CLINICAL STUDY

Characterization of the *RET* protooncogene transmembrane domain mutation S649L associated with nonaggressive medullary thyroid carcinoma

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Abstract

Context: For rare and novel *RET* mutations associated with hereditary medullary thyroid carcinoma (MTC), clinical and functional studies are needed to classify the *RET* mutation into one of the three clinical risk groups.

Objective: We analyzed proliferative properties and clinical implications associated with the *RET* protooncogene transmembrane domain mutation S649L.

Design: The transforming potential and mitogenic properties of S649L mutation were investigated clinically and by evaluating kinase activity, cell proliferation, and colony formation.

Patients: Fifteen individuals from five kindreds were identified as carriers of a *RET* protooncogene mutation in exon 11 codon 649 (TCG^{Ser} → TTG^{Leu}). In two out of five index patients, a second *RET* mutation (C634W or V804L) was detected.

Results: Eight gene carriers were operated on. Histology revealed MTC and C-cell hyperplasia in three index and three screening patients respectively. In all other gene carriers (aged 41–64 years), calcitonin levels were in the normal range, and pentagastrin-stimulated calcitonin levels were <100 pg/ml. Therefore, thyroidectomy had not yet been performed. In one index patient carrying the S649L mutation, hyperparathyroidism was confirmed histologically. *RET* S649L-expressing NIH3T3 cells exhibited a clear increase of phosphotyrosine and proliferation rate when compared with parental NIH3T3 cells but a significantly lower kinase activity and cell growth rate when compared with *RET* C634R-expressing cells. When compared with *RET* C634R, the S649L mutant showed moderate transforming potential with small-sized colonies.

Conclusions: Our clinical and in vitro findings indicate that the transmembrane *RET* S649L mutation is associated with late-onset non-aggressive disease. Recommendations for prophylactic thyroidectomy should be individualized depending on stimulated calcitonin levels.

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Introduction

Missense germline mutations in the *RET* protooncogene are associated with multiple endocrine neoplasia type 2 (MEN2) and familial medullary thyroid carcinoma (MTC) (1, 2). Detection of the mutant alleles in kindred members predicts disease inheritance and provides the basis for prophylactic thyroidectomy in children (3, 4). There is an age-related progression from C-cell hyperplasia (CCH) to MTC correlating with the transforming potential of the respective *RET* mutations (5). Codon-specific prognosis would allow individualized risk stratification (level 1–3 risk groups) for patients (6).

For newly identified or rare mutations in the *RET* gene, the causative role of the mutation and the

genotype–phenotype relationship must be evaluated to define the mutation's codon-specific risk level. Here, we describe 15 individuals from five kindreds who were identified as carriers of a *RET* mutation in exon 11 codon 649 (TCG → TTG). Our clinical analysis and functional studies indicate that this S649L mutation in the transmembrane domain of *RET* is characterized by low penetrance of MTC, a relatively low aggressive potential of the disease (level 1), and rare further endocrinopathies.

Subjects and methods

Clinical work-up and *RET* mutation analysis were performed on five kindreds from two centers that had been diagnosed with MTC or hyperparathyroidism.

Informed consent was obtained from all subjects. Fifteen individuals were identified as mutant *RET* gene carriers (S649L). The study population consisted of five index and ten screening patients.

Calcitonin levels were measured by chemiluminescence assay (Nichols Institute, Bad Vilbel, Germany). The normal basal levels were <10.3 and <4.3 pg/ml for males and females respectively. After total thyroidectomy, no increment in calcitonin after pentagastrin administration (0.5 µg/kg body weight i.v.) was considered normal.

In all patients, germline *RET* mutation analysis was performed as described previously (7). Allele-specific PCR for the linkage analysis of exon 11 mutations was performed with normal (5'-GAAGGCAGACAGCAG-CACCG-3') or mutant (5'-GAAGGCAGACAGCAG-CACCA-3') codon 649-specific primers and exon 11 forward as a counterprimer. The product was sequenced as previously described (7).

Histology

CCH was defined as 50 C-cells per low-power field. Tumor staging was performed according to the International Union Against Cancer tumor-node-metastasis (TNM) classification from 1997.

