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Differential Expression of CD34, S100, and c-Kit in Interstitial Cells of Cajal in Infantile Hypertrophic Pyloric Stenosis – Immunochemical Study

Różnicowa ekspresja CD34, S100 i c-Kit w komórkach śródmiąższowych Cajala we wrodzonym przerostowym zwężeniu odźwiernika – badanie immunochemiczne

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Abstract

Background. The pathogenesis of infantile hypertrophic pyloric stenosis (IHPS) is poorly understood although many hypotheses have been proposed.
Objectives. Assessment whether the differential expression of c-Kit, CD34, and S100 may be involved in the development of IHPS.
Material and Methods. Specimens from 14 infants with IHPS and seven control subjects were immunohistochemically stained for c-Kit, CD34, and S100. The numbers of $CD34^+$, $S100^+$, and c-Kit^+ cells in five random fields per specimen were compared via light microscopy (×200).
Results. In normal pyloric tissue, specific and intense c-Kit immunoreactivity was observed in the muscle layers and moderate staining was observed around the myenteric plexus. In IHPS patients, c-Kit^+ cells were either absent or markedly reduced around the myenteric plexus. In control and IHPS patients, $CD34^+$ cells were not observed around the myenteric plexus. In the vascular endothelium, moderate CD34 staining was observed in specimens from control subjects, whereas intense staining was observed for IHPS patients. In normal pyloric tissue, moderate S100 immunoreactivity was observed in the muscle layers and intense staining was observed in the myenteric plexus. In IHPS patients, few $S100^+$ cells were observed in the pyloric muscle layers and S100 immunoreactivity decreased markedly around the myenteric plexus.
Conclusions. These results suggest that the numbers of c-Kit^+ and $S100^+$ cells are markedly decreased in the pyloric muscle layers and around the myenteric plexus in IHPS patients. Thus a lack of c-Kit and S100, but not CD34, expression may be a critical factor in the pathogenesis of IHPS and may serve as a useful prognostic tool in the treatment of this disease (**Adv Clin Exp Med 2009, 18, 1, 33–39**).

Key words: infantile hypertrophic pyloric stenosis (IHPS), CD34, S100, c-Kit, immunohistochemistry.

Streszczenie

Wprowadzenie. Patogeneza wrodzonego przerostowego zwężenia odźwiernika (w.p.z.o.) nie została dostatecznie poznana, chociaż zaproponowano wiele hipotez.
Cel pracy. Ocena znaczenia ekspresji różnicowej c-Kit, CD34 i S100 w rozwoju w.p.z.o.
Materiał i metody. Przeprowadzono badania immunohistochemicznie ekspresji c-Kit, CD34 oraz S100 w wycinkach tkankowych pobranych od 14 niemowląt cierpiących na w.p.z.o. i od 7 osób z grupy kontrolnej. W obu grupach porównano liczbę komórek $CD34^+$, $S100^+$ i komórek c-Kit^+ z 5 losowo wybranych obszarów z każdego wycinka za pomocą mikroskopu optycznego (×200).

