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The correlation of genetic markers with anatomoclinical and histopathological forms in Hirschsprung's disease

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Abstract

Hirschsprung's disease is a birth defect that affects about one out of 5000 newborns. It is one of the most common causes of intestinal obstruction at the babies. The objectives of this study are to evaluate the characteristics of Hirschsprung's disease in Dobrogea area, test of genetic markers in families and single cases, estimate the value of the test in the diagnosis and for evolution. We made a case-control study for the period 1995–2006 and analyzed 21 cases of Hirschsprung's disease, which were treated in the Emergency County Hospital, Constanta. The diagnostic methods comprised clinical and paraclinical examination. The chromosomal markers used in the study are represented by four categories of chromosome abnormalities: Trisomy 21, Del 10q, Del 13q, Del 17q. The molecular markers investigated by us are represented by: RET, EDNRB and EDN3. We made the correlation of genetic markers with the anatomopathological and histopathological forms, by measuring the level of association, expressed by the calculated relative risk (OR) and using the correlation index φ. Based on data obtained from the group investigated, we found that the indices of association and correlation are consistently higher compared to DNA-markers with chromosomal markers, both for anatomopathological forms as well as histopathological. We noticed that no chromosomes markers were recorded with indices of correlation with negative values, which means that these chromosomal abnormalities are involved with a particular quota to the release of disease.

Keywords: Hirschsprung's disease, chromosomal markers, molecular markers.

Introduction

Hirschsprung's disease, also known as congenital megacolon or aganglionic megacolon, is one of the most common causes of intestinal obstruction at the babies. In Hirschsprung's disease (HD), the ganglion cells are missing from a short or long part of the bowel, causing chronic constipation [1, 2]. The absence of ganglion cells in Hirschsprung's disease has been attributed to a failure of migration of neural crest cells.

While the internal anal sphincter is the constant inferior limit, patients can be classified as classical segment Hirschsprung's disease when the aganglionic segment does not extend beyond the upper sigmoid, long-segment Hirschsprung's disease (L-form) when aganglionosis extends to the splenic flexure or transverse colon, and total colonic aganglionosis when the aganglionic segment extends to the colon and a short segment of terminal ileum [3]. Total intestinal aganglionosis with absence of ganglion cells from duodenum to the rectum is the rarest form of Hirschsprung's disease [4, 5].

Genetic factors are implicated in the etiology of Hirschsprung's disease. A chromosomal abnormality is associated with HD in 12% of patients, trisomy 21 being by far the most frequent. Other chromosomal abnormalities that have been described in association with HD include interstitial deletion of distal 13q, partial deletion of 2p, reciprocal translation, and mosaic trisomy 18. A number of unusual hereditary syndromes have been reported in patients with HD: multiple endocrine neoplasia (MEN) type 2 syndrome, Shah–Waardenburg syndrome, Bardet–Biedl syndrome, congenital central hypoventilation syndrome (Ondine's curse), Goldberg–Shprintzen syndrome, Cartilage-hair hypoplasia syndrome, Kaufman–McKusick syndrome, Smith–Lemli–Opitz syndrome [6–10].

Molecular-genetic analysis has identified several genes that have a role in the development of Hirschsprung's disease: the proto-oncogene RET (RET), glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN), endothelin B receptor (EDNRB), endothelin 3 (EDN3), endothelin-converting enzyme 1 (ECE1), SOX10, Phox21, GFRq1 and SIP1 genes [8, 11]. The RET proto-oncogene on chromosome 10-q was identified as major gene involved in 50% from the family forms and 40–45% from sporadic cases of Hirschsprung's disease [8, 12–14].

The incidence of Hirschsprung's disease is estimated to be one in 5000 live births and it has long been recognized that males are more commonly affected than females with a male: female ratio of 4:1 [3, 6, 15-21].

Hirschsprung's disease is usually suspected when a baby does not have a bowel movement for several days following birth. Babies with Hirschsprung's disease often have large, swollen abdomens and may vomit green bile after feeding. The diagnostic steps include a careful history and physical examination, radiographic studies, anorectal manometry and a rectal biopsy [22].

