ABSTRACT

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Pulmonary arterial hypertension (PAH) is characterized by pulmonary arterial vasoconstriction and vascular remodeling. In our previous study, novel and rare heterozygous variants in *SMAD5* and *SLIT2*, predicted to be pathogenic and involved in biological pathways potentially associated with disease pathogenesis, were identified by whole-exome sequencing in a patient diagnosed with idiopathic PAH (IPAH) at a young age. In addition, *SMAD5* expression was observed to be decreased in IPAH cases. This thesis aimed to evaluate the potential roles of the molecular alterations caused by the identified premature termination codon-forming *SMAD5*: c.694C>T, p.Gln232* variant and missense *SLIT2*:c.2852G>A, p.Gly951Glu variant in PAH pathogenesis using *in vitro* functional analyses.

The effects of the variants were evaluated *in silico* using pathogenicity prediction tools. Family segregation analysis was performed by Sanger sequencing, and *SMAD5* mRNA expression was examined in family members. For the *in vitro* studies, HEK293T cells were subjected to CRISPR/Cas9-mediated editing and single-cell cloning to establish a *SMAD5 knock-out* (KO) model. In wild-type (WT) HEK293T cells, siRNA-mediated *SLIT2* silencing and BMP-4 stimulation were performed. The effect of BMP-4 treatment on cell viability was evaluated using the CCK-8 assay. Under the established cell culture conditions, mRNA expression levels of *SLIT2* and its receptor *ROBO1*, the BMP receptor *BMPR2*, members of the R-SMAD family (*SMAD1*, *SMAD5*, and *SMAD8*), the common SMAD, *SMAD4*, and the downstream targets of the BMP signaling pathway, *ID1* and *ID3*, were analyzed by quantitative real-time PCR. In addition, protein levels of SMAD5, phosphorylated SMAD5 (pSMAD5), and phosphorylated SMAD1/5/8 (pSMAD1/5/8), indicating active BMP signaling, were evaluated by Western blot.

In silico analyses classified both variants as pathogenic. The *SLIT2* variant was predicted to decrease protein stability, whereas the *SMAD5* variant was predicted to cause nonsense-mediated mRNA decay. This predicted effect on *SMAD5* was confirmed by Sanger sequencing of cDNA obtained from the proband. The *SMAD5* variant was detected in the heterozygous state in four family members in addition to the proband, whereas the *SLIT2* variant was detected only in the proband. *SMAD5* expression was found to be decreased in the four variant-carrying family members compared with non-carriers. In the *in vitro* studies, six different colonies generated for the *SMAD5* KO model initially showed 46–87% reduction in mRNA expression and 57–92% reduction in protein expression; however, this reduction was found not to be stable in the long term. In WT HEK293T cells, *SLIT2* expression was successfully silenced by 69% following siRNA treatment. BMP-4 treatment increased *ID1* and *ID3* expression, confirming pathway activation. *SLIT2* silencing increased *BMPR2* gene expression. In addition, in *SLIT2*-silenced cells, BMP-4 treatment increased *SMAD1* and *SMAD8* expression compared with their respective controls. *SMAD8* expression was also increased in the BMP-4-only condition compared with the control. Under *SLIT2*-silenced conditions, SMAD5, pSMAD5, and pSMAD1/5/8 protein levels, as well as the pSMAD5/total SMAD5 ratio, were decreased.

In conclusion, the reduction in SMAD5 protein levels and R-SMAD phosphorylation following *SLIT2* silencing suggests that SLIT2 may have a regulatory role in the BMP/SMAD signaling pathway. Together with the *SMAD5* variant, this finding supports a shared molecular mechanism that may contribute to the severe PAH phenotype observed only in the proband.

Key Words: IPAH, BMP, BMP-4, *SMAD5*, *SLIT2*.

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