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
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.

### Table 1. Patients and results

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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.
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 50 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 50 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)

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...
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).

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

Table 2. Summary of SDHx polymorphisms

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<td>(11.1%)</td>
<td>(11.1%)</td>
<td></td>
<td>1/13</td>
</tr>
<tr>
<td>MTC MEN</td>
<td>2/9</td>
<td>1/9</td>
<td>0/11</td>
<td>(7.7%)</td>
<td>1/11</td>
</tr>
<tr>
<td>Control population</td>
<td>7/34</td>
<td>3/34</td>
<td>0/36</td>
<td>0/37</td>
<td>0/83</td>
</tr>
<tr>
<td></td>
<td>(20.6%)</td>
<td>(8.8%)</td>
<td></td>
<td></td>
<td>(1.2%)</td>
</tr>
</tbody>
</table>

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


