CLINICAL SCIENCE

Over-representation of the G12S polymorphism of the *SDHD* gene in patients with MEN2A syndrome

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OBJECTIVE: To evaluate whether germline variants of the succinate dehydrogenase genes might be phenotypic modifiers in patients with multiple endocrine neoplasia type 2. Mutations of genes encoding subunits of the succinate dehydrogenase are associated with hereditary paraganglioma/pheochromocytoma syndrome. Pheochromocytoma is one of the main manifestations of multiple endocrine neoplasia type 2 caused by germline mutation of the *rearranged during transfection* proto-oncogene.

METHODS: Polymorphisms of the succinate dehydrogenase genes were analyzed in 77 *rearranged during transfection* mutation carriers, 47 patients with sporadic medullary thyroid cancer, 48 patients with sporadic Pheo, and 100 healthy individuals. Exons 10–16 of the *rearranged during transfection* proto-oncogene were analyzed by direct DNA sequencing, and all exons of the von Hippel-Lindau, succinate dehydrogenase *B,* and succinate dehydrogenase subunit *D* genes were tested by direct DNA sequencing and multiple ligation probe analysis. The G12S polymorphism of the succinate dehydrogenase subunit *D* gene was determined by restriction fragment length polymorphism.

RESULTS: Of the 77 *rearranged during transfection* mutation carriers, 55 from 16 families had multiple endocrine neoplasia type 2A, three from three families had multiple endocrine neoplasia type 2B, and 19 from two families had familial medullary thyroid carcinoma. Eight of 55 (14.5%) patients with multiple endocrine neoplasia type 2A had this variant whereas it was absent in multiple endocrine neoplasia type 2B, familial medullary thyroid carcinoma, sporadic medullary thyroid carcinoma, and sporadic pheochromocytoma groups, and its prevalence in controls was 1% ($p<0.002$ multiple endocrine neoplasia type 2A versus controls). No associations between G12S and age of manifestation, incidence of pheochromocytoma or hyperparathyroidism, or level of serum calcitonin were observed.

CONCLUSION: The high prevalence of the G12S variant in patients with multiple endocrine neoplasia type 2A raises questions about its role as a genetic modifier, but this proposal remains to be established.

KEYWORDS: *RET* proto-oncogene; Succinate dehydrogenase subunit D; Medullary thyroid cancer; Pheochromocytoma.

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INTRODUCTION

The *RET* (*rearranged during transfection*) proto-oncogene located on chromosomal region 10q11.2 consists of 21 exons, and it encodes a receptor tyrosine kinase (1). Germline gain-of-function mutations of the *RET* proto-oncogene cause multiple endocrine neoplasia type 2 (MEN2), an autosomal dominantly inherited disease with an approximate prevalence of 2.5 per 100.000 in the general population (1). MEN2 has three subtypes: i) MEN2A, characterized by medullary thyroid carcinoma (MTC), pheochromocytoma (Pheo) and primary hyperparathyroidism; ii) MEN2B, which presents with the most aggressive MTC, pheochromocytoma, neuromas and marfanoid phenotype; and iii) familial MTC (FMTC), the mildest form of MTC. Several important genotype–phenotype associations have been determined; the most commonly affected codon, 634 (nearly 85% of MEN2A cases), frequently associates with Pheo and hyperparathyroidism, whereas mutations of codons 609, 611, 618, and 620 (accounting for 10–15% of MEN2A) usually associate with the milder form of MEN2 (1–3).

No potential conflict of interest was reported.

However, the phenotypic heterogeneity observed even in members of the same family suggests that other factors, for example genetic modifiers, may influence the clinical manifestation of the disease (4–7).

Hereditary paraganglioma/pheochromocytoma (PGL) syndrome is caused by the germline heterozygous mutations of the *SDHx* genes (*SDHB, SDHC, SDHD*, encoding subunits B, C and D, respectively) (8–10) and the newly identified *SDH5* gene (11) and *TMEM127*. *SDHx* genes encode subunits of the mitochondrial complex II (succinate dehydrogenase, SDH), an enzyme involved in oxidative phosphorylation and intracellular oxygen sensing and signaling (12).

