Up-regulated FHL1 Expression Maybe Involved in the Prognosis of Hirschsprung’s Disease

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Abstract

**Background:** In a subset of patients with Hirschsprung’s disease (HSCR), gastrointestinal motor dysfunction persisted long after surgical correction. Gastrointestinal motility is achieved through the coordinated activity of the enteric nervous system, interstitial cells of Cajal, and smooth muscle (SMC) cells. Inhibition of four-and-a-half LIM protein-1 (Fhl1) expression by siRNA significantly decreases pulmonary artery SMCs migration and proliferation. Furthermore when up-expressing FHL1 in atrial myocytes, K (+) current density markedly increases, therefore changing myocytes’ response to an electrical stimulus. However whether FHL1 in colon SMCs (the final effector organ) influences intestinal motility in HSCR patients has not been clarified.

**Methods:** FHL1 mRNA and protein expressions were analyzed in 32 HSCR colons and 4 normal colons.

**Results:** Smooth muscle layers were thicken and disorganized in HSCR. FHL1 was expressed in the ganglion cells of the myenteric plexus, submucosa, as well as in the longitudinal and circular muscle layer of the ganglionic colon. **FHL1 mRNA relative expression level in aganglionic colons was 1.06±0.49 (ganglionic colon relative expression level was 1) (P=0.44). FHL1 protein gray level relative to GAPDH in normal colons was 0.83±0.09. FHL1 expression level in ganglionic colon (1.66±0.30) or aganglionic colon (1.81±0.35) was significantly higher than that in normal colons (P=0.045 and P=0.041, respectively).** Meanwhile, we found FHL1 expression in aganglionic colon was slightly stronger than that in ganglionic colon (P=0.036).

**Conclusion:** These data suggested that up-regulated FHL1 in smooth muscle in HSCR might be associated with intestinal wall remodeling in HSCR and might be one of the risk factors for gastrointestinal motor dysfunction.

Key words: FHL1; Hirschsprung’s disease; expression; smooth muscle; prognosis

Introduction

Hirschsprung’s disease (HSCR, aganglionic megacolon, OMIM 142623) is a developmental disorder characterized by congenital absence of intrinsic ganglion cells in the myenteric (Auerbach) and submucosal (Meissner) plexuses of the gastrointestinal tract, leading to tonic contraction of the affected segment, intestinal obstruction, and massive distension of the bowel [1]. Resection of the abnormally innervated bowel is essential to avoid life threatening complications. Previous data showed despite successful surgical treatment, there was a large proportion of patients in whom constipation, soiling and abdominal pain persisted. After an average of 9 years, 53% of patients continued to suffer from fecal incontinence and 22% were still constipated [2-4]. The reasons for these persistent symptoms were not clear,
although data were emerging to suggest that those
with constipation have a neuropathy proximal to the
aganglionic segment [5]. Aganglionosis is attributed
to a failure in the time-specific migration of enteric
nerve crest-derived cells into the intestinal tract. Most
studies on the pathogenesis of HSCR were concerned
on the autologous abnormality of migrating enteric
nerve precursors (ENPs), and the results showed that
mutations of the RET, GDNF, EDNRB, SOX10, NRG1,
NKK2-1 and EDN3 genes appeared to give dominant,
recessive, or polygenic patterns of inheritance [6-9].
However, research on target cells such as smooth
muscle was rather limited. Smooth muscle thickening
and intestinal wall remodeling existed in aganglionic
and ganglionic segment of HSCR. But the influence of
intestinal wall remodeling on prognosis of Hirsch-
sprung disease, and the molecular pathways trigger-
ing the thickening and remodeling process are still
poorly understood.

Previous studies indicated that smooth muscle
had a major impact on determining the number of
innervating neurons. Jacob CL found that smooth
muscle of the aganglionic colon was less favourable
for neuronal development than that of normal colon
and the mechanism was not clear [10]. Many proteins
with abnormal function or expression level in the
smooth muscle cells of the aganglionic part of the
colon in HSCR have been identified, such as choli-
noreceptors, alpha-smooth muscle isoactin, Semap-
phin 3A, sarcoglycan subcomplex and Connexin43
etc [11- 17]. These proteins participate in the patho-
genesis through impairing intercellular communica-
tion between interstitial cells of Cajal and smooth
muscle cells or altering the cytoskeleton in smooth
muscle cells or disturbing the microenvironment
around the targets of migrating neural crest cells in
autocrine or paracrine manner during colon devel-
opment. But the mechanism of the proteins in smooth
muscle participated in intestinal dysfunction in HSCR
patients has not been clarified.

