


ORIGINAL ARTICLE

Distribution of *RET* proto-oncogene variants in children with appendicitis

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Abstract

Background: In addition to patient-related systemic factors directing the immune response, the pathomechanisms of appendicitis (AP) might also include insufficient drainage leading to inflammation caused by decreased peristalsis. Genetic predisposition accounts for 30%–50% of AP. M. Hirschsprung (HSCR), also characterized by disturbed peristalsis, is associated with variants in the *RET* proto-oncogene. We thus hypothesized that *RET* variants contribute to the etiology of AP.

Methods: DNA from paraffin-embedded appendices and clinical data of 264 children were analyzed for the *RET* c.135A>G variant (rs1800858, NC_000010.11:g.43100520A>G). In 46 patients with gangrenous or perforated AP (GAP), peripheral blood DNA was used for *RET* sequencing.

Results: Germline mutations were found in 13% of GAP, whereas no *RET* mutations were found in controls besides the benign variant p.Tyr791Phe (NC_000010.11:g.43118460A>T). In GAP, the polymorphic G-allele in rs2435352 (NC_000010.11:g.43105241A>G) in intron 4 was underrepresented ($p = 0.0317$).

Conclusion: Our results suggest an impact of the *RET* proto-oncogene in the etiology of AP. Mutations were similar to patients with HSCR but no clinical features of HSCR were observed. The pathological phenotypes in both populations might thus represent a multigenic etiology including *RET* germline mutations with phenotypic heterogeneity and incomplete penetrance.

KEYWORDS

appendicitis, general surgery, genetic association studies, pediatrics, proto-oncogene protein ret

1 | INTRODUCTION

Appendectomy is among the most frequently performed operations, with incidences peaking around the 10th year of life (Addiss et al., 1990; Anderson et al., 2012). The etiology of appendicitis (AP) is multifactorial. Cumulative

evidence from epidemiological studies (Andersson, 2007), cytokine profiles (Rubér et al., 2010), and gene expression studies (Kiss et al., 2021) suggest systemic factors determining the course of AP. However, long-standing good evidence also points to insufficient drainage leading to increased intraluminal pressure and invasive infection as

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a pathogenic sequence (Bhangu et al., 2015; Khan et al., 2019; Wangenstein & Dennis, 1939). This insufficient drainage might be caused by decreased peristalsis of the appendix that otherwise freely evacuates into the cecal lumen, as it can be observed on oral contrast studies or barium enemas (Maglinte et al., 1981; Tang et al., 2019).

A rare childhood disorder characterized by absent peristalsis due to altered enteric innervation is Hirschsprung disease (HSCR [MIM: 142623]). HSCR is a dysgenetic neurocristopathy characterized by a congenital absence of the intramural ganglia in the hindgut causing intestinal obstruction in newborns. Besides germline mutations in other genes (*EDNRB*, *EDN3*, *GDNF*, *NTN*, *SOX10*, *ECE1*, *SIP1*, and *NRG1*) and other linked genetic loci (3p12, 19q, and 9q31), the *RET* (OMIM 164761) gene locus at 10q11.2 is the major gene locus for HSCR. *RET* germline mutations, which are primarily point mutations scattered throughout the extracellular domain and within the intracellular tyrosine kinase domain, have been detected in up to 20% of sporadic and in 50% of familial cases (Amiel et al., 2008; Bolk et al., 2000). Furthermore, *RET* variants that are acting by modulation of the gene expression are strongly associated with the HSCR phenotype. These observations support the concept of synergistic heterozygosity for HSCR, which considers the disease phenotype as a result of cumulative effects of at least two mutations or variants in different genes (Amiel et al., 2008; Borrego et al., 1999; Emison et al., 2005; Fitze, Cramer, et al., 2003; Fitze et al., 1999).

