Department of Medical Genetics
University of Helsinki
Finland

THE ROLE OF *FUMARASE* (*FH*) IN TUMORIGENESIS

Rainer Lehtonen

Academic dissertation

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Supervised by

Lauri A. Aaltonen, MD, PhD
Research Professor of the Finnish Academy of Sciences
Department of Medical Genetics
Haartman Institute and Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Virpi Launonen, PhD
Docent, Academy Research Fellow
Department of Medical Genetics
Haartman Institute and Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Reviewed by

Olli Ritvos, MD, PhD
Docent, Senior Scientist
Department of Bacteriology and Immunology
Haartman Institute and Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Maija Wessman, PhD
Docent, Academy Research Fellow
Folkhälsan Research Center and
Finnish Genome Center
University of Helsinki
Helsinki, Finland

Official opponent

Kirsi Huoponen, Ph.D
Docent, Clinical Molecular Geneticist
Department of Medical Genetics
DNA Diagnostics
University of Turku
Turku, Finland

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LIST OF ORIGINAL PUBLICATIONS

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The Multiple Leiomyoma Consortium.
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Germline mutations in *FH* predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. *Nature Genetics* (2002), 30, 406-410.

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Lehtonen R, Kiuru M, Rökman A, Ikonen T, Cunningham JM, Schaid DJ, Matikainen M, Nupponen NN, Karhu A, Kallioniemi O-P, Thibodeau SN, Schleutker J, and Aaltonen LA.
No *fumarate hydratase (FH)* mutations in hereditary prostate cancer. *Journal of Medical Genetics* (2003), 40, e19.

III.
Kiuru M, **Lehtonen R**, Eerola H, Aittomaäki K,Blomqvist C, Nevanlinna H, Aaltonen LA, and Launonen V.
No germline *FH* mutations in familial breast cancer patients. *European Journal of Human Genetics* (2005), 13, 506-509.

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Few *FH* mutations in sporadic counterparts of tumor types observed in hereditary leiomyomatosis and renal cell cancer families. *Cancer Research* (2002), 62, 4554-4557.

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Biallelic Inactivation of Fumarate Hydratase (FH) Occurs in Nonsyndromic Uterine Leiomyomas but Is Rare in Other Tumors. *American Journal of Pathology* (2004), 164, 17-22.

* Equal contribution
Publications I and IV are part of Maija Kiuru's academic dissertation (Helsinki 2002)

ABBREVIATIONS

3-NPA	3-nitropropionic acid
4EBP1	eukaryotic intitiation factor 4E (eIF4E) binding protein 1
AATF	*apoptosis-antagonizing factor*
ACTH	adrenocorticotrophic hormone
ADH	alcohol dehydrogenase
ADH1	*alcohol dehydrogenase 1A*
AI	allelic imbalance
ALDH1	aldehyde dehydrogenase 1
BER	base-excision repair
BHD	Birt-Hogg-Dudé syndrome
BRCA1/2	*Breast cancer1/2 gene*
CAPB	*prostate and brain cancer susceptibility locus*
CDK4	*cyclin-dependent kinase 4*
CDKN1A	*cyclin-dependent kinase inhibitor 1A*
cDNA	complementary deoxyribonucleic acid
CDP	*CCAAT displacement protein gene*
CHML	*CHM-like*
CS	Cushing syndrome
CYP17	*cytochrome P450c17*
DHPLC	denaturing high-performance liquid chromatography
DIRC2/3	*disrupted in renal carcinoma 2/3*
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DPT	dermatopontin
eEF2	eukaryotic elongation factor 2
eIF2α	translation eukaryotic initiation factor-2-alpha
EXO1	*exonuclease 1*
FH	*fumarase, fumarate hydratase*
FHD	FH deficiency
FHIT	*fragile histidine triad*
FISH	fluorescence *in situ* hybridization
FLCN	*folliculin*
FMM	familial malignant melanoma
GLUT1/3	glucose transporter 1/3
HIF	hypoxia-inducible factor
HIF1α	hypoxia-inducible factor 1 alpha
HLRCC	hereditary leiomyomatosis and renal cell cancer
HMG	high mobility group
HMGA1	*high mobility group AT-hook 1*
HMGA2	*high mobility group AT-hook 1*
HPC1	*first putative hereditary prostate cancer locus*
HPC20	hereditary prostate cancer locus at chromosome 20q13
HPCX	hereditary prostate cancer X linked locus
HPGL	hereditary head and neck paraganglioma
HPRC	hereditary papillary renal cell carcinoma
HPRCA	hereditary prostate cancer
HSPBAP1	heat-shock 27-kd protein-associated protein 1
HUS	Hospital District of Helsinki and Uusimaa
IGF1/2	*insulin growth factor 1 or 2*
IGFBP	IGF binding protein
KDH	ketoglutarate dehydrogenase
KIT	*v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog*
KMO	*kynurenine 3-monooxygenase*
K-RAS	*Harvey sarcoma virus homolog, Kirsten type*
LOD	logarithm of odds
LOH	loss of heterozygosity