Wyniki. W prawidłowej tkance odźwiernika stwierdzono swoistą i silną immunoreaktywność c-Kit w warstwach mięśniowych. Nie stwierdzono komórek $CD34^+$ w okolicy splotów nerwowych błony mięśniowej, a w okolicy splotów nerwowych błony mięśniowej barwienie było umiarkowane. U pacjentów z w.p.z.o. komórki $c\text{-}Kit^+$ były albo nieobecne, albo było ich znacząco mniej w okolicy splotów nerwowych błony mięśniowej zarówno w grupie kontrolnej, jak i u pacjentów z w.p.z.o. W śródbłonku naczyń krwionośnych zaobserwowano umiarkowane barwienie CD34 w wycinkach pochodzących z grupy kontrolnej, a w wycinkach pochodzących od pacjentów z w.p.z.o. barwienie było intensywne. W prawidłowej tkance odźwiernika obserwowano umiarkowaną immunoreaktywność S100 w warstwach mięśniowych oraz intensywne barwienie w okolicy splotów nerwowych błony mięśniowej. U pacjentów z w.p.z.o. wykryto niewiele komórek $S100^+$ w warstwie mięśniowej odźwiernika oraz zmniejszoną reaktywność S100 w okolicy splotów nerwowych błony mięśniowej.
Wnioski. Przedstawione wyniki sugerują, że liczba komórek $c\text{-}Kit^+$ i $S100^+$ jest znacząco mniejsza w warstwach mięśniowych odźwiernika oraz w okolicy splotów nerwowych błony mięśniowej pacjentów z w.p.z.o. Dlatego brak ekspresji c-Kit i S100, ale nie CD34 może być uznany za ważny czynnik patogenetyczny w.p.z.o. i może być pożytecznym narzędziem diagnostycznym w leczeniu tej choroby (**Adv Clin Exp Med 2009, 18, 1, 33–39**).

Słowa kluczowe: wrodzone przerostowe zwężenie odźwiernika, CD34, S100, c-Kit, immunohistochemia.

Infantile hypertrophic pyloric stenosis (IHPS) is a common condition requiring surgery in infants [1]. It occurs in approximately three of every 1000 live births, but the incidence of IHPS varies widely according to geographic area, season, and ethnic origin [2]. IHPS is characterized by hypertrophy of the pyloric muscle, resulting in narrowing and elongation of the pyloric channel [1]. The pathogenesis of IHPS remains poorly understood although many hypotheses have been proposed. These hypotheses involve the loss of nerve terminals [3], markers for nerve-supporting cells [4], peptide-containing nerve fibers [5], nitric oxide synthase activity [6], or interstitial cells of Cajal [7]; increases in insulin-like and platelet-derived growth factors [2]; or increased expression of insulin-like growth factor-I messenger RNA [8].

Interstitial cells of Cajal (ICCs) are non-neuronal cells that function as pacemaker cells and are responsible for the spontaneous, rhythmic, electrical excitatory activity of the gastrointestinal smooth muscle [9–12]. Mesenchymal ICC precursors that carry the c-Kit receptor bind the Kit ligand, which is produced by neuronal or smooth muscle cells [13]. $CD34^+$ stromal cells, which are termed dendritic interstitial cells, are distributed throughout the human body. Vascular endothelial cells are used as an internal positive control when immunostaining for CD34. $CD34^+$ stromal cells are not only distinct from ICCs [14], which are $c\text{-}Kit^+$, but are also distinct from smooth muscle cells, glial cells, and macrophages and are known to express the fibroblast marker prolyl 4-hydroxylase [15]. S100 proteins are small acidic proteins with molecular weights of approximately 11 kDa [16] that bind calcium at two EF-hand binding sites per molecule. This protein family regulates a number of biological activities via interactions with target proteins, including annexins [17], cytosolic phospholipase A2 [18], the sarcoplasmic reticulum Ca^{2+} release channel [19], and myosin [20]. The present authors hypothesized that the differential expression of CD34, S100, and c-Kit may be involved in the development of IHPS.

