Few FH Mutations in Sporadic Counterparts of Tumor Types Observed in Hereditary Leiomyomatosis and Renal Cell Cancer Families

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Abstract
Loss of function mutations in the fumarate hydratase (fumarase, FH) gene were recently identified as the cause for dominantly inherited uterine and cutaneous leiomyomas and renal cell cancer. To further evaluate the role of FH in tumorigenesis, we screened FH mutations from tumor types seen in hereditary leiomyomatosis and renal cell cancer mutation carriers—41 uterine and 10 cutaneous leiomyomas, 52 renal cell carcinomas, 53 sarcomas, 29 prostate carcinomas, and 15 lobular breast carcinomas. Few mutations were detected. Biallelic inactivation of FH was found in one uterine leiomyosarcoma, one cutaneous leiomyoma, and one soft tissue sarcoma. Whereas the two former lesions were shown to originate from a germ-line mutation, the soft tissue sarcoma is to our knowledge the first example of purely somatic inactivation of FH in tumors.

Introduction
Germ-line mutations of the FH gene were recently shown to cause hereditary predisposition to uterine and cutaneous leiomyomas and renal cell cancer [HLRCC (OMIM 605839) or MCL (OMIM 150800); Ref. 1]. In HLRCC/MCL, affected individuals typically develop leiomyomas of the skin, and females also develop leiomyomas of the uterus [fibroids (2–4)]. Some families include individuals affected with renal cell carcinoma of a specific papillary type II histology (2, 4). In addition, a predisposition to uterine leiomyosarcoma has been suggested (2, 4). Mutations of the FH gene were found in 25 of 42 probands; sequence changes included truncating mutations (deletions of various size and premature termination codons) as well as non-truncating changes (missense mutations and in-frame deletions). The great majority of tumors from the affected individuals had acquired two hits as predicted by Knudson’s hypothesis of inactivation of a tumor suppressor gene (5); tumors displayed loss of the wild-type allele or harbored somatic mutations. The genetic data were further confirmed by demonstrating significantly reduced fumarase activity in FH deficiency (OMIM 136850), which is characterized by neurological impairment and delay of growth and development. No tumors have been reported in the affected individuals, who often survive only a few months, or in their first-degree relatives (8–11). A significant difference in the location and nature of mutations between FH deficiency families and leiomyomatosis families has been observed; in the latter group, sequence changes were more frequently situated in the 3’ end of the gene and were also often truncating. However, there is similarity in the phenotypes of heterozygous mutants in these two conditions because leiomyomas of the skin were observed by careful clinical and histopathological examination in a FH deficiency patient’s mother, who had no history of skin lesions or fibroids (1).

To evaluate the role of FH in the development of sporadic tumors, we screened FH mutations by genomic sequencing from 41 uterine leiomyomas, 10 cutaneous leiomyomas, and 52 renal cell carcinomas, tumors that are characteristic of HLRCC/MCL. Fifty-three sarcomas, 29 prostate carcinomas, and 15 lobular breast carcinomas were also included in the study because these tumor types have occurred in mutation-positive individuals of HLRCC families (2, 4). Materials and Methods

Tumor Material. Tumors from 194 patients were included in FH mutation screening. None of the patients was known to have a family history of malignancy. Samples included 41 uterine leiomyomas, 10 cutaneous leiomyomas, 52 renal cell carcinomas, 53 sarcomas including 18 uterine leiomyosarcomas, 29 prostate carcinomas, and 15 lobular breast carcinomas (see Table 1 for details). Five of the renal cell carcinomas were specifically selected because they displayed the rare papillary type II histology, which is associated with HLRCC.

