Gene 575 (2016) 144–148

Contents lists available at ScienceDirect

Gene

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

Research paper

Genetic variation in the *GDNF* promoter affects its expression and modifies the severity of Hirschsprung's disease (HSCR) in rats carrying *Ednrb*^{sl} mutations

Jieping Huang [a,b,1], Ruihua Dang [b,*,1], Daisuke Torigoe [c,e], Anqi Li [b], Chuzhao Lei [b,*], Nobuya Sasaki [d], Jinxi Wang [c], Takashi Agui [c]

[a] College of Life Sciences, Northwest A&F University, Yangling, Shaanxi, China
[b] College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi, China
[c] Laboratory of Laboratory Animal Science and Medicine, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido, Japan
[d] Laboratory of Laboratory Animal Science and Medicine, School of Veterinary Medicine, Kitasato University, Aomori, Japan
[e] Division of Microbiology and Genetics, Center for Animal Resources and Development, Kumamoto, Japan

ARTICLE INFO

Article history:
Received 24 June 2015
Received in revised form 12 August 2015
Accepted 24 August 2015
Available online 28 August 2015

Keywords:
HSCR
Gdnf
Promoter
Genetic variation
Dual-luciferase assay

ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) is necessary for the migration of neural crest stem cells in the gut. However, mutations in *GDNF* per se are deemed neither necessary nor sufficient to cause Hirschsprung's disease (HSCR). In a previous study, a modifier locus on chromosome 2 in rats carrying *Ednrb*^{sl} mutations was identified, and several mutations in the putative regulatory region of the *Gdnf* gene in AGH-*Ednrb*^{sl} rats were detected. Specifically, the mutation −232C>T has been shown to be strongly associated with the severity of HSCR. In the present study, the influence of genetic variations on the transcription of the *Gdnf* gene was tested using dual-luciferase assay. Results showed that the mutation −613C>T, located near the mutation −232C>T in AGH-*Ednrb*^{sl} rats, decreased *Gdnf* transcription in an in vitro dual-luciferase expression assay. These data suggested an important role of −613C in *Gdnf* transcription. Expression levels of the *Gdnf* gene may modify the severity of HSCR in rats carrying *Ednrb*^{sl} mutations.

1. Introduction

Hirschsprung's disease (HSCR) is a congenital disease characterized by the absence of enteric ganglia along a variable length of the hindgut, which results in obstruction of normal intestinal motility in the aganglionic gut segment and distention of the foregut. Aganglionosis is observed in about 1/5000 live births (Amiel et al., 2008) and its genetics is highly complex and not strictly Mendelian (Belknap, 2002). Aganglionosis shows sexual and racial variation in penetrance. More than a dozen genes have been identified to be associated with HSCR [*RET*, *EDNRB*, *GDNF*, *SOX10*, *NRTN* (*NTN*), *PHOX2B*, *EDN3* (*ET3*), *ECE1*, *KIAA1279*, *TCF4*, *ZFHX1B* (*SIP1* or *ZEB2*), and *NRG1*] (Wallace and Anderson, 2011). Most of these susceptibility genes belong to three major pathways associated with the development of enteric ganglia, namely, the RET receptor tyrosine kinase pathway, the EDNRB receptor pathway, and the transcriptional factor SOX10 signaling pathway.

The RET receptor tyrosine kinase pathway, namely the RET/GDNF/GFRα1 signaling pathway, plays a vital role in the development and differentiation of the enteric nervous system (ENS) (Schuchardt et al., 1994; Moore et al., 1996; Cacalano et al., 1998). Ret proto-oncogene (*RET*) is the major susceptibility gene for HSCR (Miao et al., 2010). Mutations in *RET* account for around 50% of familial HSCR patients and 7%–35% of sporadic cases (Edery et al., 1994; Attié et al., 1995; Seri et al., 1997; Svensson et al., 1998; Garcia-Barceló et al., 2004). As a receptor, RET plays a positive role in maintaining cell development and survival after binding to the GDNF and GFRα1 complex. However, in the absence of the ligand, RET can produce negative regulation signals and promote apoptosis (Mehlen and Bredesen, 2004). GDNF, the ligand for RET, is expressed by the gut mesenchyme before the entry of neural crest cells (Natarajan et al., 2002). GDNF enhances the proliferation of cells in early migratory enteric neural crest cells (Chalazonitis et al., 1998). However, mutations in *GDNF* per se are deemed neither necessary nor sufficient to cause HSCR (Martucciello et al., 1998). Clinical studies on HSCR in humans indicated that none of the *GDNF* mutations detected so far are sufficient to cause disease (Angrist et al., 1996; Salomon et al., 1996; Eketjäll and Ibáñez, 2002). Therefore, *GDNF* is considered a rare susceptibility gene for human HSCR (<5%) (Pan and Li, 2012). However, studies in the mouse model demonstrated that GDNF contributes to ENS. *Gdnf*$^{+/-}$ mice did not exhibit HSCR (Gianino

