

Original Article

MLC20, MYPT1, PP1c and CPI-17 expression and phosphorylation in intestinal smooth muscle specimens from patients with Hirschsprung disease

Jianjun Zhang^{1,2}, Shungen Huang¹, Xiaobo Liu¹, Wanliang Guo³, Jian Wang¹

Departments of ¹Pediatric General Surgery, ³Radiology, The Children's Hospital of Soochow University, Suzhou, Jiangsu, China; ²Department of Neonatal Surgery, Xuzhou Children's Hospital, Xuzhou, Jiangsu, China

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Abstract: Abnormal intestinal smooth muscle contraction in patients with Hirschsprung's disease (HSCR) is relatively uncharacterized. It is known that 20 kDa myosin light chain (MLC20) and its regulatory proteins MYPT1, PP1c and CPI-17 are associated with intestinal smooth muscle construction. Therefore, this study aimed to assess the expression and phosphorylation levels of these proteins in intestinal tract tissue samples from HSCR patients. Thirty-one pediatric patients (22 males and 9 females; 21 and 10 cases of common and long-segment types, respectively) pathologically diagnosed with HSCR were divided into normal ganglionic (N), oligoganglionic (O) and aganglionic (A) groups. Western blot and immunohistochemistry (S-P method) were used to assess protein amounts of MYPT1, MLC20, CPI-17 and PP1c at the stenotic segment of smooth muscles in HSCR patients. Immunohistochemistry was used to detect these proteins in intestinal smooth muscles. Western blot showed higher p-MLC20^{Ser19} levels and lower MLC20 amounts in HSCR group A compared with group N; meanwhile, MYPT1 and p-MYPT1^{Thr696} levels were reduced, and p-CPI-17 amounts increased. PP1c levels showed no significant difference among groups. These findings indicated that p-MLC20 and p-CPI-17 were upregulated, with MYPT1 and p-MYPT1 downregulated at the stenotic segment in HSCR patients. Changes of p-MYPT1 and p-CPI17 levels might cause p-MLC20 upregulation, and these abnormal changes directly participate in abnormal intestinal smooth muscle contraction in HSCR patients.

Keywords: Hirschsprung's disease, MYPT1, MLC20, CPI-17, intestinal smooth muscle

Introduction

Hirschsprung's disease (HSCR) is a developmental disorder occurring once every 5,000 live births [1, 2]. HSCR is characterized by an absence of the enteric nervous system in variable lengths of colon [3]. The lack of ganglion cells in the distal gut causing functional obstruction, which in turns results in severe chronic constipation and abdominal distention [1]. Currently, HSCR is treated by surgically removing the aganglionic segment, but functional outcome varies, with many patients suffering life-long complications [4]. More recently, enteric neuronal stem/progenitor cell (ENSC) transplantation was proposed for the replacement of absent ganglia [5].

Despite an impressive wealth of knowledge in the field, the exact mechanisms of abnormal

intestinal smooth muscle contraction in HSCR patients remain unclear. Indeed, whether there are protein expression variations associated with smooth muscle contraction has not been previously reported. Interestingly, studies have revealed that 20 kDa myosin light chain (MLC20) and its regulatory proteins MYPT1, PP1c and CPI-17 are associated with intestinal smooth muscle contraction. Notably, He et al. [6] demonstrated that smooth muscles in the intestinal tract of MYPT1 gene knockout mice tend to show increased, quick, violent, and sustained energy during the contraction phase. Meanwhile, smooth muscle contraction is regulated by MLC20 phosphorylation at Ser-19 [7], a regulation achieved by balancing myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) amounts [8]. The myosin phosphatase subunit is the main decider of smooth muscle contraction and generation of

Table 1. Clinicopathological characteristics of the patients

	No. of cases	Percent-age (%)
Sex		
Male	22	71%
Female	9	29%
Age		
≤1 year	19	61.3%
>1 year	12	38.7%
Pathological classification		
Common type (S)	21	67.7%
Long segment type (L)	10	32.3%

Ca²⁺-dependent energy [9]. MLCP includes the target subunit 1 (MYPT1, the main regulatory subunit), catalytic subunit PP1c (major enzyme in MLC20 dephosphorylation) and 20 kDa subunit with unclear function (M20) [10]. MYPT1 can bind PP1c to yield compounds that subsequently activate PP1c, which in turn binds p-MLC20 and affect intracellular localization of MYPT1 [11, 12].

