

Preliminary study shows novel variant detected in the screening of *RET* gene in Malaysian patients with Hirschsprung's disease

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Abstract

Hirschsprung's disease (HSCR) is a disorder associated with congenital absence of ganglion cells in the gastrointestinal tract. Molecular analyses have identified variants in various genes including *RET*, *GDNF*, *EDN3* and *EDNRB* that are involved in the development, migration and survival of neural cells. Variants in the receptor tyrosine kinase (*RET*) are most common and have been identified in 10-20% of sporadic HSCR patients. The objective of this study was to screen for *RET* gene variants in Malaysian patients with HSCR. Thirty-two patients with HSCR and 30 normal controls were recruited for this study. Mutations were screened using the Polymerase Chain Reaction – Denaturing High Performance Liquid Chromatography (PCR-dHPLC) approach. Mutations identified were then confirmed using Sanger sequencing. We identified one novel rare variant in exon 4 (A268A c807 G>C) in one patient. We also identified the common coding sequence variants A45A (c135G>A), A432A (c1296A>G), L769L (c2307 T>G) and the G691S in our cohort of patients. In conclusion, our Malaysian patients with HSCR diseases showed the presence of similar *RET* gene common variants which have been described in other populations. We have also identified a novel variant in exon 4 (A268A).

Introduction

Hirschsprung's disease (HSCR) or aganglionic megacolon is characterized by the absence of parasympathetic intrinsic ganglion cells in the submucosal and myenteric plexuses in the gastrointestinal tract [1-3]. The disease can be subdivided into 3 groups including short-segment (S-HSCR, aganglionosis up to upper sigmoid colon), long-segment (L-HSCR, aganglionosis up to the splenic flexure and beyond) and total colonic aganglionosis (TCA) [4]. HSCR presents in the neonatal period or in early childhood with symptoms ranging from chronic constipation to acute ileus. However, late manifestation in adults has also been described [5]. HSCR is the most common cause of neonatal intestinal obstruction and it affects one in 5,000 newborns with a male predominance of 3:1 to 5:1 [6-7].

HSCR is likely to be of multifactorial inheritance and associated with genes involved in the signaling

pathway of enteric nervous system (ENS) including the *RET* (REarranged during Transfection), glial derived neurotrophic factor (*GDNF*), endothelin receptor B (*EDNRB*) genes and endothelin 3 (*EDN3*) [5, 8]. These genes are interrelated and involved in the development of enteric ganglia from the specific lineage of neural crest cells [5, 8]. *RET* encodes for a receptor tyrosine kinase and is a major susceptibility gene for HSCR [9-11]. This gene is located in the 10q11.2 which transcribes for a transmembrane receptor with a cadherin-like extracellular domain, a cysteine-rich region and an intracellular tyrosine kinase domain [12]. Heterozygous variants in the *RET* gene accounts for 7-35% of sporadic HSCR and around 50% of familial HSCR cases [8]. Variants reported include missense, nonsense, deletion, insertion and frameshift mutations and are present throughout the whole *RET* gene sequences [13-17]. Some studies have also shown that certain variants of the *RET* gene serves as low susceptibility factors and modify the penetrance and severity of the HSCR phenotype [18].

There is a significant racial variation in the incidence of the disease worldwide, with the highest prevalence among Asians [9, 15, 19-22]. Eight heterozygous rare variants (RVs) were detected in 13 Vietnamese patients (13.40%) in which the variants were not present in healthy individuals [17]. Among those variants, 2 were novel and deleterious (R133C [c.397 C>T]; R144C [c.430 C>T]) and 4 have been described previously (R114H [c.341 G>A]; V292M [c.874 G>A]; G533S [c.1597 G>A]; R982C [c.2944 C>T]) (17). The common *RET* coding sequence variants are rs1800858 (A45A [c.135 G>A]) and rs1800861 (L769L [c.2307 T>G]) and were highly associated with the disease [17]. Other common variants associated with HSCR that have been reported previously in Taiwan were c135G>A (rs1800858, A45A) in exon 2, c1296A>G (rs1800860, A432A) in exon 7, c2307T>G (rs1800861, L769L) in exon 13 and c2712C>G (rs1800863, S904S) in exon 15 [8]. A recent study in southern Thailand showed similar sequence variants for *RET* gene [22]. They identified additional novel rare variants in exon 2, 4 and 8 which were c299G>T (S100M), c692G>A (R231H), c833C>A (T278N) and c1597G>A (G533S) [22]. About 11 different nucleotide substitutions were identified in Korean patients with HSCR [15]. Of these, 2 were new missense mutations (C558Y and R844W) and 9 were previously described variants [15]. Although the common and rare variants of *RET* have been extensively characterized in HSCR patients from various ethnic groups, the genotype data from Malaysia has yet to be investigated. Therefore, this