The FHL1 gene, located on chromosome Xq27.2,
encodes four-and-a-half LIM protein-1 (FHL1) and its
spliced isoform, SLIMMER or FHL1C. Fhl1 protein
contains four and a half tandemly repeated LIM do-
 mains and Northern blot analysis confirms strikingly
high expression of Fhl1 in skeletal muscle and heart,
with considerably lower expression levels observed in
several other tissues, including colon, small intestine,
and prostate [18,19]. Fhl1 can also promote myoblast
spreading and migration by inhibiting integrin-
mediated myoblast adhesion [20]. FHL1 mutations
have been identified in a spectrum of human skeletal
and cardiac muscle diseases [21-24]. In rat aortic
smooth muscle cells (SMCs) FHL1 knockdown can
significantly inhibit the proliferation but exert no
significant effect on cell apoptosis [25]. Kwapiszewska
G demonstrated that inhibition of Fhl1 expression by
siRNA significantly decreased pulmonary artery
SMCs migration and proliferation, so these results
suggested Fhl1 was the key factor triggering the vas-
cular remodeling process in pulmonary hypertension
[26]. However, the functions of FHL1 in colon SMCs
and its role in the HSCR have not been characterized
in studies.

Methods

Patients and controls

Colon tissues from 32 sporadic HSCR patients,
aged from one month to seven years, were obtained
from Shengjing Hospital, China Medical University.
HSCR diagnosis was based on histological examina-
tion of surgical resection for absence of enteric plex-
uses. Ganglionic colon in HSCR was the most rostral
part of the colon that was surgically removed from
patients. In addition there were 4 colons from new-
born infants, died from non-nervous or digestive
system diseases. The study was approved by the local
ethical committee and all the subjects involved in the
study gave written informed consent.

Immunohistochemistry

Sections were deparaffinized in xylene, hydrated
and incubated with 3% H2O2 in methanol for 30 min
at room temperature to block endogenous peroxidase,
then washed twice in PBS (2×5min) and incubated in
normal serum for 30 min at room temperature to
block non-specific sites. Sections were incubated
overnight at 4°C with the primary antibody against
FHL1 (Santa Cruz, California, USA; polyclonal goat,
sc-23175) at a concentration of 1µg/ml, washed twice
with PBS (2×15min min); then transferred to 1:200 v/v
biotinylated IgG anti-goat serum in PBS for 60 min at
room temperature; washed twice with PBS (2×5min);
incubated again in ABC Elite reagent in PBS for 30min
at room temperature; washed twice with PBS (3×5
min); took the final incubation in 0.02% H2O2 and
0.075% dianaminobenzidine in 0.05 M Tris buffer (pH
7.6), kept for 1min in a dark room; and rinsed in dis-
tilled water. Negative controls were obtained in each
instance by omitting the primary antibody.

Real-time PCR reaction

FHL1 gene expression in HSCR patients were
detected using SYBR-Green I real-time PCR. RNA
from aganglionic and ganglionic colon tissue of 32
HSCR patients were extracted using the TRIZol
Reagent (Invitrogen, California, USA) according to
the manufacturer’s protocol. cDNA synthesis was
performed starting from 3 µg of RNA using the
TaKaRa RNA PCR kit (Takara, Dalian, JAPAN).
Real-time PCR amplifications were performed in triplicates on Light Cycle (Roche, Basel, Switzerland) using the following oligonucleotides: FHL1-1: 5′-GTA GTCGTGCCCAGGATTGT-3′; FHL1-2: 5′-GCTGTGGAGGCCAGTATTA-3′ (product size=142bp). The housekeeping gene GAPDH (Takara DR3702) was used as an endogenous control. The relative levels of FHL1 gene expression for each sample were calculated using the 2^-△△ct method.

**Western-blot**

Antibodies against FHL1 were purchased from Sigma-Alorich (Sigma-Alorich, Saint Louis, USA; monoclonal mouse, WH0002273M1). Aganglionic and ganglionic colon segments of HSCR samples and colon segments of newborn infants were frozen and lysed in buffer. The protein concentration of each lysate was determined using the bicinchoninic acid (BCA) kit according to the manufacture’s protocol. Total protein (90µg) was applied to each lane on 12% SDS-polyacrylamide gels. After electrophoresis, the polyvinylidene fluoride (PVDF) membranes were washed in Tris-buffered saline containing 0.1% Tween-20, and then incubated with primary antibody (diluted 1:2000) followed by secondary antibody (diluted 1:2000). Immunostained bands were detected with a ProtoBlot II AP System with a stabilized substrate (Promega, Madison, USA). GAPDH protein was used as internal control.