Abbreviations: HSCR, Hirschsprung's disease; GDNF, glial cell line-derived neurotrophic factor; ENS, enteric nervous system; RET, Ret proto-oncogene; LEF1, lymphoid enhancing factor-1.

E-mail addresses: dangruihua@nwsuaf.edu.cn (R. Dang), leichuzhao1118@126.com (C. Lei).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.gene.2015.08.051
0378-1119/

et al., 2003), whereas *Gdnf*-null mice lacked enteric neurons from the gastrointestinal caudal to the stomach and died within 24 h after birth (Pichel et al., 1996).

In a previous study, three HSCR rat strains carrying *Ednrb*^{sl} mutations, namely, AGH-*Ednrb*^{sl}, F344-*Ednrb*^{sl}, and LEH-*Ednrb*^{sl}, were established. AGH-*Ednrb*$^{sl/sl}$ rats showed the most serious aganglionosis, followed by LEH-*Ednrb*$^{sl/sl}$ rats and F344-*Ednrb*$^{sl/sl}$ rats (Dang et al., 2011. A modifier locus of HSCR on chromosome 2 using the F_2 (AGH-*Ednrb*^{sl} × F344-*Ednrb*^{sl}) rats and F_2 (AGH-*Ednrb*^{sl} × LEH-*Ednrb*^{sl}) rats (Dang et al., 2011; Huang et al., 2015) was also identified, and several potentially causative mutations on the putative regulatory region of the *Gdnf* gene in AGH-*Ednrb*$^{sl/sl}$ rats were detected (Huang et al., 2015). The functions for these mutations in the modification of HSCR phenotypes remain unknown. In the present study, different promoter fragments of the *Gdnf* gene were cloned, and dual-luciferase assay was used to assess the activity of these promoter mutations. Further, possible binding sites of transcription factors were predicted using gene-regulation public website (http://www.biobase-international.com/gene-regulation) and the functions of the candidate transcription factors were tested subsequently.

2. Subjects and methods

2.1. DNA constructs

In a previous study, two mutations (g.76896910C>T and g.76897291C>T) in the promoter and three mutations in the first intron (g.76901040-76901042TTA>—, g.76901607G>A, and g.76901863G>—) were identified in the *Gdnf* gene (Huang et al. 2015). Among these mutations, g.76896910C>T and g.76897291C>T were characterized at positions −613 and −232 upstream from the start point of transcription (+1) of the *Gdnf* gene. *F-A1* (−232T) and *F-A2* (−232T/−613T) were amplified from the genomic DNA of AGH-*Ednrb*^{sl} rats. *F-L1* (−232C) and *F-L2* (−232C/−613C) were amplified from the genomic DNA of LEH-*Ednrb*^{sl} rats. Two fragments, *F-L* (TTA/G/G) and *F-A* (−/A/−), which contain three mutations of the first intron, *F-L* (TTA/G/G) and *F-A* (−/A/−) were cloned from LEH-*Ednrb*^{sl} rats and AGH-*Ednrb*^{sl} rats, respectively. Four different lengths of promoter fragments, namely, P(−417/+178), P(−772/+50), P(−1325/+178), and P(−1812/+178), were also cloned from the wild-type rats. Fragments are shown in Fig. 1. All fragments were inserted into the *Hind III* and *Mlu I* restriction sites of the pGL3-basic vector (Promega), which was used as a luciferase reporter to measure the promoter activity of the *Gdnf* gene. The pLR-TK vector was used as an internal control. CDS of transcription factor LEF1 (+1/+1210) was amplified from rat C6 cells cDNA and cloned into the *Hind III* and *Xho I* restriction sites of the pCDNA3.1(+) vector. Constructs were verified by direct sequencing. Primers used in this assay are shown in Table 1.

