR2* mutations subject to NMD (NMD^+) tend to have milder disease (later age of diagnosis) than individuals with mutations that produce stable transcripts (NMD^-)(Austin, et al., 2008). Failure to eliminate PTC containing mRNAs can result in synthesis of abnormal proteins that can be toxic to cells through dominant negative (DN) or gain of function effects. Thus, individuals with PAH and NMD^+ *BMPR2* mutations have disease due to HI whereas patients whose mutations are NMD^- may have disease due to a DN mechanism. Affected individuals with *BMPR2* NMD^+ mutations have a 12 year later age of onset than those with NMD^- mutations, making *BMPR2* NMD status an important modifier in the disease process (Austin, et al., 2008).

One of the major impediments to identify genes that cause or modify FPAH pathogenesis is the lack of easy availability of the tissue of interest (the smallest pulmonary arteries). It has been hypothesized that vascular smooth muscles of the small arteries are the disease initiating tissue in FPAH but uncertainty remains and It is now believed to be likely that multiple cell types in the pulmonary arterial wall and pulmonary arterial circulation contribute to the development of vessel remodeling (for a review see (Chan and Loscalzo, 2008; Pietra, et al., 2004). Also, the isolation and eventual study of the pulmonary vascular smooth muscle cells remains difficult if not essentially impossible due to the sporadic availability of tissue from affected patients at the time of lung transplant or post mortem and the poor condition secondary to disease related pathogenesis rendering them less than ideal for laboratory use. Furthermore the tissue of interest cannot be obtained from carriers and non-affected individuals secondary to the significant risks associated with lung biopsy.

In order to overcome these issues we have used patient-derived lymphoblastoid cell (LB) lines to study disease pathogenesis and gene expression. LB lines offer several advantages such as unlimited amount of tissue carrying the mutation, elimination of possible effects on gene expression form drugs and disease pathogenesis. While it is true that the culture of cells may introduce changes in cell physiology these alterations are uniform across the samples. Moreover LB cell lines have been successfully used in gene expression studies for a wide variety of disease processes including schizophrenia (Kakiuchi, et al., 2007), drug resistance (Huang, et al., 2007), autism (Baron, et al., 2006), and asthma (Dixon, et al., 2007). Lastly, we believe that comparing expression between affected and unaffected *BMPR2* mutation carriers is the most effective way to identify and study genetic modifiers associated with FPAH and LB cell lines are the most practical way to perform these studies.

Based on our studies demonstrating that NMD^+ *BMPR2* mutations tend to be later onset and follow HI model of inheritance, we reasoned that the level of expression of the wild type (WT) *BMPR2* allele could also influence disease development. To determine the possible role of the WT *BMPR2* allele as a modifier of disease, we measured expression levels of WT *BMPR2* in patient-derived LB lines from selected NMD^+ FPAH kindreds, by real time RT-PCR, and compared the expression of *BMPR2* transcripts from the normal alleles of mutation carriers with and without disease. Our study demonstrated the utility of LB cell lines in the study of PAH and suggested that higher expression levels of WT alleles correlate with non-penetrant carrier status while lower levels predicted penetrance.

MATERIALS and METHODS

FPAH Kindreds

Study subjects included individuals with FPAH and *BMPR2* mutation carriers without disease from 4 different kindreds (Cogan, et al., 2006; Machado, et al., 2001). All subjects are heterozygous for *BMPR2* mutations (Table 1) that resulted in frame shifts leading to PTCs that were shown to be subject to NMD as described previously (Cogan, et al., 2006; Cogan, et al., 2005). The *BMPR2* mutation in family-20 had deletion of exons 4–5 (c.419-?_621+?del) causing an out of frame deletion beginning at codon 140 with a PTC at codon 151. Families 59 & 80 were heterozygous for an IVS8 C to G transversion at the –3 position of the acceptor splice site (c.1129-3C>G) which resulted in deletion of exon 9 with a frameshift at codon 377 and generation of a PTC at codon 424. Family 127 had an insertion of a single adenosine in exon 9 (c.1141_1142insA) altering the predicted amino acid sequence starting at codon 381 and causing a PCT at codon 398. Nucleotide numbering reflects cDNA numbering, where +1 corresponds to the A of the ATG translation initiation codon in the reference sequence (GenBank NM_001204.6), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Establishment of LB cell lines

Lymphocytes were isolated from anticoagulated whole blood within 48 hrs of collection and exposed to Epstein-Barr Virus (EBV) to induce cell immortalization as previously described (Oh, et al., 2003) (Bird, et al., 1981).