**Statistical analysis**

FHL1 expression values are expressed as mean±SEM. Data were analyzed with Student’s T test. P values less than 0.05 were considered to be statistically significant.

**Results**

**Immunostaining of FHL1 in HSCR patients**

The HE and immunostaining of FHL1 in 4 HSCR colons and 4 normal colons were accomplished. Circular muscle layer and longitudinal muscle layer were thickening at different extent in aganglionic and ganglionic segment of HSCR. Compared with normal colon the arrangement of circular muscle layer in aganglionic segment of HSCR was disorganized (Fig.1). Immunohistologic study revealed that in the ganglionic segment of HSCR, FHL1 was expressed in the ganglia cells in myenteric, submucosa, circular muscle layer and longitudinal muscle layer. However in the aganglionic segment of HSCR we found expression levels of FHL1 in the circular muscle layer, submucosa, and longitudinal muscle layer (Fig.1).

![Figure 1](http://www.medsci.org)
FHL1 gene expression in HSCR patients

FHL1 mRNA and protein expressions were analyzed in 32 HSCR patients and 4 normal colons. As revealed in Fig 2, the FHL1 mRNA relative expression in aganglionic colons was 1.06±0.49 (ganglionic colon relative expression level was 1) (P =0.44). FHL1 protein gray level relative to GAPDH in normal colons was 0.83±0.09. FHL1 expression level in ganglionic colon (1.66±0.30) or aganglionic colon (1.81±0.35) was significantly higher than that in normal colons (P =0.045 and P =0.041, respectively). Meanwhile, we found FHL1 expression in aganglionic colon was slightly stronger than that in ganglionic colon (P =0.036), as shown in Fig 3.

Discussion

In patients with Hirschsprung disease (HSCR), resection of the affected aganglionic bowel segment is the accepted treatment but constipation, soiling and abdominal pain are the challenging problems in some children after pull-through surgery for Hirschsprung disease (HSCR). Data showed that, in a subset of patients with HSCR, gastrointestinal motor dysfunction persisted long after surgical correction [2, 27]. The physiology underlying the persistent symptoms in children after surgery for Hirschsprung’s disease is yet not clear.

Gastrointestinal motility is achieved through the coordinated activity of enteric nervous system (ENS), interstitial cells of Cajal (ICC), and smooth muscle (SM) cells. ICC and ENS supply SM cells with the necessary stimuli to contract and generate motility. The response of SM cells to an electrical stimulus provided by the neighboring ICC network is mediated by the activation of a wide variety of voltage-dependent ion channels within their cell membrane. Voltage-activated ion channels expressed in ICC and smooth muscle cells, such as Ca2+ channels, Na+ channels or K+ channels, are contributed to the electrical activity and subsequent contractile activity of intestinal smooth muscle [28-30].

A decreased number of c-kit positive cells (interstitial cells of Cajal) in the normoganglionic segment is regarded as a clue to predict a poor clinical outcome after surgery, probably due to poor intestinal motility [31, 32]. Smooth muscle thickening and intestinal wall remodeling exist in aganglionic and ganglionic segment of HSCR. An additional enteric smooth muscle layer was firstly reported in a patient with Mowat-Wilson syndrome and Hirschsprung’s disease. After the resection of the aganglionic colon at the age of 5 months, this patient started to suffer from intermittent constipation. Although the exact mechanism of abnormal gut motility in this case was unknown, the supernumerary muscle and its associated neural plexus might be responsible for the patient's late complication [33]. In addition, impairment of cytoskeleton in SMC of aganglionic bowel may be associated with abnormal gut motility. Since cytoskeletal proteins are required for the coordinated contraction of muscle cells, their absence or notable reduction in the aganglionic bowel of HSCR may be responsible for the motility dysfunction in the aganglionic segment [34]. Sarcoglycan subcomplex (SG) support the development and maintenance of muscle cells, therefore up-regulation of α-Sarcoglycan subcomplex in SMC is probably an acquired phenomenon relating to the intestinal dysmotility which persists in 20% of patients after resection of the aganglionic bowel [14]. But whether or not other proteins in smooth muscle...
participate in intestinal dysfunction in HSCR patients is not clear.