The common complications of this disease are: inflammatory septic lesions secondary of infection with specific microorganisms, rectal prolapse, intestinal occlusion, pseudomembranous enterocolitis, irritable bowel syndrome, peritonitis.

The only way to treat this disease is through surgery [16, 23]. Untreated aganglionic megacolon in infancy may result in a mortality rate of as much as 80%. Operative mortality rates are very low and most children have normal lives after surgery.

The aim of this study was to evaluate the genetic features of congenital megacolon found in our area for improving the primary, secondary and tertiary prevention of this disease difficult to predict and particularly disabling.

Material and Methods

We made a retrospective study for the period 1995–2006 and analyzed 21 cases of Hirschsprung's disease, which were treated in the Emergency County Hospital, Constanta. These 21 patients (14 males and seven females) represent the group of cases, each of them representing the proband in family investigations that we conducted in all cases. The eligibility criteria: probands diagnosed, treated and monitored in their progress in Emergency County Hospital, Constanta, since debut until present, probands with belonging in life who agreed to donate biological samples required in this study, probands whose parents gave written consent to participate in the study.

The witness groups are represented by: the witness group of parents, the witness group in fratry and the witness group from the general population. The witness group of parents is formed by 42 people. The witness group in fratry is represented by 26 people, brothers of the probands including 20 males and 6 females. The witness group from the general population was composed of patients with intestinal occlusion syndrome and it has a total of 84 persons of which 56 males and 28 females. For this third group were selected persons that meet the following eligibility criteria: be of the same sex, weight at birth and APGAR close, the same rank of birth, born in the same quarter in which it was born the proband.

The diagnostic methods comprised clinical and paraclinical examination: rectal biopsy, anorectal manometry, simple abdominal radiography, irigography, ultrasonography, endoanal ultrasound, nuclear magnetic resonance (NMR) imaging, histopathological diagnosis, molecular genetic techniques [22, 24, 25].

A special place in our study was the family investigation, which is a specific method of investigation in clinical genetics and aims the assessment of the family nature of the disease and then the hereditary transmission of that normally or abnormally character.

The chromosomal markers used in the study are represented by four categories of chromosome abnormalities: Trisomy 21, Del 10q, Del 13q, Del 17q. To reveal the genetic markers involved in the etiology of congenital megacolon, karyotype with marker G from lymphocyte culture of peripheral blood was carried out to all patients, because of the family investigation and clinical symptoms. The samples were processed in the cytogenetic laboratory [26–30].

The molecular markers investigated by us, are represented by: RET, EDNRB and EDN3 [26–30]. DNA-extraction was performed by the methods recommended by the suppliers kits namely the method of phenol-chloroform extract. The purification of genomic DNA Promega Ready Amp provides a simple, efficient, safe and inexpensive way to isolate genomic monocatenar DNA from whole blood for analysis of amplification. The amplification was performed using Taq DNA-polymerase standard. All subsequent steps respected the protocol Promega's DNA Silver Staining System [29–31].

We made the correlation of genetic markers with the anatomopathological and histopathological forms, by measuring the level of association, expressed by the calculated relative risk (OR) and using the correlation index φ, based on research protocol type case-control [24].

We have calculated the level for association and correlation between the DNA-markers and two types of parameters by taking each of the markers and chromosomal DNA as exposure (risk factor) and morphological parameters as associates or not with these in the presence of the congenital megacolon.

The level of association shows how many times the presence of a particular DNA marker is more frequent, simultaneously with a parameter in the presence of congenital megacolon.

The correlation index (φ) expresses the level of dependence and intensity of association, both positively and negative, and it may take any value in the range from -1 to +1.

As long as the values are removed from extreme to zero the level of correlation decreases.

A positive value of the index of correlation (φ) means that the two variables (parameters, characters, markers) are found together and do not exclude as in case of negative values.