2.2. Cell culture

The rat C6 glioma cell line was purchased from Shanghai Tongpai Biotechnology Company (China). 293T cells were kindly provided by Dr. Qin (Northwest A&F University, Yangling, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco) plus 10% fetal bovine serum and 1% penicillin–streptomycin (Gibco) in 5% CO_2 at 37 °C. Cells were plated in a 24-well plate with a density of 2 × 10^5 cells/well 24 h prior to transfection.

2.3. Transfection and luciferase reporter assays

Upon reaching ~90% confluence, cells were co-transfected with 73 ng of pLR-TK plasmid as internal control and 727 ng of pGL3-basic-*Gdnf*-promoter or the first intron fragment using 2.0 μL of LipoFECTAMINE™ 2000 (Invitrogen) following the manufacturer's protocol. After transfection for 48 h, cells were washed with PBS three times and then harvested by adding 100 mL of 1× passive lysis buffer. The activity of each fragment was analyzed by a Dual-Luciferase Reporter Gene Assay System (Promega) on a MicroLumatPlus LB 96 V instrument. About 5 μL of cell lysate was mixed with 25 μL of Luciferase Assay Reagent II for firefly luciferase assay and then mixed with 25 μL of Stop&Glo® for the *Renilla* luciferase assay. The ratio of firefly luciferase

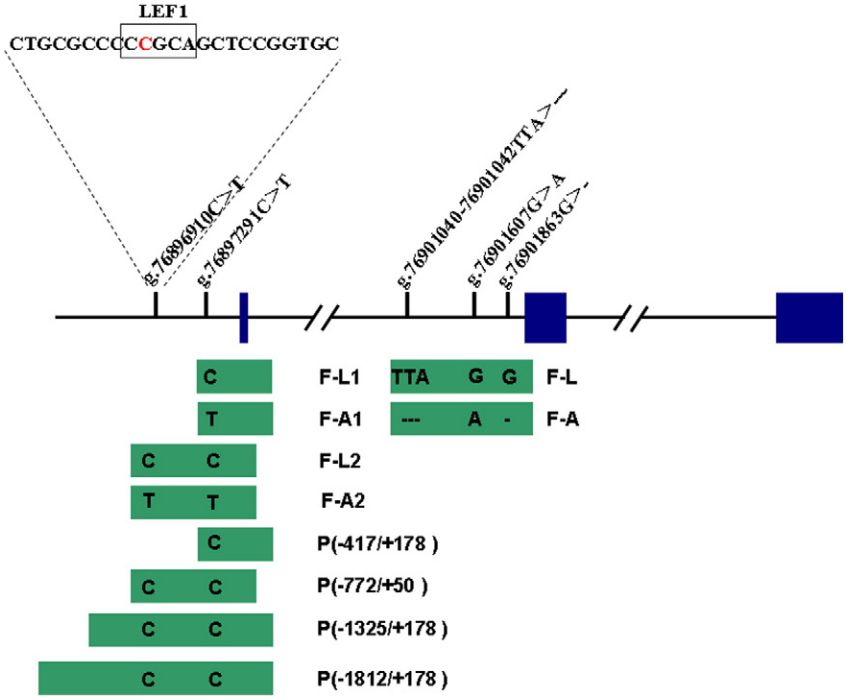

Fig. 1. Visualization of candidate variants, promoter fragments, and putative transcription factor over the mutation -613C>T of the *Gdnf* gene. Exons are shown as boxes, non-coding regions as thin lines, and the positions of variants as vertical bar (medium panel). The promoter fragments are indicated by boxes in the corresponding area, and the bases of variants are labeled in the boxes (lower panel). Putative transcription factor over the mutation -613C>T is boxed (upper panel).