LSAMP	limbic system-associated membrane protein
MCUL	multiple cutaneous and uterine leiomyomata
MDH	malate dehydrogenase
MDM2	*TP53-binding protein gene*
MEN2	multiple endocrine neoplasia type II
MET	*met protooncogene*
MMAD	massive macronodular adrenocortical disease
MMR	mismatch repair
mRNA	messenger ribonucleic acid
MSI	microsatellite instability
mTOR	mammalian target for rapamycin
NADP+	nicotinamide adenine dinucleotide
NER	nucleotide-excision repair
NIX	Nucleid Acid Identification Program
NORE1	RAS associated domain family protein 5
NPL	non-parametric LOD
OMIM	Online Mendelian Inheritance in Man
OPN3	*opsin 3*
$p70^{S6K}$	$p70^{S6K}$ kinase
PCAP	predisposing for prostate cancer
PCAP	*putative predisposing gene for cancer of prostate*
PCR	polymerase chain reaction
PHD	HIF1α prolyl hydroxylase
RB1	*retinoblastoma*
RB1	*retinoblastoma*
RCC	renal cell cancer
RET	*Rearranged during transfection protooncogene*
RGS7	*regulator of G protein signaling 7*
RNA	ribonucleic acid
RNASEL	r*ibonucleaseL*
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SDH	succinate dehydrogenase
SDHA	*succinate dehydrogenase A*
SDHB	*succinate dehydrogenase B*
SDHC	*succinate dehydrogenase C*
SDHD	*succinate dehydrogenase D*
SRD5A	*5α reductase type II* gene
SSCP	single-stranded conformational polymorphism
TCAC	tricarboxylic acid cycle (Krebs cycle)
TGFB	transforming growth factor beta
TK	*transketolase*
TP53	*tumor protein 53*
TRC8	*translocation in renal carcinoma on chromosome 8*
tRNA	total ribonucleic acid
TSC	tuberous sclerosis complex
TSC	tuberous sclerosis complex (syndrome)
TSC1	*tuberous sclerosis complex-2*
TSC2	*tuberous sclerosis complex-2*
TSP1	*trombospondin 1*
U	units
ULMS	uterine leiomyosarcoma
UTR	untranslated region
VEGF	*vascular endothelial growth factor*
VHL	von Hippel-Lindau syndrome
VHL	*von Hippel-Lindau syndrome gene*

ABSTRACT

Studies in hereditary cancer syndromes have been crucial in the localization and identification of many cancer genes involved in both familial and sporadic cancers. Detection of genetic alterations in cancer susceptibility genes has provided implements for genetic testing and follow-up of individuals with increased cancer risk. Understanding of the molecular mechanism of tumorigenesis has provided tools for the development of better treatment and targeted drugs for cancer.