To measure the level of correlation in the family and single cases we proceeded to determination of the chromosomal and DNA genetic markers mentioned above.

Results

Incidence of Hirschsprung's disease in Constanta County was 1.2 to 5000 newborn babies, in the analyzed period. Gender-specific incidence is characterized by a higher share of male patients who, in our study are in a

ratio of 66% (14 cases) of all patients taken into study; seven cases were female, which is an incidence of 34%. Sex ratio (M:F) – 2:1.

Regarding the incidence of started disease on age groups we found that it occurred at a rate equal to 48% in newborn and infant, one case was diagnosed at age of one year.

Predominant symptom in cases of our group was severe constipation associated with abdominal distension of varying degrees.

On the histopathological point of view prevailed aganglionosis limited to rectosigmoidian junction, even if from the microscopic point of view was total or partial aganglionosis (with rare cell).

In the study of genealogical tree we found that all cases of congenital megacolon treated in the Department of Pediatric Surgery and Orthopedics of Constanta are the unique cases in their families, some probands having heredo-collateral history loaded on one or both parents, with colic obstructive pathology (chronic constipation), other probands having parents with diverse pathology but also by digestive nature.

Chromosomal abnormalities were not detected in all cases of congenital megacolon. These were represented by trisomy 21 (one case), del 10q (six cases), del 13q (five cases), del 17q (three cases).

Karyotype analysis of parents revealed the presence of chromosomal abnormalities such as deletions in one of the parents, only in four cases. All parents with the chromosomal anomaly had a history of pathological chronic constipation.

On the fratry karyotype, analysis shows the presence of a chromosomal abnormality – 13q deletion, in one case, at one of the brothers, who present history of chronic constipation.

All probands with congenital megacolon were identified with mutations of the RET-gene (in nine cases), EDNRB (six cases), EDN3 (seven cases).

Only seven cases showed mutation only in the RET gene, the remaining 14 cases were associated with mutations in several genes known to be involved in the etiology of the disease, which suggests the multifactorial disease.

The analysis of mutations in fratry revealed the presence of mutations in a single case, to one of the brothers; he inherited the mutation of EDNRB gene from his mother.

Correlation of chromosomal and DNA genetic markers with anatomopathological and histopathological forms, have made it by measuring the level of association expressed by the calculated relative risk (OR) and using the correlation φ-index.

The strongest association we found in Del 10q (Table 1).

In Tables 1 and 2 we marked with different colors only the calculated values of the coefficient of correlation φ which correspond to values of the threshold of significance (Pv) equal to or less than 0.05 (corresponding to a limits of confidence interval exceeding 95%). We used green color to mark positive φ-values, and red for negative values with statistical significance. We used a gray color for positive values but without statistical significance.

Analyzing the values of correlation indices (Figure 1), we find that in case of Del 10q we meet the highest values of φ (0.33) for the anatomopathological form of short segment and φ=0.35 for the anatomopathological form of long segment. Relatively high values were recorded for the marker 13q.

We can say that for any chromosomal markers have not been recorded indices of correlation with negative values.

Analyzing the correlation between chromosome markers and anatomopathological forms, we can see that the highest values are found in Del 10q and in decreasing order being Del 13q, Trisomy 21 and Del 17q. For the long segment forms (L-forms), the values are similar but increased comparative with those of the short segment (S-forms) (Figure 2).

Table 1 – ***The values of the level of association and correlation between chromosomal markers with anatomopatological and histopathological forms***

		Anatomoclinical forms		Histopathological forms	
		Form of short segment	***Form of long segment***	***Rare ganglionar cells***	***Total aganglionosis***
Trisomy 21	O.R.	2.25	2.28	0.50	0.88
	F.R.A. [%]	55	56	–	–
	χ^2	0.95	0.92	0.42	0.02
	φ	+0.14	+0.16	-0.14	0
Del 10q	O.R.	7.11	9.00	4.32	1.94
	F.R.A. [%]	85	88	76	48
	χ^2	5.23	4.07	1.89	0.30
	φ	+0.33	+0.35	+0.20	0
Del 13q	O.R.	2.92	2.00	4.50	0.75
	F.R.A. [%]	65	50	81	–
	χ^2	2.27	0.57	3.49	0.11
	φ	+0.21	+0.13	+0.27	0
Del 17q	O.R.	0,10	0,19	1,39	0,00
	F.R.A. [%]	–	–	28	–
	χ^2	4.90	1.93	0.10	1.99
	φ	-0.32	-0.24	0	+0.24

