No mutations but an increased frequency of *SDHx* polymorphisms in patients with sporadic and familial medullary thyroid carcinoma

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Abstract

Germline mutations of the three succinate dehydrogenase subunits *SDHB*, *SDHC* and *SDHD* have recently been associated with familial pheochromocytoma and paraganglioma. Several reasons make these genes candidate tumor suppressor genes for medullary thyroid carcinoma (MTC): (1) *SDHB* lies on chromosome 1p, the region known to be deleted most frequently in MTC, (2) MTCs develop from neural crest-derived cells, as do pheochromocytomas and paragangliomas and (3) patients with germline mutations of the *Ret*-protooncogene develop MTCs as well as pheochromocytomas, indicating a relationship of these tumors on a genetic level. Therefore, we attempted to determine whether the tumor suppressor genes *SDHB*, *SDHC* and *SDHD* are involved in sporadic and familial MTC. Somatic mutations of the *SDH* subunits were absent in all 35 investigated MTCs. Loss of heterozygosity was found in 27% (*SDHB*) and 4% (*SDHD*) respectively. While the frequency of non-coding, intronic polymorphisms did not differ in MTC patients compared with a control population, an accumulation of amino-acid coding polymorphisms (S163P in *SDHB* as well as G12S and H50R in *SDHD*) was found among MTC patients especially patients with familial tumors, suggesting a functional connection of coding *SDH* polymorphisms to activating *Ret* mutations.

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Introduction

Medullary thyroid carcinomas (MTCs) account for about 10% of thyroid malignancies. Little is known about the genetics of MTC: germline mutations of the *Ret*-protooncogene are found in approximately 25% of all MTC patients in the setting of multiple endocrine neoplasia type 2 (MEN2). Additionally, 30 to 80% of sporadic tumors harbor somatic *Ret* mutations, most frequently C618Y in exon 16, at least in subclones (Eng *et al*. 1996, Wiench *et al*. 2001, Jindrichova *et al*. 2003). However, no causative genes in sporadic MTC lacking a *Ret* mutation have been described to date. Mutations of the tumor suppressor genes *SDHD* (Baysal *et al*. 2000), *SDHC* (Niemann & Muller 2000) and *SDHB* (Astuti *et al*. 2001) have recently been described in familial paragangliomas and pheochromocytomas. Several reasons make these genes candidate tumor suppressors for MTC: (1) allelic loss of the short and long arm of chromosome 1, the localization of *SDHB* and *SDHC*, is found in 23% of both familial and sporadic MTCs and makes this the most frequent genomic change of these neoplasms (Mathew *et al*. 1987, Khosla *et al*. 1991, Mulligan *et al*. 1993), (2) MTCs share similarities with paragangliomas/pheochromocytomas regarding their common development from neural crest-derived precursor cells, immunohistochemical phenotype and common genetic

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Table 1. Patients and results

