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Study of a Hirschsprung’s Disease Patient Cohort Using Multiplex Ligation-Dependent Probe Amplification

Badania wybranych genów u pacjentów z chorobą Hirschsprunga z użyciem metody MLPA

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Abstract

Background. Hirschsprung’s disease is a congenital disorder with an incidence of 1 per 5000 live births, characterized by the absence of intestinal ganglion cells. The etiology of Hirschsprung’s disease is very heterogeneous (chromosomal, monogenic, oligo- and polygenic, as well as multifactorial). Various genes play a role in its etiology, i.e. RET, EDNRB, EDN3, GDNF, SOX10, and ZEB2; new susceptible loci have also been identified.

Material and Methods. The characterized mutations in these genes are mostly small deletions and point mutations in both coding and non-coding regions. Also microdeletions of exons of the critical genes have been reported. The goal of this study was to screen 71 patients with Hirschsprung’s disease for micro-rearrangements in four genes (RET, ZEB2, EDN3, and GDNF) using the MLPA method.

Results and Conclusions. In one case, deletion of all ZEB2 exons was detected and Mowat-Wilson syndrome was diagnosed. In the rest of the patients, no genomic rearrangements were observed in any of the four genes (Adv Clin Exp Med 2010, 19, 1, 83–88).

Key words: Hirschsprung’s disease, MLPA, deletions, duplications, RET, ZEB2, EDN3, GDNF, Mowat-Wilson syndrome.

Streszczenie

Wprowadzenie. Choroba Hirschsprunga jest wrodzonym schorzeniem jelita występującym z częstością 1 na 5000 żywo urodzonych dzieci, charakteryzuje się brakiem komórek zwojowych w warstwie mięśniowej błony śluzowej oraz w błonie podśluzowej. Etiologia choroby Hirschsprunga jest bardzo heterogenna genetycznie (czynniki chromosomowe, monogenowe, oligo- i poligenowe, a także wieloczynnikowości), Zidentyfikowano wiele genów odgrywających znaczącą rolę w etiologii choroby: RET, EDNRB, EDN3, GDNF, SOX10, ZEB2, są opisywane także nowe, istotne loci. Charakterystyczne mutacje w tych genach to najczęściej małe delekcje oraz mutacje punktowe, zarówno regionach kodujących, jak i niekodujących. Opisuje się także mikrodelecje w regionach chromosomowych, gdzie są zlokalizowane geny krytyczne dla choroby Hirschsprunga.

Materiał i metody. Ocena molekularna 71 pacjentów z chorobą Hirschsprunga w kierunku mikrorearanżacji genomowych w czterech genach (RET, ZEB2, EDN3 i GDNF) z użyciem metody MLPA. W jednym przypadku wykazano delekcje wszystkich egzonów genu ZEB2, rozpoznając jednocześnie u pacjenta zespół Mowat i Wilsona.


Słowa kluczowe: choroba Hirschsprunga, MLPA, delekcje, duplikacje, RET, ZEB2, EDN3, GDNF, zespół Mowat-Wilson.

Hirschsprung’s disease (HSCR, OMIM 142623), or aganglionic megacolon, is a birth defect characterized by a complete absence of neuronal ganglion cells in the myenteric and submucosal plexuses along a variable length of the intestine. This disease occurs in one out of 5000 live births in an isolated or syndromic form. Two main types of Hirschsprung’s disease have been described. The
short-segment form accounts for 80% of cases and is restricted to the rectosigmoid colon. The long-segment form extends proximal to the sigmoid colon and accounts for the remaining 20%. Aganglionosis rarely extends into the entire large intestine (total colonic aganglionosis) or even the entire bowel (total intestinal aganglionosis). The incidence of short-segment disease is four times greater in males than in females, while equal numbers of males and females present with long-segment HSCR [1]. Clinically, HSCR is characterized by failure to pass stool, a distended abdomen, vomiting, and enterocolitis. Chronic constipation is a major feature of HSCR in childhood [2].

