Gene 598 (2017) 79–83

Contents lists available at ScienceDirect

Gene

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

Research paper

Characterization of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor gene *STX18* variations for possible roles in congenital heart diseases

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ARTICLE INFO

Article history:
Received 24 August 2016
Received in revised form 19 October 2016
Accepted 31 October 2016
Available online 2 November 2016

Keywords:
Congenital heart disease
STX18
Human embryonic stem cells
SNP
VSD

ABSTRACT

Congenital heart disease (CHD) is among the most prevalent and complex congenital anatomic malformations in newborns. Interactions of cardiac progenitor with a broad range of cellular regulatory factors play key roles in the formation of mammalian heart and pathogenesis of CHD. STX18 is a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor, which is involved in numeral cellular activities such as organelle assembly and the cell cycle. The aim of this work was to find evidence on whether *STX18* variations might be associated with CHD in Chinese Han populations. We evaluated SNPs rs2044, rs33952588, rs61740788, rs12504020 and rs12644497, which are located within the exon or intron sequences of the *STX18* gene, for 310 Chinese Han CHD patients and 400 non-CHD controls. Using SPSS software (version 19.0) and the online software OEGE, we conducted statistical analyses and Hardy-Weinberg equilibrium test, respectively. Among the five SNPs identified in the *STX18* gene, rs33952588 and rs61740788 had very low genetic heterozygosity. In contrast, the genetic heterozygosity of the remaining three variations rs12504020 and rs12644497 near the 5'UTR and rs2044 within 3'UTR of the *STX18* gene was considerably high. Analysis of associations of these genetic variations with the risk of CHD showed that rs12644497 (P value = 0.017 < 0.05) was associated with the risk of CHD, specifically VSD and ASD, whereas rs12504020 (P value = 0.560 > 0.05) and rs2044 (P value = 0.972 > 0.05) were not. The SNP rs12644497 in the *STX18* gene was associated with CHD in Chinese Han populations.

1. Introduction

Congenital heart disease (CHD) has many subtypes, including atrial septal defect (ASD), ventricular septal defect (VSD), tetralogy of Fallot, mitral valve insufficiency, pulmonary stenosis, patent ductus arteriosus etc. (Deng et al., 2014). It is among the most frequent and complex congenital anatomic malformations in newborns and a significant cause of childhood deaths (Tennant et al., 2010). The prevalence of the disease is about 7.5% in the newborns (Hoffman and Kaplan, 2002), with 1% of the CHD patients needing clinical intervention (Hoffman et al., 2004). Many genetic changes, such as polymorphisms, chromosomal variants, rare genomic copy number variants, and Mendelian disorders, have been identified in familial and sporadic CHD cases (Pierpont et al., 2007; Bruneau, 2008; Richards and Garg, 2010; Soemedi et al., 2012).

The mammalian heart is a complex organ and its formation is strictly regulated by many genes, such as those encoding transcription factors, epigenetic factors, miRNAs and signaling pathways, which form complex regulatory networks for the development of the heart (Buckingham et al., 2005; van Weerd et al., 2011; Deng et al., 2014). In previous studies, we characterized variations in *LEFTY* and *SMAD3*

Abbreviations: CHD, congenital heart disease; ASD, atrial septal defect; VSD, ventricular septal defect; STX18, syntaxin18; HES, human embryonic stem; LD, linkage-disequilibrium.

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http://dx.doi.org/10.1016/j.gene.2016.10.043
0378-1119/

genes for their association with CHD, especially VSD (Deng et al., 2014; Li et al., 2015b). LEFTY and SMAD3 play important roles in the Nodal/TGF-β signaling pathway (van de Laar et al., 2011; van der Linde et al., 2012) by regulating differentiation of human embryonic stem (HES) cells and directing the cells to develop into different embryonic lineages (Postovit et al., 2008; Costa et al., 2009; Malchenko et al., 2010). In mammalian heart development, many cell types are involved, such as myocardium, aorticopulmonary septum, cardiac neural crest and membranous ventricular septum cells (Cheng et al., 1999; Schuldiner and Benvenisty, 2003; Chen et al., 2007), and any mistakes in the process may result in congenital heart malformations (Hoffman and Kaplan, 2002). As important transcriptional regulators, the muscle segment homeobox family proteins regulate cell morphogenesis and growth and control cellular proliferation, differentiation and apoptosis (Han et al., 2007). In a previous study, we found that variations in the *MSX1* gene are closely associated with the risk of CHD, specifically VSD (Li et al., 2015a).

As a soluble *N*-ethylmaleimide sensitive factor attachment protein receptor, syntaxin18 (STX18) plays key roles in transporting vesicle membrane fusion with target compartments (Hong, 2005), such as membrane trafficking of endoplasmic reticulum and Golgi (Hatsuzawa et al., 2000) and ER-mediated phagocytosis (Hatsuzawa et al., 2006). STX18 also forms complexes with cell cycle-related proteins, which ensure proper chromosome segregation during cell division and prevent cells from prematurely exiting mitosis (Hirose et al., 2004; Varma et al., 2006; Lin et al., 2007; Bassett et al., 2008; Famulski et al., 2008). A genome-wide association study of CHD showed that a region on chromosome 4p16, adjacent to *STX18* gene, is obviously associated with the risk of atrial septal defect (ASD) (Cordell et al., 2013).