Sequencing. Mutation screening was performed by sequencing of genomic DNA. DNA was extracted from the tissues by the standard procedures with slight modifications. Ten intronic pairs of PCR primers were designed to amplify the 10 FH exons (Table 2). PCR reactions were carried out in a 50-μl reaction volume containing 100 ng of genomic DNA, 1× PCR buffer (Applied Biosystems, Branchburg, NJ), 300 μM each deoxynucleotide triphosphate (Finnzymes, Espoo, Finland), 1 μM forward and reverse primer, and 2.5 units of AmpliTaq Gold polymerase (Applied Biosystems). MgCl2 concentrations and annealing temperatures for each exon are provided in Table 1. The following general cycling conditions were used: 10 min at 95°C, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at the corresponding

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4 The abbreviations used are: FH, fumarate hydratase; HLRCC, hereditary leiomyomatosis and renal cell cancer; MCL, multiple cutaneous and uterine leiomyomatosis; SSCP, single-strand conformational polymorphism; DHPLC, denaturing high-performance liquid chromatography.

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FH donors were used to investigate the Finnish population frequency of FH chemistry (Applied Biosystems, Foster City, CA) with ABI 3100 Genetic sequencing of PCR products was performed using the BigDye3 termination. 541delAG in exon 4, a putative Finnish founder mutation (1). SSCP analysis, DHPLC analysis were generated in 50-°C for 12 min; followed by 35 cycles of denaturing for 30 s, 58°C for 1 min; Clontech, Palo Alto, CA), and AmpliTaq Gold (0.6 unit; Applied Biosystems). The hot-start PCR cycling conditions were as follows: 94°C for 12 min; 94°C for 12 min; followed by 35 cycles of denaturing for 30 s, 58°C for 1 min, and reannealed by gradually cooling down temperature was lowered from 94°C to 89°C after 10 cycles. Samples were denatured at 95°C for 5 min and reannealed by gradually cooling down 1°C/min for 50 min. DHPLC heteroduplex analysis was performed at 55°C using automated HPLC instrumentation with Eclipse double-stranded DNA 2.1 × 75-mm analytical column (Agilent Technologies, Palo Alto, CA). The analytical gradient was created by mixing buffer A (100 mM triethylammonium acetate and 0.10 mM EDTA) and 57.4–71.9% buffer B (100 mM triethylammonium acetate, 0.10 mM EDTA, and 25% acetonitrile) at a flow rate of 0.4 ml/min.

### Results and Discussion

To clarify the role of FH in tumorigenesis of sporadic tumor types associated with HLRCC/MCL, we screened FH mutations from sporadic uterine and cutaneous leiomyomas, renal cell carcinomas, sarcomas, prostate carcinomas, and lobular breast cancers. FH mutation frequency was generally low in these tumors, but 2 of 53 sarcomas (a uterine leiomyosarcoma and a soft tissue sarcoma, respectively) and 1 of 10 cutaneous leiomyomas had acquired somatic mutations.

### Table 1: Tumor types included in FH mutation analyses

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>N</th>
<th>Histological subtype</th>
<th>PH/PHG</th>
<th>N</th>
<th>Samples from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine leiomyoma</td>
<td>41</td>
<td>Clear cell</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>52</td>
<td>Papillary type I</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>53</td>
<td>MFS/MGH</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Prostate carcinoma</td>
<td>29</td>
<td>Adenocarcinoma</td>
<td>29</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>15</td>
<td>Lobular</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

*PH, Department of Pathology at Helsinki University Central Hospital; OGH, Department of Obstetrics and Gynecology at Helsinki University Central Hospital; PO, Department of Pathology at Oulu University Hospital; SH, Department of Surgery at Helsinki University Central Hospital; PUO, Department of Pathology at the University of Otago; MGH, Department of Medical Genetics at University of Helsinki; IMTT, Institute of Medical Technology at Tampere University Hospital; MFH, malignant fibrous histiocytoma.*

### FH mutations. Moreover, the uterine leiomyosarcoma and the cutaneous leiomyoma also harbored a germ-line mutation.

In the uterine leiomyosarcoma from patient LS10, a missense mutation His153Arg in exon 4 and a premature termination codon Leu240Stop in exon 5 were detected (Fig. 1, A and B). The mutation His153Arg was also found in the patient’s normal tissue DNA, indicating a germ-line change. The change occurred at a highly conserved region of FH and was not detected by DHPLC in a panel of 268 control chromosomes. Through examination of patient records, the patient’s disease history was clarified. Patient LS10 was first diagnosed with uterine leiomyomatosis at the age of 30 years and had had two myomecystomas in the following 2 years. The tumor excised in the second operation turned out to be malignant uterine leiomyosarcoma in the histopathological evaluation, and thus a hysterectomy was performed. Compatible with hereditary cancer predisposition, the patient was only 32 years old.