study was performed to determine the variation in the *RET* gene of Malaysian HSCR patients.

Materials and methods

Patient collection and DNA samples

A total of 32 HSCR patients and 30 normal controls (healthy unselected and unrelated individuals) were recruited into this study. Of the 32 HSCR patients included in the study, 26 were Malays, 5 were Chinese and 1 was Indian. Twenty-five patients were male and 7 patients were female. The mean age of the patients was 2.98 years (1 month – 11 years). Controls were collected from patients who came to the hospital for diagnosis other than HSCR. Of the 30 non-HSCR controls, 20 were Malays, 8 were Chinese and 2 were Indians. There were fifteen male and 15 female controls. The mean age of the normal controls was 7.83 years (3 – 11 years). Written informed consent was obtained from the parents of all patients and the study was approved by the UKM Medical Centre (UKMMC) Ethics Committee. About 3ml of peripheral blood was drawn from the subjects into the EDTA blood container for DNA isolation using the conventional ‘salting out’ method with slight modification [23]. The clinical details of the 32 HSCR patients are given in Table 1. Mean age for HSCR group are younger than the control group, due to the early presentation of the disease in a patient’s life. Majority of the patients (81.25%) were diagnosed as with aganglionosis to the recto-sigmoid HSCR.

Table 1. Clinical data of 32 patients with Hirschsprung’s disease.

Description	HSCR (n=32)	Controls (n=30)
<i>Demographic data</i>		
a) Mean age \pm SD (years)	2.98 \pm 3.2	7.83 \pm 2.520 : 8 : 2
b) Ethnic ratio (Malay:Chinese:Indian)	26 : 5 : 1	15 : 15
c) Gender (Male, female)	26 : 6	
<i>Clinical Data</i>		
a) Total colonic aganglionosis (TCA)	4/32 = 12.5%	no related
b) Recto-sigmoid	26/32 = 81.25%	not related
c) Up to proximal end of sigmoid	2/32 = 6.25%	not related

Polymerase chain reaction (PCR)

PCR amplification of 20 exons of the *RET* gene was performed as previously described with slight modification [24-26]. HotStar Taq polymerase was used for PCR amplification. The PCR reactions and conditions differed depending on the size of the PCR products. The PCR reactions consisted of 0.4 - 25 M of forward and reverse primers, 2.0 - 2.5U of HotStar

Taq DNA polymerase, 50 - 250 ng of template DNA, 1.5 - 3.0 mM of MgCl₂, 0.16 - 0.4 mM of dNTP’s and 1x of PCR buffer. The PCR conditions were as follows: initial denaturation at 95°C for 4 min, 35 cycles of denaturation step at 95°C for 4 min, annealing temperature from 50 - 60°C, extension at 72°C for 45 seconds and followed by final extension at 72°C for 1 minute.

Denaturing high performance liquid chromatography (dHPLC)

Analysis by dHPLC was carried out using the HelixTM Varian System (Varian Analytical Instrument, Palo Alto, CA, USA). Approximately 10 to 20 µl of the PCR products (50 - 100 ng of DNA) were denatured for 3 minutes at 95°C, and gradually reannealed at decreasing temperature from 95°C to 65°C for 30 minutes to form the heteroduplexes molecules. The PCR products were separated at 0.9 ml/min linear acetonitrile gradient. The column mobile phase consisted of 0.1 M triethylamine acetate (pH 7.0) with Buffer B.