O.R. – calculated relative risk; F.R.A. – etiological fraction of attributable risk, χ^2 – comparison index; φ – correlation index.

Table 2 – ***The values of the level of association and correlation between DNA-markers with anatomopatological and histopathological forms***

		Anatomoclinical forms		Histopathological forms	
		Form of short segment	*Form of long segment*	*Rare ganglionar cells*	*Total aganglionosis*
RET	O.R.	0.36	1.47	0.75	4.48
	F.R.A. [%]	–	–	–	77
	χ^2	2.51	0.28	0.22	3.97
	φ	-0.23	+0.07	-0.06	**+0.35**
EDNRB	O.R.	0.31	0.36	1.05	1.75
	F.R.A. [%]	–	–	–	42
	χ^2	2.53	0	0.01	0.53
	φ	-0.23	0	-0.01	+0.12
EDN3	O.R.	0.45	0.50	0.32	2.50
	F.R.A. [%]	–	–	–	60
	χ^2	1.79	0.91	3.6	1.5
	φ	-0.19	-0.16	-0.27	+0.21

O.R. – calculated relative risk; F.R.A. – etiological fraction of attributable risk, χ^2 – comparison index; φ – correlation index.

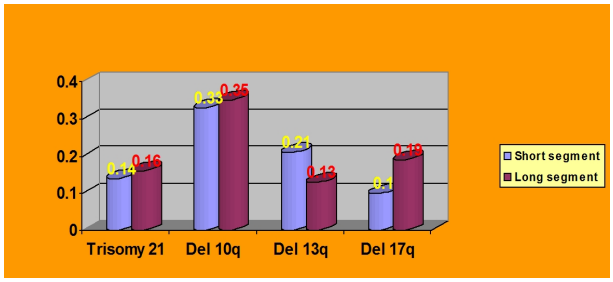

Figure 1 – ***The level for association between chromosomal markers and anatomopathological forms.***

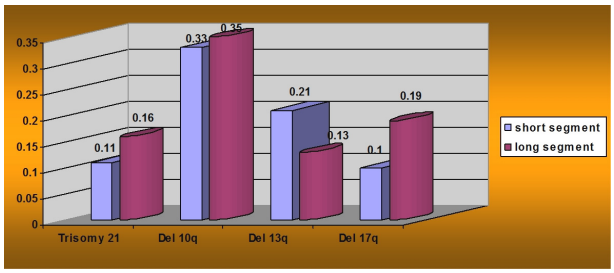

Figure 2 – ***The values of the level of correlation between chromosome markers with anatomopathological forms.***

The values of the level of association between chromosome markers with histopathological forms are positive in the following order: Del 13q, Del 10q, and Trisomy 21 for rare cells types and for aganglionosis type it is positive only Del 17q (Figure 3).

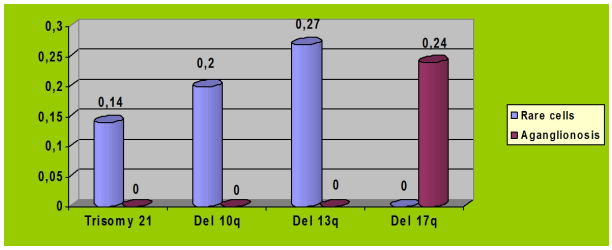

Figure 3 – ***The values of the level of association between chromosome markers with histopathological forms.***

For the correlation between chromosome markers and histopathological forms, the values are represented in the Figure 4.

