Novel RET Mutation Produces a Truncated RET Receptor Lacking the Intracellular Signaling Domain in a 3-Generation Family with Hirschsprung Disease, Vincent C.H. Lui, Thomas Y.Y. Leon, Maria-Mercedes Garcia-Barcelo, Raymond W. Ganster, Benedict L.S. Chen, John M. Hutson, and Paul K.H. Tam (1) Department of Surgery and 2 Genome Research Centre, The University of Hong Kong Pokfulam, Hong Kong SAR, China; 3 Department of General Surgery, Royal Children’s Hospital, Melbourne, Victoria, Australia; * address correspondence to this author at: Division of Paediatric Surgery, Department of Surgery, University of Hong Kong, Pokfulam, Queen Mary Hospital, Hong Kong SAR, China; fax 852-28193155, e-mail paultam@hkucc.hku.hk)

The RET gene encodes a transmembrane receptor tyrosine kinase, RET (1, 2), which is produced by enteric nervous system progenitors and functions, together with glial cell-line–derived neurotrophic factor (GDNF) family receptors, as the receptor for GDNF family ligands (3). Ligand binding induces RET dimerization, autophosphorylation of the tyrosine kinase (TK) domains, and intracellular signaling (3). RET mutations cause enteric nervous system anomalies in patients with Hirschsprung disease (HSCR), which is characterized by a deficiency of ganglion cells (aganglionosis) in the intramural plexuses of the colon (4–6).

It is not always easy to offer biological evidence of alteration of the RET function for a large number of RET mutations in HSCR patients. Generally, mutations affecting the extracellular domain of RET could cause RET haploinsufficiency (7, 8) and/or interference of normal RET, causing RET signaling deficiency in a dominant fashion (9). Mutations affecting the intracellular domain of RET lead to interference of normal RET, causing RET functional deficiency in a dominant fashion (8, 10–12). The biological consequences of truncating mutations of RET is relatively little studied. In this study, we identified a novel truncating mutation of the RET gene and provided evidence indicating that the mutation could cause RET signaling deficiency in a dominant fashion and RET haploinsufficiency (for a description of the materials and methods used, see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue8/).

A 3-generation HSCR family with 6 members was studied (Fig. 1A). Members II2, III1, and III2 are affected with variable length of aganglionic bowel. The index case (II2) presents with short-segment HSCR (anterior limit of aganglionic bowel at the sigmoid colon). Her daughters present with long-segment HSCR [anterior limit of aganglionic bowel at the transverse colon (III1) or jejunum (III2)]. All of the affected members show no other syndromes. Using previously described primers and conditions (13), we screened all exons of the RET, GDNF, endothelin 3 (EDN3), and endothelin receptor B (EDNRB) genes, including intron/exon boundaries, for mutations and polymorphisms in all family members.

We identified a 5-bp deletion in the coding region of exon 11 of the RET gene in all affected family members, indicating that this was a causative mutation (see Fig. 1S of the online Data Supplement). Possible locations of the deletion from c1927–1931 (5’-CTCTT-3’) or c1930–1934 (5’-TTCTC-3’) were localized (Fig. 1B; see also Fig. 1S of the online Data Supplement). These deletions overlap with the sequence palindromic (5’-TCCTTCCTCTC-3’; c1925–1936), causing the same frame shift and a termination codon after amino acid 643 in the mutant RET (Fig. 1S of the online Data Supplement). Exon 11 of the RET gene encodes the transmembrane domain (TMD); amino acids 636–657 of RET (Fig. 1S of the online Data Supplement). The signal peptide, the extracellular domain, and the first 8 amino acid residues of the TMD, but not the TK, domain were encoded in the mutant RET protein. No mutations were found in the GDNF, EDNRB and EDN3 genes (data not shown).

Reverse transcription-PCR analysis, Western blotting, and immunofluorescence analyses showed that the mutant RET transcript was transcribed and that the mutant RET protein was translated in mutant RET-transfected cells (Fig. 25 of the online Data Supplement). Cellular distributions of wild-type and mutant RET were analyzed by immunofluorescence. Full-length (wild-type) RET (Fig. 1C) was localized to the endoplasmic reticulum (open arrowheads) and at the cell membrane (filled arrowheads). In contrast, mutant RET (Fig. 1D) was localized mainly to the endoplasmic reticulum (open arrowheads). However, weak immunofluorescence of mutant RET could also be detected at the cell membrane (filled arrowheads). The weak immunofluorescence of mutant RET at the cell membrane but strong immunofluorescence at the endoplasmic reticulum suggests that cell membrane transport of mutant RET is not efficient. Western blotting analyses on purified surface proteins of transfected cells showed that truncated RET was detectable as cell membrane protein (Fig. 1E, arrowhead).

The mutation identified in this study produces a trun-
cated RET containing the extracellular domain and part of the TMD, but not the TK, domain. The truncated RET on the cell membrane could interfere with normal RET signaling in a dominant fashion (Fig. 3S of the online Data Supplement), but membrane transport of the mutant RET is not efficient. Intracellular accumulation of mutant RET could lead to degradation of mutant RET and RET haploinsufficiency in carriers. The grandmother (I2) carries the mutation but does not display a HSCR phenotype, suggesting that RET haploinsufficiency alone is necessary but not sufficient to cause aganglionosis. We postulated that carriers will develop HSCR disease if there is sufficient amount of mutant RET to interfere with the normal RET.