Analysis of *BMPR2* transcripts by real-time

cDNA was synthesized from total cellular RNA (1 μg) using (Superscript III cDNA Synthesis Kit (Invitrogen). Taqman real-time assay were designed for exon-9-deletion and wild type (WT) BMPR2 transcripts (NM_001204.6) using the Primer Express software package. Primer and probe sequences are as follows. WT-forward primer 5-TTAGTGACTTTGGACTGTCCATGAG-3′, WT-reverse primer 5′-TCTAGCACTTCTGGTGCCATATATCT-3′, WT-Taqman probe 5′-FAM-TAAGCGAGGTTGGCACT-3′, exon-9-deletion forward primer 5′-TTATTAGTGACTTTGGACTGTCCATGA-3′, exon-9-deletion reverse primer 5′-GAGAACCTGCATATCCTCAAAAGTC-3′, exon-9-deletion Taqman probe 5′-FAM-ATAAGCAAGGGGAATCC-3′. Assays were optimized using the standard curve method as previously described (Bustin, 2002; Livak and Schmittgen, 2001) and all had amplifications efficiencies of > than 99.5%. Real-time PCR analysis was carried out using Taqman Universal Master Mix and a 7500 Real-Time PCR system according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). We used the TaqMan human endogenous control plate (catalog number 4309199) to analyze 13 genes as potential house keeping genes for data normalization as previously described (Hamid, et al., 2008). Amplification parameters consisted of initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. Relative expression levels were calculated using the comparative Ct method (Bustin, 2002; Livak and Schmittgen, 2001).

Western analysis

Membranes were probed with primary antibody ab-10862 (Abcam, Cambridge, MA) for one hour and with secondary antibody 111-035-003 (Jackson ImmunoResearch, West Grove, PA) for an additional one hour. Detection was done using the Immobilon Chemiluminescent HRP substrate (Millipore, Billerica, Massachusetts). β-Actin was used as a loading control.

Cell culture

Control LB cell lines were obtained from the Coriell Cell Repositories (Camden, N.J). All LB cell lines were grown in 15% fetal bovine serum in RPMI 1640 with 2mM L-Glutamine.

Statistics

Data are expressed as means ± SD. Comparisons between groups were performed with one-way analysis of variance (ANOVA) as well as Mann-Whitney U (Wilcoxon rank-sum) test. All experiments were repeated 3 times and done in replicates of 3 or more. A *P* value less than 0.05 was considered significant. JMP software package version 6.0 (SAS Institute Inc. Cary NC) was used for all analysis.

RESULTS

BMPR2 transcripts can be detected in LB cell lines

To determine if LB cell lines express *BMPR2* we used Taqman-based relative real-time PCR analysis on RNA isolated from a number of LB cell lines from normal individuals. Our results showed that *BMPR2* transcripts can be detected in LB cell lines and that our real-time PCR assay detected relative differences in expression between samples (Fig. 1A). We then determined if similar differences in *BMPR2* transcript expression can also be detected in PAH patient LB cell lines. Our data showed that *BMPR2* RNA could be detected in LB cell lines derived from FPAH samples (Fig. 1B). Importantly, as was the case with the controls, we detected differences in total *BMPR2* levels between different patients.

Variation in *BMPR2* expression is not secondary to LB immortalization

Our finding that FPAH patient derived LB cell lines had different levels of *BMPR2* transcripts could either be secondary to the LB immortalization process or, more likely, represented true variation in *BMPR2* transcripts expression across patient samples. To test the first we established multiple LB cell lines using lymphocytes isolated from a single patient but with different stocks of EBV viruses in three separate experiments. After immortalization total cellular RNA was isolated and used in a cDNA reaction and then subjected to real-time PCR analyses for total cellular *BMPR2* transcript levels. The results showed that *BMPR2* transcript expression did not vary between the three LB cell lines established from a single patient (Fig. 2). This suggests that the differences seen in *BMPR2* transcript levels between patients with either SPAH or FPAH were not an artifact of LB immortalization process but likely represent true differences in transcript levels.