Both MTC and paraganglioma/pheochromocytoma arise from neural crest-derived precursor cells. Accumulation of amino-acid coding polymorphisms (S163P in *SDHB*, G12S, and H50R in *SDHD*) has been found among patients with MTC, especially in those with familial tumors (13). In addition, these rare genetic variants have been identified in patients with Cowden-like syndrome (14) and the H50R polymorphism has been described in six members of a family with non-*RET*-associated C-cell hyperplasia and hypercalcitoninemia (15). These previous data may suggest a possible connection between *SDHx* polymorphisms and familial MTC and/or C-cell hyperplasia/hypercalcitoninemia; however, the occurrence of the *SDHx* variants among patients harboring germline *RET* mutations has not been previously examined. Therefore, the aim of the present study was to investigate whether polymorphisms of the *SDHx* genes could influence clinical manifestations of the disease in a cohort of subjects harboring *RET* mutations. In addition, we wanted to determine whether the prevalence of *SDHx* polymorphisms in patients with sporadic MTC, sporadic Pheo and healthy subjects might be different from that found in *RET* mutation carriers.

PATIENTS AND METHODS

Written informed consent was obtained from all patients and family members who participated in the study. Patients underwent a complete clinical examination, laboratory testing, including serum basal calcitonin measurement [hCalcitonin IRMA kit (Diagnostic Systems Laboratories, Inc., Budapest, Hungary), reference range: male <15 pg/ml, female <10 pg/ml until December 2007; and Liaison (Diasorin SPA, Stillwater, MN, USA), reference value: male <18.9 pg/ml and female <6 pg/ml after January 2008], plasma parathyroid hormone (Elecsys; Roche Diagnostics, Basel, Switzerland), urinary catecholamine metabolites (high pressure liquid chromatography with electrochemical detection), and imaging studies, including cervical ultrasonography, thoracal and abdominal computed tomography (CT), and whole-body metaiodobenzylguanidine scintigraphy (MIBG).

Patients with MEN2 syndrome

In total, 77 patients with germline *RET* proto-oncogene mutations who were members of 21 unrelated families with MEN2 syndrome were identified by genetic screening at our center. Of the 77 patients, 55 had MEN2A (mean age at diagnosis: 33.4 ± 17 years; range: 7–76 years), three had MEN2B (mean age at diagnosis: 15.6 ± 5 years; range: 10–20 years), and 19 had FMTC (mean age at diagnosis: 23.7 ± 16.8 years; range: 2–57 years). The presence of Pheo and MTC were confirmed by histologic examination of surgically removed tumors. Total thyroidectomy was performed in all patients with germline *RET* mutation in the symptomatic group and was also offered to all individuals from the asymptomatic group.

Patients with sporadic MTC

The study included 47 unrelated patients with histologically confirmed MTC evaluated consecutively at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University between 1998 and 2010. There were 15 men (age, mean $\pm$ SD, 44.7 ± 13.3; range: 28–82 years) and 32 women (age, mean $\pm$ SD, $47.7 \pm 12,3$; range: 23–76 years). Pre-operative evaluation included medical history, physical examination, thyroid and abdominal ultrasonography, CT or magnetic resonance imaging (MRI), MIBG-scintigraphy, routine biochemical testing, serum calcitonin measurements, and mutation analysis of exons 10–14 of the *RET* gene.