In vitro studies

The *RET*51 mutant S649L was generated from the pJ7ΩRET51wt plasmid (a kind gift from M Billaud) by site-directed mutagenesis using the QuikChange II XL kit (Stratagene, La Jolla, CA, USA). NIH3T3 stable cell lines expressing *RET* S649L and C634R were obtained and

analyzed for equal *RET* protein expression as described previously (8). The cell growth rate and transforming potential were determined as described (8). For the XTT assay, cells seeded in 96-well plates were incubated with TACS XTT labeling mixture (Trevigen Inc., Gaithersburg, MD, USA) for 4 h. Conversion of XTT to formazan was quantified by measuring the absorbance at 450 nm.

In vitro kinase activity assay

Subconfluent NIH3T3 cells, and stable cell lines expressing *RET* C634R or *RET* S649L were lysed in lysis buffer (150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM sodium vanadate, 50 mM Tris; pH 8.0) supplemented with protease inhibitors. Whole *RET* protein was immunoprecipitated from cell extracts containing 500 mg total protein with *RET* 51 (Santa Cruz, Biotechnology, Heidelberg, Germany) polyclonal antibody. Immunoprecipitation was carried out overnight at 4 °C, and immunocomplexes were harvested by addition of A/G agarose beads. After extensive washing, immunocomplexes were eluted in tyrosine kinase (TK) buffer (60 mM HEPES (pH 7.5), 5 mM MgCl₂, 5 mM MnCl₂, 3 mM Na₃VO₄, 1.25 mM DTT, and 200 mM ATP) and TK activity was measured using a TK activity assay kit (Chemicon, Temecula, CA, USA) according to the manufacturer's protocol.

Results

Fifteen individuals from five kindreds (male:female ratio 6:9; median age 44 years, range 20–75 years) were

Table 1 Data for 15 carriers (from five different families) of the *RET* S649L mutation.

Family	Age	Sex	<i>RET</i> mutations	Index	Calcitonin levels preoperatively basal/ stimulated	Histology	pTNM	Cured
1								
II,5	29	M	649+634	Index	500/—	MTC, Pheo	pT2N0M0	Yes
I,1	64	F	649		0.7/1	CCH, PTC		Yes
I,2	60	F	649		2/4	—		—
II,1	44	M	649		3/19	CCH		Yes
III,1	20	F	649		0.7/0.7	CCH		Yes
2								
I,1	69	F	649	Index	—/—	MTC	pT2N0M0	Yes
II,1	47	M	649		1.5/15	—		
II,3	44	M	649		17/81	No MTC No CCH		
II,4	43	F	649		—/—	—		
II,5	40	M	649		—/—	—		
3								
I,1	47	F	649+804	Index	—/—	MTC	pT2N0M0	No
4								
II,1	43	F	649	Index	11/65	—		
I,1	75	F	649		3/7	—		
II,2	41	F	649		3/8	—		
5								
II,1	24	M	649	Index	Normal/—	HPT		Yes

MTC, medullary thyroid carcinoma; CCH, C-cell hyperplasia; PTC, papillary thyroid cancer; Pheo, pheochromocytoma; HPT, primary hyperparathyroidism.

identified as carriers of a *RET* protooncogene mutation in exon 11 codon 649 (TCG^{Ser} → TTG^{Leu}; Table 1). In two out of the five index patients, a second mutation (C634W or V804L) was detected.

Clinical and histological findings

Out of the 15 gene carriers, 8 have been operated on (Table 1). Histology revealed MTC in three index patients, including a patient carrying the *RET* S649L mutation and two others carrying both the S649L mutations and a second mutation (C634W or V804L). Two index patients were cured: a 69-year-old woman carrying the S649L mutation (histology showed pT2NOM0) and a 29-year-old man carrying S649L and C634W mutations (histology showed pT2NOM0). The third index patient operated on, a woman having both S649L and V804L mutations located on the same allele and tumor stage pT2NOM0 at primary operation, was not cured. In this patient, pentagastrin stimulated a calcitonin increase to 39 pg/ml postoperatively and no tumor tissue could be localized.

In three screening patients of family 1, total thyroidectomy was performed and CCH was detected. In one of these patients, multifocal papillary thyroid cancer was detected. In one screening patient of family 2, histology was completely normal. In all other gene carriers, calcitonin was in the normal range, and stimulated values were <100 pg/ml despite age range of 41–64 years. Therefore, thyroidectomy has not yet been performed.

