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Biological Properties of Ret with Cysteine Mutations Correlate with Multiple Endocrine Neoplasia Type 2A, Familial Medullary Thyroid Carcinoma, and Hirschsprung’s Disease Phenotype

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Abstract

We investigated the transforming activity of the ret proto-oncogene with a mutation in cysteine 609, 611, 618, 620, 630, or 634 detected in patients with multiple endocrine neoplasia type 2A (MEN 2A), familial medullary thyroid carcinoma (FMTC), or Hirschsprung’s disease. Of these cysteine mutations, codon 634 mutations are known to be correlated with the development of MEN 2A, whereas codon 609, 618, or 620 mutations were detected in two-thirds of FMTCs and in several cases of Hirschsprung’s disease. Analysis of a total of 18 mutant genes revealed that codon 634 mutations have the highest transforming activity. The activity of ret with a codon 609, 611, 618, or 620 mutation and with a codon 630 mutation was approximately 3- to 5-fold and 2-fold lower than that of ret with a codon 634 mutation, respectively. In addition, different amino acid substitutions for the same cysteine displayed comparable transforming activity. The expression of the cell surface form of Ret with codon 609, 611, 618, or 620 mutation was very low compared with that of Ret with codon 634 mutation, indicating that the former four mutations might impair transport of Ret to the plasma membrane, as observed for several Hirschsprung mutations affecting the Ret extracellular domain. These results thus suggest that mutations in cysteine 609, 611, 618, or 620 may have the potential to develop Hirschsprung’s disease in addition to MEN 2A and FMTC.

Introduction

Germline mutations of the ret proto-oncogene are associated with the development of three hereditary neoplastic disorders: MEN2A, MEN 2B, and FMTC (1–4). These disorders are inherited in an autosomal dominant fashion; the former two share the clinical feature of medullary thyroid carcinoma and pheochromocytoma. FMTC is characterized by the development of medullary thyroid carcinoma alone. The MEN 2A and FMTC mutations involve cysteine residues present in the extracellular domain, whereas a single point mutation in the tyrosine kinase domain was found in MEN 2B. It was demonstrated that cysteine 634 mutations are strongly associated with the MEN 2A phenotype, whereas cysteine 609, 618, or 620 mutations were detected frequently in FMTC (5). In addition, two novel ret mutations were found in its kinase domain in FMTC families (6, 7). We and others demonstrated that the cysteine mutations in the extracellular domain activate Ret by inducing its coherent dimerization (8–10). In contrast, the MEN 2B mutation seems to enhance the Ret kinase activity without dimerization, probably due to a conformational change of its catalytic core region (8, 10, 11).

Germline mutations of ret also lead to the development of Hirschsprung’s disease characterized by the absence of intrinsic ganglion cells in the distal gastrointestinal tract (12, 13). Many missense, nonsense, and frameshift mutations of ret have been identified in its extracellular as well as tyrosine kinase domains in Hirschsprung’s disease (14). Pasini et al. (15) reported that the missense mutations detected in the tyrosine kinase domain result in severe impairment of the kinase activity of Ret/PTC2, a rearranged form of Ret found in human papillary thyroid carcinoma. On the other hand, most missense mutations in the extracellular domain appear to impair transport of the Ret protein to the plasma membrane or its maturation (16, 17). As a result, these mutations might cause the premature arrest of the cranio-caudal migration or differentiation of enteric neuroblasts.

Interestingly, six families who developed both MEN 2A and Hirschsprung’s disease were reported (18, 19). In these families, substitution of arginine or serine for cysteine 618 and of arginine for cysteine 620 in ret were identified. Moreover, mutations in cysteine 609 or 620 were found in sporadic cases expressing only the Hirschsprung phenotype (20).

In the present study, we introduced 18 different cysteine mutations into ret cDNA and analyzed their transforming activity. The results demonstrated that the activity of ret with a codon 634 mutation, which showed a strong association with the MEN 2A phenotype, was much higher than that of ret with other cysteine mutations, suggesting that the site of cysteine mutations correlates with the disease phenotype. In addition, biochemical analysis revealed that codon 609, 611, 618, and 620 mutations resulted in a marked decrease of cell surface expression of Ret.

Materials and Methods

Plasmid Construction. A cDNA clone containing the entire coding sequence (for 1114 amino acids) of the human c-ret gene was inserted into the APtag-1 vector containing the Moloney murine leukemia virus long terminal repeat, provided kindly by Dr. P. Leder (Harvard Medical School, Cambridge, MA) (21). Each cysteine mutation was introduced by PCR. In brief, primers containing the mutations were synthesized and used for amplification of c-ret sequences of approximately 100–150 bp. The corresponding sequences of the c-ret gene were replaced with the amplified fragments with the mutations. The amplified fragments were sequenced to confirm that proper mutations were introduced.

Transfection. Each recombinant plasmid (0.1 μg) was transfected into NIH 3T3 cells (5 × 10⁵ cells in a 60-mm-diameter dish) with 10 μg of NIH 3T3 DNA, as described previously (9). Transformed foci were scored on day 12 after transfection. Then, foci were picked up and grown into cell lines.

Western Blotting. Total cell lysates were prepared from transfecteds as described previously (9). The lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Nihon Millipore Kogyo KK, Tokyo, Japan). After membranes were reacted with anti-Ret antibody or antiphosphotyrosine antibody, the reaction was examined by enhanced chemiluminescence (Amersham Corp.).