Patients with apparently sporadic Pheo

The study included 48 unrelated patients with histologically confirmed sporadic adrenal pheochromocytomas evaluated consecutively at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University between 1998 and 2010. There were 16 men (age, mean $\pm$ SD, 36 ± 14; range: 13–66 years) and 32 women (age, mean $\pm$ S.D, 42 ± 14; range: 19–64 years). Pre-operative evaluation included medical history, physical examination, abdominal ultrasonography, CT or MRI, MIBG-scintigraphy, routine biochemical testing, and 24 h urinary catecholamine metabolite determination. The mutation analysis of *RET* exons 10–14 and the entire *VHL*, *SDHB,* and *SDHD* genes revealed no disease-causing mutations. Patients with confirmed *VHL* (five patients), *SDHB* (one patient), or *SDHD* (one patient) mutations were excluded from the study. Five patients were initially thought to have sporadic pheochromocytoma, but were later identified as having a disease-causing *RET* mutation and were included in the study as *RET* mutation carriers. MTC, either by elevated serum calcitonin or by postoperative histology, had been diagnosed in all of these patients. Genetic counseling and genetic screening for all first-degree relatives have been offered.

Germline mutation screening of the RET, *VHL, SDHB,* and *SDHD* genes

Genomic DNA was isolated from peripheral blood using the Roche DNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) and QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) in accordance with the manufacturers' instructions. *RET* proto-oncogene mutations were detected by direct sequencing as previously reported (16,17). Mutation analysis of the *VHL, SDHB,* and *SDHD* genes in cases of apparently sporadic Pheo were performed by direct sequencing of the entire coding region of the *VHL, SDHB,* and *SDHD* genes, as previously reported (17,18), and large deletion analysis of the *VHL, SDHB, SDHC,* and *SDHD* genes performed using multiplex ligation probe amplification (18).

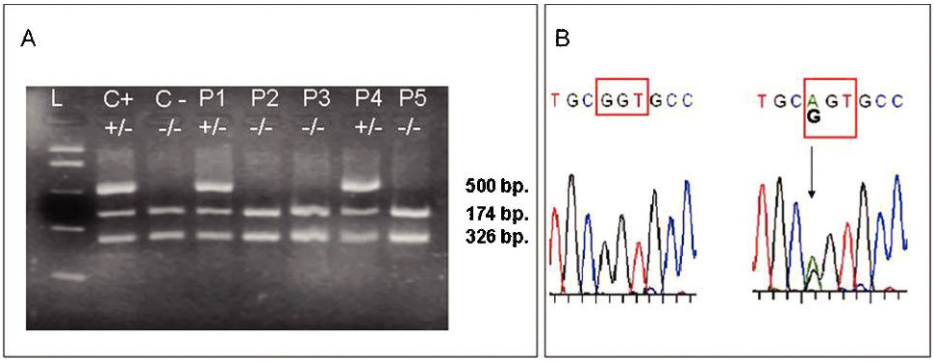

Figure 1 - (A) Gel electrophoresis of PCR fragments after digestion with *Ban*I for identification of the G12S polymorphism of the *SDHD* gene by RFLP (L = DNA ladder; C+ = positive control; heterozygote for G12S; C- = negative control; G12 normal, P1–P5 = patients. (B) Chromatograms of exon 1 of the *SDHD* gene showing the wild type and the heterozygote form of the G12S (GGT12AGT) polymorphism.

Restriction fragment length polymorphism (RFLP) analysis for identification the G12S polymorphism of the *SDHD* gene

The nucleotide change of G to A, which corresponds to the G12S polymorphism, results in the preservation of the *Ban*I restriction cleavage site. Therefore, digestion with the *Ban*I restriction enzyme (New England BioLabs Inc., Ipswich, MA, USA) for 90 min at 37℃ was performed after polymerase chain reaction (PCR) amplification of exon 1 of the *SDHD* gene for genotyping of *RET* mutation carriers, sporadic MTC patients, and 100 controls (Figure 1). Samples from patients with positive results were examined by direct DNA sequencing. The results obtained with both methods were the same in all cases.