NMD can be detected in LB cell lines by real-time PCR

We have shown previously that certain mutations in *BMPR2* can generate PTCs that result in NMD of the mutation carrying mRNA (Cogan, et al., 2006; Cogan, et al., 2005). We then determined if real-time PCR could detect NMD^+ transcripts in patient derived LB cell lines. We examined LB cells from two cohorts (family 80 and family 59) of patients in whom *BMPR2* exon 9 is deleted resulting in a frame shift and generation of a PTC. RNA was isolated from LB cells grown with or without puromycin. Real-time PCR assays were then used to quantitate relative levels of wild-type (WT) or exon-9 deleted *BMPR2* transcripts (c. 1129-3C>G). Our results showed we can detect transcripts that are subjected NMD in LB cell lines (Fig. 3A). Growth of LB cell lines in puromycin containing media [which inhibits NMD and thus would stabilize the mutant transcript (Carter, et al., 1995; Noensie and Dietz, 2001)] resulted in very significant increases (112 fold in LB cell line derived from patient 80-1409 and 23 fold in patient 80-1312; $p<.0001$ in each case) in the mutant mRNAs (exon-9 deleted *BMPR2* mRNA; c.1129-3C>G) which otherwise are present in very low quantities in LB cells grown without puromycin (Fig. 3A). As expected, puromycin did not

have a statistically significant effect on the expression of WT mRNA (data not shown). We then compared the levels of mutant (c.1129-3C>G) and WT mRNA in non-puromycin treated LBs derived from the same two kindreds (PPH-80 and PPH-59) to determine the relative contribution of either to the total cellular *BMPR2* message. The results show that, when LB cells are not grown with puromycin, the levels of WT allele are by far the major determinant of the total cellular *BMPR2* transcripts (Fig. 3B). The mutant transcript (c. 1129-3C>G) contributes 2.3%, 0.06%, 2.1% and 2.5% to the total BMPR2 levels in case in LB cell lines derived from patients 80-1409A, 80-1872, 80-1881, 80-1312 and 59-139 respectively (all with p<.0001). The real-time expression data correlated with western blot analysis which showed that carriers have higher BMPR2 protein expression than affected individuals in these two kindreds (Fig. 3C).

BMPR2 expression levels are different between patients and carriers

After characterizing and validating levels of *BMPR2* transcripts, we tested our hypothesis that the amounts of the wild type (WT) *BMPR2* transcripts may influence FPAH pathogenesis by affecting penetrance. To do this we analyzed all the available LB cell lines (grown without puromycin and thus expected to show transcripts only from the WT allele) derived from blood samples from four kindreds (PPH, 20, 59, 80 and 127). that have NMD+ mutations (Table 1). There were two affected and one carrier from kindred 80 (c. 1129-3C>G), three affected and two carriers from kindred 20 (c.419-?_621+?del), two affected and 5 carriers from kindred 127 (c.1141_1142insA) and one affected from kindred 59 (c.1129-3C>G). Total *BMPR2* transcript amounts were determined using our real-time PCR assay. Expression data from all four kindreds (PPH 20, 59, 80 and 127) were analyzed together and showed that the expression of WT *BMPR2* transcripts was significantly lower in patients with PAH than individuals who are unaffected mutation carriers (Fig. 4) ($p<0.005$). We also determine the level of statistical significance in a more conservative manner by reducing the data to remove redundant variables from the data, replacing the data file with a smaller number of uncorrelated variables. To do this, we calculated the average value of WT *BMPR2* transcript levels for each kindred in the affected group, and the average value of WT *BMPR2* transcript levels for each kindred in the unaffected group. As a result, each kindred was represented by one value that contributes to the overall data file for each group: affected versus unaffected. Data reduction to the average contribution of each kindred results in a more conservative statistical approach due to a loss of power. The data was reanalyzed for statistical significance using the conservative nonparametric approach for continuous variables: Mann-Whitney U (Wilcoxon rank-sum) test. Expression of WT *BMPR2* transcripts was still significantly lower in patients affected with PAH compared to unaffected mutation carriers (p value 0.034). These results thus suggest that the level of expression of the normal WT *BMPR2* allele may contribute to the clinical development of FPAH in individuals who carry a mutant BMPR2 allele.