					Stage		*SDHD*		*SDHB*			*SDHC*	*Ret*
No	**Ret**	**genotype**	**Age**	**Sex**	**T**	**N**	**exon**	**LOH**	**exon**	**intron**	**LOH**	**intron**	**somatic**
1	familial	M918T	x	m	x	N−	**G12S**	□			■		
2	familial	C634Y	22	m	T1(m)	N−	**H50R**	□			x		
3	familial	C620Y	62	f	T3(m)	N+		■	**S163P**	ivs2+33	□		
4	familial	C634R	39	m	T1(m)	x		□		ivs2+35	□		
5	familial	C634F	33	f	T1(m)	N−		□		ivs2+33	□		
6	familial	C634W	49	m	T3	N−		□			■		
7	familial	C634Y	19	m	T1(m)	N+		□			NI		
8	familial	M918T	34	m	T1(m)	N−		NI			■		
9	familial	x	19	m	T1	x		x			□		
10	familial	x	31	f	T1(m)	N+		NI			NI		
11	familial	C634R	48	f	T1	N−		NI			□		
12	familial	C634Y	45	f	T3	x		NI			■		
13	familial	x	53	f	T1(m)	N−		x			□		
14	spor	–	45	m	T3	N+	**H50R**	□			□		M918T
15	spor	–	45	f	T3	N+	**H50R**	□			□		M918T
16	spor	–	47	f	T2	N−		□			□	ivs2-39	M918T
17	spor	–	62	f	T1(m)	N+		□			□		M918T
18	spor	–	57	m	T3	N−		□			□		M918T
19	spor	–	72	f	x	N+		NI			■		M918T
20	spor	–	34	f	T3	x		□		ivs2+33	□		M918T
21	spor	–	79	f	T2	N−		□			■		M918T
22	spor	–	47	f	T3	N−		□	**S163P**	ivs2+33	□		–
23	spor	–	56	m	T3	N+		□		ivs2+35	□		–
24	spor	–	52	m	x	N+		□			□		–
25	spor	–	71	f	T1	N−		□			■		–
26	spor	–	76	f	T3	N+		x			NI		–
27	spor	–	32	f	T2	N+		□			□		–
28	spor	–	42	m	T2	N−		□			□		–
29	spor	x	72	m	T3	N+		x			x		–
30	spor	–	41	f	T2	N−		□			□		–
31	spor	–	37	f	T1	N−		□			□		–
32	spor	–	39	m	T3	N−		x		ivs2+33	□		–
33	spor	–	39	m	T3	N+		□			□		–
34	spor	–	76	f	T3	N+		□			□		x
35	spor	–	78	f	T3	N+		NI			■		x

T: tumor stage and N lymph node stage; −: negative; x: not available. N+: lymph node metastases; N−: no metastases found or no neck dissection performed; ■: loss of heterozygosity (LOH); □: retention of heterozygosity (ROH); NI: not informative.

background in the familial setting of MEN2, where both MTC and pheochromocytoma arise due to *Ret* germline mutations, and (3) an *SDHD*-gene germline polymorphism in exon 2 has recently been described in six members of a family with non-*Ret*-associated C-cell hyperplasia and hypercalcitoninemia (Lima *et al*. 2003). All these findings point towards a possible role of the succinate dehydrogenase (*SDH*) genes in the tumorigenesis of MTC. The aim of our study was to investigate sporadic and familial MTC for mutations and deletions of the candidate tumor suppressor genes *SDHB*, *SDHC* and *SDHD* and to explore a possible modifying role of *SDH* polymorphisms in MTC.

Materials and methods

Tissue specimens of 35 medullary thyroid carcinomas were included (22 sporadic and 13 MEN2 associated). Germline MEN2 status has previously been confirmed in all but 3 patients (9, 10 and 13 (low quality DNA)) by investigation of the *Ret* gene (Table 1). Somatic *Ret* exon 16 mutations have been sought in all sporadic tumors. C-cell hyperplasia has been re-evaluated in patients 14 and 22 on a minimum of 10 calcitonin-stained paraffin sections containing non-neoplastic thyroid tissue using the criteria proposed by Perry *et al*. (1996). Tumor tissue of 17 patients (5 MEN associated and 12 sporadic) was frozen in liquid

nitrogen and stored at −70 °C. The exclusive presence of tumor tissue was confirmed by controls on frozen sections prior to DNA extraction. The remaining 18 MTCs (8 familial and 10 sporadic), were embedded in paraffin and the tumor tissue was harvested by microdissection. Whole blood leukocytes and connective or thyroid tissues were used as non-neoplastic tissue in 20 and 14 patients respectively. No non-neoplastic tissue was available for patient no. 29 (Table 1). Blood samples from 80 unrelated Swiss individuals not suffering from endocrine disease were used as normal controls.