DNA sequence analysis

DNA sequencing was performed using the ABI 3100 Genetic Analyzer (Applied Biosystem, New York, USA). The PCR products were purified using a commercially available kit (Qiagen, Germany). The sequencing reactions were performed using the BigDye Terminator Kit V3.1 (Applied Biosystem, USA). The cycle sequencing reactions were as follows: 50 - 100 nmol of purified PCR products, 2 µl of BigDye Terminator Kit V3.1, 1 µl of Sequencing Buffer, 3.2 pmoles of primer in a total volume of 20 µl. Cycle sequencing was performed using 55 cycles and consisted of 96°C at 5 minutes, 60°C at 5 minutes and 96°C at 5 minutes. The cycle sequencing products were then purified using ethanol/sodium acetate precipitation. Finally, the products were sequenced using the standard method from Applied Biosystem,

USA. DNA sequencing was performed only in exon 2, 4, 7, 11 and 13 for control subjects as only these exons showed SNPs. DNA sequencing results were analyzed using Basic Local Alignment System Tools (BLAST). Reference sequences and SNP information of the RET gene were obtained from the Ensemble Human Genome Browser (http://www.ensembl.org/Homo_sapiens) and the National Center for Biotechnology Information, NCBI (<http://www.ncbi.nih.gov>).

Prediction of mutation pathogenicity

The Piezoelectric Micro Machine Ultrasonic Transducers (PMut) software which uses the neural network (NN) algorithm was applied to predict the pathogenicity of each variant (<http://mmb2.pcb.ub.es:8080/PMut>) [17]. The neural network (NN) predicts the outcome of a particular variant using different types of sequence information to label the variants and subsequently process the information. A variant is predicted to be pathogenic when the NN output is >0.5 [17].

Statistical analysis

Fisher's Exact and χ^2 tests were used to determine the significance of the association of the allele frequencies between patients and normal control groups. Our calculation was based on the R programming language. A p value of < 0.05 was considered as statistically significant.

Table 2. Allelic distribution frequency of the RET gene variants.

Exon	Nucleotide change	HSCR (%)	# of individuals	Allele frequencies	Control (%)	# of individuals	Allele frequencies	95% CI	OR	P-value
2	c135	A:96%	31	0.86	A:77%	23	0.52	1.0 - 437.5	9.14	0.02411
	G>A: A45A	G:4%	1	0.14	G:23%	7	0.48			
4	c807	C:3%	1	0.02	NA	NA	NA	NA	NA	NA
	G>C: A268A	G:97%	31	0.98						
7	c1296	G:96%	31	0.88	G:100%	30	0.15	0 - 41.6	0	1
	A>G: A432A	A:4%	1	0.13	A:0%	0	0.85			
11	c2073	A:3%	1	0.02	A:20%	6	0.12	0 - 1.2	0.13	0.04962
	G>A: G691S (rs1799939)	G:97%	31	0.98	G:80%	24	0.88			
13	c2307	G:96%	31	0.84	G:77%	23	0.52	1.05 - 437.5	9.14	0.02411
	T>G: L769L	T:4%	1	0.16	T:23%	7	0.48			
Total			32			30				

Table 3. Comparison of allelic distribution or frequency of the *RET* gene variants with other populations.

Exon	Nucleotide change	Spain (9)		Germany (27)		China (28)		Hong Kong (19)		Malaysia	
		HSCR (%) (n=282)	Control (%) (n=178)	HSCR (%) (n=61)	Control (%) (n=25)	HSCR (%) (n=123)	Control (%) (n=168)	HSCR (%) (n=430)	Control (%) (n=632)	HSCR (%) (n=32)	Control (%) (n=30)
2	c135 G>A: A45A (rs1800858)			73.4	23	26	50.6			96.9	76.7
4	c807 G>C: A268A									3.13	
7	c1296 A>G: A432A (rs1800860)	21.8	30	25.8	27.6	32.1	18.7	74.2	72.4	96.9	100
11	c2071 G>A: G691S (rs1799939)	12.5	23.5	10.5	20.2			89.5	79.8	3.1	20
13	c2307 T>G: L769L (rs1800861)	30.5	14	42.7	23.7	56.6	21.1	57.3	76.3	96.9	76.7

Results

We analyzed the whole exonic region of the *RET* gene in this cohort of patients. We identified 4 common variants and 1 novel variant (Table II). The common variants include A45A, A432A and L769L in exons 2, 7, and 13 respectively. Another variant involved the substitution of nucleotide G>A in exon 11, resulting in the G691S variant. The G691S variant has been predicted to be benign with a score of 0.103 (sensitivity: 0.93; specificity: 0.86) using PolyPhen 2 web-based tool [9]. We also identified a novel variant in one patient. This is the A268A (c807G>C) variant in exon 4. Table III shows the frequency of the variants in HSCR patients from different populations.

