Gene 588 (2016) 163–172

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Unveiling the interactions among *BMPR-2*, *ALK-1* and *5-HTT* genes in the pathophysiology of HAPE☆,☆☆

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ARTICLE INFO

Article history:
Received 1 October 2015
Received in revised form 5 May 2016
Accepted 12 May 2016
Available online 16 May 2016

Keywords:
High-altitude
Pulmonary hypertension
BMPR-2
ALK-1
5-HTT
BMP-2
5-HT
Genetic-interactions
High altitude pulmonary edema

ABSTRACT

Context: Few potential candidate genes coding for type I and II receptors of transforming growth factor beta signaling pathway and the serotonin transporter have been associated with pulmonary hypertension (PH). The latter being a phenotype for high altitude pulmonary edema (HAPE), these genes are hypothesized to be crucial markers to investigate under the hypobaric hypoxic environment of high altitude.
Aims: We hence aimed to investigate bone-morphogenetic protein-2 (BMP2), bone morphogenetic protein receptor type-II (*BMPR-2*), activin receptor-like kinase-1 (*ALK-1*), serotonin transporter (*5-HTT*) and serotonin (5-HT) for their contribution, individually/epistatically, to clinical endpoints by altering downstream signaling molecules.
Methods and materials: In a case–control design, interactions between/among polymorphisms of *BMPR-2*, *ALK-1* and *5-HTT* were screened in 200 HAPE-patients (HAPE-p) and 200 HAPE-free sojourners (HAPE-f). Plasma biomarker BMP-2 and 5-HT were estimated. The relative gene expression was also witnessed in 20 humans/10 rats followed by correlation analyses.
Results: The genotype/allele models revealed the prevalence of *BMPR-2* rs6717924A-rs4303700A-rs1048829A; *ALK-1* rs11169953T-rs3759178C-rs706816C and *5-HTT* rs6354C in HAPE ($P \le 0.05$). Multifactor dimensionality reduction for interactions among genes revealed a 4-locus model of BMPR-2 rs6717924G/A; *ALK-1* rs11169953C/T-rs706816T/C and *5-HTT* rs6354A/C as the best disease predicting ($P \le 0.001$); whereas HapEvolution analysis confirmed the alleles rs6717924A, rs4303700A and rs6354C as the best interacting ($P \le 0.01$). Plasma levels of BMP-2 and 5-HT were elevated in HAPE ($P \le 0.0001$). The expression of BMP-2, *ALK-1*, 5-HT, *5-HTT* was elevated and of *BMPR-2* decreased in humans and rats ($P \le 0.05$).
The risk alleles *BMPR-2* rs6717924A-rs4303700G-rs1048829A; *ALK-1* rs11169953T-rs706816C and *5-HTT* rs6354C correlated inversely with arterial oxygen saturation (SaO_2) and positively with mean arterial pressure (MAP), BMP-2 and 5-HT in HAPE. Likewise, haplotypes *BMPR-2* GGGCGAAAA, AAATAGGGA and *ALK-1* CCTCAA AG, CCTTAAAG correlated with clinical markers and biomarkers ($P \le 0.01$). BMP-2 and 5-HT correlated positively with MAP and negatively with SaO_2 ($P \le 0.01$).
Conclusions: The genetic-interactions among *BMPR-2*, *ALK-1*, and *5-HTT* polymorphisms, elevated BMP-2 and