Similar to the HSCR, a familial occurrence of AP has been observed (Baker, 1937; Li et al., 2018). Today, around 20%–50% of AP are considered to have an underlying genetic cause (Basta et al., 1990; Duffy et al., 1990; Ergul, 2007; Oldmeadow et al., 2009; Sadr Azodi et al., 2009; Simó Alari et al., 2017). Recently, genome-wide association studies (GWAS) found strong association of appendectomies with a SNP near *PITX2* (Kristjansson et al., 2017), a gene also involved in intestinal development that has been shown to be significantly less expressed in appendectomy specimen (Orlova et al., 2019). Recent studies also showed an irregular distribution of ganglia in the wall of inflamed appendices, suggesting a possible role of ganglia cell distribution and functioning in the pathogenesis of AP similar to HSCR (Kubikova et al., 2015).

According to this notion, we hypothesized that mutations of the *RET* proto-oncogene may contribute to the etiology of AP in children.

2 | PATIENTS, MATERIALS, AND METHODS

Within three consecutive years, 264 children (133 boys and 131 girls) underwent an appendectomy in our

department. Together with our pathologists, we classified appendix specimen in gangrenous and perforated appendicitis (GAP) or non-GAP, which includes all less inflamed stages. We collected their clinical data and isolated DNA from the paraffin-embedded appendices. Family history was available in 185 patients. The *c.135A>G* variant that is in linkage disequilibrium with a functional variant in the *RET* promoter (NC_000010.11:g.42787315G>A, rs10900269) as well as with a variant in the *RET* intron 1 (NC_000010.11:g.43086608T>C, rs2435357) (Emison et al., 2005; Fitze, Appelt, et al., 2003) was analyzed. For these analyses, 131 anonymous healthy blood donors (HBD control) from Germany served as controls.

In addition, we collected DNA samples from peripheral blood leucocytes within a subpopulation (46 out of the 264 patients; 25 boys and 21 girls) with a histologically confirmed gangrenous or perforated appendicitis (GAP). In this population, the complete coding region of the *RET* proto-oncogene was sequenced. Forty-seven individuals >20 years of age which had not undergone appendectomy (NA controls) served as second control. Both control populations were matched for sex and Caucasian ethnicity. In six of seven patients with a detected *RET* germline mutation, we collected clinical data of their families and sequenced the *RET* proto-oncogene in their relatives.

We isolated DNA from leukocytes of peripheral blood and from paraffin-embedded tissues according to standard protocols. The coding regions of all 21 exons including the adjacent intronic regions of the *RET* proto-oncogene were amplified from genomic DNA with subsequent sequence analysis by a direct DNA sequencing approach using the primers and methodology described previously (Fitze et al., 2002). MLPA analysis was performed on genomic DNA isolated from peripheral blood leukocytes to assess genomic rearrangements in the *RET* proto-oncogene using the MLPA test kit P169 lot 0106 (MRC-Holland, Amsterdam, The Netherlands) according to the supplied protocol and as described previously (Serra et al., 2010). The association between the genotypes of the *c.135A>G* variant and the histological evaluation of the appendix was analyzed using the Jonckheere-Terpstra-Test.

To evaluate deviation from the Hardy-Weinberg equilibrium, observed and expected genotype frequencies were compared by an exact goodness of fit test separately in cases and controls. Odds ratios and exact 95% confidence intervals from logistic regression for comparing genotype frequencies with additive genotype coding are presented together with exact *p*-values. The global significance level was set to 0.05, and no adjustments were made for multiple testing.

NC_000010.11 and NM_020975.6 were used as reference genomes.

3 | RESULTS

3.1 | Genotype analysis of the c.135A>G variant in exon 2

The analysis of the c.135A>G variant (rs1800858) as a marker for the HSCR-associated haplotype revealed no deviation of the genotype frequencies within the population of 122 patients with a non-GAP out of 264 appendectomized patients compared with those of the control population (Table 1). In contrast, the homozygous c.135AA genotype is slightly overrepresented in patients with GAP and perforated GAP in comparison to the control population, as well as compared to the non-GAP population, without reaching statistical significance ($p = 0.3246$).

Furthermore, homozygous c.135AA genotype is overrepresented in patients with a family history of the disease (Table 2). However, these data do not show statistical significance ($p = 0.1291$).