In this study we found that circular muscle layer and longitudinal muscle layer were thickening at different extent in aganglionic and ganglionic segment of HSCR and the arrangement of circular muscle layer in aganglionic segment of HSCR was disorganized (Fig.1). FHL1 was expressed in the ganglion cells of the myenteric plexus, submucosa, as well as in the longitudinal and circular muscle layer of the ganglionic colon. FHL1 expression level in ganglionic colon or aganglionic colon was significantly higher than that in normal colons. Meanwhile, we found FHL1 expression in aganglionic colon was slightly stronger than that in ganglionic colon.

Four and a half LIM domains protein 1 (FHL1) is the most widely expressed member of the FHL family of proteins, consisting of four and a half highly conserved LIM domains [35]. LIM domains have been observed to act as modular protein-binding interfaces mediating protein-protein interactions in the cytoplasm and the nucleus [36]. Northern blot analysis confirmed strikingly high expression of Fhl1 in skeletal muscle and heart, with considerably lower expression levels observed in several other tissues, including the colon, small intestine, and prostate. FHL1 mutations have been identified in a spectrum of human skeletal and cardiac muscle diseases [21-24]. FHL1 could alter cytoskeleton and cell shape by binding with PDZ and LIM domain protein 1 (PDLIM1), Gelsolin (GSN), gamma-actin (ACTG) and a-actin (ACTN1) [37,38]. In rat aortic smooth muscle cells (SMCs) FHL1 knockdown could significantly inhibit the proliferation of SMCs but exerted no significant effect on cell apoptosis [25]. Kwapiszewska G demonstrated that inhibition of Fhl-1 expression by siRNA significantly decreased pulmonary artery SMCs migration and proliferation, suggesting that Fhl1 was the key factor triggering the vascular remodeling process in pulmonary hypertension [26]. Protein–protein interactions were critical for the normal membrane trafficking, localization, and function of voltage-gated ion channels. Immunoprecipitation experiments confirmed a physical interaction of FHL1 with the K (+) channel (KCNA5) complex in human atrium. With coexpression of FHL1, K (+) current density was markedly increased in atrial myocytes [39,40].

Data suggested that the alteration of delayed rectifier (I_{Kr}) K+ current and Kv1.2 expression in DRG neurons from Irritable bowel syndrome (IBS) model rats represented a molecular mechanism underlying visceral pain and hyperexcitability in IBS [41]. In the study of chronic stress-induced colonic hypermotility, Ying Liu found that repeated water avoidance stress (WAS) treatment resulted in up-regulation of Kir6.1 and SUR2B of KATP channels in the colon devoid of mucosa and submucosa [42]. These results demonstrated that Kv channels in the DRG neurons or colonic smooth muscle cells were associated with gastrointestinal motility and might have potential clinical utility in treating gastrointestinal motility disorders.

However whether FHL1 in smooth muscle (SM) cells (the final effector organ) influences intestinal motility in HSCR patients or its mechanism in this process has not been clarified. Our results and all the above-mentioned data suggested that up-regulated expression level of FHL1 in ganglionic colon or aganglionic colon in HSCR might be associated with smooth muscle thickening and intestinal wall remodeling in HSCR through increasing the proliferation of smooth muscle cell or altering its skeleton. Furthermore when up-expressed FHL1 in smooth muscle cell, K (+) current density would also be markedly increased, like in atrial myocytes, thus changing their response to an electrical stimulus provided by the neighboring ICC. In summary we hypothesize that FHL1 in smooth muscle (SM) cells (the final effector organ) could influence intestinal motility in HSCR patients by remodeling intestinal wall and changing their electrical response to stimulus provided by the neighboring ICC. Moreover since this experiment revealed that FHL1 expression in ganglionic colon, reserved in the operation, was much higher than that in the normal colon from newborn infants died from non-nervous or digestive system diseases, we believed that molecular abnormality also existed in this relatively normal colon in HSCR. So after resection of the affected aganglionic bowel segment, constipation, soiling and abdominal pain (intestinal dysfunction) are the subsequent problems in some children after pull-through surgery for Hirschsprung’s disease (HSCR). In further research we intend to screen gene abnormality in ganglionic colon in HSCR and investigate the mechanism of intestinal dysfunction existing after surgery which will provide a fresh clue for clinical therapy for HSCR.

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Abbreviations

HSCR: Hirschsprung’s Disease; SMC: smooth muscle
muscle cells; FHL1: four-and-a-half LIM protein-1; ENPs: enteric neural precursors; ENS: enteric nervous system; ICC: interstitial cells of Cajal; PDLIM1: PDZ and LIM domain protein 1; GSN: Gelsolin; ACTN1: a-actin.

Competing Interests
The authors have declared that no competing interest exists.

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