In the soft tissue sarcoma, a missense mutation Arg8Glu in exon 1 was observed. The mutation was not present in the patient’s normal tissue DNA (Fig. 1C). The sequence change targeted a residue conserved in yeast and Escherichia coli. Tumor sequencing also revealed loss of the wild-type FH allele. Compatible with this finding, the tumor had displayed a deletion of the FH region in comparative genomic hybridization. Thus, both FH alleles were inactivated in the tumor. The patient was 48 years old at the time of diagnosis of high-grade sarcoma in her right lower limb. In histopathological examination, this soft tissue sarcoma could not be classified in more detail.

In the cutaneous leiomyoma from patient IL10, a 2-bp deletion 541delAG in exon 4 and a premature termination codon Arg300Stop in exon 6 were found (Fig. 1, D and E). The mutation 541delAG was also detected in the patient’s normal tissue DNA. This mutation has been described previously in two Finnish HLRCC families (1), although no common ancestry was known for patient IL10 and the families. To determine the frequency of the 541delAG allele in the Finnish population, we analyzed the sequence change from 896 control chromosomes by SSCP. 541delAG alleles were not found in the control individuals. The somatic change Arg300Stop detected in the cutaneous leiomyoma had also been described previously in individuals of one HLRCC family. Sample mix-up was excluded by ream-

### Table 2: Oligonucleotide primers used for FH mutation analysis

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence (5'→3')</th>
<th>Product size (bp)</th>
<th>MgcL2 (mM)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0th</td>
<td>FH0F: TGTGGGCTGTAGTTGAGTGTG</td>
<td>299</td>
<td>1.5</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>FH0R: GUGGCGGCTAGACGCTIGC</td>
<td>256</td>
<td>2.25</td>
<td>57</td>
</tr>
<tr>
<td>1st</td>
<td>FH1F: AAGTGCGGATTTCTGCTCAC</td>
<td>309</td>
<td>1.5</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>FH1R: TGAATACGCTTCTCATCACAAG</td>
<td>349</td>
<td>1.5</td>
<td>58</td>
</tr>
<tr>
<td>2nd</td>
<td>FH2F: CTGCAAAATAAATAACCTGCTG</td>
<td>381</td>
<td>2.25</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>FH2R: GGGACATTTCACGAGCTCT</td>
<td>329</td>
<td>2.25</td>
<td>59</td>
</tr>
<tr>
<td>3rd</td>
<td>FH3F: AAAACCTGTGCTGATATTGACCA</td>
<td>300</td>
<td>1.5</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>FH3R: CCAAGATAAAACGCTTGTCA</td>
<td>317</td>
<td>2.25</td>
<td>57</td>
</tr>
<tr>
<td>4th</td>
<td>FH4F: ATGTTGGAGCCTCCTTCTAT</td>
<td>312</td>
<td>3.0</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>FH4R: CAGTITCCCTTCAACCTATC</td>
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<td></td>
</tr>
</tbody>
</table>

*An exon upstream (GenBank U59309) of the nine FH exons (GenBank NM_000143).*

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5. S. Knuttila, unpublished data.