In this study, a predisposing gene for a recently characterized cancer syndrome, hereditary leiomyomatosis and renal cell cancer (HLRCC), was identified and the role of the gene was investigated in other familial cancers and in nonsyndromic tumorigenesis. HLRCC is a dominantly inherited disorder predisposing predominantly to uterine and skin leiomyomas, and also to renal cell cancer and uterine leiomyosarcoma. The disease gene was recently localized in Finnish families to 1q42-q43 by a genome-wide linkage search. Independently in the UK, a clinically similar condition, multiple cutaneous and uterine leiomyomata (MCUL), was linked to the same chromosomal region, strongly suggesting that HLRCC and MCUL are actually a single syndrome. Linkage results were confirmed by detecting loss of heterozygosity (LOH) at the disease locus in most of the patients' tumors, suggesting that this predisposing gene acts as a tumor suppressor. Through detailed investigation by genotyping of microsatellite markers and haplotype construction in Finnish and UK HLRCC/MCUL families we were able to narrow the disease locus down to 1.6 Mb. Extensive mutation screening of known and predicted transcripts in the target region resulted in identification of the HLRCC predisposing gene, *fumarase* (*fumarate hydratase*, *FH*). FH is a key enzyme in energy metabolism, catalyzing fumarate to malate in the tricarboxylic acid cycle (TCAC) in mitochondria. Germline alterations in *FH* segregating with the disease were detected in 25 of 42 HLRCC/MCUL families including whole-gene deletions, truncating small deletions/insertions and nonsense mutations, as well as substitutions or deletions of highly conserved amino acids. Biallelic inactivation was detected in almost all studied tumors of HLRCC patients. Furthermore, FH enzyme activity was reduced in the patients' normal tissues and was completely or virtually absent from tumors. Based on these findings, we extensively demonstrated that mutations in *FH* underlie the HLRCC/MCUL syndrome. In our studies of other familial cancers, evidence for involvement of *FH* defects was not found in familial prostate and breast cancers.

To investigate the role of FH in sporadic tumorigenesis, we analyzed 652 lesions, including a series of 353 nonsyndromic counterparts of tumor types associated with HLRCC. Mutations in nonsyndromic tumors were rare and appeared to be limited to tumor types observed in the hereditary form of the disease. Biallelic inactivation of FH was detected in a uterine leiomyosarcoma, a cutaneous leiomyoma, a soft-tissue sarcoma, and in two uterine leiomyomas. In the uterine leiomyosarcoma and the cutaneous lesion *FH* mutations originated from the germline whereas the soft-tissue sarcoma harbored purely somatic changes. In uterine leiomyomas somatic mutations were detected in the two out of five tumors with LOH at the *FH* locus. Our findings demonstrate that FH

inactivation is also involved in nonhereditary tumor development, and further support the hypothesis that FH acts as a tumor suppressor. The role of FH in predisposition to malignancies, renal cell carcinoma and leiomyosarcoma is important in the diagnosis and prevention of cancer among HLRCC patients. This study is of general clinical interest, because prior to our findings, little was known about the molecular genetics of uterine leiomyomas, the most common tumors of women. Discovery of FH and other TCAC defects in tumor predisposition adds to our knowledge of the contribution of basic metabolism to tumor pathogenesis.

REVIEW OF THE LITERATURE

1. Genes and cancer

Cancer is a genetic disease. A malignant tumor develops through accumulation of genetic changes in proliferating cells (Fearon and Vogelstein 1990). These changes include activation of oncogenes, dysfunction of stability genes (caretakers), or inactivation of tumor suppressor genes (Vogelstein and Kinzler 2004). Although multiple changes are required for malignant transformation, only a limited number of molecular pathways contribute to most cancers (Hahn and Weinberg 2002).

The major knowledge of cancer genes is still based on rare, high penetrance alleles which have been identified using genetic linkage and positional cloning. Although the contribution of these genes in sporadic lesions has been extensively evaluated, the molecular events in common cancer have remained unclear.