Various genes, such as RET, EDNRB, GDNF, EDN3 and SOX10, NTN3, ECE1, and ZEB2 are involved in the etiology of Hirschsprung’s disease. Mutations in these genes may result in dominant, recessive, or multifactorial patterns of inheritance. The complex nature of HSCR could suggest an oligogenic etiology [3–7]. The diverse models of inheritance, the coexistence of numerous genetic disorders, and the detection of numerous chromosomal aberrations together with the involvement of various genes confirm the genetic heterogeneity of Hirschsprung’s disease [4–8].

Most defects characterized in the genes involved in HSCR are small (point) mutations. The aim of this study was to identify genomic micro-rearrangements in RET, ZEB2, EDN3, and GDNF involved in Hirschsprung’s disease. To perform this study, multiplex ligation-dependent probe amplification (MLPA), a method designed to detect small deletions and duplications of one or more exons in the genes of interest, was used.

## Material and Methods

The study was carried out on a group of 71 patients with Hirschsprung’s disease. The outline of the study was accepted by the University Ethics Committee. The Hirschsprung’s disease patients were classified according to the length of the aganglionic colon into two groups: long segment (L-HSCR) and short segment (S-HSCR, rectosigmoid). The histopathological criteria used for inclusion in the study were histological evaluation of the aganglionic tract (absence of neuronal ganglia) and histochemical staining of nerve fibers (ACTC, S-100, and NSE) in suction biopsies of the rectal submucosa [9].

### Multiplex Ligation-Dependent Probe Amplification (MLPA)

DNA was isolated from peripheral blood lymphocytes using the standard proteinase K/phenol method [10]. MLPA was performed according to the attached manufacturer’s protocol using a SALSA P169 Hirschsprung MLPA kit (lot 0706; MRC-Holland). Genomic DNA (100 ng) was denaturated in 5 µl of Tris EDTA (TE) at 98°C for 5 min and then hybridized with the SALSA probemix for 16–18 h at 60°C. After sample hybridization, ligation in Ligase-65 mix (15 min in 54°C) was performed. Then the samples were heated for 5 min at 98°C. The last step was amplification of the obtained ligation products using polymerase chain reaction (PCR) with the SALSA-PCR set of primers (one FAM labeled). PCR was carried out for 35 cycles (30 s at 95°C, 30 s at 60°C, 60 s at 72°C, and a final extension of 20 min at 72°C) in a TC-512 thermocycler (Techne) in 50 µl of reaction mix.

The fragments were separated on an ABI 310 capillary sequencer with Genescan software version 3.1.2 (Applied Biosystems) using the LIZ 500 size standard. Data were analyzed using GeneMarker software version 1.85 (SoftGenetics LLC). Probe ratios between 0.7–1.3 were defined as normal. For statistical analysis, a population normalization test was used, but samples with a wide random spread of signals were excluded.

## Results

A short segment of aganglionic gut was found in 61 patients (85.59%) and a long segment in 10 patients (14.1%). Additional major congenital defects were observed in 32% of the patients, such as chromosomal aberrations, single gene mutation, urinary tract defects, cardiac defect, alimentary defect, deafness, ophthalmologic problems, and intellectual disability.

Seventy-one patients with Hirschsprung disease were tested using MLPA. Micro-rearrangements in four genes (RET, ZEB2, EDN3, and GDNF) were screened (Fig. 1). Only in one case, a patient with multiple congenital defects and psychomotor retardation, were deletions of all exons of the ZEB2 gene found. Mowat-Wilson syndrome was diagnosed in this patient. In the other 70 cases with long- and short-segment aganglionosis, no deletions or amplifications were identified in these four genes.

## Discussion

Hirschsprung’s disease is a model for a complex oligogenic disorder with several identified loci contributing to susceptibility. The relationships among the genes creating a non-Mendelian inher-
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Persistence pattern remain unclear and should be elucidated. Several authors noted that although at least eight genes with mutations that could be associated with Hirschsprung’s disease have been identified, mutations at individual loci are neither necessary nor sufficient to cause clinical disease. These eight genes involved in the etiology of Hirschsprung’s disease are \( \text{RET} \), \( \text{GDNF} \), \( \text{EDNRB} \), \( \text{EDN3} \), \( \text{NTN} \), \( \text{ECE1} \), \( \text{SOX10} \), and \( \text{ZEB2} \) (SIP-1) [5–7, 11].