Modes of diagnosis of the five index cases

The index patient of family 1 (1 II,5) was diagnosed with multiple endocrine neoplasia type 2 (MEN2A) before surgery (Fig. 1). Diagnostic work-up of chronic diarrhea unveiled pathological carcinoembryonic antigen (CEA) and basal calcitonin levels with nodular goiter. Subsequently, the patient was diagnosed as being a heterozygous carrier of a *RET* C634W mutation in coincidence with a S649L mutation. Mutant allele-specific PCR followed by sequencing confirmed that the mutations at C634W and S649L are located on different alleles. Furthermore, the patient had elevated urinary catecholamines and underwent right adrenalectomy for pheochromocytoma. Calcium and parathyroid hormone levels were within normal limits. Molecular genetic screening of the parents of this patient for C634W revealed no such mutation. Sequencing unveiled a heterozygous S649L mutation in the patient's mother but no further mutation. Therefore, the C634W mutation is *de novo* in this patient.

For the index patients of families 2 and 3, operation of nodular goiter revealed MTC (Fig. 1). In family 4, calcitonin determination in a patient with a small thyroid nodule showed slight elevation, but pentagastrin-stimulated calcitonin was <100 pg/ml. In family 5, the index

Figure 1 Pedigrees of families (A) 1, (B) 2, and (C) 4. Black symbols depict carriers of *RET* protooncogene mutation S649L, grey symbols signify individuals with negative molecular genetic test results, while white symbols are those who await to be tested or were not available for it. Hatched symbols signify patients with S649L and an additional *RET* protooncogene mutation. Arrows depict index patients.

case was diagnosed at 24 years of age in the work-up of an operation for primary hyperparathyroidism.

Functional characterization of the S649L mutation

To determine whether the *RET* S649L mutation is capable of converting *RET* into a dominantly transforming oncogene, NIH3T3 cells were transfected with mutant cDNA to generate stable cell lines. Comparable expression of *RET*51 mutant proteins (C634R and S649L) in NIH3T3 stable cell lines is shown in Fig. 2A. The cell growth rate was evaluated by direct counting of cell numbers at the indicated time points, after exclusion of dead cells by trypan blue staining. As shown in Fig. 2B, cells expressing *RET*51 C634R (a predominant mutation in MEN2A) grew rapidly, reaching 1.03×10^6 cells after 4 days. *RET* S649L-expressing NIH3T3 cells showed a decreased proliferation rate (8×10^5 cells after 4 days) compared with cells expressing *RET* C634R.

To confirm these data, the viabilities of NIH3T3 cell lines expressing the various *RET*51 mutants were determined by XTT assay. Relative cell viability was calculated after standardization with parental NIH3T3 cells. As shown in Fig. 2C, all *RET*51 mutants exhibited comparable cell growth during the initial 3-day

Figure 2 *RET* kinase activity and the influence on cell proliferation. (A) Expression of *RET*51 mutant proteins (C634R and S649L) in NIH3T3 cells. (B) Growth rates of cells expressing *RET* mutant proteins. (C) Cell viability (white bars, NIH3T3; black bars, *RET*51 C634R; grey bars, *RET* S649L; * $P < 0.05$, ** $P < 0.01$). (D) Colony formation of cells expressing *RET* mutant proteins; upper panel, single colony size; bottom panel, number of colonies.

observation time. At day 4, there were more viable cells expressing *RET*51 C634R than cells expressing *RET* S649L, which is most likely due to the lower proliferation rates of cells expressing the S649L mutant.

Moreover, the transforming capacities of the mutant *RET* proteins were assessed by their abilities to promote anchorage-independent growth of NIH3T3 cells (Fig. 2D). A strong transforming activity was observed for the *RET* C634R mutation, resulting in a high number of large colonies. The S649L mutant showed a moderate transforming potential with small-sized colonies.

The TK activity of *RET* S649L and C634R was validated by an *in vitro* kinase assay (Fig. 3). In both NIH3T3 clones, *RET* was constitutively active and exhibited a clear increase of phosphotyrosine when compared with parental NIH3T3 cells. Comparison of both the mutants revealed a significantly higher kinase activity for C634R ($P = 0.041$).