DISCUSSION

While all the members of FPAH kindreds with NMD+ *BMPR2* mutations could potentially develop disease due to HI, only 20% actually develop FPAH (Austin and Loyd, 2007). Thus, the mutation-induced HI alone is not sufficient to cause FPAH in most individuals. We hypothesized that the levels of expression of normal (WT) transcripts could modify the degree of HI and contribute to penetrance in individuals, carrying the NMD^+ mutations.

To test our hypothesis we first characterized WT and mutant *BMPR2* transcript levels in LB cell lines to show that LB cell lines can be used to study *BMPR2* expression. This was important since as explained in the introduction tissues from the affected site, i.e. lung are not available for study, particularly, from unaffected mutation carriers. Utilizing LBs derived from normal controls and PAH patients, we showed that WT *BMPR2* transcripts can

be detected and reproducibly quantitated by real-time PCR and that expression levels vary between individuals. We further show that these differences in transcript amounts were not secondary to the immortalization process and that we could quantify and differentially detect WT *BMPR2* and mutant (NMD+) transcripts in LB cells derived from affected and non-affected *BMPR2* mutation carriers. Further more our data show that in the absence of puromycin, the WT transcripts constitute the great bulk of the detected transcripts.

After validation of the LB cell line as a reproducible model for BMPR2 expression we then compared WT *BMPR2* expression in four kindreds with NMD^+ mutations and found that LB cells from the affected family members have lower levels of WT *BMPR2* transcripts compared to those found in LB cells from unaffected relatives who carry the same *BMPR2* mutation. This association of transcript levels with penetrance is not limited to a single NMD^+ mutation since all four of the kindreds analyzed have different NMD^+ mutations. Our data thus support the notion that variation in WT *BMPR2* transcript amounts may act as a modifier that influences the penetrance of FPAH at least in individuals who carry HI causing mutations.

There is growing body of work that suggests that modifier genes play an important role in complex disease pathogenesis (Nadeau, 2001). In lung disease the best studied example for the role of modifiers is in cystic fibrosis (for a review see (Boyle, 2007)). However, in many other chronic lung diseases, including PAH, these modifiers are poorly understood. The findings from our study suggest that the levels of WT transcripts may be one such modifier of PAH. Clues as to how levels of WT transcripts might affect PAH penetrance come from the finding that HI (secondary to NMD+ mutations) of *BMPR2* is responsible for nearly 50% FPAH. Since BMPR2 receptor forms a heteromeric complex with BMPR1a and BMRP1b the degree of deficiency of normal BMPR2 receptor could affect the stoichiometric imbalance in the BMPR2 heteromeric complex on the cell surface leading to decreased signaling through the receptor and thus disease. Thus, decreased expression of the normal allele in a HI background (caused by the heterozygosity for NMD^+ mutation) may further lower *BMPR2* expression, below a critical threshold, needed for proper receptor stoichiometry and function thus impacting clinical expression of the disease. Such modulation of disease penetrance by WT transcripts is thought to be a rare phenomenon that has been previously reported in only three genetic disorders such as dominantly inherited Erythropoietic Protoporphyria, Hereditary Elliptocytosis and autosomal dominant Retinitis Pigmentosa (Gouya, et al., 2002; Gouya, et al., 2004; Vithana, et al., 2003; Wilmotte, et al., 1993). Such a hypothetical threshold effect for *BMPR2* in FPAH pathogenesis suggests novel therapeutic approaches such as the use of agents that promote read-through of the PTCs of the mutant *BMPR2* transcripts resulting in an increase in expression, enough, to exceed the threshold of HI and prevent FPAH may have some clinical utility. Preliminary data from ongoing research suggest that aminoglycosides allow read through of the PTCs in LB cell lines (Hamid et al. unpublished data).