Statistical analysis

Baseline characteristics were compared using the chi-squared test or Fisher's exact test for qualitative variables, and Student's t test or Mann-Whitney's U test for quantitative variables. The statistical package SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used and $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Eight of the 55 patients with MEN2A (15.5%) had the G12S variant, whereas it was absent in the MEN2B and FMTC groups. No patient with sporadic MTC and/or sporadic Pheo carried this variant. Among the 100 population-based, healthy control individuals, only one individual carried this variant (prevalence, 1%) (Table 1). No association between the G12S polymorphism of the *SDHD* gene and the incidence of Pheo or hyperparathyroidism in *RET* mutation carriers was observed, and the age of disease manifestation was similar in G12S carriers and in non-carriers (43 ± 9 versus 40 ± 3 years in probands and 29.6 ± 19.3 versus 32.5 ± 20.5 years in non-carriers). Among probands with *RET* mutations, carriers of the G12S had higher serum calcitonin levels compared with those who did not carry the *SDHD* G12S variant ($6{,}864\pm11{,}111$ versus $1{,}250\pm932$ pg/ml), but the difference was not significant. Among family members with *RET* mutations, serum calcitonin levels were similar in G12S carriers and non-carriers 436 ± 876 versus 393 ± 556 pg/ml) (Table 2).

The phenotypic heterogeneity seen in families with different *RET* mutations, the variation of clinical course within families with the same *RET* mutation, and the results from *RET* transgenic mouse models suggest a potential role of genetic components in phenotype modulation (2,19). Polymorphisms of the *RET* gene have been analyzed as such genetic modifiers, but the results from these studies are conflicting. Robledo et al. showed that two *RET* variants (G691S and S904S) may modify the age of onset of MTC in family members (5); and Tamanaha et al. reported that two intronic polymorphisms of *RET* may modify the phenotype in a large family with G533C *RET* mutation (7), while Baumgartner-Parzer found that the L769L and the IVS14-24 may act as modifiers in some forms of hereditary and sporadic MTC (20). However, Lesueur et al. were unable to replicate this association in a large cohort of 384 members of MEN2 families from four different European populations. This latter study showed that of the several polymorphisms of genes encoding the RET protein, its co-receptors and ligands, only the synonymous polymorphism A432A of the *RET* gene associated weakly with tumor spectra in patients with MEN2A (6). MEN2-related MTC *RET* variants have been analyzed as genetic susceptibility factors for the development of sporadic MTC: polymorphisms located in coding regions of *RET*, G691S, L769L, S836S, and S904S have been shown to be over-represented in patients with sporadic

Table 1 - Prevalence of the G12S polymorphism of the *SDHD* gene among germline *RET* mutation carriers, patients with sporadic medullary thyroid cancer, patients with sporadic pheochromocytomas, and healthy controls.

MEN2A	FMTC	MEN2B	Spoaradic MTC	Spoaradic Pheo	Control
8/55 (15.5%)*	0/19 (0%)	0/3 (0%)	0/47	0/48	1/100 (1%)

*$p<0.002$ versus control group.
FMTC = familiar medullary thyroid cancer; MEN2A = multiple endocrine neoplasia type 2A; MEN2B = multiple endocrine neoplasia type 2B; MTC = medullary thyroid cancer; Pheo = pheochromocytoma.

Table 2 - Clinical presentation, serum calcitonin concentration and the G12S status of patients with MEN2A.

MEN2A (n = 55)	G12S negative	G12S positive
Probands (n = 16)	n = 13	n = 3
Age of presentation (years)	40.1 ± 9	43 ± 3
Prevalence of:		
MTC	12/13	3/3
Pheo	6/13	2/3
PHPT	4/13	1/3
Serum calcitonin; mean ± SD (range)	1,206 ± 932 (13–2400)	6,864 ± 11,111 (124–19,690)
Affected family members (n = 39)	n = 34	n = 5
Age of presentation	32.5 ± 19.3	29.6 ± 20.5
Prevalence of:		
MTC	22/34	3/5
Pheo	8/34	2/5
PHPT	4/34	1/5
Serum calcitonin mean ± SD (range)	393.8 ± 556 (0–1978)	436.2 ± 876 (0–2000)

SD = standard deviation; MEN2A = multiple endocrine neoplasia type 2A; MTC = medullary thyroid cancer; Pheo = pheochromocytoma; PHPT = primary hyperparathyroidism.