Denaturing gradient gel electrophoresis (DGGE)-based mutation analysis

Genomic DNA from fresh tissue (fresh frozen tumor tissue and fresh frozen non-neoplastic tissue of peripheral blood as normal controls) was isolated using the D-5000 Purgene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Where no fresh tissue was available, DNA was extracted from paraffin blocks for mutation and loss of heterozygosity (LOH) analysis. For this purpose, 10-µm sections were microdissected and DNA extraction was performed as described (Perren *et al*. 1998, Gortz *et al*. 1999). Primers for PCR have been designed based on Genbank sequences using the Primer 3 software (Rozen & Skaletsky 2000); all exons as well as intron-exon boundaries have been included. PCR using genomic DNA as template was carried out in a 50-µl mixture of 1 × PCR buffer (Perkin Elmer Europe, Rotkreuz, Switzerland) containing 400 ng template DNA, 200 µM dNTP (Roche Diagnostics, Rotkreuz, Switzerland), 1 µM of each primer and 1 µl Taq polymerase (Ampli Taq Gold, Perkin Elmer Europe). A touch-down procedure was used consisting of 5 s at 95 °C, annealing for 60 s at temperatures decreasing from 60 °C to 55 °C during the first 11 cycles (with 0.5 °C decremental steps in cycles 2 to 11), and ending with an extension step at 72 °C for 60 s. Ten cycles with annealing temperature of 55 °C and 15 cycles with annealing temperature of 45 °C followed with extension times of 90 s. After a final extension for 10 min at 72 °C, heteroduplex formation was induced after 10 min denaturation at 98 °C by incubations at 55 °C for 30 min and 37 °C for 30 min. For DGGE analysis, 10 µl of the PCR product were loaded with 3 µl Ficoll based loading buffer onto 10% polyacrylamide gels containing a urea-formamide gradient in 0.5 × TAE (tris-acetate-EDTA buffer). The amplicons were electrophoresed at 60 °C and 100 V for 16 h with the exception of exon 1 of *SDHD*, where the electrophoresis was run at 60 V for 15 h. The fragments were visualized using silver staining as described (Komminoth *et al*. 1994). Samples exhibiting additional bands were cycle sequenced. Assessment of *Ret* status in blood cells or tumor tissue has been performed using PCR, single strand conformation polymorphism (SSCP) and DGGE as described (Komminoth *et al*. 1995, Marsh *et al*. 1997, Mihic-Probst *et al*. 2004). (Fig. 1)

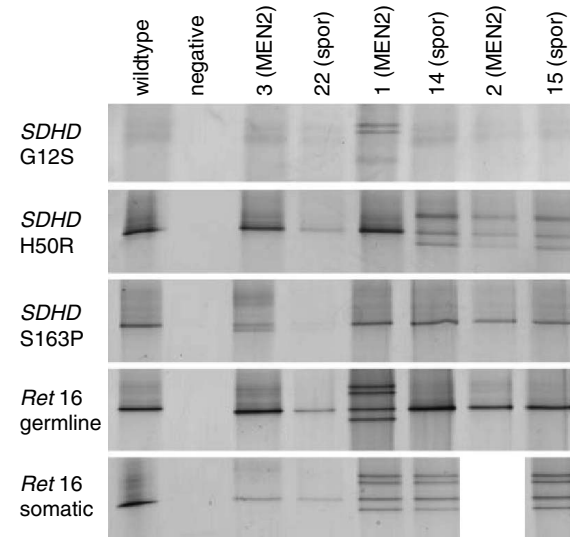

Figure 1 DGGE of *SDHD* (exons 1 and 2), *SDHB* (exon 5) and *Ret* exon 16 (tumor and control tissue) of patients with amino acid-coding polymorphisms in either *SDHD* or *SDHB*. MEN2 samples lacking a somatic *Ret* exon 16 mutation have *Ret* mutations in different exons (not shown; see Table 1). spor, sporadic. Numbers above indicate patient numbers.

LOH analysis

The genomic DNA was used to amplify the polymorphic markers D1S402 (telomeric), D1S199 and D1S2644 (centromeric) flanking the *SDHB* gene as well as D11S900 (centromeric) and D11S1347 (telomeric) flanking the *SDHD* gene. The forward primers were 5′ labeled with either HEX or 6-FAM fluorescent dyes. Fragment size analysis was performed with the 3100 Genetic Analyzer, Applied Biosystems/Hitachi and Gene-Scan software (Applied Biosystems, Foster City, CA, USA). Ratios >2 or <0.5 were designated as LOH.