3.2 | Sequence analyses of *RET* proto-oncogene

In 46 of the 264 appendectomy samples with GAP or perforated GAP, we analyzed the complete coding region of the *RET* proto-oncogene from peripheral leukocyte samples. Of these 46 patients, 6 (13%) carried *RET* germline mutations (Table 3). Because of this unexpected finding, we decided to analyze the complete coding region of the *RET* proto-oncogene in 47 healthy individuals older than 20 years who have not had an appendectomy (NA) as control. In contrast to the GAP population, no *RET* germline mutations were found in NA controls besides two NA controls who carried the p.Tyr791Phe variant. We detected this NC_000010.11:g.43118460A>T variant in exon 13 not only in two NA controls but also in one GAP patient. In the past, this mutation has been considered a disease causing mutation in MEN2 (Berndt et al., 1998b), but none of these three individuals showed any MEN2 symptoms.

In five of six patients who carried *RET* germline mutations, we could perform DNA sequencing in first-degree

relatives. In four of these families, the mutation was inherited from the parents (two paternal and two maternal) but only in family 1 the mutation co-segregated with appendectomy (Table 3). We identified a de novo mutation in the patient of family 2. In family 3, the analysis was incomplete due to an unknown father. In family 2 with the de novo mutation in the patient, three family members had an appendicitis, in family 5 there was no appendicitis in family members, and in family 6 the mutation was inherited maternally and the father has had an appendicitis (Table 3). Furthermore, none of the investigated mutation carriers showed any clinical symptoms of HSCR, particularly no chronic constipation. Finally, haplotype reconstruction was possible in five of the six families in which a *RET* germline mutation was detected. In four families the c.135A allele carried the *RET* mutation and only in one family the mutation was located on the c.135G allele (Table 3).

Similar to the variants found in the 264 appendectomized patients, in 46 patients who had undergone sequence analysis of *RET*, we found an overrepresentation of the homozygous c.135AA genotype of the exon 2 variant compared with the NA controls (Table 4). Except for the intron 4 variant NC_000010.11:g.43105241A>G (rs2435352) that shows a significant overrepresentation of the wild-type allele in the patient population compared with the NA controls, the genotype distribution of none of the analyzed variants deviated significantly from the patient population (Tables 4 and 5).

4 | DISCUSSION

Although the etiology of AP is multifactorial, heredity is a significant factor in pediatric patients. Already in 1937, Baker (1937) described a family in which 50% of members had undergone appendectomy. Gauderer et al. (2001) found that children with AP are twice as likely to have a positive family history compared to patients with right lower quadrant pain without AP. Ergul (2007) revealed in a representative retrospective study on 2670 patients that a positive family history increases the relative risk of

TABLE 1 Distribution of c.135A>G genotypes among the different histopathological types of appendicitis in comparison with normal controls

Exon 2 c.135	Histopathological evaluation				Normal controls (n = 131)
	Non-gangrenous (n = 122)	Gangrenous (n = 115)	Perforated (n = 27)	Total (n = 264)	
GG	67 (54.9%)	58 (50.4%)	15 (55.6%)	140 (53.0%)	69 (52.7%)
GA	50 (41.0%)	46 (40.0%)	7 (25.9%)	103 (39.0%)	55 (42.0%)
AA	5 (4.1%)	11 (9.6%)	5 (18.5%)	21 (8.0%)	7 (5.3%)

TABLE 2 Distribution of c.135A>G genotypes among the different histopathological types of appendicitis in relation to the family history for appendicitis

Histopathology	Familial history for appendectomy and c.135G>A genotypes							
	Positive (n = 110)				Negative (n = 75)			
	GG	GA	AA	Σ	GG	GA	AA	Σ
Non-GAP	22	17	2	37.3%	20	16	0	49.3%
GAP	23	28	6	51.8%	20	8	3	40.0%
Perforated	7	3	2	10.9%	5	1	2	10.7%
	47.3%	43.6%	9.1%		60.0%	33.3%	6.7%	

