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LETTER

# A novel structural variant of *RET* causes familial Hirschsprung's disease via nonsense-mediated mRNA decay

Hirschsprung's disease (HSCR) is the most common type of developmental gastrointestinal malformation causing intestinal obstruction in children, with an incidence of 1/5000 in live births.[1] It is characterized by the absence of intrinsic ganglion cells in the myenteric and submucosal plexuses of the distal gastrointestinal tract, leading to the dysregulation of smooth muscle contraction/relaxation and resulting in intestinal obstruction.[1] Depending on the length of the aganglionic intestine, HSCR can be categorized into short-segmental HSCR (S-HSCR), which affects up to the sigmoid colon from the anus, and long-segmental HSCR (L-HSCR), which affects beyond the sigmoid colon.[1] In some rare conditions, aganglionic lesions can affect the full length of the colon or even the whole intestine.[1]

HSCR is considered to be an inherited disease with a familial incidence range from 5% to 20%.[1] To date, more than 20 genes have been found to be associated with the onset of HSCR.[1] Among them, ret-proto oncogene (*RET* ) was the first identified and was considered to be the major pathogenic gene. The severity of HSCR was reported to have a direct relationship with the decrease in the expression of *RET* gene.[1] Recently, three functional *RET* enhancer variant sites (*rs2506030*, *rs7069590*, and *rs2435357*) in the regulatory region were also reported to have a significant impact on the risk of HSCR.[2] Additionally, another genetic study, which included over one hundred HSCR cases, indicated that at least 48.4% of European ancestry HSCR patients had a structural or regulatory deficiency in the *RET* gene.[3] All these findings indicate that the onset of HSCR is complex, and apart from the exploration of novel pathogenic genes, research on the classic HSCR risk gene *RET* is still insufficient and necessary.

In this study, we start with a three-generation Chinese HSCR family with two affected and five unaffected members (Fig. 1A). Subject III-1 was the proband with total colonic HSCR (Fig. 1B), and subject II-2 was the proband's father with S-HSCR. The other 5 family members were healthy with no signs of HSCR or constipation.

In the primary exome sequencing (ES) of the proband, no pathogenic variant was found (Fig. S1). In subsequent genome sequencing (GS), the deletion of the (*chr10:43613122-43615021*, *hg19)* fragment in *RET* gene was found among two HSCR patients and subject I-2 (Fig. 1C; Fig. S1). Further studies have been performed to reveal the pathogenic mechanism of the candidate variant and the reasons for the vastly different clinical phenotypes among three variant gene carriers.

To confirm the sequencing result and the exact variant of the *RET* gene, primers *RET-del-F/R* and *RET-F/R* for variant fragments were designed for PCR and Sanger sequencing (Table S1). The genomic DNA PCR test confirmed that three variant *RET* gene carriers had two bands (*2562 bp* and *668 bp*) (*bp*, base pair), while other members were wild type (WT) with only one band (*2562 bp*) in the agarose gel electrophoresis results (Fig. 1D). Sanger sequencing results of the PCR products demonstrated that the variant in *RET* was a *1900 bp* fragment deletion (from the 12th intron to the 14th exon) with a *6 bp* (*TCCTCC* ) insertion, which should be described as (*NC_000010.10:g.43613122_ 43615021delinsTCCTCC* ) (Fig. 1E). Subsequently, we isolated total RNA from the blood sample of subjects I-1, I-2, II-2, and III-1, and subject I-1 was set as the normal control. cDNA was obtained through reverse transcription PCR. The following cDNA PCR test also indicated that, unlike the normal control, three variant *RET* carriers have another band with a smaller size and a much weaker brightness (Fig. 1F). Then, Sanger sequencing indicated that the larger and brighter band represented the WT *RET* mRNA fragment with 419 nts (nucleotides), while the weaker and smaller band represented the variant *RET* mRNA fragment with 333 nts. Consequently, the exact variant in *RET* mRNA was the deletion of the whole