The mechanisms underlying variable expression of the WT *BMPR2* allele are not known at this time. Altered expression could be secondary to a *cis* effect i.e. variation in regulatory regions, or a *trans* effect i.e. variation of expression of genes or factors that control *BMPR2* expression. Thus, our findings suggest the importance of detailed characterization of the proximal regulatory regions *BMPR2* and of WT *BMPR2* alleles that have different expression levels.

In summary, our data show the validity of using LB cell lines to study *BMPR2* expression and importantly, show for the first time that variations in the amounts of WT *BMPR2* transcripts may modulate the HI that causes FPAH pathogenesis in all four kindreds studied.

A future expanded study (already underway in our laboratory) is to determine if this association is seen consistently in all FPAH kindreds with NMD^{+}/HI *BMPR2* mutations.

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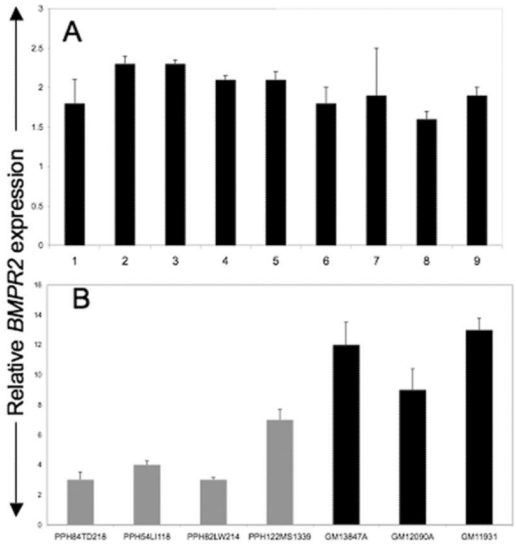

Fig. 1.

BMPR2 transcript levels, as measured by relative real-time PCR analysis, in CEPH control LB samples (A) and FPAH derived LB samples (B). Samples from control individuals (non-mutation carriers) are shown in black and from affected individuals in grey. Each experiment was done at least three times in replicates of 3. Error bars represent SD of the replicates.