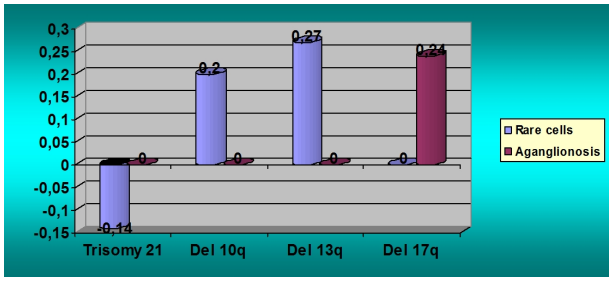

Figure 4 – ***The values of the level of correlation between chromosome markers with histopathological forms.***

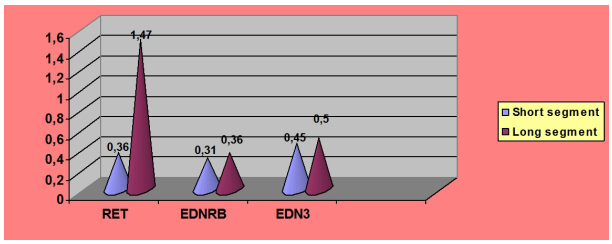

Figure 5 – ***The values of the level of association between DNA-markers with anatomopathological forms.***

The correlation of the DNA-molecular markers with anatomopathological and histopathological forms is shown in the following lines.

The molecular markers investigated in study are represented by: EDNRB (endothelin B receptor 13q22), EDN3 (endothelin 3 – 20q13), and RET (encodes a receptor tyrosine kinase and its ligand 10q11.2). The values for χ^2, OR and φ are presented below (Table 2).

The analysis of the association between DNA-markers and anatomoclinical forms (Figure 5) has the following features:

- The long segment form has the highest values of the index of association for RET-marker (OR=1.47) followed by EDN3 (OR=0.5) and EDNRB (OR=0.36). Supra-unitary value presents only RET, which suggest a higher risk of developing congenital megacolon in the presence of this mutation.
- The higher risks of developing congenital megacolon with anatomoclinical form of short segment present the next markers EDN3 (OR=0.45), RET (OR= 0.36), EDNRB (OR=0.31).

Analyzing the association between DNA-markers and histopathological forms (Figure 6), we found that:

▪ All investigated DNA-markers are associated more strongly with histopathological form of aganglionosis; especially RET which has values two times higher compared to EDN3.

▪ RET with an OR value of 4.48 represents the mutation which makes the risk of congenital megacolon with aganglionosis to be more than four times higher than in its absence.

▪ Double risks induce the presence of the mutations EDN3 (OR=2.2) and EDNRB (OR=1.7) too.

The correlation of DNA-markers with anatomopathological forms (Figure 7) showed that these three markers correlate positively (but with low values) only with the anatomopathological form of short segment namely RET (φ=-0.23), EDNRB (φ=-0.23) and EDN3 (φ=-0.19).

The correlation of DNA-markers with histopathological forms (Figure 8) has the following characteristics:

▪ RET (φ=+0.35), EDN3 (φ=+0.21) and EDNRB (φ=+0.12) have large positive values of index of correlation of these markers with the histopathological form with aganglionosis.

▪ There have not been positive correlations of DNA-markers with the histopathological form with rare cells.

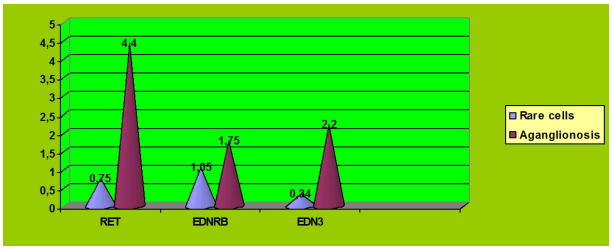

Figure 6 – ***The values of the level of association between DNA markers with histopathological forms.***

