Letters to the Editor

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A Loss-of-Function Mutation in the Endothelin-Converting Enzyme 1 (ECE-1) Associated with Hirschsprung Disease, Cardiac Defects, and Autonomic Dysfunction

To the Editor:

Hirschsprung disease (HSCR [MIM 142623]) is a congenital disorder characterized by an absence of enteric ganglia over various lengths of the bowel, leading to functional obstruction and resulting in life-threatening bowel distension shortly after birth. The incidence is 1 in 5,000 live births. In ~80% of cases, the rectosigmoid colon is the only part affected, whereas in 15%–20% of cases, the aganglionosis extends to the ileocecal junction. In a small percentage of cases, the entire small bowel and colon are aganglionic, and in some rare cases, so-called skip-lesions occur, in which ganglionic and aganglionic bowel segments alternate.

HSCR is considered to be genetically heterogeneous (Edery et al. 1994; Puffenberger et al. 1994; Romeo et al. 1994; Angrist et al. 1996; Edery et al. 1996; Hofstra et al. 1996; Salomon et al. 1996; Pingault et al. 1998) and even polygenic (Puffenberger et al. 1994; Angrist et al. 1996; Salomon et al. 1996; Bolk et al. 1997). Mutations in five genes, *RET* (Edery et al. 1994; Romeo et al. 1994), *GDNF* (Angrist et al. 1996; Salomon et al. 1996), *EDNRB* (Puffenberger et al. 1994), *EDN3* (Edery et al. 1996, Hofstra et al. 1996), and *SOX10* (Pingault et al. 1998) have been shown to give rise, separately or in combination (Angrist et al. 1996; Salomon et al. 1996), to HSCR. They account for 60%–70% of the familial cases and 10%–30% of the sporadic cases (R. M. W. Hofstra, unpublished data). Conceivably, mutations in other genes that might be part of the signalling pathways to which these proteins belong may also lead to the HSCR phenotype. Here we describe the involvement of one such gene, the gene encoding the endothelin-converting enzyme I. This enzyme, ECE-1, is involved in the proteolytic processing of big endothelin 1, 2, and 3, encoded by genes *EDN1, EDN2,* and *EDN3,* to the biologically active peptides, endothelins ET1, ET2, and ET3, respectively.

For the purpose of the present paper, it is important to summarize the phenotypes of *Edn1, Edn3,* and *Ece1* knockout mice. In $Edn1^{+/-}$ mice, blood pressure is mildly but significantly elevated, whereas $Edn1^{-/-}$ mice are characterized by abnormal development of the pharyngeal arches, cleft palate, and small mandibula; abnormalities in the outflow tract of the heart; and abnormal thymus and thyroids (Kurihara et al. 1994, 1995). Similar abnormalities are also seen in the human DiGeorge syndrome (MIM 188400). Genetically, however, these are unrelated, as *EDN1* is located on the short arm of chromosome 6, whereas the locus for DiGeorge syndrome is mapped to the long arm of chromosome 22. $Edn1^{-/-}$ mice die shortly after birth (within hours). $Edn3^{+/-}$ mice are normal, whereas $Edn3^{-/-}$ mice die within a few weeks after birth and have pigment anomalies and aganglionosis in the distal colon (Baynash et al. 1994). Similar abnormalities are seen in the human Shah-Waardenburg syndrome (MIM 277580) (Edery et al. 1996; Hofstra et al. 1996). $Ece1^{+/-}$ mice are normal, whereas $Ece1^{-/-}$ mice exhibit neonatal lethality due to craniofacial and cardiac defects identical to those seen in $Edn1^{-/-}$ mice. In addition, $Ece1^{-/-}$ newborns lack enteric ganglia in the terminal colon (Yanagisawa et al. 1998). Thus, *Ece1* knockout mice seem to present a combination of features characteristic for the *Edn1* and *Edn3* knockout mice.