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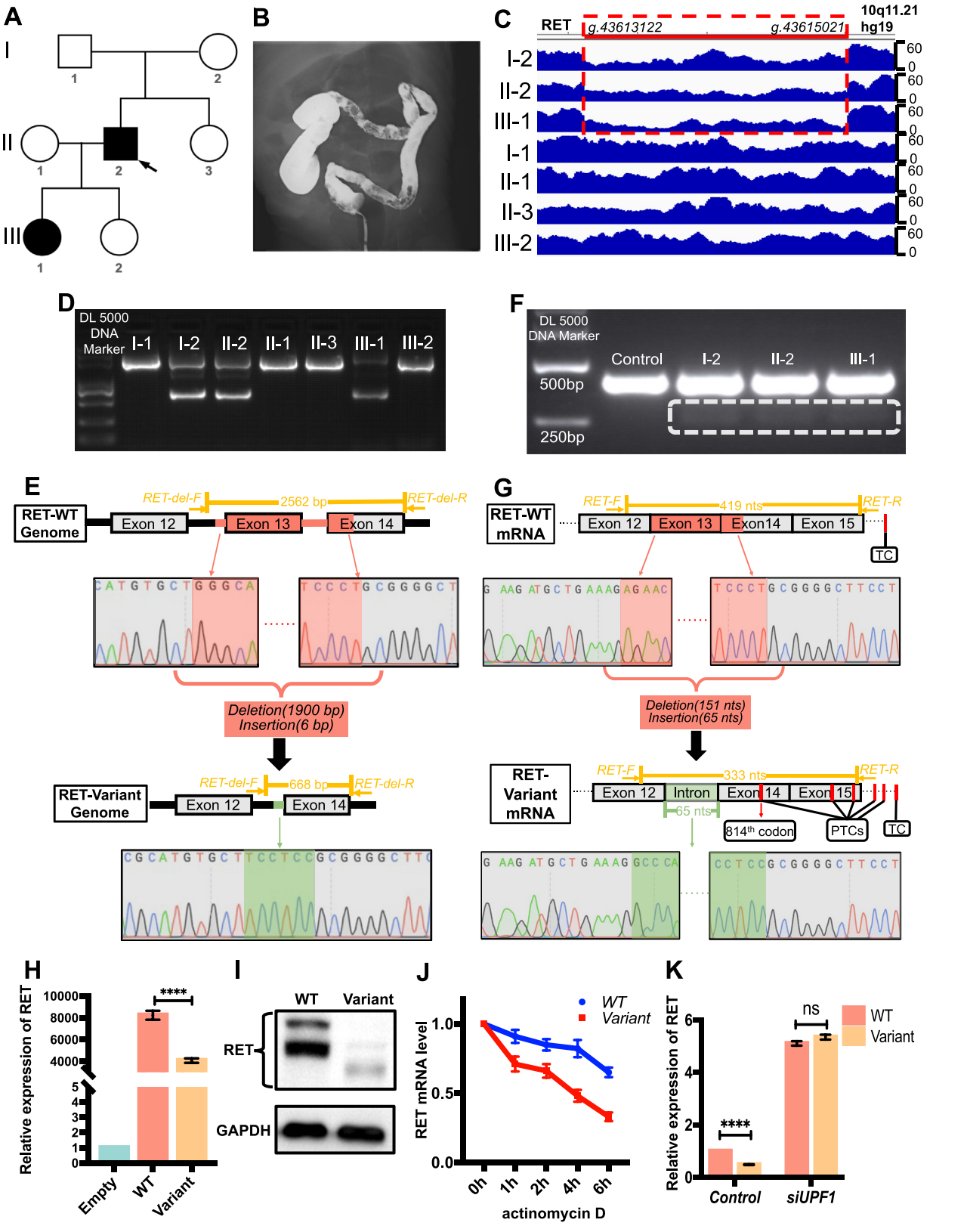


**Figure 1** Clinical data, genetic analysis, and functional research of this study. **(A)** Pedigree of this HSCR family. Subjects diagnosed with HSCR were represented as black symbols (II-2, III-1), unaffected subjects were represented as white symbols, and the proband was indicated by a black arrow. **(B)** The colon X-ray image of the proband, who was diagnosed as total colonic HSCR. **(C)** The genome sequencing results indicated that the variant in the genome was the deletion of the (*chr10:43613122-43615021*,

*exon 13* and part of *exon 14* with a 65 nts intron sequence insertion, resulting in premature termination codons (PTCs) after the inserted sequence, and the first one locates in *exon 14* at the 814th codon (Fig. 1G). We, therefore, predict that the variant *RET* protein may be truncated after the 813rd amino acid (Aa) in the intracellular tyrosine kinase domain (Fig. S2). In addition, the weaker brightness of the variant *RET* mRNA also indicated that the PTCs may have triggered nonsense-mediated mRNA decay (NMD)[4] in these subjects.

To test the above hypothesis, two plasmids containing the WT or Variant *RET* gene were constructed (Fig. S3), and were transiently transfected into HEK-293 T cells with equal quality to simulate the expression of *RET* gene. The following RT—qPCR and Western blot results demonstrated that the expression of the variant *RET* gene was much fewer than the WT (Fig. 1H, I). Then after adding actinomycin D (10 μg/mL) to the transfection system to inhibit transcription, we noticed that the degradation of variant *RET* mRNA was faster than WT over time (Fig. 1J), suggesting that the stability of variant *RET* mRNA with PTCs was disrupted. Additionally, we designed a siRNA (siUPF1) to knock down *UPF1* (a key component of NMD pathway[4]) in HEK-293 T cells (Table S1 and Fig. S4). After adding siUPF1 (15 nM) to the transfection system, we found that the relative expression of *RET* could be elevated to a similar level between the WT and variant group (Fig. 1K), demonstrating that it was NMD that contributed mainly to the rapid degradation of variant *RET* mRNA. With these results, the NMD of variant *RET* mRNA with PTCs that can lead to the dose insufficiency of functional *RET* protein, resulting in HSCR, has been confirmed.