HSCR occurs as an isolated trait in 70% of Hirschsprung patients, while it is associated with a chromosomal anomaly in 12% of cases and occurs with additional congenital anomalies in 18% of cases. While all Mendelian modes of inheritance have been described for syndromic HSCR, isolated HSCR is a model for oligogenic disorders with low sex-dependent penetrance [6, 12].

The gene most involved in HSCR’s etiology is \( \text{RET} \), in which mutations are identified in about 15–20% of patients with sporadic HSCR and 50% of patients with familial HSCR cases [13–17]. \( \text{RET} \), a member of the cadherin superfamily, encodes one of the receptor tyrosine kinases, which are cell-surface molecules that transduce signals for cell growth and differentiation and which play a crucial role in neural crest development, i.e. the migration and differentiation of specific cells into enteric neurons [13, 14]. Numerous loss-of-function germline mutations have been found in this gene [13, 18]. Further studies showed that a common non-coding \( \text{RET} \) variant within a conserved enhancer-like sequence in intron 1 is significantly associated with HSCR [12]. Recent advances have shown a new aspect in the etiology of Hirschsprung’s disease. Variants of several \( \text{RET} \) polymorphisms are over- or under-represented in the HSCR population and appear to modify the HSCR phenotype [19–21]. The severity of the clinical manifestation of HSCR correlates with the frequency of the 135A allele and seems to be inversely associated with the frequencies of the 2071A and 2712G alleles [20, 21]. Moreover, studies of families with HSCR suggest that \( \text{RET} \) penetrance is dependent on allele dosage and other modifier loci [5, 22, 23]. Gabriel et al. found new susceptibility loci at 3p21 and 19p12 engaged in Hirschsprung’s disease etiology together with 10q11, where the \( \text{RET} \) gene is located [23]. Bolk et al. suggested that Hirschsprung’s disease requires both \( \text{RET} \) and a newly identified locus at 9q31 [22]. Emison et al. concluded that \( \text{RET} \) mutations, coding and/or non-coding, are probably a necessary feature in all cases of HSCR [12]. However, \( \text{RET} \) mutations are not sufficient for HSCR and are dependent on other genetic modifiers (the oligogenic model of inheritance) [5–7].

Further mutations in several genes, such as \( \text{EDNRB} \), \( \text{GDNF} \), \( \text{NTN} \), \( \text{ECE1} \), and \( \text{SOX10} \) (either...
isolated or combined with \textit{RET} mutation), have been identified in up to 5\% of HSCR cases, supporting the genetic heterogeneity of this disorder, which may result from a cumulative effect of at least two mutations in different genes [5–7, 17, 22–24].

The \textit{EDNRB} gene is located on the 13q22 chromosomal region and encodes endothelin receptors belong to the G protein-coupled receptor family. \textit{EDNRB} gene mutations were found also in Waardenburg syndrome type IV, also called Shah-Waardenburg syndrome, characterized by a sensorineural hearing deficiency and pigmentation defects linked with HSCR. Several authors observed mutations in both \textit{RET} and \textit{ENDRB} in the same HSCR patients [25, 26]. Mutations in \textit{EDNRB} were also reported to be linked to mutations in \textit{EDN3} and \textit{SOX10}.

\textit{EDN3} protein (ligand endothelin 3) is a receptor for \textit{EDNRB} and was shown to be involved in the development of the neural crest. Hirschsprung’s disease is an example of a neurocristopathic disorder causing disturbances in innervation of the gastrointestinal tract. \textit{EDN3} gene is located on chromosome 20q13. \textit{EDN3} could be mutated in a small number of patients with HSCR and could be linked to mutations in \textit{EDNRB} [27]. Mutations in the \textit{EDN3} gene can result in Shah-Waardenburg syndrome and congenital central hypoventilation syndrome (CCHS), assigned to neurocristopathies as well [6, 28].

The \textit{GDNF} gene is located on 5p12-p13 and encodes glial cell-derived neurotropic factor (GDNF). Mutations in \textit{GDNF} were found solely in sporadic HSCR patients and were also found in connection with \textit{RET} mutations [29].

