Blood Cells, Molecules and Diseases 58 (2016) 21–25

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

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

Association of adenylyl cyclase 6 rs3730070 polymorphism and hemolytic level in patients with sickle cell anemia

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ARTICLE INFO

Article history:
Submitted 21 December 2015
Revised 16 February 2016
Accepted 17 February 2016
Available online 18 February 2016

Editor: Mohandas Narla

Keywords:
Sickle cell anemia
Hemolysis
Adenosine pathway
Adenylyl cyclase

ABSTRACT

A recent study suggested that adenosine signaling pathway could promote hemolysis in patients with sickle cell anemia (SCA). This signaling pathway involves several gene coding enzymes for which variants have been described. In this study, we analyzed the genotype–phenotype relationships between functional polymorphisms or polymorphisms associated with altered expression of adenosine pathway genes, namely adenosine deaminase (*ada;* rs73598374), adenosine A_{2b} receptor (*adora2b*; rs7208480), adenylyl cyclase 6 (*adcy6*; rs3730071, rs3730070, rs7300155), and hemolytic rate in SCA patients. One hundred and fifty SCA patients were genotyped for *adcy6*, *ada*, and *adora2b* variants as well as alpha-globin gene, a genetic factor known to modulate hemolytic rate. Hematological and biochemical data were obtained at steady-state. Lactate dehydrogenase, aspartate aminotransferase, reticulocytes and total bilirubin were used to calculate a hemolytic index. Genotype–phenotype relationships were investigated using parametric tests and multivariate analysis. SCA patients carrying at least one allele of *adcy6* rs3730070-*G* exhibited lower hemolytic rate than non-carriers in univariate analysis ($p = 0.006$). The presence of *adcy6* rs3730070-*G* variant was associated with a decreased hemolytic rate in adjusted model for age and alpha-thalassemia ($p = 0.032$). Our results support a protective effect of *adcy6* rs3730070-*G* variant on hemolysis in SCA patients.

1. Introduction

Sickle cell anemia (SCA) is a hereditary disorder of hemoglobin (Hb) caused by a single missense mutation in the human β-globin gene leading to an amino acid substitution (Glu → Val) in the sixth position of the β-globin chain. The polymerization of the resulting abnormal hemoglobin, HbS, under hypoxic conditions is a key event in the complex pathophysiology of SCA, inducing a mechanical distortion of red blood cells (RBCs) [1]. Sickle RBCs exhibit decreased deformability, reduced life span and may adhere to the vascular endothelium and other cells, hence triggering vaso-occlusive crisis (VOC). In addition, the increased fragility of sickle RBCs is at the origin of enhanced hemolysis, which plays a central role in the development of vasculopathy [2,3].

Several parameters modulating the extent of HbS polymerization are known since decades such as intracellular hemoglobin concentration and composition, oxygen saturation, as well as intracellular pH and 2,3-diphosphoglycerate (2,3-DPG) concentration [4]. A recent study performed in mouse models has shown that hypoxia-mediating adenosine signaling may also play an important role in hemolysis by promoting HbS polymerization [5]. Indeed, ATP derived from hemolysis and tissue damage caused by vaso-occlusive events is rapidly converted to adenosine by the combined action of the 5′-ecto-nucleotidase CD39 and CD73. It has been shown that adenosine binding to RBC adenosine A_{2b} receptor (ADORA2B), a seven-transmembrane-spanning G protein-coupled receptor [6], stimulates adenylyl cyclase activity, and leads to cAMP-dependent protein kinase A signaling and ultimately an increase of RBC production of 2,3-DPG. The resulting decrease of Hb oxygen affinity favors the development of deoxyhemoglobin S polymers and consequently promotes further RBC sickling and hemolysis [5].

Several functional gene variants involved in the adenosine metabolic pathway have been previously described. Adenosine deaminase (ADA) is one of the key enzymes of this pathway. Bound at the cell surface of

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http://dx.doi.org/10.1016/j.bcmd.2016.02.006
1079-9796/

monocytes and lymphocytes via CD26, ADA regulates plasma concentration of adenosine by irreversibly converting adenosine into inosine and thus lowering ADORA2B signaling [7,8]. In contrast to the rare and mostly 'private' (occurring in single families) *ada* mutations associated with lymphopenia and immunodeficiency [9], the guanine to adenine substitution at nucleotide 22 of exon 1 (rs73598374) of ADA gene has been described as a common polymorphism in several populations [10] leading to the substitution of aspartic acid by asparagine at amino acid 8 (Asp8Asn) [11]. Individuals carrying one copy of rs73598374-A allele display 15 to 20% lower ADA activity and higher plasma adenosine level compared to rs73598374-G homozygotes [12,13]. To our best knowledge, no variant altering *adora2b* expression has been described except a cis-acting eQTL (rs7208480) associated with the peripheral blood mononuclear cell *adora2b* expression [14], a gene potentially involved in the modulation of clinical variability in SCA [15,16]. Finally, Eyler et al. have shown that two non-coding *adcy6* polymorphisms (rs3730070 and rs7300155) were associated with elevated adherence of sickle RBCs to laminin [17] while the non-coding rs3730071-T allele has been associated with an increased adenylyl cyclase activity and function [18].

In the present gene-candidate study, we analyzed the genotype–phenotype relationship between the previously described genetic variants of the adenosine metabolic pathway and hemolytic rate in 150 SCA patients regularly followed-up by the sickle cell center of Guadeloupe, a French Caribbean island. We also included the α-globin gene status in the genetic factors analyzed since α-thalassemia is a well-established genetic factor modulating the hemolytic rate in SCA [19,20].