1.2. Oncogenes

Oncogenes are excessively activated counterparts of protooncogenes, which are involved in normal cellular signaling. In cancer, protooncogenes are activated through chromosomal amplification, rearrangements, or activating point mutations, leading to excessive cell proliferation or aberrant differentiation (Bishop 1991).

Most of oncogene alterations occur at the somatic level and are common in many tumor types (Tumor Gene Database, http://condor.bcm.tmc.edu/oncogene.html). RAS genes are the most frequently mutated oncogenes in human cancer cells (Giehl 2005). Aberrant activation of RAS proteins is involved in practically all processes of malignant transformation, such as gene expression, cell cycle progression, apoptosis, exocytosis, aberrant angiogenesis, tissue invasion, and metastasis (Symons and Takai 2001, Campbell and Der 2004). RAS proteins are members of a large superfamily of over 100 small GTP-binding proteins, commonly classified into six subfamilies (Takai *et al.* 2001). In human tumors, the majority of RAS mutations occur in the *Kirsten type Harvey sarcoma virus homolog* (*K-RAS)* gene (Giehl 2005). Mutational *K-RAS* activation is involved in most of pancreatic adenocarcinomas and in third of lung and colorectal cancers (Almoguera *et al.* 1988, Rodenhuis *et al.* 1988, Bos *et al.* 1987).

Inherited oncogene mutations are rare compared to somatic alterations. *Rearranged during transfection protooncogene (RET), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT), met protooncogene (MET*), and *cyclin-dependent kinase 4 (CDK4)* are probably the only known high-penetrance oncogenes mutated in familial cancers. Multiple endocrine neoplasia type II (MEN2) is characterized by medullary thyroid carcinoma and pheochromocytoma (Schimke and Hartmann 1965). A predisposing germline mutation, and subsequently additional somatic changes, were found

in the *RET* protooncogene of MEN2 families (Mulligan *et al.* 1993, Quadro *et al.* 2001). Mutations in the *KIT* gene can be found either somatically or in the germline, underlying gastrointestinal stromal tumor predisposition (Hirota *et al.* 1998, Nishida *et al.* 1998). *MET* mutations were first found in the germline of affected members of hereditary papillary renal cell carcinoma (HPRC) families, and subsequently also in sporadic cases (see section 3.2.3) (Schmidt *et al.* 1997). CDK4 is included in protein complexes controlling passage through the G1 checkpoint of the cell cycle (Sherr 1993). Germline mutations in *CDK4* have been detected, though very rarely, in familial malignant melanoma (FMM) (Zuo *et al.* 1996).

1.3. Stability genes

Stability genes, also referred to as caretakers, promote tumorigenesis in a totally different way than do oncogenes and tumor suppressors. Stability genes keep genetic alterations at a minimal level, and when inactivated mutations in other genes occur at higher levels, whereas tumor suppressors and oncogenes accelerate neoplastic growth by increasing tumor cell number through stimulation of cell proliferation or the inhibition of apoptosis or cell-cycle arrest (Friedberg 2003, Vogelstein and Kinzler 2004). Although potentially all genes are targets of increased genomic instability, only somatic mutations in oncogenes, tumor suppressor genes, or other stability genes are critical for tumor development (Akiyama *et al.* 1997).

Stability genes include mismatch repair (MMR), nucleotide-excision repair (NER), and base-excision repair (BER) genes responsible for repairing normal DNA replication errors (Friedberg 2003). Failures in these systems can lead to accumulation of somatic mutations in cells and, accordingly, increased predisposition to severe conditions. Germline mutations in the MMR genes, *MLH1, MSH2, PMS2*, and *MSH6* have been found to predispose to HNPCC (Fishel *et al.* 1993, Leach *et al.* 1993, Bronner *et al.* 1994, Papadopoulos *et al.* 1994). Because simple repetitive sequences are particularly prone to replication errors, microsatellite instability (MSI) can be used as a landmark in HNPCC (Ionov *et al.* 1993). Other stability genes control chromosomal stability, through such processes as mitotic recombination and chromosomal segregation. Mutations in, for example, *BRCA1* and *BRCA2* predispose to familial breast cancer (Miki *et al.* 1994, Wooster *et al.* 1995).