Protein kinase C-related (PKC-related) phosphatases suppress the phosphorylation of protein-17 kDa (CPI-17) and PKC at Thr38 site to suppress PP1cδ, and ultimately MLCP activation [13]. Reduced MLCP activation decreases MLC20 dephosphorylation, thereby promoting smooth muscle contraction; thus, MYPT1 and CPI-17 phosphorylation is the main inducing factor in sensitizing intestinal smooth muscles to Ca²⁺ [14].

MYPT1 knockout HeLa cells show reduced MLCP activation and MLC20 dephosphorylation, causing active myosin accumulation, thereby leading to focal adherence of stress fibers, and ultimately inhibiting cell migration [15]. Phosphorylation of MLC20 at antrum, fundus and proximal colon smooth muscles in mice is regulated by MYPT1 and CPI-17 [16].

Based on the above findings, we hypothesized that abnormal contraction of stenotic segment of smooth muscles in HSCR patients may be associated with MYPT1, the major regulatory subunit of MLCP and pivot in contraction and relaxation pathways. Therefore, this study aimed to assess the expression and phosphorylation levels of MLC20 as well as its regulatory proteins MYPT1, PP1c and CPI-17, in intestinal tract tissue samples from HSCR patients.

Materials and methods

Patients

Thirty-one children (22 males and 9 females; 21 common and 10 long segment types, respectively), pathologically diagnosed with HSCR, were included in this study. The pediatric patients underwent surgical resection of HSCR colon in the Children's Hospital of Soochow University, Soochow, China. Surgical specimens obtained along the longitudinal axis of the bowels were divided into 3 groups, including aganglionic (A), oligoganglionic (O), and normal ganglionic (N) groups. Patients' parents provided written informed consent on behalf of the children enrolled in this study. The study protocol was approved by the ethics committee of the Children's Hospital of Soochow University.

Western blot

The narrow intestinal segment in each surgical specimen was included in the experimental narrow group (A group); the control group comprised subjects matched for age and sex that were confirmed HSCR-free (N-group). Surgical specimens were snap frozen in liquid nitrogen, followed by homogenization with a tissue grinder. Tissue samples (50 mg) were lysed in 50 mL lysis buffer containing protease and phosphatase inhibitors. Equal amounts of proteins were separated and transferred onto PVDF membranes. After blocking with 5% skim milk in PBS-T (0.05% Tween in PBS), the membranes were incubated with rabbit anti-human MYPT1 polyclonal (AB59235, Abcam; 1:1000), MLC20 monoclonal (AB137063, Abcam; 1:1000), CPI-17 mono-/polyclonal (AB32213 or AB52170, Abcam; 1:1000), PP1c polyclonal (AB169976 or AB93864, Abcam; 1:1000), p-MYPT1^{Thr696} polyclonal (AB59202 or AB59203, Abcam; 1:1000), p-MLC20^{Ser19} polyclonal (AB2480, Abcam; 1:1000), p-CPI-17^{Thr38} polyclonal (AB52174, Abcam; 1:1000) primary antibodies, overnight at 4°C. This was followed by incubation with secondary antibodies for 1 h at room temperature. ECL™ Western Blotting Detection Reagents (GE, USA) were used for detection. GAPDH was used as an internal control.