Material and Methods

Full-thickness muscle biopsy specimens were obtained from 16 infants treated for IHPS (14 boys and 2 girls). None of these babies were born prematurely. The median age at the time of presentation was 4.1 weeks (range: 2–8 weeks). Control tissues were obtained from seven neonates and infants (5 boys and 2 girls, age range: 1 day to 1 year) without pyloric disease. The ischemic interval varied from 2–4 h and the mucosa remained intact in all samples. All pyloric specimens were embedded in optimum cutting temperature compound (Tissue-Tek; Miles Inc., Elkhart, IN, USA), frozen in liquid nitrogen, and stored at −80°C until sectioning. Alternate 4-μm-thick cryostat sections were stained with hematoxylin and eosin. Immunohistochemical staining was performed on the remaining tissue sections to visualize c-Kit, CD34, and S100 expression in pyloric tissues. Sections were cut at 4-μm thickness, de-waxed in xylene, and incubated for 20 min in 0.3% H_2O_2 to block endogenous peroxidase activity. The sections were then microwaved for 4 min in phosphate-buffered saline (PBS) and incubated with primary antibodies (rabbit polyclonal antibody against CD117/c-Kit, mouse monoclonal antibody against CD34 Ab-1, mouse monoclonal antibody against S100 Ab-1; Lab Vision, Fremont, CA, USA) overnight at room temperature. The primary antibody was visualized using diaminobenzidine (DAB) as a chromogen. The tissue sections were examined using a light microscope interfaced with a Zeiss color camera. Five fields per specimen were chosen at random and the numbers of $c\text{-}Kit^+$, $CD34^+$, and $S100^+$ cells were counted under a light

microscope at a magnification of ×200. The specimens were evaluated blindly by two examiners and graded from 0 to 3+, where 0 = no cells visible, 1+ = few cells (light staining), 2+ = moderate staining, and 3+ = numerous cells (intense staining). The results, expressed as the mean ± standard deviation (*SD*), were compared using analysis of variance (ANOVA), as noted in Table 1. Values of p less than 0.05 were considered significant.

Results

The results are summarized in Table 1. In normal pyloric tissue, numerous c-Kit$^+$ cells were observed in the muscle layers (Fig. 1A) and the myenteric plexus showed a moderate number of c-Kit$^+$ cells (Fig. 1B). In IHPS patients, few c-Kit$^+$ cells were observed in pyloric muscle compared with the controls ($p < 0.0001$, Fig. 2C) and c-Kit immunoreactivity was either absent or markedly reduced around the myenteric plexus compared with the controls ($p < 0.001$, Fig. 2D).

CD34 immunoreactivity was not observed in the myenteric plexus in either the control or IHPS specimens (Fig. 2A). A small increase in the number of CD34$^+$ cells was observed in pyloric muscle, but this change was not statistically significant. Moderate CD34 immunoreactivity was observed in vascular endothelial cells in the control specimens, whereas intense staining was observed in IHPS patients (Fig. 2B); however, the number of CD34$^+$ vascular endothelial cells was not significantly different between the groups.

In normal pyloric tissue, moderate S100 immunoreactivity was observed in the muscle layers (Fig. 3A), and intense staining was observed in the myenteric plexus (Fig. 3B). In IHPS patients, few S100$^+$ cells were present in the pyloric muscle layers compared with the controls ($p < 0.01$, Fig. 3C) and S100 immunoreactivity was markedly decreased around the myenteric plexus compared with the controls ($p < 0.003$, Fig. 3C).

Table 1. Average number of c-Kit$^+$, CD34$^+$, and S100$^+$ cells per random field (×200)

Tabela 1. Średnia liczba komórek c-Kit$^+$, CD34$^+$ i S100$^+$ w polu widzenia (×200)

	c-Kit$^+$	CD34$^+$	S100$^+$
Control (n = 7)			
Muscle layers	2.5 ± 0.5	0.1 ± 0.3	1.1 ± 0.3
Myenteric plexus	1.4 ± 0.5	–	2.7 ± 0.4
Vascular endothelia	–	1.5 ± 0.5	–
IHPS (n = 14)			
Muscle layers	1.1 ± 0.3*	0.7 ± 0.7	0.4 ± 0.5$^\#$
Myenteric plexus	0.2 ± 0.3$^\&$	–	1.4 ± 0.7†
Vascular endothelia	–	2.2 ± 0.7	–

$^*p < 0.0001$ compared with control.
$^\#$ $p < 0.01$ compared with control.
$^\&$ $p < 0.001$ compared with control.
† $p < 0.003$ compared with control.