an AP 3.15 times. In our own population, 110 (60%) out of 185 patients with available family history had a family history for AP supporting the etiological importance of heritability. Analyzing the Swedish twin register, Sadr Azodi et al. (2009) described larger genetic and non-shared environmental factors in females, and stronger shared environmental effects in males in the underlying etiology of AP. For males and females combined, genetic effects explained 30% of the variation in AP risk. Complex segregation analysis supported the genetic component of the disease (Basta et al., 1990). A genome-wide linkage analysis of a large Australian twin dataset identified linkage of AP to chromosome 1p34.3. Other supportive regions of interest were located on chromosomes 11 and 14 (Oldmeadow et al., 2009). Investigations into the nature of AP showed that it may be a result of an inappropriate immune response which may have a genetic basis (Rivera-Chavez et al., 2004). This corresponds with an association of *NOD2/CARD15* variants with AP in a large Danish study with 43,600 individuals (Yazdanyar & Nordestgaard, 2010) and with recent data on the differential expression of immune factors long after progressive GAP or often self-limiting non-GAP had been diagnosed (Kiss et al., 2021; Rubér et al., 2010).

In parallel to HSCR, an altered distribution of intramural ganglia has been found in the appendices of AA patients (Kubikova et al., 2015). Furthermore, up to 50% of HSCR patients are suffering from a recurrent colitis that also persists after resection of the aganglionic colon segment in about half of these patients. Interestingly, an association of this HSCR related colitis with the mentioned *NOD2/CARD15* variants was excluded recently (Lacher et al., 2010). Because the *RET* proto-oncogene is the major susceptibility gene for HSCR we have hypothesized that this gene could have an etiological effect in AP, as well.

In line with previous data that have shown the wild-type allele in NC_000010.11:g.43105241A>G (rs2435352) to be strongly associated with HSCR (Fitze, Cramer, et al., 2003), the wild-type A-allele was significantly over-represented in our GAP-populations, as well. Similarly, the G-allele in NM_020975.6:c.135A>G (rs1800585) in

RET exon 2 and the C-allele in c.2944C>T (rs17158558) in *RET* exon 18 which are associated with HSCR (Fitze, Appelt, et al., 2003; Luzón-Toro et al., 2012; Zhang et al., 2017) were overrepresented in our GAP populations, however, without reaching statistical significance. Although not statistically significant either, the distribution of NC_000010.11:g.43100731 G>A (rs2345351) was also similar to frequencies that were previously found to be strongly associated with HSCR (Fitze, Schierz, et al., 2003).

Interestingly, germline mutations of the *RET* proto-oncogene were found in 13% (6 of 46) of the patients with GAP, which further emphasizes a possible impact of the *RET* proto-oncogene in the etiology of the disease. Besides the frequency of *RET* mutations, the distribution of mutations scattered throughout the whole gene and the types of mutations comprising missense, nonsense and silent mutations are similar to that found in patients with HSCR (Amiel et al., 2008; Auricchio et al., 1999; Fitze, Appelt, et al., 2003). We excluded the occurrence of these mutations in a control population of healthy controls (NA controls) comprising 47 patients >20 years of age without appendectomy and without a history for HSCR or MTC. The p.Arg163Trp-variant (rs371153966) inherited in family 1 from the father, who had been appendectomized at age 16 for AP to the affected daughter who was appendectomized at age 13 for GAP, lies in exon 3 of the extracellular *RET*-domain. This exchange of a basic and hydrophilic amino acid with the neutral and hydrophobic tryptophan has so far only been reported as a variant in clinical testing. The p.Pro476Thr-de novo mutation in family 2 has not been reported before. However, the p.Ser649Leu-mutation (rs148935214) in the trans-membrane domain in exon 11 did not result in MTC, even though it has been described in patients with elevated serum calcitonin and hereditary MTC (Colombo-Benkmann et al., 2008; Vierhapper et al., 2004; Wei et al., 2016). European guidelines even recommend total thyroidectomy before age of 10 depending on serum calcitonin levels in patients carrying this mutation (Niederle et al., 2014). However, in view of functional in vitro studies that showed only moderately