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FEW FH MUTATIONS IN HLRCC-TYPE SPORADIC TUMORS

By analyzing tumors from 194 patients, we detected somatic FH mutations in one uterine leiomyosarcoma, one soft tissue sarcoma, and one cutaneous leiomyoma. The leiomyosarcoma and the cutaneous leiomyoma also harbored germ-line mutations. This effort adds to the knowledge of the role of FH in tumorigenesis. First, mutations of the FH gene appear to be rare in the sporadic tumor types included in the study. Second, two of three cases with a somatic mutation also harbored an inherited mutation. Whereas this observation may be incidental, it raises the hypothesis that germ-line mutations of FH are primarily required for effective tumorigenesis. Third, one mutation-positive lesion, a soft tissue sarcoma, had acquired both inactivation events, a mutation and allelic loss, at the somatic level. This is to our knowledge the first evidence that FH is also involved in the development of nonhereditary tumors. Studies on mechanisms other than mutational inactivation, such as transcriptional silencing through promoter hypermethylation, will further clarify this issue. Fourth, in all cases, tumors followed the two-hit model of inactivation of a tumor suppressor gene (5), confirming the previous findings (1–4). The inactivation pattern of FH is remarkably robust; two hits can almost always be demonstrated in a tumor. Fifth, detection of a missense mutation in the germ line of a patient affected with uterine leiomyosarcoma demonstrates that missense mutations of FH can predispose individuals to malignant neoplasms. Previously, families segregating missense mutations of FH have not included affected individuals diagnosed with cancer (1, 10, 11). Demonstration of a somatic nonsense mutation in a leiomyosarcoma, in addition to the germ-line defect, provides conclusive evidence that leiomyosarcoma predisposition is associated with HLRCC. Sixth, multiple cutaneous leiomyomas might be a useful sign of hereditary tumor susceptibility because we detected a germ-line FH mutation in a patient with cutaneous leiomyomas. Careful clinical and histopathological evaluation of skin lesions could be valuable in identifying patients with inherited predisposition to tumor development.

Much further work is needed to determine the role of FH mutations in tumorigenesis and the tumor spectrum and other clinical features associated with hereditary FH defects. More accurate identification of HLRCC/MCL should result in improved diagnosis, detection, and prevention of tumors in these patients.

single-bp substitution C to T, resulting in a premature termination codon Arg300Stop, occurred in a CG dimer, a sequence that is underrepresented in the human genome and specifically associated with C to T transitions by deamination of methylated cytosine (12). Patient records revealed that patient IL10 had been operated on several times due to multiple leiomyomas of the skin but had no history of malignancy.

Mutations of hereditary cancer genes do not always explain the development of corresponding sporadic tumors, as demonstrated in reports on the low rate of BRCA1 and BRCA2 mutations detected in sporadic breast and ovarian carcinomas (13–15). By contrast, the VHL tumor suppressor gene seems to play a significant role also in tumorigenesis of sporadic renal cell carcinomas. VHL mutations are found in as many as 57% of sporadic renal cell carcinomas (16), and in 19% of tumors, VHL is inactivated by loss of one allele and DNA methylation of the other allele (17). Methylation, an alternative mechanism for inactivation of a tumor suppressor gene, could also be the mechanism for FH inactivation in a subset of tumors included in this study. After identification of the FH promoter region, studies on promotor hypermethylation will further clarify whether FH is inactivated in those tumors, which harbored no FH sequence changes.

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Much further work is needed to determine the role of FH mutations in tumorigenesis and the tumor spectrum and other clinical features associated with hereditary FH defects. More accurate identification of HLRCC/MCL should result in improved diagnosis, detection, and prevention of tumors in these patients.

Fig. 1. FH mutations detected in a uterine leiomyosarcoma (A and B), a soft tissue sarcoma (C), and a cutaneous leiomyoma (D and E); the respective normal tissue DNA sequences are depicted under tumor sequences. A, a germ-line His153Arg mutation. B, somatic mutation Leu240Stop resulting in a premature termination codon. C, somatic missense mutation Arg8Glu and loss of the wild-type allele (low wild-type signal is visible due to normal tissue contamination; allelic loss was confirmed in comparative genomic hybridization; data not shown). D, 2-bp deletion 541delAG present also in the germ line. E, somatic nonsense mutation Arg300Stop.
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References


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