$^*p < 0.0001$ w porównaniu z grupą kontrolną.
$^\#$ $p < 0.01$ w porównaniu z grupą kontrolną.
$^\&$ $p < 0.001$ w porównaniu z grupą kontrolną.
† $p < 0.003$ w porównaniu z grupą kontrolną.

Discussion

ICCs are found throughout the gut, from the esophagus to the anus [21, 22]. A decrease in the number of ICCs has been identified in some disorders of human intestinal motility, including hypertrophic pyloric stenosis [23], Hirschsprung disease [24], intestinal pseudo-obstruction [25], slow-transit constipation [26], and diabetic enteropathy [27]. In IHPS patients, ICCs are absent from sections of circular muscle [23], although cells with some ultrastructural features of ICCs have been observed, suggesting some inhibition of ICC development [7]. The lack of ICCs in the pylorus muscle may prevent normal propagation of electrical impulses, thus resulting in abnormal peristaltic motion [12]. Current studies have established that c-Kit, a transmembrane tyrosine kinase receptor, is necessary for the development of ICCs [28]. Immunohistochemical staining using an anti-c-Kit antibody is a sensitive technique for the identification of ICCs in animal [29] and human tissues [23] and facilitates the study of ICCs in the gastrointestinal tract. Yamataka et al. [30] suggested that the lack of c-Kit expression (as an indicator of intestinal pacemaker activity) in the hypertrophic pyloric smooth muscle may be an important factor in the pathogenesis of IHPS. Their results showed that c-Kit$^+$ cells were either absent or markedly reduced in pyloric muscle from IHPS patients and that no c-Kit$^+$ cells were present around the myenteric plexus. Langer et al. [7] demonstrated that ICCs were almost totally absent from patients with hypertrophic pyloric stenosis, although a group of cells resembling ICCs (ICC-like cells) were observed. Piotrowska et al. [22] suggested that the lack of ICCs and heme oxygenase-2 expression in IHPS indicates impaired intracellular communication between ICCs and smooth muscle cells, which would contribute to motility dysfunction.