Discussion

In total, we identified 4 common variants and 1 novel variant in the *RET* gene from our HSCR patients. The A45A (G>A) variant in exon 2 was identified in 97% of our patients and in 77% of the normal controls. Almost all patients with A45A are those with short segment aganglionsis (SSA). This silent A45A variant has been reported in HSCR patients of different ethnic background including German and Chinese [11, 30]. Wu and colleagues suggested that the presence of A45A and L769L polymorphisms were significantly higher in patients with LSA compared to SSA [8]. Fitze and colleagues has reported the association between the c135G/A genotype and *RET* germline mutations with the phenotype of HSCR. [31].

Our results are generally in agreement with other studies. In Taiwan, the A45A, A432A, G691S and L769L variants were identified in both patients and controls [8, 18]. The A45A was identified in 68% of HSCR and 54% of controls while the L769L variant

was found in 72% of HSCR and 53% of controls [18]. Our study showed that the A45A and L769L variants were present at a higher frequency in Malaysian HSCR patients compared to patients from Germany and China although our sample size is smaller (Table 3).

Only 1 (3%) of our HSCR patients showed the presence of the G691S variant. This frequency is much lower than the other populations and possibly due to the fact that our patients were mostly sporadic cases. Fitze and colleagues suggested that the mutation in exon 11, may possibly result in complete or partial loss of *RET* kinase activity [31]. Borrello and colleagues proposed a modifier role for the *RET* G691S where together with K666E, it enhances the transforming activity which results in increased downstream signaling of the *RET* gene [30]. The c807G>C (A268A) is a novel variant which was detected in a Malay patient. Given that this variant was not detected in the control group, the association of this variant with HSCR requires further evaluation in a bigger set of patients.

Advances in sequencing technologies have made mutational screening more robust where exome or targeted sequencing may be used to characterize mutations in several genes simultaneously. Current higher throughput sequencing platforms would potentially yield more information compared to the dHPLC and DNA sequencing techniques we used for this study.

In conclusion, we have identified 5 variants, including the novel A268A variant. The DHPLC-DNA sequencing proves to be a feasible approach to detect mutations and polymorphisms in patients with HSCR.