\textit{ZEB2} (\textit{ZFHX1B}, zinc finger homeobox 1b gene) has been recently described in a patient with Mowat-Wilson syndrome with multiple congenital anomalies and mental retardation [30–33]. \textit{ZEB2} is located on chromosome 2q22 and encodes a protein product, Smad interacting protein 1 (SIP1). Expression of SIP1 protein is detected in nearly all human tissues, such as the heart, brain, placenta, lung, liver, and skeletal muscle [34]. SIP1 expression is also detected in human fetal tissues, including brain, kidney, liver, and spleen. It is speculated that SIP1 has a crucial role in embryonic development, also in the development of the enteric nervous system [31, 34]. Several autosomal dominant mutations and different sized heterozygous deletions have been described in patients with Mowat-Wilson syndrome leading to allelic loss and resulting loss of function (protein shortening or inactivation) [35–37]. It is supposed that cases with deletions of the \textit{ZEB2} gene are similar to those with point mutations with non-allelic modifiers, explaining the clinical variability [36, 37]. Larger deletions have been shown to be associated with a more severe course [37, 38].

To detect deletions and duplications of the exons of the four genes critical to Hirschsprung’s disease, MLPA was used containing 41 probes with amplification products for all 20 exons of \textit{RET} gene [10q11.21], all 5 exons of \textit{EDN3} gene [20q13.32], all 4 exons of \textit{GDNF} gene [5p13.2], and all 10 exons of \textit{ZEB2} (\textit{ZFHX1B}) gene [2q22.3]. The analysis of the 70 patients with short- and long-segment as well as sporadic and syndromic Hirschsprung’s disease showed no significant genomic rearrangements in the tested genes. These data confirm previous results obtained by a German group [39].

Only in one case was deletion of all \textit{ZEB2} exons detected and Mowat-Wilson syndrome was diagnosed [30, 35–37]. Mowat-Wilson syndrome is characterized by distinctive facial features, short stature, structural anomalies including Hirschprung’s disease, genitourinary anomalies, congenital heart defects, agenesis or hypogenensis of the corpus callosum, and eye defects as well as moderate to severe intellectual disability and severe speech impairment. The patient with MWS diagnosed in the MLPA analysis showed Fallot tetralogy, Hirschsprung’s disease, hepatic cell lesion, α-1 antitrypsin deficiency (ATD), and liver cirrhosis in the course of ATD as well as delayed psychomotor development, hypotonia, and a variety of dysmorphic features [38].

A chromosomal abnormality is present in approximately 12\% of individuals with HSCR [1, 6]. The most common chromosomal abnormality associated with HSCR is Down’s syndrome (trisomy 21), which occurs in 2–10\% of all individuals with HSCR [6–8, 40]. In the present cohort of patients with HSCR, Down’s syndrome was diagnosed in 4 cases (5\% of all patients). Although individuals with Down’s syndrome are at a 100-fold higher risk of HSCR than the general population [40], none of the established “HSCR genes” reside on chromosome 21; thus the association between trisomy 21 and HSCR remains unexplained. Other chromosomal aberrations include deletions that encompass HSCR-associated genes. These are deletion of the 13q22 region (\textit{EDN3B} gene), deletion of the 10q11.2 region (\textit{RET}), and deletion of the 2q22 region (\textit{ZEB2}) [5–7, 13, 14, 30]. The identification of individuals with HSCR and such deletions aided in the discovery of these genes and reinforces the haplo-insufficiency model of HSCR pathogenesis. Other chromosomal anomalies have been described in individuals with HSCR, but the relevant gene(s) of interest have not been identified.

It is evident that Hirschsprung’s disease displays a complex genetic etiology, with particular
genes contributing as separate entities or in combination. It has been demonstrated that the RET, EDN3, GDNF, and ZEB2 genes are involved in HSCR’s etiology, but changes in these genes are often insufficient for HSCR and are dependent on different modifiers. Further genetic studies of known and new genes are needed to elucidate the complex nature of Hirschsprung’s disease as an oligogenic disorder.

References


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