In the present study, intense c-Kit staining was

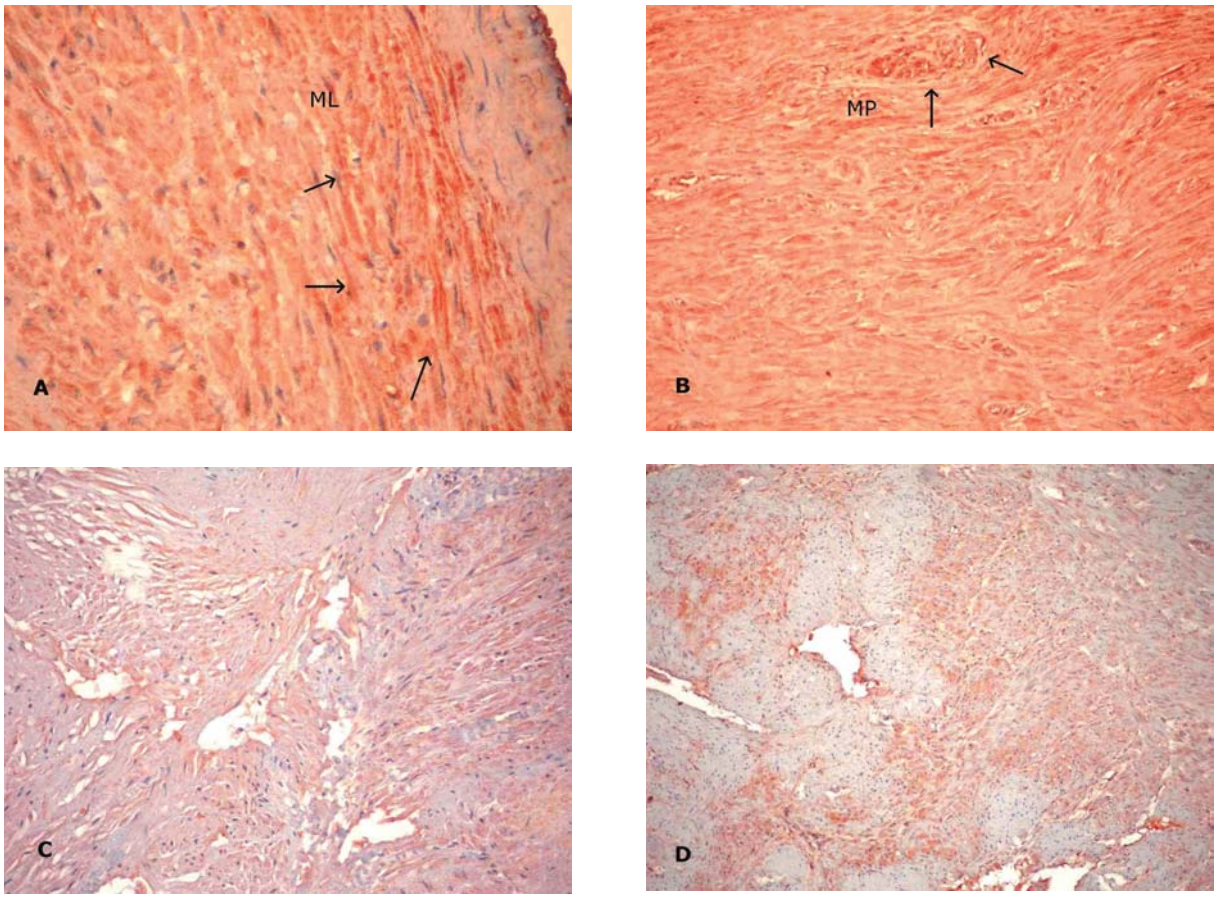

Fig. 1. c-Kit immunohistochemical staining. Numerous c-Kit$^+$ cells were observed in the pyloric muscle layers (A: ML, arrows) and a moderate number of c-Kit$^+$ cells were observed around the myenteric plexus (B: MP, arrows) in control specimens. In IHPS specimens, few c-Kit$^+$ cells were observed in the muscle layers (C) and no c-Kit$^+$ cells were present in the myenteric plexus (D: original magnification ×200)

Ryc. 1. Barwienie immunohistochemiczne komórek c-Kit. Liczne komórki c-Kit$^+$ są widoczne w warstwie mięśniowej odźwiernika (A; ML, strzałki), a umiarkowana liczba komórek c-Kit$^+$ wokół splotów nerwowych (B; MP, strzałki) w grupie kontrolnej. W wycinkach pacjentów z w.p.z.o. wykryto niewiele komórek c-Kit+ w warstwie mięśniowej (C) oraz ich brak w okolicy splotów nerwowych (D, oryginalne powiększenie ×200)

found in normal pyloric muscle tissue from control subjects. The myenteric plexus was demarcated by a moderate number of c-Kit$^+$ cells. In the IHPS patients, however, few c-Kit$^+$ cells were observed in pyloric muscle. In addition, c-Kit$^+$ cells were either absent or markedly reduced around the myenteric plexus.

CD34, a sialylated transmembrane glycoprotein (sialomucin), is present in myeloid progenitor cells, vascular endothelium [31], and a variety of mesenchymal cells [32]. CD34$^+$ dendritic interstitial cells are thought to play an important supporting role in the maturation and proliferation of neighboring mesenchymal and epithelial stem cells and in immune-mediated responses [33, 34]. However, it is unclear whether CD34$^+$ staining represents a subpopulation of ICCs or perineural fibroblasts. Recently, Vanderwinden et al. [14] demonstrated that interstitial CD34-IR$^+$ cells are present in the muscularis propria of the human and murine GI tracts. These cells did not show Kit immunoreactivity, but were closely adjacent to Kit$^+$ ICCs. In the present study the number of CD34$^+$ cells increased among vascular endothelial cells in the control and IHPS patients. However, in both the control and IHPS patients, no CD34$^+$ cells were observed around the myenteric plexus or pyloric muscle. This may explain recent findings in which the comparison of consecutive sections revealed only a small fraction of c-Kit$^+$ cells that were also CD34$^+$ [35].