Immunohistochemistry

The narrow intestinal segment in each surgical specimen was included in the experimental

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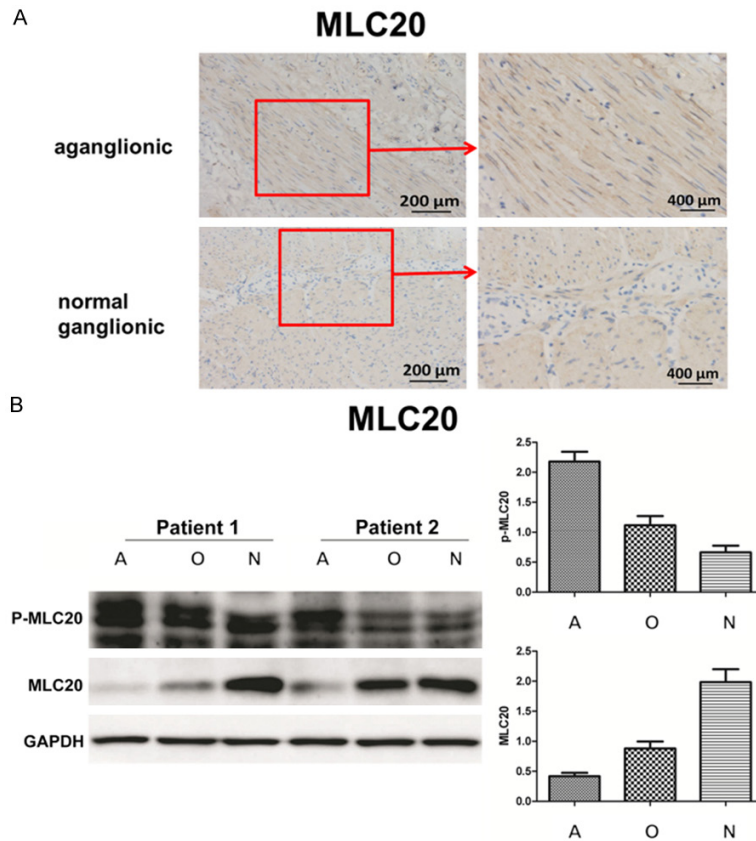


Figure 1. Detection of protein levels of MLC20 in non-phosphorylated and phosphorylated forms. A. Immunohistochemistry data revealing MLC20 in intestinal smooth muscle samples from patients of both N and A groups. B. Western blot showing increased p-MLC20^{Ser19} levels ($P < 0.01$) and decreased MLC20 amounts ($P < 0.01$) in the HSCR A group.

narrow group (A group); the control group comprised subjects matched for age and sex that were confirmed HSCR-free (N-group).

Tissue specimens were collected and fixed in neutral formalin and paraffin embedded. After deparaffinization, the tissue sections were submitted to antigen retrieval. Then, endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide, and non-immunized animal serum was used to block non-specific antigens. This was followed by incubation with primary antibodies (MYPT1, 1:50; MLC20, 1:100; CPI-17, 1:50; p-MYPT1^{Thr69}, 1:50; p-CPI-17^{Thr38}, 1:50; PP1c, 1:100-500) at 4°C overnight. After incubation with secondary antibodies and subsequently streptavidin-peroxidase (SP), the 3,3'-Diaminobenzidine (DAB)-peroxidase substrate solution was used for color development. Hematoxylin was used for counterstaining. Finally, the stained sections were

dehydrated in gradient ethanol, cleared in xylene, and mounted in neutral gum. PBS was used in the negative control in lieu of primary antibodies.

Statistical analysis

Data are mean \pm standard deviation (SD). Grayscale values in Western blot and histological scores were assessed by t-test or one way analysis of variance (ANOVA). The SPSS 17.0 software (SPSS, Chicago, IL) was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

The characteristics of study subjects are summarized in **Table 1**. The 31 patients included 22 (71%) and 9 (29%) males and females, respectively. Among them, 19 were one year or less, while 12 were above 1 year old. As for disease classification, there were 21 and 10 common and long segment types, respectively.

MLC20 expression and phosphorylation in HSCR

MLC20 expression was detected immunohistochemically in intestinal smooth muscle samples from patients of both N and A groups (**Figure 1A**). Western blot showed that p-MLC20^{Ser19} levels were higher, with reduced MLC20 amounts in HSCR group A, compared with group N values (**Figure 1B**).