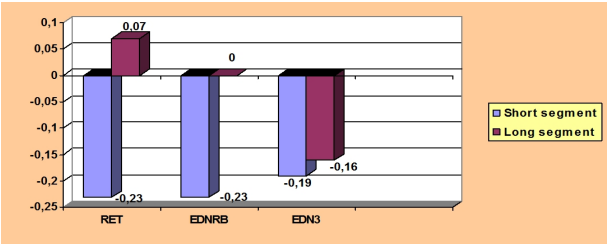

Figure 7 – ***The values of the level of correlation between DNA-markers with anatomopatological forms.***

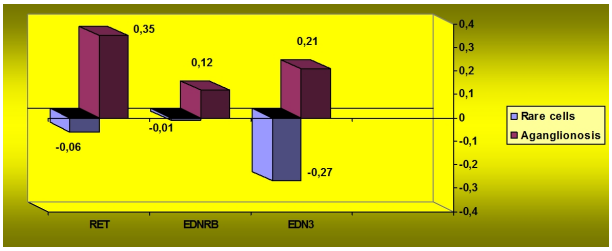

Figure 8 – ***The values of the level of correlation between DNA-markers with histopathological forms.***

Discussion

Congenital megacolon is a disease affecting one child in 5000 newborn babies (in a general statistics). The incidence in the Constanta County is close to that seen in other studies [3, 6].

Gender-specific incidence is treated differently in the literature. After Prof. Dr. T. Zamfir, the report sex-ratio M:F should be 6:1. After Prof. Dr. A. Pesamosca, the specific incidents have values close to those reported by us namely 73.4% in boys and 26.6% in girls, so a sex ratio of 3:1. Other authors (Kusafuka T *et al.*, 1996) reported a sex ratio value of 2.5:1, for boys, having the same correspondent in Kruglyac L *et al.* study. High frequency reported by Prof. T. Zamfir is because at the time of reporting (1980) in many sections from the country, there shall be no operation for megacolon [32–34].

Molecular-genetic analysis has identified that the most important gene involved in producing of Hirschsprung's disease is the proto-oncogene RET (RET). The data are close to that seen in other studies [8, 12–14].

It should be noted that the presence of mutations it correlate with detected chromosomal abnormalities.

Correlation of chromosomal and DNA-genetic markers with anatomopathological and histopathological forms, indicates that all values calculated for relative risks were low and this aspect should be interpreted as: no single mutation has the ability to generate one of the anatomoclinical forms of congenital megacolon considered for the study.

In one study published in 2008, the correlation of chromosomal markers with anatomopathological forms is significant for patients with S-form and trisomy 21 [35].

In other studies the correlation of DNA-markers with anatomopathological forms has been shown to be higher for RET-mutation in the L-form than in the S-form, suggesting the effects of multiple genes which would work particularly in the least severely affected, and providing an explanation for the still very poor genotype–phenotype correlation in Hirschsprung's disease [35].

Because for any chromosomal markers have not been recorded indices of correlation with anatomopathological forms with negative values we can say that means that these chromosomal abnormalities are involved with a certain quota to disease outbreak.

For the values of the level of correlation between chromosomal markers with anatomopathological forms we can say that take it individual, any of this chromosomal markers investigated are not risk factors.

Compared with the values of association of the chromosome markers, the values of the level of association of DNA-markers with anatomoclinical forms of congenital megacolon are consistently higher.

From the clinical point of view, our study has major repercussions for diagnosis, by investigating preoperative genetic markers, corroborating such genetic investigation with other diagnostic methods. Therefore, it can guide us in the first place on whether conservative or surgical treatment. Also knowing these informations, we would appreciate optimal surgical technique.

Conclusions

Based on data obtained from the group investigated, we found that the indices of association and correlation

are consistently higher compared to DNA-markers with chromosomal markers, both for anatomopathological forms as well as histopathological.

Improving the genetic investigations we can replace the invasive diagnostic methods which can cause serious complications taking into account the small age of the patients (8 months–2 years).

Not having claim that this work exhausted the entirely subject, which has not been approached until now in Romanian Medicine, we express our conviction that the issue will be approached by other colleagues and will be perfected.