TABLE 3 Characterization of six *RET* germline mutations identified in 46 patients with GAP (aminoacid change according to NP_066124.1), transmission of these mutations, the family history for appendicitis, and the analysis which c.135G>A allele harbors the mutation

No.	Exon	Mutation	Nucleotide change (NC_000010.11)	Type of mutation	Histological evaluation of appendix	Family history for appendicitis	Transmission of the mutation	Mutated c.135G>A allele
1	3	Arg 163 Trp	CGG → TGG g.43102491C>T (rs371153966)	Missense	Gangrenous	Positive (father)	Paternal	A
2	7	Pro 476 Thr	CCC → ACC g.43111369C>A	Missense	Gangrenous	Father, mother, brother	De novo	G
3	11	Ser 649 Leu	TCG → TTG g.43114546C>T (rs148935214)	Missense	Gangrenous	n.d.	n.d.	n.d.
4	13	Asn 777 Ser	AAC → AGC g.43118418A>G (rs377767415)	Missense	Gangrenous	2 Sisters	Maternal	A
5	18	Pro 996 =	CCG → CCA g.43124931G>A (rs145798106)	Silent	Gangrenous	Negative	Paternal	A
6	19	Arg 1050 *	CGA → TGA g.43126683C>T (rs767479170 but only C>G recorded so far)	Nonsense (>STOP)	Perforated	Father	Maternal	A

Abbreviation: n.d., not defined.

TABLE 4 Genotype distribution (percentages in parenthesis) of eight exonic *RET* proto-oncogene variants of 46 GAP patients compared to the control population (positions according to reference genome NM_020975.6)

SNP-position	Genotype	Patients (n = 46)	Controls (n = 47)	Cochran–Armitage trend test		
				OR	CI	ex. p-value
c.135A>G	GG	21 (45.70%)	25 (53.20%)			
rs1800858	GA	18 (39.10%)	20 (42.60%)	0.6512	0.325–1.271	
	AA	7 (15.20%)	2 (4.30%)	0.4241	0.106–1.615	0.2104
c.375C>A	CC	43 (93.50%)	46 (97.90%)			
rs1800859	CA	3 (6.50%)	1 (2.10%)	0.3152	0.006–4.097	
	AA	0 (0.00%)	0 (0.00%)			0.3613
c.1296A>G	GG	23 (50.00%)	16 (34.00%)			
rs1800860	GA	17 (37.00%)	26 (55.30%)	1.353	0.704–2.649	
	AA	6 (13.00%)	5 (10.60%)	1.831	0.495–7.018	0.3576
c.2071G>A	GG	27 (58.70%)	28 (59.60%)			
rs1799939	GA	18 (39.10%)	16 (34.00%)	1.104	0.514–2.388	
	AA	1 (2.20%)	3 (6.40%)	1.219	0.265–5.703	0.8591
c.2307G>T	TT)	23 (50.00%)	27 (57.40%)			
rs1800861	TG	21 (45.70%)	16 (34.00%)	0.9177	0.447–1.874	
	GG	2 (4.30%)	4 (8.50%)	0.8421	0.200–3.513	0.8675
c.2508C>T	CC	43 (93.50%)	45 (95.70%)			
rs1800862	CT	3 (6.50%)	2 (4.30%)	0.6401	0.051–5.873	
	TT	0 (0.00%)	0 (0.00%)			0.6771
c.2712C>G	CC	26 (56.50%)	28 (59.60%)			
rs1800863	CG	19 (41.30%)	16 (34.00%)	1.035	0.482–2.228	
	GG	1 (2.20%)	3 (6.40%)	1.071	0.232–4.965	1
c.2944C>T	CC	45 (97.80%)	42 (89.40%)			
rs17158558	CT	1 (2.20%)	5 (10.60%)	5.275	0.557–258.9	
	TT	0 (0.00%)	0 (0.00%)			0.2035