MYPT1 expression and phosphorylation in HSCR

To explore the mechanism behind increased p-MLC20^{Ser19} in the A group, several proteins related to p-MLC20^{Ser19} regulation were assessed for their levels. Immunohistochemistry revealed that MYPT1 was expressed in intesti-

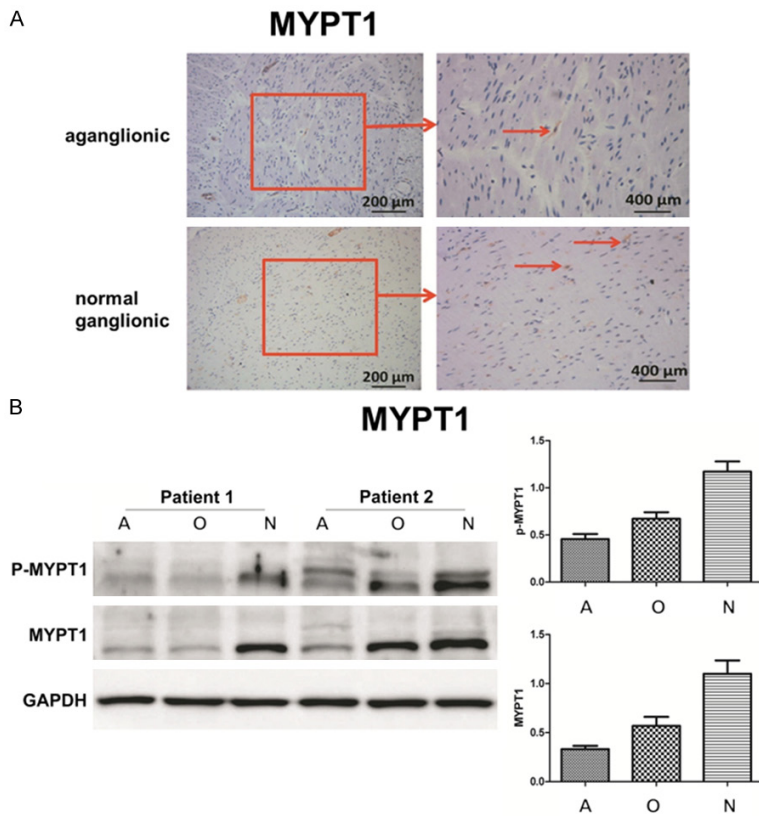


Figure 2. Detection of protein levels of MYPT1 in non-phosphorylated and phosphorylated forms. A. Immunohistochemistry data showing MYPT1 detection in intestinal smooth muscle samples from patients of both N and A groups. B. Western blot demonstrating that both p-MYPT1^{Thr696} and MYPT1 levels are reduced in the HSCR A group compared with the N group (P<0.01).

nal smooth muscle specimens from N and A group patients (**Figure 2A**). In addition, Western blot showed that MYPT1 and p-MYPT1^{Thr696} levels were reduced in the A group compared with N group values (**Figure 2B**).

CPI-17 expression and phosphorylation in HSCR

Immunohistochemistry demonstrated CPI-17 expression in intestinal smooth muscle samples from patients of both N and A groups (**Figure 3A**). Meanwhile, Western blot demonstrated reduced CPI-17 levels and increased p-CPI-17^{Thr38} amounts in the A group compared with N group values (**Figure 3B**).

PP1c expression and phosphorylation in HSCR

PP1c was detected immunohistochemically in intestinal smooth muscle samples from patients of both N and A groups (**Figure 4A**). Meanwhile, a non-significant difference in PP1c

levels between the N and A groups was obtained (**Figure 4B**).

Discussion

This study demonstrated that expression of MLC20 and its regulatory proteins MYPT1, PP1c and CPI-17 were generally altered in stenotic segment of smooth muscles in HSCR patients, which might result in abnormal smooth muscle contraction.

As shown above, p-MLC20 levels were increased in HSCR patients. These findings corroborated previous reports demonstrating that MLC20 phosphorylation might play a key role in regulating smooth muscle contraction [17, 18].