Abbreviations: ALK-1, activin A receptor type II-like 1; ACVRL-1, activin A Receptor Type II-Like 1; ANOVA, analysis of variance; ANCOVA, analysis of covariance; BMI, body mass index; BMP-2, bone morphogenetic protein-2; BMP-4, bone morphogenetic protein-4; BMPR-2, bone morphogenetic protein receptor-2; BP, blood pressure; CCA, cooperative coevolutionary algorithm; Co-SMADs, collaborating SMADs; CI, confidence interval; CVC, cross-validation consistency; C_t, threshold cycle; EGLN-1, Egl nine homolog 1; eNOS, endothelial nitric oxide synthase; FDR, false discovery rate; HWE, Hardy-Weinberg equilibrium; H_2O_2, hydrogen peroxide; HA, high altitude; HAPE, high-altitude pulmonary edema; HPVR, hypoxic pulmonary vasoconstriction response; HVR, hypoxic ventilatory response; HRR, haplotype risk ratio; HRP, horseradish peroxidase; I-SMADs, inhibitory SMADs; *D′*, Lewontin's disequilibrium coefficient; LD, linkage disequilibrium; mnSOD, manganese superoxide dismutase; MAF, minor allele frequency; SNPs, single nucleotide polymorphisms; NOS3, endothelial nitric oxide synthase; OR, odds ratio; $ONOO^{\bullet-}$, peroxynitrite; *P*-value, level of significance; PEG, polyethylene glycol; PH, pulmonary hypertension; PR, pulse rate; PPH, primary pulmonary hypertension; PAH, pulmonary arterial pressure; R-SMADs, receptor SMADs; PASMC, pulmonary artery smooth muscle cell; QTLs, quantitative trait loci; rSNPs, regulatory SNPs; r^2, correlation coefficient; RR, respiratory rate; $O_2^{\bullet-}$, superoxide; SISA, simple interactive statistical analysis; SMADs, mothers against DPP homolog 4; SOD, superoxide dismutase; SBP, systolic blood pressure; SPSS, statistical package for social sciences; SSc, systemic sclerosis; TA, testing accuracy; TB, testing balance; TGF-β, transforming growth factor-β; TPH-1, tryptophan hydroxylase-1; 5-HT, serotonin; 5-HTT, hydroxytryptamine transporter; 8-isoPGF2α, 8-isoprostaglandin F2α; χ^2, chi square.

☆ Source of support: The Council of Scientific and Industrial Research, India, financially supported under the Supra-Institutional Project — SIP0006.

☆☆ Key message: The outcome highlighted that HAPE is the consequence of cross talk among clinical, genetic markers of more than one gene and biomarkers and hence is a multifactorial disorder. There is disrupted balance between *BMP* and 5-HT/*5-HTT* signaling, which may act as crucial events to the susceptibility to HAPE.

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http://dx.doi.org/10.1016/j.gene.2016.05.016
0378-1119/

5-HT levels and differential gene expression substantiated the strong genetic contribution in HAPE pathophysiology.

1. Introduction

High altitude pulmonary edema (HAPE) is a noncardiogenic form of pulmonary edema characterized by increased capillary permeability and fluid in the lungs and uneven vasoconstriction that results in exaggerated capillary pressure (Swenson et al., 2002; Hlastala et al., 2004; Hopkins et al., 2005; West et al., 1991). It is a potentially fatal disease that occurs in otherwise healthy sojourners after ascent to altitudes of >2500 m (Swenson et al., 2002; Hlastala et al., 2004; Hopkins et al., 2005; West et al., 1991). In addition to the clinical and physiological understanding, the last one decade has provided substantial knowledge on the genetic basis of HAPE. Investigators used molecular approaches such as the candidate gene and genome-wide association to identify the variants in polymorphisms on genes or chromosomes. Several candidate genes of various physiological pathways have shown potential to be candidate markers for HAPE, albeit genetic understanding of HA disorders still is in its infancy.

Pulmonary hypertension (PH), also one of the hallmark features of HAPE, has been found to associate with mutations in two genes of the transforming growth factor-β (*TGF-β*) receptor superfamily, namely the bone morphogenetic protein receptor type-2 (*BMPR-2*) (Deng et al., 2000; International PPH Consortium et al., 2000; Machado et al., 2001) and the activin receptor-like kinase 1 (*ACVRL1* or *ALK-1*) (Abdalla et al., 2004; Chaouat et al., 2004). Signal transduction through this pathway involves heterodimerization of *BMPR-2* with *BMPR-1*. Activated *BMPR-1* initiates intracellular signaling by phosphorylating specific receptor-regulated (R-SMAD) proteins, which translocate to the nucleus, where they regulate the transcription of target genes. The ligands for the *BMPR-2* receptor are the bone morphogenetic proteins (BMPs). Genetic susceptibility in this signaling system may affect vascular differentiation, cell proliferation giving rise to lesions in pulmonary arteries and overall, increases the pulmonary vascular resistance to cause severe vasoconstriction. Among the vasoconstrictors, serotonin or 5-hydroxytryptamine (5-HT) is a potent mitogen crucially involved in proliferation of smooth muscle cells and pulmonary fibrosis. 5-HT exerts its vasoconstrictory effect through its transporter, 5-hydroxytryptamine transporter (*5-HTT*) (Long et al., 2006; MacLean et al., 2000, 2004; Machado et al., 2006). It appears to result from the overexpression of tryptophan hydroxylase-1 enzyme and 5-HTT, causing an abnormal 5-HT production by endothelial cells and an enhanced pulmonary proliferative response. Several studies have reported that pulmonary arterial *5-HTT* and 5-HT concentration was increased in PH (Eddahibi et al., 1999, 2003; Morecroft et al., 1999; Ali et al., 2012). It has been functionally demonstrated in PH but its effect is yet to be established in the hypobaric hypoxia associated disorder namely, HAPE.