Calcium-binding proteins from the S100 family are potential transducers and are thought to be involved in the regulation of cytoplasmic calcium concentrations and calcium signal-related processes [36, 37], including secretion, proliferation, differentiation, transcription, apoptosis, and muscle contraction [38, 39]. Calcium-binding sites in most effector molecules are associated with EF-hand structures [16]. Daub et al. [36] reported that S100A4, S100A6, S100A1, and S100A10 are expressed in

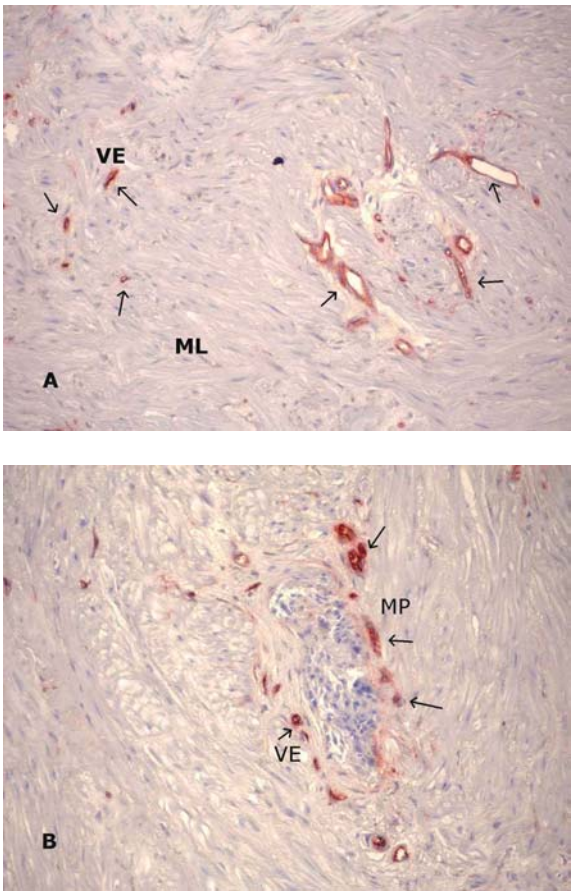

Fig. 2. CD34 immunohistochemical staining. CD34 immunoreactivity was not observed in the myenteric plexus or pyloric muscle in specimens from control or IHPS patients. CD34$^+$ immunoreactivity was observed in vascular endothelial cells in specimens from both control (A: arrows) and IHPS patients (B: arrows, original magnification ×200)

Ryc. 2. Barwienie immunohistochemiczne komórek CD34. Nie obserwowano immunoreaktywności w okolicy splotów nerwowych oraz mięśni odźwiernika w wycinkach pochodzących z grupy kontrolnej i z grupy z w.p.z.o. Immunoreaktywność CD34$^+$ stwierdzono w śródbłonku naczyń krwionośnych w wycinkach z grupy kontrolnej (A, strzałki) i z grupy z w.p.z.o. (B, strzałki, oryginalne powiększenie ×200)

guinea pig smooth muscle tissues, where they are thought to contribute to the regulation of cytoplasmic Ca^{2+} concentrations and/or signal transduction. Sy et al. [40] demonstrated that all IHPS patients show strongly positive S-100 staining within the muscle and minimal staining in the myenteric plexus. However, the investigators did not examine normal pyloric muscle as a control. In the present study, relatively few S100$^+$ cells were observed in the pyloric muscle layers in the IHPS patients and S100 staining was markedly decreased around the myenteric plexus compared with the controls.