1.4. Tumor suppressor genes and loss of heterozygosity (LOH)

Most hereditary cancer syndromes are caused by mutations in tumor suppressor genes. These genes are needed to keep cells under control (Marshall 1991). Compared to sporadic cases, patients with germline mutations have a higher risk of developing secondary malignancies, and are prone to get cancer at a younger age. Tumor suppressor genes are

typically involved in the transduction of extra cellular signals, regulation of transcription, or controlling the cell cycle (Smith *et al.* 1998).

Several tumor suppressor genes predisposing to hereditary cancer syndromes have been shown to be a key element also in nonsyndromic cancers (Bishop 1991, Soussi *et al.* 2005). Many of these genes have been found to be mutated and involved in cancer progression at a somatic level. Tumor protein TP53 is one of the best examples of these functions. The *TP53* gene is inactivated in approximately 50% of human cancers, in most cases only in somatic cells (Harris 1996, Soussi *et al.* 2005, Soussi and Lozano 2005). Germline mutations in *TP53* predispose to Li-Fraumeni syndrome, but have also been reported in many sporadic cancers (Malkin *et al.* 1990, Camplejohn and Rutherford 2001).

The main evidence for the presence of tumor suppressor genes was provided by a study by Knudson (1971), where he suggested that two events were required for retinoblastoma development. In hereditary cases, one mutation has occurred in the germline and is inherited. The second mutation has taken place at the somatic level and is typically a deletion of a larger chromosomal region containing the respective gene. Such chromosomal events result in homozygosity of the region, i.e. loss of heterozygosity (LOH) (Figure 1). These two genetic events in the Knudson's model were afterwards shown to represent biallelic inactivation of a tumor suppressor gene, human *retinoblastoma* (*RB*) (Lee *et al.* 1987).

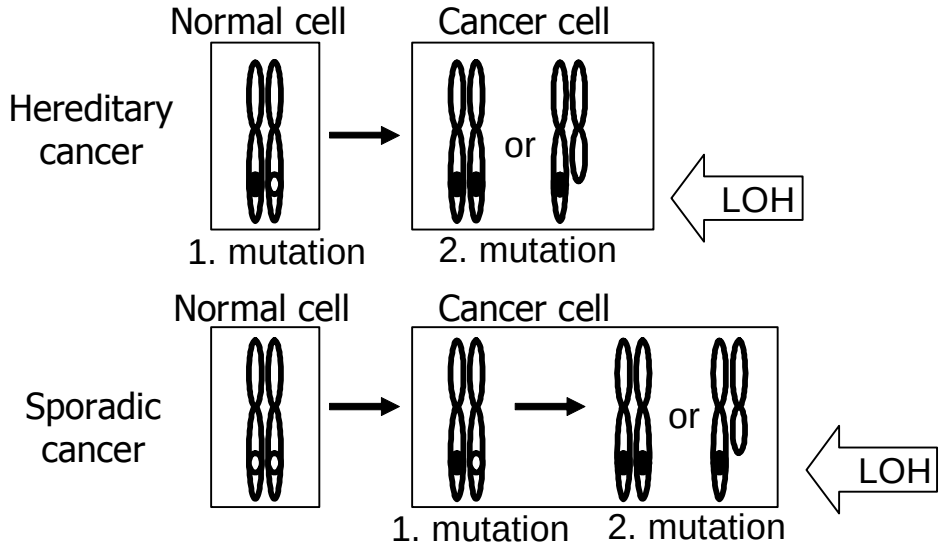

Figure 1. *Loss of heterozygosity (LOH) in hereditary and sporadic cancer. Typically, two events are needed to inactive a tumor suppressor gene. In hereditary cancers, the first mutation is inherited in all cells. In a tumor cell of the target tissue a loss of a wild type allele through deletion of the chromosomal material leads to inactivation of a possible tumor suppressor gene. The s hit can also be a point mutation in another allele. In sporadic cancers, both events take place in a somatic cancer cell.*