MYPT1 is of central importance in modulating smooth muscle myosin phosphorylation [19, 20]. Eikichi Ihara et al. [21] and Trent Butler et al. [22] outlined the mechanisms of smooth muscle contraction. As shown above, MYPT1

and PP1c were downregulated at the stenotic segment in HSCR patients, and might be involved in smooth muscle contraction. In agreement, previous reports indicated that CPI-17 binds the PP1 active site for dephosphorylation, with this association yielding the MYPT1.PP1C.P-CPI-17 complex, which increases smooth muscle contraction [23, 24]. We propose the following reasons to explain why MYPT1 downregulation causes abnormal contraction at stenotic segment of smooth muscles in HSCR patients. 1) MYPT1 and PP1c downregulation reduces MLCP activation, thereby decreasing MLC20 dephosphorylation [21, 22]. 2) MYPT1 downregulation increases MLCK phosphorylation, subsequently suppressing MLC20 dephosphorylation [9]. 3) PP1c is the main enzyme in MLC20 dephosphorylation [5], and its reduction would decrease MLC20 dephosphorylation [12]. 4) He et al. [6] found that intestinal smooth muscles increase in energy during the contraction phase

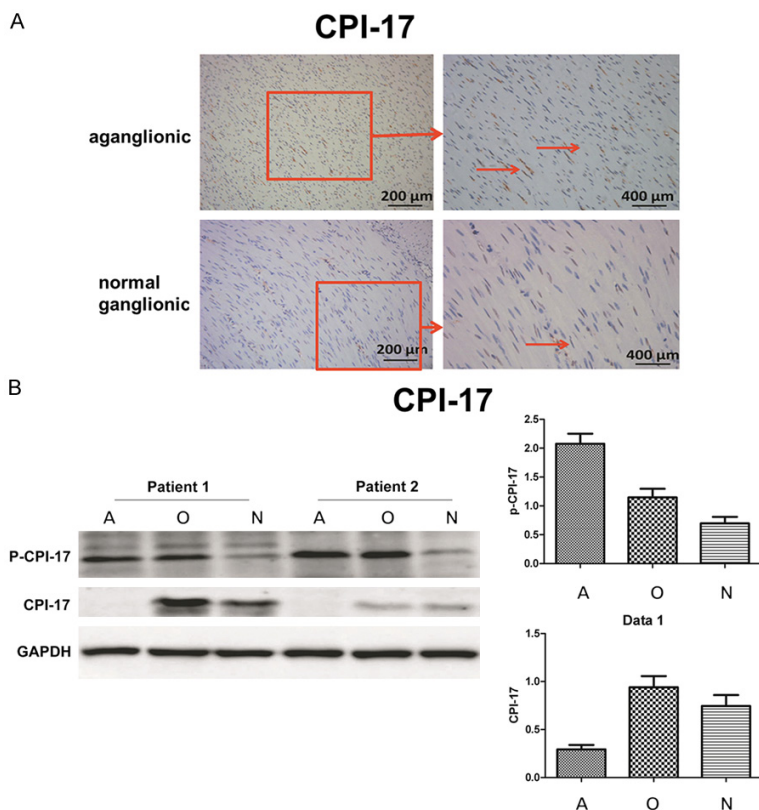


Figure 3. Detection of protein levels of CPI-17 in non-phosphorylated and phosphorylated forms. A. Immunohistochemistry data revealing CPI-17 expression in intestinal smooth muscles specimens from patients of both N and A groups. B. Western blot showing increased p-CPI-17^{Thr38} levels ($P < 0.01$) and decreased CPI-17 amounts ($P < 0.01$) in the HSCR A group compared with the N group.

in MYPT1 knockout mice, providing a quick, violent, and sustained energy. In this study, reduced MYPT1 expression was found at the stenotic segment in HSCR patients, which, according to He et al., might also increase the energy of smooth muscles during the contraction phase, consistent with the contraction and stiffness state of the stenotic segment of intestinal canal in HSCR patients. 5) He et al. [6] also discovered that the Interstitial Cells of Cajal (ICC) network is decreased in MYPT1 gene knockout mice, causing abnormal myoelectric activity and altered peristalsis of intestinal smooth muscles. It was shown that ICC and their precursor cells are significantly decreased at the stenotic segment in HSCR patients [25]; combined with our results of downregulated MYPT1 expression at the stenotic segment in HSCR patients, this might cause abnormal contraction at the stenotic segment of smooth muscles as well as intesti-

nal peristalsis in HSCR patients, thus resulting in low-level intestinal obstruction symptoms.