References

[1] KLEIN MD, CORAN AG, WESLEY JR, DRONGOWSKI RA, *Hirschsprung's disease in the newborn*, J Pediatr Surg, 1984, 19(4):370–374.

[2] CASS D, *Aganglionosis: associated anomalies*, J Paediatr Child Health, 1990, 26(6):351–354.

[3] PURI P, Hirschsprung's disease: clinical generalities. In: HOLSCHNEIDER AM, PURI P (eds), *Hirschsprung's disease and allied disorders*, Harwood Academic Publishers, Amsterdam, 2000, 129–135.

[4] SENYÜZ OF, BÜYÜKÜNAL C, DANIŞMEND N, ERDOĞAN E, OZBAY G, SÖYLET Y, *Extensive intestinal aganglionosis*, J Pediatr Surg, 1989, 24(5):453–456.

[5] SHARIF K, BEATH SV, KELLY DA, MCKIERNAN P, VAN MOURIK I, MIRZA D, MAYER AD, BUCKELS JA, DE VILLE DE GOYET J, *New perspective for the management of near-total or total intestinal aganglionosis in infants*, J Pediatr Surg, 2003, 38(1):25–28; discussion 25–28.

[6] RYAN ET, ECKER JL, CHRISTAKIS NA, FOLKMAN J, *Hirschsprung's disease: associated abnormalities and demography*, J Pediatr Surg, 1992, 27(1):76–81.

[7] CHAKRAVARTI A, LYONNET S, Hirschsprung disease. In: SCRIVER CR, BEAUDET AL, VALLE D, SLY WS, CHILDS B, KINZLER KW, VOGELSTEIN B (eds), *The metabolic and molecular basis of inherited disease*, 8th edition, McGraw–Hill Professional, New York, 2001, 6231–6255.

[8] AMIEL J, LYONNET S, *Hirschsprung disease, associated syndromes, and genetics: a review*, J Med Genet, 2001, 38(11):729–739.

[9] BROWN RA, CYWES S, Disorders and congenital malformations associated with Hirschsprung's disease. In: HOLSCHNEIDER AM, PURI P (eds), *Hirschsprung's disease and allied disorders*, Harwood Academic Publishers, Amsterdam, 2000, 137–145.

[10] GARIEPY CE, *Genetic basis of Hirschsprung disease: implications in clinical practice*, Mol Genet Metab, 2003, 80(1–2):66–73.

[11] PURI P, SHINKAI T, *Pathogenesis of Hirschsprung's disease and its variants: recent progress*, Semin Pediatr Surg, 2004, 13(1):18–24.

[12] BODIAN M, CARTER OO, *A family study of Hirschsprung's disease*, Ann Hum Genet, 1963, 26(3):261–277.

[13] GARVER KL, LAW JC, GARVER B, *Hirschsprung disease: a genetic study*, Clin Genet, 1985, 28(6):503–508.

[14] PARISI MA, KAPUR RP, *Genetics of Hirschsprung disease*, Curr Opin Pediatr, 2000, 12(6):610–617.

[15] ORR JD, SCOBIE WG, *Presentation and incidence of Hirschsprung's disease*, Br Med J (Clin Res Ed), 1983, 287(6406):1671.

[16] IKEDA K, GOTO S, *Diagnosis and treatment of Hirschsprung's disease in Japan. An analysis of 1628 patients*, Ann Surg, 1984, 199(4):400–405.

[17] RUSSELL MB, RUSSELL CA, NIEBUHR E, *An epidemiological study of Hirschsprung's disease and additional anomalies*, Acta Paediatr, 1994, 83(1):68–71.

[18] GOLDBERG EL, *An epidemiological study of Hirschsprung's disease*, Int J Epidemiol, 1984, 13(4):479–485.

[19] SUITA S, TAGUCHI T, IEIRI S, NAKATSUJI T, *Hirschsprung's disease in Japan: analysis of 3852 patients based on a nationwide survey in 30 years*, J Pediatr Surg, 2005, 40(1):197–201; discussion 201–202.