Inactivation of *VHL*, the second example of classical tumor suppressor genes, has been shown to predispose to von Hippel-Lindau syndrome (VHL, OMIM 193300) (Latif *et al.* 1993, Crossey *et al.* 1994). VHL is a dominantly inherited familial cancer syndrome predisposing to a variety of malignant and benign neoplasms, most frequently renal cell, retinal, and spinal hemangioblastoma carcinoma, pheochromocytoma, and pancreatic tumors (see section 3.2.1.) (Neumann and Wiestler 1991).

2. Uterine leiomyomas

Uterine leiomyomas (fibroids) are the most common tumors in women, being present in at least 25% of women (Buttram 1986). Most myomas are symptomless leading to possible underestimation of the prevalence. Careful pathological examination with new imaging techniques suggests that the prevalence is as high as 77% (Cramer and Patel 1990). Despite their benign nature, uterine fibroids are a significant health issue. Although most myomas are symptomless, many women suffer from abdominal pain, abnormal bleeding, and reproductive dysfunction (Stewart 2001). Uterine myomata is the leading cause for hysterectomy in many countries.

Myomas arise from the smooth muscle cells of the uterus. The pathophysiology of myomas is not well understood. It is known that myomas follow life-cycle changes of the reproductive hormones estrogen and progesterone (Rein 2000). It has been proposed that risk factors associated with uterine leiomyomas, such as early menarche, obesity, diet, and smoking act by regulating these hormones (Flake *et al.* 2003). The most common pathway to myoma development includes the transformation of normal myocytes into abnormal ones (Cramer and Patel 1990). Mosaicism is a frequent finding in uterine leiomyomas with chromosomal rearrangements (Xing *et al.* 1997, Gross *et al.* 2004).

Little is known about the molecular background of these lesions. Mutation analyses of candidate genes such as *TP53, P53-binding protein* gene *(MDM2), K-RAS*, and *CCAAT displacement protein (CDP)* have been negative ((Hall *et al.* 1997, Patrikis *et al.* 2003). Other than activating *K-RAS* mutations reported in one study, no specific point mutations underlying nonsyndromic leiomyomas have been identified. However, the contribution of chromosomal rearrangements to the development of these lesions has long been evident (Pandis *et al.* 1991, Hall *et al.* 1997).

Nonrandom chromosomal abnormalities have been observed in 40 to 50% of surgically removed uterine leiomyomas (Flake *et al.* 2003). The most common cytogenetic changes are translocations between chromosomes 12 and 14 (20%), deletions and translocations in chromosome 7q (17%), trisomy of chromosome 12, and a wide variety of aberrations of 6p21 (< 5%) (Meloni *et al.* 1992, Ozisik *et al.* 1993, Sargent *et al.* 1994, Ishwad *et al.* 1995, Sornberger *et al.* 1999, Ligon and Morton 2000). Cytogenetic abnormalities similar to fibroids have also been observed in other mesenchymal tumors, such as lipomas and hamartomas (Dal Cin *et al.* 1997, Tallini *et al.* 1997, Xiao *et al.* 1997). Rearrangements in