Figure 3 *In vitro* tyrosine kinase assay. Protein extracts from NIH3T3 transfectants stably expressing mutated forms of *RET* were immunoprecipitated with anti-*RET* and subjected to a kinase activity assay. The kinase activity was determined by differences in absorbance at OD 450 nm. Data represent three independent experiments, bar $\pm$ S.D. Student's *t*-test was performed to compare the kinase activity of *RET* mutants with parental NIH3T3 cells. * $P < 0.01$.

Discussion

We describe the clinical data of five families carrying a rare and not yet well-defined mutation in the transmembrane domain of the *RET* protooncogene in exon 11 codon 649 (TCG^{Ser} → TTG^{Leu}) and the *in vitro* characterization of this mutation.

Other *RET* transmembrane domain mutations have been identified in codons 640 (9) and 643 (9, 10); however, affected individuals carried additional, highly penetrant germline missense *RET* mutations. Moreover, a germline substitution in codon 648 coinciding with a codon 634 mutation has not shown oncogenic activity clinically (11). Thus, the phenotypic expression of mutations in the *RET* transmembrane domain has remained obscure.

The present data indicate that the *RET* S649L mutation harbors a low but still significant risk of MTC. Four of our patients carrying S649L alone had MTC or CCH. Moreover, in one index patient hyperparathyroidism was the leading symptom.

Gene carriers harboring two *RET* mutations simultaneously may have a more aggressive disease than those with the 649 *RET* mutation alone. In our series, the two patients with the double *RET* mutation showed the same tumor stage of MTC as the patient with 649 *RET* mutation, but age at diagnosis was more advanced in the 649 *RET* mutation carrier. Therefore, we conclude that disease phenotype in double *RET* mutation is dominated by the more severe mutations, like 634 *RET* mutation. In our series, there is no aggravation of disease in the patients carrying double *RET* mutations.

Our findings are supported by other reports of *RET* S649L mutations (12, 13). In these publications, the MTC-carrying index patients were 44 and 47 years old when first diagnosed. One 22-year-old mutation carrier had normal thyroid sonography and pentagastrin-stimulated calcitonin levels. Like other level 1 mutations identified in patients with hereditary MTC, the S649L mutation displayed a low transforming potential and limited constitutive TK activity, although it did stimulate the growth of NIH3T3 cells.

In patients with MTC or MEN2A, mutations of cysteine residues in the extracellular juxtamembrane region of the *RET* receptor TK cause the formation of covalent receptor dimers linked by intermolecular disulfide bonds between unpaired cysteines, followed by oncogenic activation of the *RET* kinase. Recent observations support an active role of the transmembrane domain in non-covalent receptor–receptor interactions that may contribute to keeping receptor molecules in close proximity to each other, allowing *RET* homodimers to be formed by MEN2A mutations or ligand binding.

Mutations in the *RET* transmembrane domain such as S645A, S649A, and S653A lead to impaired self-association (14). When introduced in the context of C634R mutation, A639G/A641R mutations were also shown to abrogate *RET* transmembrane domain

interactions, significantly reducing dimer formation and transforming activity of this highly oncogenic mutant. However, NIH3T3 cells expressing the S649L mutant exhibit accelerated proliferation and display a moderate transforming potential, consistent with the oncogenic activity of *RET* S649L in patients. Therefore, the oncogenic mechanism for the *RET* S649L mutation might be similar to the model derived for erythropoietin and epidermal growth factor receptors, in which activation of preformed, inactive receptor dimers is achieved via rotational changes of transmembrane and intracellular domains that bring kinases into a preferred orientation for signaling (15, 16).

Our clinical and *in vitro* data support the classification of the *RET* transmembrane domain mutation S649L as a level 1 mutation (6). The optimal timing for surgery in patients carrying level 1 *RET* mutations remains controversial. In the decision-making process, the risk of early metastases and the small risk of surgical sequelae in young children must be balanced against the biological behavior of MTC in other family members. There is good evidence in favor of thyroidectomy before the age of 6 years in patients carrying level 2 mutations, especially for a mutation in *RET* codon 634 (4–6). However, the decision for thyroidectomy in level 1 mutation carriers depends on personal experience and must be individualized until more data are available (17). The results of this study suggest that recommendations for prophylactic thyroidectomy in patients carrying *RET* mutation S649L should be individualized depending on levels of stimulated calcitonin. Further studies are necessary to confirm and extend these findings.

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