These observations prompted us to scan the human ECE-1 gene (Valdenaire et al. 1995) for mutations in a patient with skip-lesions HSCR, cardiac defects (ductus arteriosus, small subaortic ventricular septal defect, and small atrial-septal defect), craniofacial abnormalities (cupped ears: immature, and posteriorly rotated; and small nose with a high bridge and bulbous tip), and other dysmorphic features (tapered fingers with hyperconvex nails; a single left palmar crease; contractures at the DIP joints of the thumbs; PIP joints of the fingers, bilaterally; and micropenis) and autonomic dysfunction (episodes of severe agitation in association with significant tachycardia, hypertension, and core temperatures as high as 40.5°C; and status epilepticus). The patient had a normal karyotype without a 22q11 deletion.

We screened all 19 exons of the gene, using denaturing gradient-gel electrophoresis (DGGE) (GenBank accession numbers: cDNA sequence, Z35307; exon and in-

tron boundaries, X91922–91939). For DGGE analysis, a 9% PAA gel (acrylamide-to-bisacrylamide, 37.5:1) containing a 40%–80% UF (100% UF = 7 M urea and 40% deionized formamide) was used. Electrophoresis was performed in 0.5 × TAE (1 × TAE = 40 mM Tris, HAC pH 8.0; 20 mM NaAc; 1 mM Na EDTA) at 11 V/cm and 58°C for 18 h. An aberrant DGGE pattern was detected in exon 19 (fig. 1*a*). For the analysis of exon 19, the following primers were used: ECE-1/19F, 5′-ACAGTGACCCTGGCCTCTCC-3′, and ECE-1/19R, 5′-(40-bp GC clamp)TCTCGTCCTCAGCCCCTTCC-3′. The aberrant PCR products were purified and sequenced. A heterozygous C→T transition, resulting in the substitution of cysteine for arginine at 742, was detected (fig. 1*b*). Unfortunately, the patient's parents were not available for testing. In 100 control individuals, this mutation was never found. Furthermore, no *ECE-1* mutations were found in a further 110 HSCR patients screened. None of them, however, had the phenotype of the described patient.

Amino acid position 742 is in the vicinity of the active site of ECE-1 (Valdenaire et al. 1995). The observed mutation results in the replacement of a basic amino acid by a neutral polar amino acid. Moreover, this might result in the formation of an alternative disulfide bridge. In humans, three ECE-1 isoforms are generated from the same gene (Schweizer et al. 1997). They differ only in their first N-terminal amino acid residues; they share the same extracellular domain (which includes the enzyme active site) and cleave big endothelins with similar efficiencies.

To investigate the functional consequences of the mutation on ECE-1 activity, we introduced it into the human ECE-1b isoform (Valdenaire et al. 1995; Schweizer et al. 1997). A PCR approach was used to construct the mutant (Cys742). Fidelity of the mutants was checked by sequencing. Wild-type and mutant proteins were produced by transient expression of the above-described expression constructs in Chinese hamster ovary cells (CHO-K1). ECE-1 activity was measured on cell membrane preparations by means of a specific radioimmunoassay and quantitative immunoblotting as described elsewhere (Schweizer et al. 1997). The specific ECE-1 activity was calculated as nanomoles of endothelin 1 produced per minute per milligram of expressed ECE-1 ($nM/min^{-1}/mg^{-1}$). A more detailed protocol of this functional assay can be found elsewhere (Löffler and Maire 1994; Schweizer et al. 1997).