elevated kinase-activity of the p.Ser649Leu-mutant protein (Colombo-Benkmann et al., 2008), these guidelines have to be carefully interpreted. Similarly, the p.Asn777Ser-missense-mutation in exon 13 within the intracellular domain has been associated with low-penetrance non-aggressive familial MTC (D'Aloiso et al., 2006). Recently updated American guidelines recommend prophylactic thyroidectomy or yearly screenings including basal and stimulated serum calcitonin and neck ultrasound in carriers of this level A pathogenic variant p.Asn777Ser (Eng, 1993–2021). Neither our patient nor the mutation-carriers in his family (mother and two sisters) suffered from MTC. The silent p.Pro996 = variant (AAC to AGC) was inherited in family 5 from the unaffected father to the appendectomized son as well as to the healthy daughter. The p.Arg1050* (CGA to CCA) nonsense mutation that inserts a STOP-codon in the intracellular domain found in family 6 was inherited from the healthy mother to her two sons, one of them healthy the other one appendectomized at

age 3 with a perforated GAP. This variant has not been reported before. While having negative histories and negative family histories for HSCR and MTC in all detected mutation carriers, we did not measure neither Calcitonin nor Pentagastrin-provoked Calcitonin levels.

Except for the p.Tyr791Phe variant that we have found twice in the controls and once in the GAP population, this analysis did not identify any *RET* germline mutation in our specific NA control population. The p.Tyr791Phe mutation has been first described in HSCR patients (Fitze et al., 2002; Seri et al., 1997) and later associated with MEN2A syndrome (Berndt et al., 1998a). Since then, this mutation was described in patients with congenital central hypoventilation syndrome (Fitze, Appelt, et al., 2003), gastric and pancreatic cancer as well as glioblastoma, pheochromocytoma, and neuroblastoma (Rückert et al., 2011; Toledo et al., 2010). The RET receptor-tyrosine kinase is activated by dimerization of two receptor protein chains and a possible pathomechanism for RET activation by the p.Tyr791Phe mutation due

TABLE 5 Genotype distribution (percentages in parenthesis) of seven intronic *RET* proto-oncogene variants of 46 GAP patients compared to the control population (nucleotide positions according to NC_000010.11)

SNP position	Genotype	Patients (<i>n</i> = 46)	Controls (<i>n</i> = 47)	Cochran–Armitage trend test		
				OR	CI	ex. <i>p</i> -value
g.43100731	GG	30 (65.20%)	22 (46.80%)			
G>A	GA	15 (32.60%)	22 (46.80%)	1.992	0.913–4.548	
rs2435351	AA	1 (2.20%)	2 (6.40%)	3.969	0.833–20.69	0.0745
g.43105241	AA	24 (52.20%)	13 (27.70%)			
A>G	AG	16 (34.80%)	24 (51.10%)	1.91	1.026–3.689	
rs2435352	GG	6 (13.00%)	10 (21.30%)	3.65	1.052–13.61	0.0317
g.43116778	CC	21 (45.70%)	27 (57.40%)			
C>T	CT	22 (47.80%)	18 (38.30%)	0.6739	0.314–1.412	
rs760466	TT	3 (6.50%)	2 (4.30%)	0.4541	0.098–1.993	0.301
g.43120057	GG	34 (73.90%)	30 (63.80%)			
G>A	GA	11 (23.90%)	15 (31.90%)	1.525	0.666–3.643	
rs2472737	AA	1 (2.20%)	2 (4.30%)	2.325	0.443–13.27	0.34
g.43126769	CC	27 (58.70%)	28 (59.60%)			
C>T	CT	17 (37.00%)	17 (36.20%)	0.9716	0.451–2.090	
rs2075912	TT	2 (4.30%)	2 (4.30%)	0.944	0.204–4.368	1
g.43128364	CC	3411 (73.90%)	29 (61.70%)			
C>T	CT	1 (23.90%)	16 (34.00%)	1.636	0.715–3.921	
rs17028	TT	–2.20%)	2 (4.30%)	2.678	0.512–15.38	0.2542
g.43129775	AA	26 (56.50%)	27 (57.40%)			
A>G	AG	17 (37.00%)	18 (38.30%)	0.9154	0.436–1.912	
rs2742241	GG	3 (6.50%)	2 (4.30%)	0.837	0.190–3.656	0.8639

to a ligand-independent activated monomeric protein was proposed (Menacho et al., 2005).