Results

To compare the transforming activity of Ret with cysteine mutations detected in MEN 2A, FMTC, and Hirschsprung’s disease, we...
introduced a total of 18 mutations in ret cDNA and transfected the mutant cDNAs into NIH 3T3 cells. The mutations include two mutations at codon 609, two at codon 611, four at codon 618, three at codon 620, one at codon 630, and six at codon 634 (Fig. 1). All mutant cDNAs had the ability to transform NIH 3T3 cells, although the degree of the transforming activities varied among the mutant cDNAs (Table I). The codon 634 mutations had the highest activity in this assay. The activity of ret with codon 609, 611, 618, or 620 mutation and with codon 630 mutation was approximately 3- to 5-fold and 2-fold lower than that of ret with codon 634 mutation, respectively. In addition, different amino acid substitutions for the same cysteine displayed comparable transforming activity (data not shown), indicating that the degree of the activity depends on the position of cysteine mutations rather than the substituted amino acids.

We next examined the expression of the mutant Ret protein in each transfectant by Western blotting. As described previously (9, 22), Ret is expressed as the Mr 155,000 and 175,000 proteins that correspond to the immature glycosylated form present in the endoplasmic reticulum and the mature glycosylated form present at the plasma membrane, respectively. We also demonstrated that the MEN 2A mutations affecting cysteine 634 induce disulfide-linked homodimers of the Mr 175,000 Ret form responsible for transforming activity (9). Interestingly, it turned out that the expression level of the Mr 175,000 Ret protein was in direct proportion to the transforming activity of each mutant protein (Table 1; Fig. 2A). According to analysis by densitometry, the amounts of the Mr 175,000 form with a codon 609, 611, 618, or 620 mutation were 5- to 10-fold lower than those with a codon 634 mutation. The amount of this form with a codon 630 mutation was about half of that of codon 634 mutant proteins. These results thus suggest that codon 609, 611, 618, and 620 mutations severely impair the transport of Ret to the cell surface or its correct maturation. As a consequence, the levels of tyrosine phosphorylation of the Mr 175,000 form of these mutant proteins as well as the amounts of its homodimers were significantly reduced, leading to low transforming activity (Fig. 2B; Fig. 3).

It was reported that substitutions of arginine or serine for cysteine 618 (C618R or C618S) and of arginine for cysteine 620 (C620R) were identified in six families expressing both MEN 2A and Hirschsprung phenotypes (18, 19). In addition, two codon 609 mutations (C609Y and C609W) shown in Fig. 1 were present in sporadic Hirschsprung patients (18, 20). When the expression levels of the Mr 175,000 proteins with these cysteine mutations were compared with those of Ret with a short- or long-segment Hirschsprung mutation (Fig. 1, Pro 64 → Leu and Phe 393 → Leu), both of which were shown to impair the cell surface expression of Ret (17), they were comparable to the level of the Mr 175,000 form with a short-segment Hirschsprung mutation (Fig. 4).

**Discussion**

Germline mutations of the ret proto-oncogene are responsible for the development of MEN 2A, MEN 2B, FMTC, and Hirschsprung’s disease (1–4, 12, 13). The mutations were identified in one of six cysteines close to the cell membrane in most cases of MEN 2A and FMTC and in some Hirschsprung patients. To assess the genotype/phenotype correlations in MEN 2A, FMTC, and Hirschsprung’s disease, we investigated the transforming activity of Ret with each cysteine mutation. Codon 634 mutations are the most frequent in MEN 2A (about 80%), whereas codon 609, 611, 618, and 620 mutations account for more than 60% of FMTC families and were also found in patients expressing the Hirschsprung phenotype (5). Our present results showing the highest transforming activity of codon 634 mutations are consistent with the observation that there is a strong correlation between these mutations and the development of pheochromocytoma in MEN 2A. On the other hand, the low activity of
Designated transfectants were separated under nonreducing conditions and reacted with anti-Ret antibody. The location of M, 150,000 Ret homodimers is shown. The bottom band may be dimers consisting of the M, 155,000 form or of the M, 155,000 and 175,000 form.

The development of FMTC. Carlomagno et al. (23) have recently reported a similar result by replacing cysteine 620 with tyrosine.

Ret with codon 609, 618, or 620 mutation may be associated with the development of FMTC. Carlomagno et al. (23) have recently reported a similar result by replacing cysteine 620 with tyrosine.

Different amino acid substitutions for the same cysteine residue showed comparable transforming activity. Taken together this finding, the fact that the same mutations affecting cysteine 618, 620, or 634 were identified in both MEN 2A and FMTC families suggests that the development of pheochromocytoma or parathyroid hyperplasia is also possible in FMTC families. In addition, our results predict that the position of cysteine mutations might correlate with the clinical course of MEN 2A and FMTC besides their phenotypes.

The expression levels of the M, 175,000 Ret proteins present on the cell surface were in direct proportion to the transforming activity of each mutant protein. We and others (16, 17) reported recently that cell surface expression of Ret with the designated cysteine mutants or Hirschsprung mutations were subjected to Western blotting with anti-Ret antibody. P64L and F393L short- and long-segment Hirschsprung mutations, respectively.

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References


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