Disease expressivity of RET mutations is affected by RET polymorphisms; in particular, RET A45A (c135 G>A) exhibits a phenotype-modifying effect in conjunction with RET mutation [Ref. (14) and the references within]. The grandmother (I2) and the affected daughter (II2) and granddaughters (III1 and III2) are homozygous for the RET c135G genotype (our unpublished data), indicating that the RET mutation and the G allele are inherited on the same chromosome. Affected members who inherited the deletion and the G allele on the same chromosome display aganglionosis, varying from short- to long-segment aganglionosis. Thus, we did not observe the association of RET c135G allele and the RET mutation with long-segment HSCR in this family. The GDNF, ENDRB, and EDN3 genes also affect the penetrance of RET mutations (4, 5, 14). We found no mutations in these

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**Fig. 1.** RET mutation produces a truncated RET receptor lacking the intracellular domain.

(A), HSCR family pedigree: circles, females; squares, males; ◊, HSCR patients carrying the mutation; ♂, healthy carriers; ◎, deceased members; "?", unknown genotype. (B), DNA sequence of wild-type (Normal) RET exon 11. The nucleotide sequences are depicted from 5' to 3' and are numbered. The deletions are indicated by solid or dashed boxes. Translated amino acids are numbered and shown below the nucleotide sequences. (C and D), immunofluorescence analysis in cells transfected with 0.25 μg of wild-type (Normal) RET or mutant RET. Nu, nucleus. (E), Western blotting analysis of purified cell surface proteins (Mu) and cytoplasmic proteins (Ve) of HTB11 cells transfected with mutant RET (Mu) or empty vector (Ve); anti-RET-N antibody was used. Truncated RET is indicated with arrowhead.
Homocysteine Stability in Heparinized Plasma Stored in a Gel Separator Tube, Roger R. Calam,* Ibrahim Mansoor, and James Blaga (St. John Hospital and Medical Center, Detroit, MI; * address correspondence to this author at: Department of Pathology, St. John Hospital and Medical Center, 22101 Moross Rd., Detroit, MI 48236; fax 313-881-4727)

Recent studies have shown that increased blood concentrations of homocysteine (Hcy) have been linked to increased risk of premature coronary artery disease, stroke, and thromboembolism (venous blood clots), even among people with cholesterol values within the appropriate reference intervals (1–3). As testing for Hcy has become more widespread, it is apparent there are preanalytical issues with respect to specimen type and analyte stability. It is well documented that erythrocytes continue to produce and export Hcy into the plasma after venipuncture (4, 5). This can lead to an artifactual increase in Hcy the longer the plasma is allowed to remain in contact with the cells. Several studies have addressed this issue, with EDTA plasma established as the recommended sample, the collected specimens placed on ice and/or centrifuged within 1 h, and the plasma physically removed from cells (6). Because the specimen is anticoagulated, it can be centrifuged immediately, contributing to rapid handling.

It has been documented that expeditious transport and handling of Hcy specimens for epidemiologic studies is difficult because of preanalytical stability requirements (7, 8). We present the analogy that collecting blood for a test from a predominately outpatient population is the same challenge, the same concern, because these samples are usually collected at sites remote from the testing laboratory. Hence, the requirement for prompt removal and freezing of the plasma until testing is difficult to achieve.

The preanalytical variables for Hcy can be controlled either by inhibiting cellular production and release of Hcy or by prompt removal of plasma from the cells. Studies have looked at the possibility of stabilizing Hcy in the collected blood before plasma separation. Citrate has been evaluated (9, 10), as has fluoride, the latter alone or in combination with EDTA. The combination of sodium fluoride and EDTA has been shown to be effective at preserving Hcy in unseparated blood for up to 7 days at 2–8 °C (7), whereas sodium fluoride alone stabilized Hcy at room temperature for only 3 h (11). Serum separation in a gel tube has been studied, with Hcy stable on the gel for 48 h (12). However, serum preparation requires additional time for the blood to clot before centrifugation. We studied the application of a heparin gel tube [plasma separator tube (PST); BD Preanalytical Solutions] to determine Hcy stability when the plasma was stored on the gel barrier at 2–8 °C. Anticoagulated, the collected blood could be centrifuged immediately, contributing to rapid processing. One study was planned, but an additional study was necessary.

For the first study, 21 paired blood specimens, one EDTA and one PST (tubes from BD Preanalytical Solutions), were drawn from apparently healthy laboratory staff (11 females and 10 males). All collected specimens were centrifuged at room temperature at 1200 g within 30 min from time of collection. EDTA plasma was removed from the cells and frozen (−20 °C) in an aliquot tube; heparinized plasma was frozen on the gel. All samples were thawed at room temperature for assay on day 1. Samples were refrigerated until being reassayed on days 3 and 6.

For the second study, 15 paired blood specimens were collected, one EDTA and one PST. In this study, after centrifugation the EDTA-plasma aliquot was not frozen but was refrigerated at 2–8 °C along with the heparinized plasma, which remained on the separator gel. Day 1 testing was the day of collection with plasma refrigerated after the initial assay until being reassayed on days 3 and 4.

All testing was performed with the Carolina Liquid Chemistries Hcy assay, which uses a recombinant enzy-