We assessed MYPT1 protein levels at stenotic, transitional and expansion segments in HSCR patients, and found successively increased values, which reflected the differences of intestinal ganglion cell numbers at the three segments, further indicating that MYPT1 might be involved in abnormal intestinal smooth muscle contraction in HSCR patients. Our findings suggested that p-MYPT1 downregulation might also be involved in abnormal contraction. In agreement, MYPT1 phosphorylation at Thr-696 and Thr-853 was shown to promote MLCP autoinhibition that drives calcium ion sensitization of the smooth muscle force [26]. Sole up-regulation of CPI-17 was shown to enhance smooth muscle contraction [27]. As shown above, CPI-17 was upregulated at stenotic segment in HSCR

patients, and might competitively bind PP1c, thereby resulting in decreased MLCP activation, reduced MLC20 dephosphorylation, and increased smooth muscle contraction. Therefore, abnormal contraction at stenotic segment of smooth muscles in HSCR patients may be associated with self-reinforced smooth muscle contraction by CPI-17 upregulation.

Conclusion

This study revealed the possible mechanisms of abnormal contraction in HSCR patients: 1) MYPT1 and p-MYPT1 downregulation at stenotic segment may lead to decreased MLCP activation, reducing MLC20 dephosphorylation, and increasing contraction; 2) decreased PP1c and p-PP1c δ levels may cause reduced MLCP activation, which leads to decreased MLC20 dephosphorylation, and increased contraction. Meanwhile, p-MLC20 upregulation at the ste-

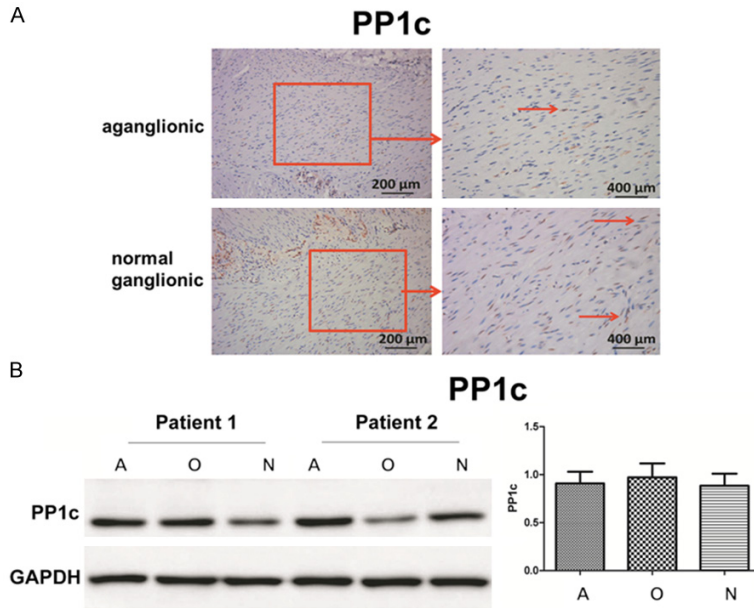


Figure 4. Detection of protein levels of PP1c in non-phosphorylated and phosphorylated forms. A. Immunohistochemistry data revealing PP1c expression in intestinal smooth muscle specimens from patients of both N and A groups. B. Western blot showing a non-statistically significant difference in PP1c levels between the N and HSCR A groups ($P>0.05$).

notic segment increases contraction energy of smooth muscles during the contraction phase, which may lead to abnormal contraction in HSCR patients. However, further studies are required to clarify the mechanisms underlying MYPT1 and p-MYPT1 downregulation as well as PP1c and p-PP1c upregulation in HSCR patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Jian Wang, Department of Pediatric General Surgery, The Children’s Hospital of Soochow University, Suzhou 215003, Jiangsu, China. Tel: +86-13606213191; Fax: +86-512-80693599; E-mail: wj196312@vip.163.com

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