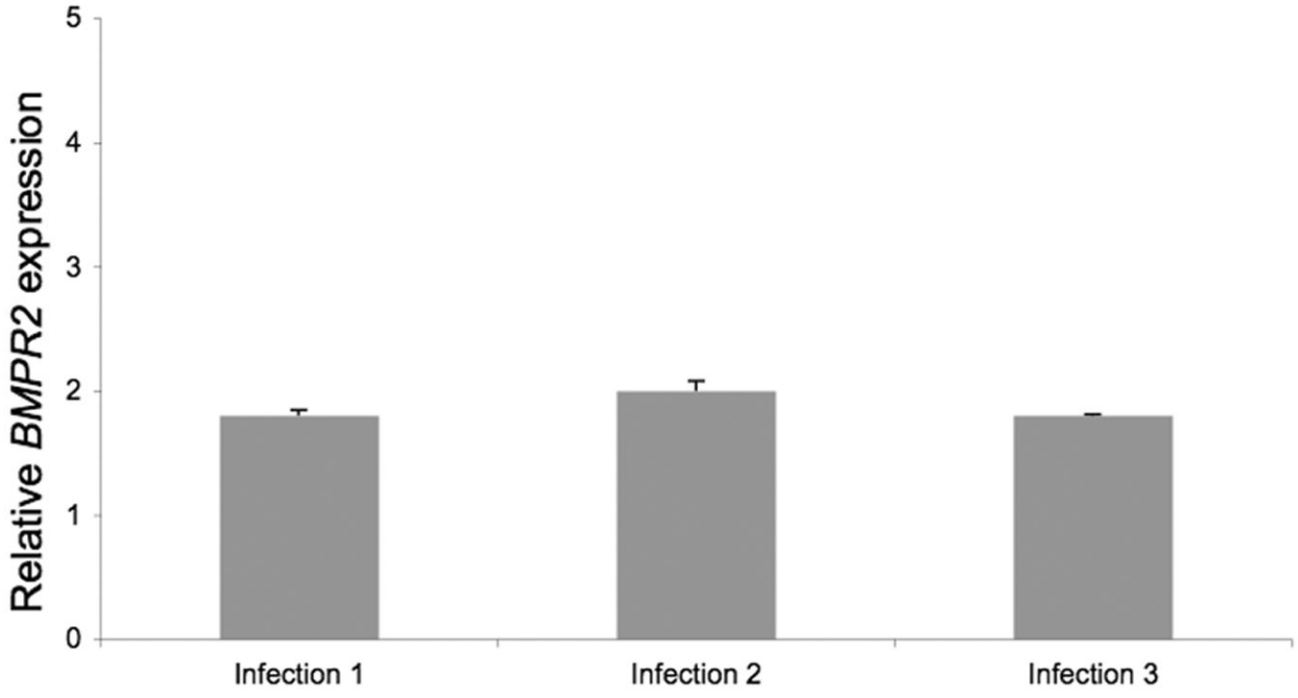

Fig. 2.

BMPR2 transcript amounts as measured by relative real-time PCR analysis from three LB cell lines established from three separate infections of lymphocytes from one affected FPAH patient from kindred 80. Each experiment was done at least three times in replicates of 3. Error bars represent SD of the replicates.

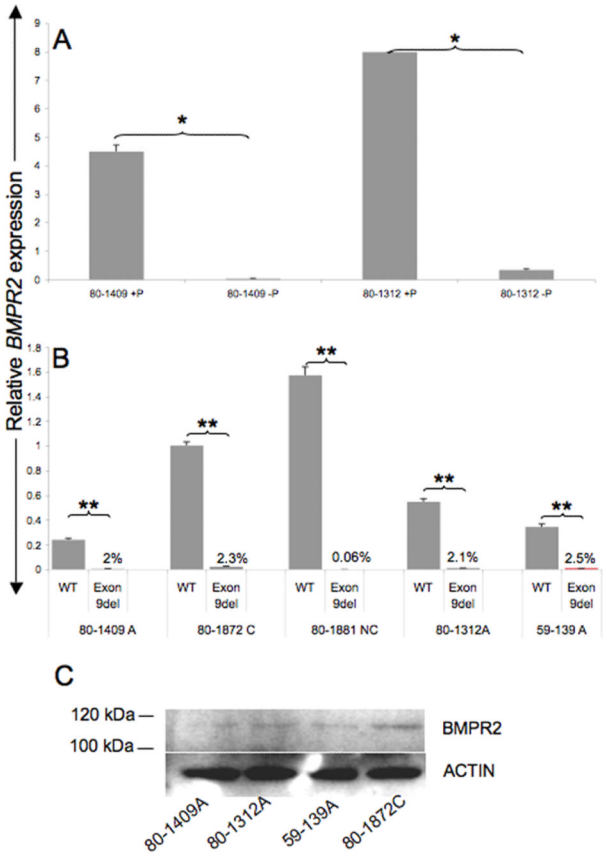

Fig. 3.

Real-time PCR analysis of the relative amounts of NMD$^+$-mutant *BMPR2* transcripts (exon 9del resulting in frame shift and PTC) in LB cell lines derived from kindred 80, with (+P) and without puromycin (−P). Puromycin stabilizes the mutant product, resulting in 112 fold increase in case of patient 80-1409 (*p<.0001) and 23 fold in case of patient 80-1312 (*p<.0001) and thus allowing for detection by real-time PCR (A). Without puromycin the bulk of the BMPR2 transcript is WT transcript (B) (**p<.0001). The percentage numbers represent the contribution of the mutant transcript to the total BMPR2 level in the absence of puromycin treatment. Western blot analysis shows that the BMPR2 transcript differences are also present at the protein level (C). A (Affected)= mutation positive and clinically affected, C (carriers)=mutation positive but clinically unaffected, NC (non carriers) non-mutation carriers and not clinically affected. Each experiment was done at least three times in replicates of 3. Error bars represent SD of the replicates.