Results

Mutation analysis was performed in 35 MTCs. Due to formalin fixation and paraffin embedding, DNA could

Table 2. Summary of SDHx polymorphisms

	SDHB			SDHC	SDHD	
	Exon 2		Exon 5	Exon 3	Exon 1	Exon 2
Alteration	ivs2+33A>G	ivs2+35G>A	**S163P**	ivs2-39T>C	**G12S**	**H50R**
dbSNP: rs	2647169	–	–	–	–	11214077
MTC Sporadic	3/19 (15.8%)	1/19 (5.3%)	1/17 (5.9%)	1/20 (5%)	0/22	2/22 (9.1%)
MTC MEN	2/9 (22.2%)	1/9 (11.1%)	1/9 (11.1%)	0/11	1/13 (7.7%)	1/11 (9.1%)
Control population	7/34 (20.6%)	3/34 (8.8%)	0/36	0/37	0/83	1/83 (1.2%)

Coding polymorphisms are indicated in bold letters. Numbers do not add up, some exons could not be amplified by PCR.

successfully be amplified by PCR (depending on the exon) in 15 (42.8%) to 35 (100%) tumors (see Table 2). Somatic mutations were absent in all informative exons of *SDHB*, *SDHC* and *SDHD*. However, 12 of 35 patients (34.3%) showed *SDHx* germline alterations, present in neoplastic as well as in non-neoplastic tissue.

SDHB

In intron 2 of *SDHB*, germline alterations were present in 7 of 28 patients (25%), consisting of a nucleic acid replacement of adenine by guanine (ivs2+33A>G) in 5 patients and a guanine to adenine exchange (ivs2+35G>A) in 2 patients. In exon 5 of *SDHB*, a germline thymine to cytosine nucleotide exchange leading to a serine to proline amino acid exchange at codon 163 (S163P) was detected in 1/17 (5.9%) patients suffering from a sporadic tumor and in 1/9 (11.1%) patients suffering from familial tumors (Fig. 1). This change was absent in 36 control patients. Eight of thirty informative tumors (26.7%) (4 familial and 4 sporadic tumors) showed a chromosomal loss of at least one polymorphic marker of the *SDHB* locus. Two of the four sporadic MTCs with *SDHB*-LOH harbored a somatic *Ret* mutation. Notably, none of the patients with the above *SDHB* germline alterations revealed an LOH (Table 1).

SDHD

A nucleotide exchange guanine to adenine, leading to a replacement of glycine by a serine on codon 12 (G12S), was found in one of thirteen MEN2 patients (7.7%) whereas no alterations were found in exon 1 of the *SDHD* gene among 22 sporadic MTC samples and 83 control samples (Fig. 1). In exon 2 a nucleotide exchange adenine to guanine, leading to a replacement of the amino-acid histidine by an arginine (H50R) was found in 2 of 22 (9.1%) sporadic and in 1 of 11 (9.1%) familial tumors, (Fig. 1) whereas this alteration occurred in only 1 of 83 (1.2%) control samples (Table 2). Twenty-four (68.6%) patients (7 with familial and 17 with sporadic tumors) were informative for at least one polymorphic marker of the *SDHD* locus. Only one patient with a familial tumor showed LOH (4.2%). This patient revealed no *SDHD* germline alteration (Table 1).

SDHC

One intronic germline alteration on position ivs2-39, replacing thymine by cytosine (ivs2-39T>C) was found in a patient with a sporadic tumor.