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References

- Whitehouse F and Kernohan J. Myenteric plexuses in congenial megacolon; study of 11 cases. *Arch Int Med* 1948; 82:75-111.
- Kenny SE, Tam PK, Garcia-Barcelo M. Hirschsprung's disease. *Semin Pediatr Surg* 2010; 19(3):194-200.
- Badner JA, Sieber WK, Garver KL, Chakravarti A. A genetic study of Hirschsprung disease. *Am J Hum Genet* 1990; 46(3):568-580.
- Emison ES, Garcia-Barcelo M, Grice EA, Lantieri F, Amiel J, Burzynski G, Fernandez RM, Hao L, Kashuk C, West K, Miao X, Tam PK, Griseri P, Ceccherini I, Pelet A, Jannot AS, de Pontual L, Henrion-Caude A, Lyonnet S, Verheij JB, Hofstra RM, Antiñolo G, Borrego S, McCallion AS, Chakravarti A. Differential contributions of rare and common, coding and noncoding Ret mutations to multifactorial Hirschsprung disease liability. *Am J Hum Genet* 2010; 9; 87(1): 60-74.
- Amiel J, Sproat-Emison E, Garcia-Barcelo M, Lantieri F, Burzynski G, Borrego S, Pelet A, Arnold S, Miao X, Griseri P, Brooks AS, Antinolo G, de Pontual L, Clement-Ziza M, Munnich A, Kashuk C, West K, Wong KK, Lyonnet S, Chakravarti A, Tam PK, Ceccherini I, Hofstra RM, Fernandez R. Hirschsprung disease, associated syndromes and genetics: a review. *J Med Genet* 2008; 45:1-14.
- Angrist M, Kauffman E, Susan AS, Matise TC, Erik GP, Washington SS, Lipson A, Daniel TC, Reyna T, Daniel EW, Sieber W, Chakravarti A. A gene for Hirschsprung disease (megacolon) in the pericentromeric region of human chromosome 10. *Nat Genet* 1993; 4(4):351-356.
- Lyonnet S, Bolino A, Pelet A, Abel L, Nihoul-Fékété C, Briard ML, Mok-Siu V, Kaariainen H, Martucciello G, Lerone M, Puliti A, Luo Y, Weissenbach J, Devoto M, Munnich A, Romeo G. A gene for Hirschsprung disease maps to the proximal long arm of chromosome 10. *Nat Genet* 1993 4(4):346-350.
- Wu TT, Tsai TW, Chang H, Su CC, Li SY, Lai HS, Li C. Polymorphisms of the RET gene in Hirschsprung disease, anorectal malformation and intestinal pseudo-obstruction in Taiwan. *J Formos Med Assoc* 2010; 109(1):32-38.
- Núñez-Torres R, Fernández RM, Acosta MJ, Enguix-Riego Mdel V, Marbá M, Carlos de Agustín J, Castaño L, Antiñolo G, Borrego S. Comprehensive analysis of RET common and rare variants in a series of Spanish Hirschsprung patients confirms a synergistic effect of both kinds of events. *BMC Med Genet* 2011; 12:138-144.
- Ruiz-Ferrer M, Torroglosa A, Luzón-Toro B, Fernández RM, Antiñolo G, Mulligan LM, Borrego S. Novel mutations at RET ligand genes preventing receptor activation are associated to Hirschsprung's disease. *J Mol Med (Berl)* 2011; 89(5):471-480.
- So MT, Leon TY, Cheng G, Tang CS, Miao XP, Cornes BK, Diem NN, Cui L, Ngan ES, Lui VC, Wu XZ, Wang B, Wang H, Yuan ZW, Huang LM, Li L, Xia H, Zhu D, Liu J, Nguyen TL, Chan IH, Chung PH, Liu XL, Zhang R, Wong KK, Sham PC, Cherny SS, Tam PK, Garcia-Barcelo MM. RET mutational spectrum in Hirschsprung disease: evaluation of 601 Chinese patients. *PLoS One* 2011; 6(12):e28986.
- Ceccherini I, Boccardi R, Luo Y, Pasini B, Hofstra R, Takahashi M, Romeo G. Exon structure and flanking intronic sequences of the human RET proto-oncogene. *Biochem Biophys Res Commun* 1993; 196:1288-1295.
- Burzynski GM, Nolte IM, Bronda A, Bos KK. Identifying candidate Hirschsprung disease-associated RET variants. *Am J Hum Genet* 2005; 76(5):850-858.
- Chin TW, Chiu CY, Tsai HL, Liu CS, Wei CF, Jap TS. Analysis of the RET gene in subjects with sporadic Hirschsprung's disease. *J Chin Med Assoc* 2008; 71(8):406-410.
- Kim JH, Yoon KO, Kim JK, Kim JW, Lee SK, Kong SY, Seo JM. Novel mutations of RET gene in Korean patients with sporadic Hirschsprung's disease. *J Pediatr Surg* 2006; 41(7):1250-154.
- Leon TY, So MT, Lui VC, Hofstra RM, Tam PK, Ngan ES, Garcia-Barcelo MM. Functional analyses of RET mutations in Chinese Hirschsprung disease patients. *Birth Defects Res A Clin Mol Teratol* 2012; 94(1):47-51.
- Ngo DN, So MT, Gui H, Tran AQ, Bui DH, Cherny S, Tam PK, Nguyen TL, Garcia-Barcelo MM. Screening of the RET gene of Vietnamese Hirschsprung patients identifies 2 novel missense mutations. *J Pediatr Surg* 2012; 47(10):1859-1864.
- Wu TT, Tsai TW, Chu CT, Lee ZF, Hung CM, Su CC, Li SY, Hsieh M, Li C. Low RET mutation frequency and polymorphism analysis of the RET and EDNRB genes in patients with Hirschsprung disease in Taiwan. *J Hum Genet* 2005; 50:168-174.
- Cornes BK, Tang CS, Leon TY, Hui KJ, So MT, Miao X, Cherny SS, Sham PC, Tam PK, Garcia-Barcelo MM. Haplotype analysis reveals a possible founder effect of RET mutation R114H for Hirschsprung's disease in the Chinese population. *PLoS One* 2010; 5(6):e10918.
- Liu CP, Tang QQ, Lou JT, Luo CF, Zhou XW, Li DM, Chen F, Li X, Li JC. Association analysis of the RET proto-oncogene with Hirschsprung disease

in the Han Chinese population of south eastern China. *Biochem Genet* 2010; 48(5-6):496-503.