Additionally, to explain that why variant carrier subject I-2 has no signs of HSCR or constipation, we collected her hair follicle and oral mucosa samples for ddPCR (droplet digital PCR) test to verify the putative mosaic phenomenon. The results demonstrated that unlike other variant carriers or other samples from her, the ratio of variant to WT *RET* gene of the hair follicle cells from subject I-2 was 0.389, which was far from 1.000 and indicated that subject I-2 was mosaic, and the content of variant *RET* gene was much lower in her hair follicles (Fig. S5). Consequently, we speculated that the enteric nervous system (ENC) in subject I-2, which is also derived from ectoderm as hair follicles, may also contain little or even no variant *RET* gene, which may not be enough to cause HSCR. Thus, this individual was normal.

Moreover, based on GS results, the combinations of three functional *RET* enhancer variant sites (*rs2506030*, *rs7069590*, and *rs2435357*) were "ACC, ATC, and GTC" in the chromosome containing the WT *RET* gene among subjects I-2, II-2, and III-1, respectively (Fig. S6). As reported previously, the enhancer activity of these three combinations was gradually weakened in the expression of *RET*.[2] Thus, this polymorphism in the regulatory region can also partially explain the increasingly severe clinical phenotypes in subjects I-2, II-2, and III-1.

Recently, several studies have found that the contribution of *RET* is potentially even larger in the onset of HSCR. Because several recently identified HSCR-related genes are associated with the regulation of *RET*, such as *UBR4* and *SOD1*.[3] Other researchers also found a large burden of *de novo* and parental mosaic variants in *RET* that may associate with HSCR.[3,5] So, Tilghman et al stated that genetic testing for at least *RET* regulatory network is warranted for HSCR.[3]

In the present study, we found no candidate variants in the initial ES, while through subsequent GS and ddPCR, a novel pathogenic variant in *RET* that originates as a mosaic mutation has been identified. This can remind us about the necessity of deeper sequencing technologies in the research of complex genetic diseases like HSCR.

In general, based on GS results, we found a novel structural HSCR-related *RET* variant *(NC_000010.10:g.43613122_43615021delinsTCCTCC)* that was hard to identify by ES and proved that its pathogenic mechanism might be the NMD of *RET* mRNA.

*hg19)* fragment in *RET* gene (inside the red dotted box). **(D)** The agarose gel electrophoresis result of the genomic DNA PCR test using primers *RET-del-F/R* (marked with orange color in (E)). Subjects (I-2, II-2, and III-1) who carried the variant *RET* allele had two bands (*2562 bp* and *668 bp*), while other members have only one band (*2562 bp*). **(E)** Sanger sequencing results of the PCR products in (D) indicated that the variant in *RET* gene was *1900 bp* fragment deletion from the 12th intron to the 14th exon (marked with red color), with a *6 bp* (*TCCTCC*) insertion (marked with green color), which should be described as *NC_000010.10:g.43613122_43615021delinsTCCTCC*. **(F)** The agarose gel electrophoresis result of the cDNA PCR test using primers *RET-F/R* (marked with orange color in (G)). The normal control (subject I-1) only had one band, while subjects (I-2, II-2, and III-1) carrying the variant *RET* allele had an additional smaller band with a much weaker brightness (inside the dotted box). **(G)** Sanger sequencing results of the cDNA PCR products indicate that the larger band represented the wild type of *RET* mRNA fragment with 419 nts, while the weaker and smaller band represented the variant type of *RET* mRNA fragment with 333 nts. The exact variant sequence in *RET* mRNA was the deletion of the whole *exon 13* and part of the *exon 14* (marked with red color) with a 65 nts intron sequence insertion (marked with green color), resulting in premature termination codons (PTCs) and the first one locates at the 814th codon (red arrow). **(H, I)** The RT-qPCR and Western blot results indicate that the expression of the variant *RET* gene was much fewer than the wild type in HEK-293 T cells after being transfected with equal quality of plasmid. **(J)** After adding actinomycin D (10 μg/mL) to the transfection system for inhibiting transcription, the subsequent RT-qPCR results indicated that the degradation of variant *RET* mRNA was faster than WT over time. **(K)** After adding siUPF1 (15 nM) to the transfection system, the RT-qPCR results demonstrated that the relative expression of *RET* could be elevated to a similar level between the WT and variant groups. WT, wild type; bp, base pair; nts, nucleotides; TC, termination codon; PTCs, premature termination codons; Aa, amino acid; ****$P < 0.0001$.

## Ethics declaration

This study was approved by the Institutional Review Board and Ethical Committee at the West China Hospital of Sichuan University in China (No. 1082, December 31, 2019). All procedures following the research protocols were approved by the West China Hospital of Sichuan University and were conducted according to the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent for participation in this study was provided, and the use of clinical records and biological samples was approved by the individuals or guardians.

## Author contributions

QW and SP collected the clinical data and biological samples. JC constructed the research concept and designed the research plan. QW, SP, and JC performed laboratory experiments and analyses. QW drafted the initial version of the manuscript. SP, BX, and JC critically reviewed and revised the initial and final version of the manuscript.

## Conflict of interests

The authors declare no conflict of interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.03.008.

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