The present findings suggest that c-Kit and S100 expression are markedly decreased in the

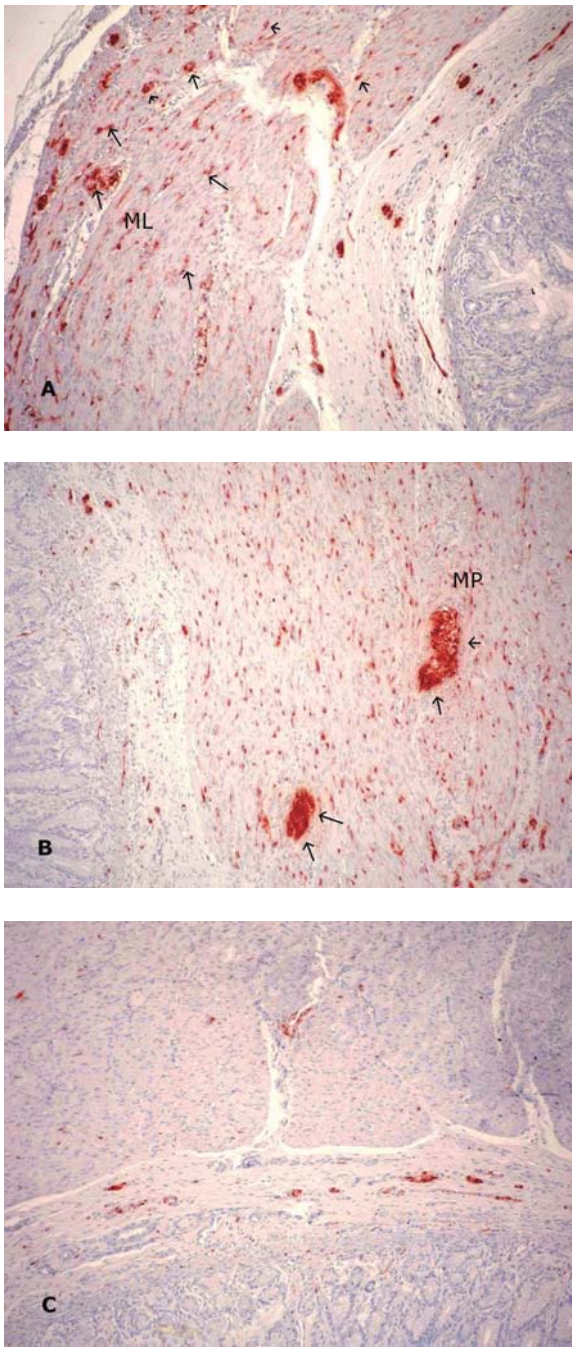

Fig. 3. S100 immunohistochemical staining. In normal pyloric tissue from control subjects, moderate S100 immunoreactivity was observed in the muscle layers (A) and the myenteric plexus showed numerous S100$^+$ cells (B). In IHPS patients, few S100$^+$ cells were observed in the pyloric muscle layers and the number of S100$^+$ cells was markedly decreased around the myenteric plexus (C: original magnification ×200)

Ryc. 3. Barwienie immunohistochemiczne S100. W prawidłowej tkance odźwiernika z grupy kontrolnej stwierdzono umiarkowaną immunoreaktywność S100 w warstwie mięśniowej (A) oraz liczne komórki S100$^+$ w splotach nerwowych (B). U pacjentów z w.p.z.o. stwierdzono nieliczne komórki S100$^+$ w warstwie mięśniowej odźwiernika, a w okolicy splotów nerwowych liczba komórek S100$^+$ była znacząco mniejsza (C, oryginalne powiększenie ×200)

pyloric muscle layers and around the myenteric plexus in the IHPS patients. Thus the lack of c-Kit and S100 expression, but not CD34 expression, may be a critical factor in the pathogenesis of IHPS and may serve as a useful prognostic tool in the treatment of this disease.

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