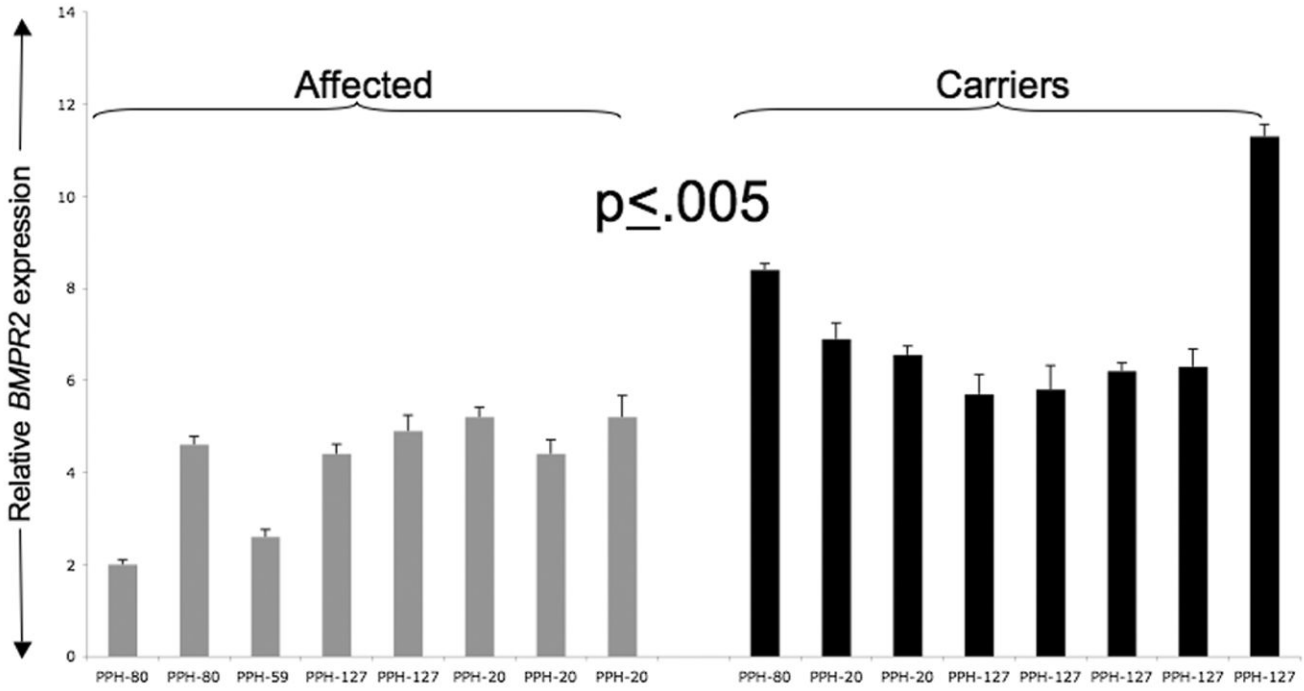

Fig. 4.

BMPR2 WT transcript levels in affected (grey) and carrier (black) individuals as detected by relative real-time PCR analysis. Affected individuals (grey) have lower levels of expression of WT allele as compared to carriers (black) of the same mutation. Analysis was done on all the available LB cells from 4 kindreds (numbers under the bars represent the kindred number in our registry database; PPH-20, PPH-59, PPH-80, PPH-127). Please see Table 1 for list of mutation present in each kindred. Each experiment was done at least three times in replicates of 3. Error bars represent SD of the replicates. One-way analysis of variance (ANOVA) and Mann-Whitney U (Wilcoxon rank-sum) test was used to determine significance with the help of JMP software (SAS Institute Inc. Cary NC) package.

Table 1

BMPR2 DNA Mutations Studied

Family	Location	Nucleotide Change	Amino Acid change
20	Exons 4–5	c.419-?_621+?del	p.S140fsX12
59 & 80	Intron 8	c.1129-3C>G	p.V377fsX48
127	Exon 9	c.1141_1142insA	p.R381fsX18

Nucleotide numbering reflects cDNA numbering, where +1 corresponds to the A of the ATG translation initiation codon in the reference sequence (GenBank NM_001204.6), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.