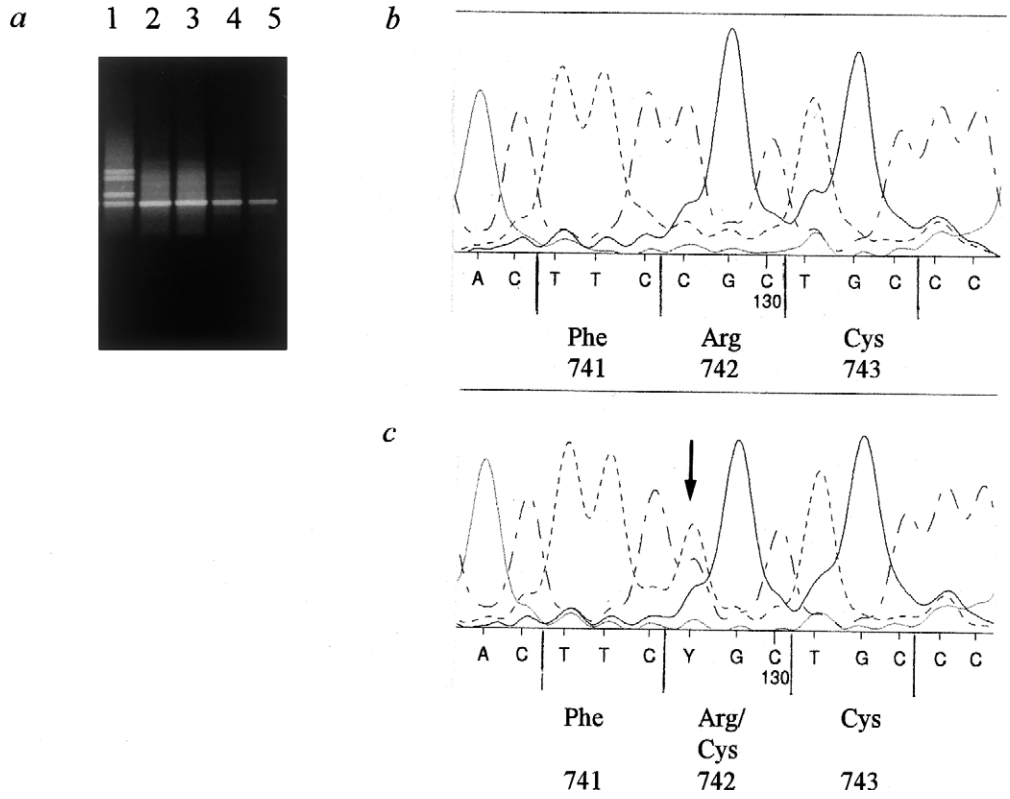

Figure 1 DNA analysis. *a,* DGGE patterns of exon 19 of *ECE-1*. A heterozygous variant can be seen in lane 1. Four normal controls are shown in lanes 2–5. Sequence analysis of *b,* the normal *ECE-1* exon 19 PCR product and *c,* the exon 19 PCR product of the patient described. The PCR primers and conditions and DGGE conditions are available upon request.

An example of a western blot used for quantitative immunoblotting is shown in figure 2*a*. The outcome of the radioimmunoassay is show in figure 2*b*. The specific activity measured in three independent transfections was for the wild-type ECE-1b, 314 ± 44 nM/min^{-1}/mg^{-1} (mean ± SD), and for the Arg742Cys mutant ECE-1b, 14.7 ± 9.8 nM/min^{-1}/mg^{-1} (mean ± SD). Thus, the activity of the mutant ECE-1 is only 4.7% of that of wild-type ECE-1. To determine whether this effect was due to this specific amino acid substitution or more generally to an effect on the catalytic site, we also generated an Arg742Ala mutant. This Arg742Ala mutant ECE-1 had a specific activity of 12.4 ± 0.3 nM/min^{-1}/mg^{-1} (mean ± SD), demonstrating that the position of the mutation is more important than its specific nature.

In addition, from a developmental point of view, there are arguments suggesting that the phenotype described might be caused by reduced activity of the ECE-1 enzyme. The vertebrate enteric nervous system is large and independent. It develops from cells that migrate to the gut from three regions of the neural crest. The cells from the vagal neural crest colonize the enteric bowel below the rostral foregut, the sacral neural crest cells colonize only the postumbilical bowel, and the cells of the truncal neural crest colonize only the rostral foregut primordia of the esophagus and the cardiac stomach (Gershon 1997). Vagal neural crest cells are also crucial for the development of the outflow tract of the heart, thymus, and parathyroid glands. Neural crest cells from more anterior hindbrain regions play a key role in the patterning of the pharyngeal arches and their derivatives. The phenotypes in spontaneous and induced *Edn3, EdnrB,* and *Ece1* mutant mice are all related to the developmental fate of hindbrain neural crest cells and to the formation of melanocytes, also derived from the neural crest.