[20] SPOUGE D, BAIRD PA, *Hirschsprung disease in a large birth cohort*, Teratology, 1985, 32(2):171–177.

[21] KLEINHAUS S, BOLEY SJ, SHERAN M, SIEBER WK, *Hirschsprung's disease – a survey of the members of the Surgical Section of the American Academy of Pediatrics*, J Pediatr Surg, 1979, 14(5):588–597.

[22] PENSABENE L, YOUSSEF NN, GRIFFITHS JM, DI LORENZO C, *Colonic manometry in children with defecatory disorders: role in diagnosis and management*, Am J Gastroenterol, 2003, 98(5):1052–1057.

[23] MARTIN LW, TORRES AM, *Hirschsprung's disease*, Surg Clin North Am, 1985, 65(5):1171–1180.

[24] BROASCĂ MADAR VV, *Sănătate publică şi management. Lucrări practice,* Ed. Ex Ponto, Constanța, 2003, 102–119.

[25] DASGUPTA R, LANGER JC, *Hirschsprung disease*, Curr Probl Surg, 2004, 41(12):942–988.

[26] ATTIÉ T, PELET A, EDERY P, ENG C, MULLIGAN LM, AMIEL J, BOUTRAND L, BELDJORD C, NIHOUL-FÉKÉTÉ C, MUNNICH A *et al.*, *Diversity of RET proto-oncogene mutations in familial and sporadic Hirschsprung disease*, Hum Mol Genet, 1995, 4(8):1381–1386.

[27] BADNER JA, SIEBER WK, GARVER KL, CHAKRAVARTI A, *A genetic study of Hirschsprung disease*, Am J Hum Genet, 1990, 46(3):568–580.

[28] GATH R, GOESSLING A, KELLER KM, KOLETZKO S, COERDT W, MÜNTEFERING H, WIRTH S, HOFSTRA RM, MULLIGAN L, ENG C, VON DEIMLING A, *Analysis of the RET, GDNF, EDN3, and EDNRB genes in patients with intestinal neuronal dysplasia and Hirschsprung disease*, Gut, 2001, 48(5):671–675.

[29] INOUE K, SHIMOTAKE T, IWAI N, *Mutational analysis of RET/GDNF/NTN genes in children with total colonic aganglionosis with small bowel involvement*, Am J Med Genet, 2000, 93(4):278–284.

[30] SYRRIS P, CARTER ND, PATTON MA, *Novel nonsense mutation of the endothelin-B receptor gene in a family with Waardenburg–Hirschsprung disease*, Am J Med Genet, 1999, 87(1):69–71.

[31] BOLK S, PELET A, HOFSTRA RM, ANGRIST M, SALOMON R, CROAKER D, BUYS CH, LYONNET S, CHAKRAVARTI A, *A human model for multigenic inheritance: phenotypic expression in Hirschsprung disease requires both the RET gene and a new 9q31 locus*, Proc Natl Acad Sci U S A, 2000, 97(1):268–273.

[32] ZAMFIR T, *Curs de chirurgie şi ortopedie pediatrică*, vol. I, Ed. I.M.F. Bucureşti, 1980, 101–115.

[33] KUSAFUKA T, WANG Y, PURI P, *Novel mutations of the endothelin-B receptor gene in isolated patients with Hirschsprung's disease*, Hum Mol Genet, 1996, 5(3):347–349.

[34] KRUGLYAK L, DALY MJ, REEVE-DALY MP, LANDER ES, *Parametric and nonparametric linkage analysis: a unified multipoint approach*, Am J Hum Genet, 1996, 58(6):1347–1363.

[35] MOORE SW, Congenital anomalies and genetic associations in Hirschsprung's disease. In: HOLSCHNEIDER AM, PURI P (eds), *Hirschsprung's disease and allied disorders*, Springer Verlag, Berlin–Heidelberg, 2008, 115–124.

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