Taking the lead from the available literature, but recognizing a lack of direct linkage of events in PAH to HAPE, we nonetheless hypothesized that *BMPR-2*, *ALK-1*, *5-HTT*, BMP-2 and 5-HT may contribute individually/epistatically to clinical endpoints by altering downstream signaling molecules in HAPE. To address these issues, (1) thirteen genetic variants of bone morphogenetic protein receptor-2 (*BMPR-2*), ten of activin receptor-like kinase 1 (*ALK-1*) and ten of 5-hydroxytryptamine transporter (*5-HTT*) were selected to investigate in well-characterized cohorts/subjects with case–control design. The single nucleotide polymorphisms (SNPs) were investigated (a) individually, (b) as haplotypes and (c) in combinations, (2) performed functional assessment of the significantly associated SNPs, to understand the extent of involvement of each allele in the interacting mode within and among the elements of selected pathways; (3) measured plasma levels of BMP-2 and serotonin (5-HT), (4) evaluated differential gene expression of *BMP-2*, *BMPR-2*, *ALK-1* and *5-HTT* both in (a) HAPE-p and HAPE-f, and (b) Wistar rats (animal study); and finally (5) performed detailed correlation analyses.

2. Materials and methods

2.1. Selection of subjects

The study protocol and consent forms were approved by institutional ethical committees of Council of Scientific and Industrial Research-Institute of Genomics and Integrative Biology (CSIR-IGIB), Delhi and the Sonam Norboo Memorial (SNM) Hospital, Leh (3500 m), Jammu and Kashmir, India. Subjects were categorized into two well-defined groups: (1) HAPE-patients (HAPE-p), $n = 200$ and (2) HAPE-free sojourners (HAPE-f), $n = 200$. All the subjects were recruited through SNM hospital. HAPE-p and HAPE-f, residing at an altitude of <200 m, were unrelated sojourners of Indo-Aryan origin. Subjects were airlifted to Leh and from there they ascended or descended to different heights (3500–5600 m). Few of them suffered mountain disorders and were immediately transferred to SNM hospital for confirmation of diagnosis and treatment. HAPE was confirmed by performing the clinical tests such as measurement of arterial oxygen saturation (SaO_2) and the chest x-ray. The patients had SaO_2 of ≤70%. Of note, the HAPE-p on their very first visit developed HAPE. On the contrary, HAPE-f remained fit throughout their stay at HA and carried out normal physical activities; their SaO_2 levels were >90%. These individuals either accompanied patients or were the volunteers, who donated blood at the blood bank of SNM hospital and had at least 2 visits above 3500 m. Table S1 represents the baseline and clinical characteristics of HAPE-p and HAPE-f.

2.2. Patient's inclusion & exclusion criteria

Trained clinicians administered a detailed questionnaire on environmental factors, life style, health, clinical history of family, migration status and hemodynamic parameters of the participants. Diagnosis of HAPE was based on the clinical symptoms that included hypoxemia, cough and dyspnea at rest, presence of pulmonary rales, cyanosis and chest radiography that revealed infiltrates consistent with pulmonary edema. Pulmonary artery systolic-pressure (PASP) was measured using Sonos-5500 echocardiography (Hewlett-Packard, USA). The clinical parameters e.g., body mass index (BMI), blood pressure (BP), SaO_2 and pulse rate (PR) were measured. The subjects were given rest prior to BP measurement. Three measurements of BP, in supine position, using a calibrated mercury sphygmomanometer with appropriate adult cuff size were recorded. The SaO_2 and PR were measured thrice using Finger-Pulse Oximeter 503 (Criticare Systems Inc., USA). The subjects on any medication were excluded. Any previous history of cardiopulmonary and other diseases was ruled out through questionnaire. Lake Louise scoring was applied to look for any symptoms of acute mountain sickness (AMS). A significant number of subjects could not be included because either they did not consent, were already on medication or for various other limiting factors.

2.3. Gene sequencing

Ten mL of blood was drawn in an acid-citrate-dextrose (ACD) anticoagulant tube in supine position from each subject. Healthy subjects' blood was drawn after overnight fasting; patients' blood was drawn immediately after diagnosis. Plasma and peripheral blood leukocyte cells were separated; the cells were used for DNA isolation. Plasma and DNA were stored at −70 °C and −20 °C, respectively.