21. Moore SW and Zaahl MG. A review of genetic mutation in familial Hirschsprung's disease in South Africa: towards genetic counseling. *J Pediatr Surg* 2008; 43(2):325-329.

22. Phusantisampan T, Sangkhathat S, Phongdara A, Chiengkriwate P, Patrapinyokul S, Mahasirimongkol S. Association of genetic polymorphisms in the RET-*proto* oncogene and NRG1 with Hirschsprung disease in Thai patients. *J Hum Genet* 2012; 57(5):286-293.

23. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Res* 1998; 16:1251.

24. Ceccherini I, Bocciardi R, Luo Y, Pasini B, Hofstra R, Takahashi M, Romeo G. Exon structure and flanking intronic sequences of the human RET *proto*-oncogene. *Biochem Biophys Res Commun* 1993; 196:1288-95.

25. Ceccherini I, Hofstra RM, Luo Y, Stulp RP, Barone V, Stelwagen T, Bocciardi R, Nijveen H, Bolino A, Seri M, et al. DNA polymorphism and conditions for SSCP analysis of the 20 exons of the *ret proto*-oncogene. *Oncogene* 1994; 9: 3025-3029.

26. Sakai T, Nirasawa Y, Itoh Y, Wakizaka A. Japanese patients with sporadic Hirschsprung: mutation analysis of the receptor tyrosine kinase *proto*-oncogene, endothelin-B receptor, endothelin-3, glial cell line-derived neurotrophic factor and neurturin genes: a comparison with similar studies. *Eur J Pediatr* 2000; 159:160-167.

27. Romero P, Niesler B, Schmitz-Winnenthal H, Fitze G, Holland-Cunz S. Is there a link between the calcium sensing receptor and Hirschsprung's disease? A mutational analysis. *J Pediatr Surg* 2012; 47(3):551-555.

28. Tou J, Wang L, Liu L, Wang Y, Zhong R, Duan S, Liu W, Xiong Q, Gu Q, Yang H, Li H. Genetic variants in RET and risk of Hirschsprung's disease in Southeastern Chinese: a haplotype-based analysis. *BMC Med Genet* 2011; 12:32-37.

29. Gray VE, Kukurba KR, Kumar S. Performance of computational tools in evaluating the functional impact of laboratory-induced amino acid mutations. *Bioinformatics* 2012; 28(16):2093-2096.

30. Borrello MG, Aiello A, Peissel B, Rizzetti MG, Mondellini P, Degl'Innocenti D, Catalano V, Gobbo M, Collini P, Bongarzone I, Pierotti MA, Greco A, Seregni E. Functional characterization of the MTC-associated germline RET-K666E mutation: evidence of oncogenic potential enhanced by the G691S polymorphism. *Endocr Relat Cancer* 2011; 18(4):519-527.

31. Fitze G, Cramer J, Serra A, Schreiber M, Roesner D, Schackert HK. Within-gene interaction between c.135 G/A genotypes and RET *proto*-

oncogene germline mutations in HSCR families. *Eur J Pediatr Surg* 2003; 13(3):152-157.

32. Gath R, Goessling A, Keller KM, Koletzko S, Coerdts 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.

33. Luzón-Toro B, Torroglosa A, Núñez-Torres R, Enguix-Riego MV, Fernández RM, de Agustín JC, Antiñolo G, Borrego S. Comprehensive analysis of NRG1 common and rare variants in Hirschsprung patients. *PLoS One* 2012; 7(5):e36524.

34. Wallace AS and Anderson RB. Genetic interactions and modifier genes in Hirschsprung's disease. *World J Gastroenterol* 2011; 17(45):4937-44.