In this study, we aimed to validate possible associations of *STX18* with CHD, especially ASD. We compared the gene sequences between 310 Chinese Han CHD patients and 400 controls and found that variations rs12644497 near the 5'UTR and Exon1 of the *STX18* gene was associated with the risk of CHD.

2. Materials and methods

2.1. The study population

From the years 2009 to 2016, we collected blood samples from 310 CHD patients and 400 normal controls for this study at the Second and the Fourth Affiliated Hospitals of Harbin Medical University, Harbin, China (Table 1). Except for 23 CHD specimens that we collected specifically for this study, the remaining samples were used in previous work (Li et al., 2015a; Li et al., 2015b). All the patients and normal controls received comprehensive physical examination and this study was approved by the Ethics Committee of the Harbin Medical University as described in detail previously (Li et al., 2015a; Li et al., 2015b).

2.2. DNA analysis

Genomic DNA was extracted from the peripheral blood leukocytes of all the participants as described before (Li et al., 2015a; Li et al., 2015b) and stored at −20 °C prior to use. The *STX18* gene consists of 15 exons and is located on 4p16.3-p16.2. To determine the SNP genotypes, we first amplified the exons and splicing sites of the *STX18* gene using polymerase chain reaction method (Table 4), and the products were sequenced using standard protocols (Tan et al., 2012). After that, the genotypes of the SNP were determined using PCR and gene sequencing methods (Deng et al., 2014).

Table 1
General information of the study population.

Parameter	CHD	Control	F	t	P	95% CI-up	95% CI-low
Sample (n)	310	400	–	–	–	–	–
Male/female (n)	140/170	173/227	–	–	0.611	–	–
Age (years)	15.11 ± 17.32	13.68 ± 10.22	100.877	1.475	0.141	-0.47510	3.34877

Data are shown as mean ± SD; between the two groups, there were no statistical differences of the age and gender composition.

2.3. Rs12504020, rs2044, rs12644497 genotyping analysis and statistical methods

We determined genotypes of the rs12504020, rs2044 and rs12644497 in the *STX18* gene (Fig. S2), and all the measurements were conducted by two independent researchers (Table 5). And then overall CHD genetic correlation analysis was conducted according to CHD types and sample sizes. The statistical analyses and Hardy-Weinberg equilibrium test were conducted as described previously (Tan et al., 2012; Deng et al., 2014; Li et al., 2015a; Li et al., 2015b). Using the Haploview software (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview) (Barrett et al., 2005), we also determined haplotype diagram of linkage-disequilibrium (LD) structure.

3. Results

3.1. Patients

The clinical diagnosis for all participants was confirmed at the Second and Fourth Affiliated Hospitals of Harbin Medical University. The CHD patients had no history or manifestations of any other systemic abnormalities, and their mothers did not take medications or attract infections during gestation; such factors have been found to be associated with heart malformation in pregnancy (van Driel et al., 2008; Kebed et al., 2014). The 310 CHD patients contained 130 with ventricular septal defects (VSD), 115 with atrial septal defects (ASD), 48 with patent ductus arteriosus (PDA), 14 with tetralogy of Fallot, and 3 with other types of congenital heart defects. The 310 CHD patients (male 140, female 170, the min and max age were 0.2 and 61.0 respectively, and the average age was 15.11 years) and 400 unrelated controls (male 173, female 227, the min and max age were 0.3 and 60.0 respectively, and the average age was 13.68 years) were recruited for this study, and there was no statistical differences in gender composition or age between the two groups (Table 1).

3.2. STX18 gene analysis

We sequenced the *STX18* gene to test the hypothesis that germline common genetic variants in *STX18* may confer susceptibility to CHD. In the comparisons of the transcribed regions and splicing sites of *STX18* between the patients and controls, we identified variations rs33952588 and rs61740788 within the translated region, and rs12504020, rs2044 and rs12644497 within the intron region of the gene (Fig. S1). Further analysis showed that the genetic heterozygosity was very low in rs33952588 and rs61740788 but remarkably high in rs12504020, rs2044 and rs12644497 (Fig. S2).

3.3. Characterization of SNPs rs12504020, rs2044 and rs12644497

To further test any possible associations between *STX18* and CHD, we conducted SNP analyses on rs2044, located within the 3′-UTR region, and rs12504020 and rs12644497, located near the 5′-UTR region of the gene, and found that rs12644497 was associated with the risk of CHD, primarily VSD and ASD, in Chinese Han population (Tables 2, 3). Haploview software was used to conduct LD analysis. The results from the LD analysis of the variants (rs12504020, rs2044 and rs12644497) in the present study and the data from the HapMap CHB