Discussion

We examined 35 medullary thyroid carcinomas for mutations of the three succinate dehydrogenase subunits *SDHB*, *SDHC* and *SDHD*. Somatic mutations were absent both in sporadic (22 tumors) and MEN2-associated MTCs (13 tumors). It is unlikely that these results represent an artifact: contamination by non neoplastic tissue can be excluded due to careful microdissection of tumor tissue and it is unlikely that significant mutations were missed, since the DGGE method is highly sensitive (Trulzsch *et al.* 1999). This is underlined by the detection of all reported polymorphisms in these genes. Additionally, all samples with faint additional bands have subsequently been cycle-sequenced to confirm negative results. We cannot exclude large homozygous deletions or deletions encompassing single exons of the genes; however, at least in a familial setting, such alterations are reported to be rare (McWhinney *et al.* 2004). Hemizygous

deletions of the *SDHB* locus on 1p36.1-35 were detected by LOH analysis in a significant proportion of MTCs. Eight of thirty informative MTCs (26.7%) showed LOH of at least one 1p marker. This finding is similar to previously published rates of 1p LOH (Mathew *et al*. 1987, Khosla *et al*. 1991, Mulligan *et al*. 1993). The absence of mutations suggests that genes other than *SDHB* are the targets of this deletion. Other mechanisms of *SDHB* inactivation such as promotor methylation cannot be excluded by our analysis. Methylation of one *SDHB* allele has recently been described in sporadic pheochromocytomas and neuroblastomas (Astuti *et al*. 2004), but this methylation did not lead to a decreased enzymatic activity and therefore seems not to be of functional significance. Only one of twenty-four informative tumors (4.2%) revealed LOH of the *SDHD* region on 11q23, arguing strongly against the presence of an important tumor suppressor gene for MTC on this locus. Using DGGE and sequencing, we detected seven different germline nucleic acid changes. The intronic sequence variants ivs2+33A>G and ivs2+35G>A are known polymorphisms (Benn *et al*. 2003) with a reported allelic frequency of 4% and 12% respectively (Benn *et al*. 2003). We detected an almost identical frequency of these polymorphisms in sporadic and familial MTC patients as well as in our control individuals (Table 2). In contrast, all coding polymorphisms leading to an amino acid change (S163P in *SDHB* as well as G12S and H50R in *SDHD*) detected in this study were more common in MTC patients (6 of 35; 17.1%) than in the control population (1 of 83; 1.2%). Neoplastic C-cell hyperplasia could not be detected in patients 14 and 22 with coding polymorphisms H50R and S163P of the *SDHD* and *SDHB* genes. This result is in contrast to the previously published family reported by Lima *et al*. (2003), where the H50R variant of the *SDHD* gene was associated with familial C-cell hyperplasia. It remains speculative whether, in this family, the H50R variant might exert some hitherto unknown influence on C-cell hyperplasia in a specific genetic setting. The rate of coding polymorphisms is also increased in MEN2-patients (3 of 13; 23.1%) as in patients with sporadic MTC (3 of 22; 13.6%) (Table 2). As five of six coding *SDH* polymorphisms were associated with either a germline or a somatic *Ret* mutation, a functional relationship of these polymorphisms to the mutated form of the *Ret* gene could be possible. *SDH* polymorphisms might lead to hypoxia-induced apoptotic signals (Eng *et al*. 2003) counteracting a *Ret*-induced resistance to apoptosis (Maeda *et al*. 2004) and could therefore be more frequent in MEN2 patients. The reported coding SDHx polymorphisms could also be genetic modifiers for MTC. Such modifying genetic factors are suspected in the familial setting of MEN2, explaining a variable clinical disease penetrance (Feldman *et al*. 2000, Fitze *et al*. 2002, Lombardo *et al*. 2002). Studies in a mouse model of MEN2 also suggest the presence of further genes modifying penetrance and expressivity of MTCs in MEN2 (Cranston & Ponder 2003). Further studies, including *in vitro* studies, are therefore needed to reveal whether coding polymorphisms of the *SDH* genes have such a function in MTCs.

In summary, somatic mutations of the *SDHB*, *SDHC* and *SDHD* genes appear to be absent in both MEN2-associated and sporadic medullary thyroid carcinomas, but the accumulation of coding polymorphisms of these genes in sporadic and familial MTC patients suggests a possible role for *SDH* polymorphisms as susceptibility/disease modifying factors in familial and sporadic MTC.

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