Further evidence that reduced levels of ET3 might contribute to the development of HSCR comes from expression studies of this gene in both ganglionic and aganglionic colon segments of HSCR patients and control individuals. Both aganglionic colon and ganglionic colon of HSCR patients show reduced levels of *EDN3* transcripts regardless of the mutation status of genes known to be involved in HSCR (S. E. Kenny, R. M. W. Hofstra, Y. Wu, C. H. C. M. Buys, C. Vaillant, D. A. Lloyd, and D. H. Edgar, unpublished data). This suggests that a low level of ET3 might be a condition for the development of HSCR.

In view of (1) the function of ECE-1 during murine development suggested by the mouse models, (2) the overlap in phenotypic features of these mouse models and our patient, and (3) the functional consequences of the mutation on the enzyme activity, we propose that the Arg742Cys mutation caused or at least contributed to the phenotype of our patient by producing reduced levels of ET1 and ET3.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for the human cDNA of ECE-1, accession number Z35307; for the nucleotide sequences of all intron-exon boundaries, accession numbers X91922–91939)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Hirschsprung [MIM 142623], for Shah-Waardenburg [MIM 277480], and for DiGeorge [MIM 188400] syndromes)

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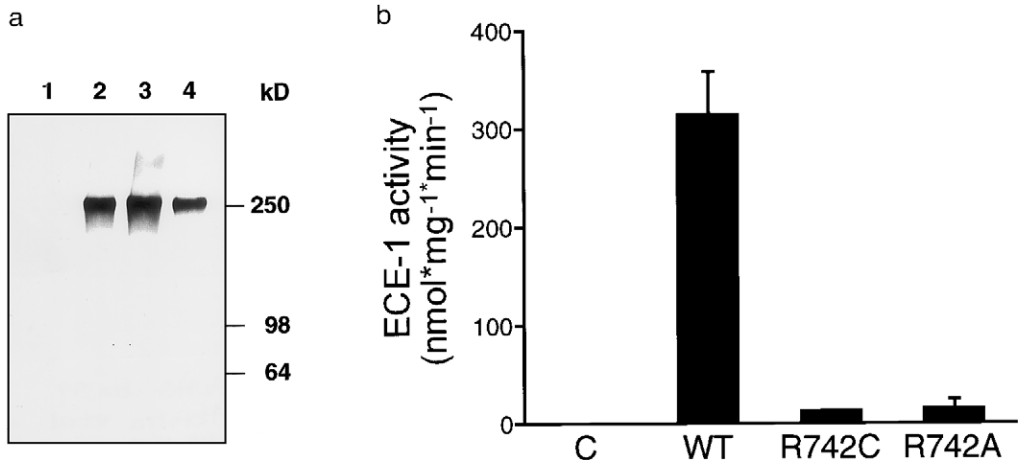

Figure 2 Western blot and activity measurements. *a,* Mutant and native ECE-1b isoforms were transiently transfected into CHO-K1 cells with Lipofectamine (Life Technologies) according to the manufacturer recommendations. Confluent cells in 100 mm plates were harvested 60 h after transfection, and membranes were prepared. Protein levels of expressed ECE-1 were measured on membranes by quantitative immunoblotting as described (Schweizer et al. 1997). Western blot of one set of transfections is shown: lane 1, nontransfected cells; lane 2, wild-type ECE-1b; lane 3, R742C mutant; lane 4, R742A. *b,* ECE-1 activity was assessed by means of a specific radioimmunoassay as described elsewhere (Schweizer et al. 1997). The results shown are the mean ± SD of at least three independent experiments, in nanomoles of produced ET-1 per minute per milligram of ECE-1 protein. C = nontransfected cells; WT = wild-type ECE-1b.

thymus in endothelin-1 knock-out mice. J Cardiovasc Pharmacol 26(suppl 3):S13–S16

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