2. Methods

2.1. Population study

One hundred and fifty SCA patients (57 children, 93 adults) regularly followed by the sickle cell center of the University Hospital of Pointe-à-Pitre (Guadeloupe) were included in this cross-sectional study. None of these SCA patients were under hydrocarbamide (HC) treatment since this condition is known to modulate the adenosine level [21]. All patients were at steady state at the time of the study, i.e., no blood transfusion in the previous three months and absence of acute episodes (infection, VOC, acute chest syndrome, stroke, priapism) at least two months before inclusion into the study.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Regional Ethics Committee (CPP Sud-Ouest Outre-Mer III, Bordeaux, France, registration numbers: 2009-A00211-56/2013, 2010-A00244-35). Children and their parents as well as adult patients were informed of the purpose and procedures of the study, and gave written informed consent.

2.2. Biological parameters

SCA diagnosis was made by isoelectrofocusing (Multiphor II™ System, GE HEALTH CARE, Buck, UK), citrate agar electrophoresis, and cation-exchange high performance liquid chromatography (VARIANT™, Bio-Rad Laboratories, Hercules, CA, USA) allowing fetal hemoglobin (HbF) quantification. In addition to Hb level, white blood cell (WBC), platelet (PLT) and reticulocyte (RET) counts, were determined with an automated cell counter. Measurements of the three following biochemical markers, i.e. bilirubin (BIL); lactate dehydrogenase (LDH); and aspartate aminotransferase (ASAT), were performed using standard methods.

2.3. Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by standard procedures and DNA was quantified using NanoDrop 2000 (ThermoFisher, Wilmington, USA). Single nucleotide polymorphisms from *ada, adora2b, and adcy6* genes were screened using SNaPshot technique. The PCR amplification conditions for *adcy6* (rs3730070, rs7300155, rs9804777, rs3730071) and *adora2b* (rs7208480) were as followed: 5 min at 95 °C followed by 35 cycles of 95 °C for 30 s, 58 °C for 45 s and 72 °C for 45 s. PCR conditions for *ada* (rs73598374) were similar, except for higher annealing temperature of 62 °C. PCR products were controlled on a 2% agarose gel and purified using Exonuclease I and Shrimp Alkaline Phosphatase (USB Corporation, High Wycombe, UK). To extend primers, SNaPshot reactions were performed using SNaPshot® Multiplex Kit (Applied Biosystems, Warrington, UK). The labeled extension products were detected by capillary electrophoresis on an ABI 3130 sequencing analyzer (Applied Biosystems, Foster City, USA). For quality control, a subsample of 10% was genotyped twice. Chain Reaction (Gap-PCR) was used to detect the 6 common α-thalassemia deletions, including $-\alpha^{3.7}$ and $-\alpha^{4.2}$ alleles, and triplication defects of α-globin genes [22].

2.4. Statistical analysis

Results are presented as means ± SD or proportions. The Chi-square test was used to test for deviation of genotypes distribution from Hardy Weinberg Equilibrium (HWE). SNPs found to deviate from HWE were excluded from analysis. Pairwise correlation was run to display coefficients between the hemolytic parameters studied (Fig. 1). A principal component analysis (PCA) was used to create a summary variable (hemolytic component) using four hemolytic parameters (BIL, LDH, ASAT and RET). It has been previously shown that the hemolytic component values obtained using such standard statistical data reduction approach reflected intravascular hemolysis [23]. Unpaired student *t*-test and Chi-square test were used for continuous covariates and for categorical covariates respectively, to compare hematological and biological parameters between the different groups obtained after patient stratification according to the genotype of the gene studied.

The transmission inheritance was assessed as followed; for each gene studied, we fitted linear regression models with genotype considered as categorical variable to test association between hemolytic components using the Wald test. If association was detected, we fitted the regression model by assuming the dominant and the recessive model and selecting the best model on the basis of the Wald test measuring the goodness of fit [24].

To identify factors independently associated with the hemolytic rate, we used a multivariate linear logistic model. Variables associated with hemolytic rates in univariate analysis such as age, α-thalassemia and *adcy6* rs3730070 were included as covariates in the multivariate regression model. Significance level was defined as $p < 0.05$. Analyses were conducted using STATA® 11.2 (StataCorp, Texas, USA).

3. Results

Baseline hematological and biochemical parameters of the 150 SCA patient included in the study are presented in Table 1. As expected, all patients presented a marked anemia and a significant hemolytic rate reflected by elevated mean values for LDH, ASAT, RET and BIL. Alpha-thalassemia was detected in fifty seven of these patients (38%).

The genotypic distribution observed for *adcy6* (rs3730070, rs3730071, rs9804777, rs7300155), *adora2b* (rs7208480) and *ada* (rs73598374) polymorphisms are summarized in Table 2. No minor variant was detected for *ada* rs73598374 and *adcy6* rs3730071. Furthermore, the genotype distribution of *adcy6* rs7300155 was inconsistent with HWE and this SNP was not further studied.

The PCA component (hemolytic component), which reflected 53% of the variability of the four variables studied (BIL, RET, LDH and ASAT), was then compared between patients classified according to the genotypic distribution of the adenosine-pathway gene polymorphisms analyzed. This analysis was performed with the 96 SCA patients for which all the hemolytic markers were available. As shown in Table 3, only rs3730070 exhibited a significant relationship with hemolytic component ($p = 0.023$).