Even though the p.Tyr791Phe variant was inherited within the families analyzed in this study, the phenotypes associated with this mutation appear as sporadic diseases and show no genotype–phenotype correlation. In view with recent clinical data as well as recent mutational studies in mice (Høxbroe Michaelsen et al., 2019; Nakatani et al., 2020; Toledo et al., 2015), we consider the p.Tyr791Phe variant rather a non-functional *RET* variant than a specific disease associated mutation. Thus, the lack of specific *RET* germline mutations in our specific control population substantiates the possible role of *RET* mutations for the development of AP.

Four of our six *RET* mutation carriers have a positive family history for AP. In four of six families, the mutation was inherited—twice paternally and twice maternally. However, only in one paternally inherited mutation we have observed evidence for a co-segregation of the mutation with AP. This lack of a genotype–phenotype correlation may be caused by an incomplete penetrance of the appendicitis phenotype similar to the situation in HSCR (Amiel et al., 2008).

It could be suspected that these *RET* mutation carriers—particularly the patient with the nonsense mutation—were suffering from Hirschsprung disease. However, in these six families we have neither a history nor any clinical features of HCSR that would justify a rectal biopsy. Therefore, we have no explicit information about the intramural ganglia in our patients, but lack of intramural ganglia without any clinical features is unlikely. This observation could be explained only by a cumulative effect of germline mutations in several genes comprising the identified *RET* mutations as one component of a synergistic heterozygosity (Bolk et al., 2000). Generally, the occurrence of a *RET* germline mutation is significantly associated with a long-segment HSCR. Previously, we have shown that *RET* haplotypes harboring a mutation in cis-position could modify the HSCR phenotype. Whereas the combination of the *RET* c.135G haplotype with a germline mutation on the same *RET* allele is associated with a long-segment HSCR phenotype, the combination of the *RET* c.135A haplotype with a mutation at the same allele shows a short-segment HSCR with a low penetrance in the families (Fitze et al., 2002). In this study, all inherited

RET germline mutations (four of six) in patients with GAP are located on *RET* c.135A alleles which could be one reason for a missing HSCR phenotype in these families.

Here, we identify germline mutations in 13% of patients with GAP as a possible genetic background of AP in childhood. Previously, we demonstrated *RET* germline mutations in 23% of HSCR patients (Fitze et al., 2002). Since no clinical features of HSCR were observed in our GAP patients, *RET* germline mutations should be considered to result in a phenotypical heterogeneity with incomplete penetrance similar to HSCR. Generally, these findings support the idea of a polygenic inheritance for both, HSCR and GAP. In the future, we need more extensive studies that include the histological appearance of ganglia in appendectomy specimens and the sequencing data of AP patients and relatives, as well as functional assessments of identified variants to elicit the genetic background and pathomechanisms of AP. In addition, we should extend sequencing to genes known to modulate the immune response and supplement cytokine profiles to detect genetic synergies in the GAP phenotype.

CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

ETHICAL COMPLIANCE

All patients and parents in case of minors gave written informed consent to participate in the study. The ethics committee of the University of Technology of Dresden approved the study protocol (EK470498).

CONSENT TO PARTICIPATE

All patients and parents in case of minors gave written informed consent to participate in the study.

CONSENT FOR PUBLICATION

All patients and parents in case of minors gave written informed consent to the publication of this study.

DATA AVAILABILITY STATEMENT

All relevant data are included in this text, tables, and figures. Primary sequencing results are available upon request anytime. Newly discovered variants have been reported to ClinVar (accession numbers: SCV001469008, SCV001469009, SCV001469010, SCV001469011, and SCV001469012).

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How to cite this article: Schultz, J., Freibothe, I., Haase, M., Glatte, P., Barreton, G., Ziegler, A., Görgens, H., & Fitze, G. (2022). Distribution of RET proto-oncogene variants in children with appendicitis. *Molecular Genetics & Genomic Medicine*, 10, e1864. <https://doi.org/10.1002/mgg3.1864>