MTC (21–23) compared with the general population, but others were unable to confirm these associations (24,25).

Germline mutations of *SDHx* genes encoding subunits of the mitochondrial complex II represent a genetic susceptibility for Pheo/PGL. These tumors are derived from cells of the neural crest, similar to MTC. *RET* mutations also cause Pheo, again suggesting a link between the genetic background of Pheo and MTC. Therefore, it has been assumed that mutations of these genes may be involved in the pathogenesis of MTC. Lima et al. reported a family with C-cell hyperplasia, a pre-cancerous state of MTC, who were proved to have the H50R variant of the *SDHD* gene (15). Montani et al. demonstrated an increased frequency of amino acid-coding *SDHx* polymorphisms in patients with sporadic and familial MTC (13). In addition, a systemic evaluation of genetic variants of the *SDHx* genes among patients with sporadic MTC showed a significant association between the H50R variant and sporadic MTC in Spanish patients, although this observation was absent in an English cohort (26). Variants of the *SDHx* genes have been implicated in the pathogenesis of various endocrine and non-endocrine tumors, such as Merkel cell carcinoma, carcinoid, papillary thyroid cancer, and renal cell cancer found in patients with Cowden-like syndrome (14).

In the present study, we found that the G12S variant was significantly over-represented among *RET* mutation carriers compared with sporadic MTC, sporadic Pheo, or control individuals. In our study, this variant occurred mainly in patients with MEN2A, while Montani et al. detected G12S in a patient with MEN2B harboring the M918T mutation of the *RET* gene (13). Interestingly, the prevalence of alterations of the *SDHx* genes in patients with *RET* mutations was similar in our study and the study of Montani et al. (13).

The prevalence of the G12S in the general population is between 2.5% and 5% (27) according to the Leiden Open Variation Database (http://chromium.liacs.nl) (28), which is somewhat higher than in our control population (1%). This difference may be due to differences in the selection criteria applied for controls. Our control group were evaluated for endocrine dysfunction; none of them had signs or symptoms characteristic of thyroid cancer or Pheo. By contrast, population-based controls, frequently anonymous blood donors, have never been tested for these rare conditions. Alternatively, the difference between the studies in prevalence of G12S can also be attributed to the ethnic background of the different populations tested. Our patients and controls were of Hungarian origin, representing an independent entity among Caucasian populations. More importantly, in our study, the high incidence of the G12S variant among *RET* carriers, especially in those with the MEN2A phenotype, raised the possibility that this variant may have a role in the phenotypic modulation of the disease. However, we were unable to detect significant differences in the clinical presentation between G12S carriers and non-carriers. Whether this failure was a result of the relatively small size of our patient cohorts remains to be further investigated. Interestingly, Waldmann et al. reported an increased prevalence of intronic *SDHB* polymorphisms among patients with malignant Pheo compared with patients with benign tumors (29).

In conclusion, we found a significantly higher prevalence of the G12S variant of the *SDHD* gene among germline *RET* mutation carriers presenting with MEN2A compared with the control group. The high prevalence of the G12S variant in these patients supports its genetic modifier role, but this proposal remains to be established.

AUTHOR CONTRIBUTIONS

Patócs A was responsible for the statistical analysis and study design, molecular biological analysis, evaluation and collection of clinical data, genetic counseling, and manuscript writing and critical review. Lendvai N has contributed to the statistical analysis and study design, molecular biological analysis, and manuscript writing and critical review. Kovács B and Kriszt B have contributed to the molecular biological analysis and to the manuscript writing and critical review. Tóth M, Szücs N, Csajbók É and Rácz K have contributed to the evaluation and collection of clinical data and to the manuscript writing and critical review. Igaz P has contributed to the evaluation and collection of clinical data, genetic counseling, and manuscript writing and critical review. Bekő G was responsible for the measurement of serum calcitonin levels and to the manuscript writing and critical review.

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