chromosomes 12 and 14 typically include the translocation t(12;14)(q15;q23-q24), involving a chromosomal segment of a gene encoding a member of the high mobility group proteins, *HMGA2* (Pandis *et al.* 1990, Mine *et al.* 2001). Altered gene expression, the aberrant splicing of *HMGA2*, or cytogenetic translocation leading to chimeric fusion of *HMGA2* and a target gene are frequent events in uterine leiomyomas (Tallini and Dal Cin 1999). *HMGA2* and the DNA repair gene *RAD51L1* have been hypothesized to create pathologically significant fusion transcripts (Takahashi *et al.* 2001). Although *HMGA2-RAD51L1* fusion transcripts or their incorrectly spliced products have been detected, the pattern of rearrangements suggests that dysregulated expression of *HMGA2*, not the formation of fusion transcripts, is the principle mechanism in the development of uterine leiomyomas (Quade *et al.* 2003). Elevated expression of the *high mobility group AT-hook 1 (HMGA1)* gene has also been observed in uterine leiomyomas. The dysregulating mechanism may be similar to *HMGA2* including post-translational modifications or cytogenetic rearrangements at the *HMGA1* locus on 6p21 (Sornberger *et al.* 1999).

Consistent with cytogenetic studies, molecular genetic analyses have confirmed the loss of genetic material on 7q21-q32, suggesting the localization of a tumor suppressor gene important for myoma development in this region (Ishwad *et al.* 1997, Mao *et al.* 1999). In a genome wide allelotyping effort, LOH was observed on 7q22-q23 (9%), 1q42-q42 (2%), and 16q12-q22 (2%) (Mao *et al.* 1999). Although the minimal commonly deleted region on 7q22 has been recently reduced to 2.3 Mb, no causative gene to uterine leiomyoma predisposition has been successfully identified in this region (Vanharanta *et al.* 2005).

The animal model for human tuberous sclerosis, the Eker rat, shows additional evidence that HMGA2 plays a key role in human uterine leiomyomata (Everitt *et al.* 1995). In the majority of Eker rat myomas HMGA2 protein is expressed, while in the normal myometrium HMGA2 is completely lost (Hunter *et al.* 2002). Eker rats are heterozygous for mutations in the *tuberous sclerosis complex-2 (TSC2)* tumor suppressor gene, segregating uterine leiomyomas and leiomyosarcomas, renal cell carcinomas (RCC), and hemangiosarcomas of the spleen (see section 3.2.4.) (Hino *et al.* 1999, Cook and Walker 2004).

Genome wide gene expression studies provide new clues to pathways contributing to the pathogenesis of uterine leiomyomas. A comparative analysis of nine gene expression studies revealed common genes aberrantly expressed in myomas (Arslan *et al.* 2005). Under-expression of *alcohol dehydrogenase 1A (ADH1)* and *aldehyde dehydrogenase 1 (ALDH1)* in uterine leiomyoma compared to normal myometrium could potentially link HMGA1/2 related chromosomal aberrations to the expression data. The HMG protein competitively binds to the ADH class I *HNF-1/HMG1* promoter, affecting *ADH1* gene transcription (Edenberg 2000). Arslan *et al.* (2005) concluded that there is a co-requirement of increased estrogens and retinoid acid that promote tumor growth. Up-regulation of insulin growth factor 2 (IGF2) and down-regulation of IGF binding proteins (IGFBP) have been the most regular observation in myoma expression studies. IGF2 is known to be involved in cell proliferation and differentiation. Furthermore, extra cellular matrix formation through transforming growth factor beta (TGFB) and dermatopontin (DPT) signaling has been suggested to play an important role in uterine myoma formation.

2.1. Genetic predisposition to leiomyomas

The hereditary form of uterine leiomyoma is normally discussed in the context of multiple cutaneous and uterine leiomyomatosis syndrome (MCUL). The first report on the hereditary form of cutaneous leiomyomatosis was based on an Italian family with three affected half first cousins. (Kloepfer *et al.* 1958). A cutaneous leiomyoma family with uterine myomata was described by Mezzadra in 1965 (Mezzadra 1965). Autosomal dominant inheritance with decreased penetrance was supported by these pedigrees. The inherited disorder, characterized by the appearance of uterine leiomyoma and/or multiple cutaneous leiomyoma, was called Reed's syndrome (Reed *et al.* 1973). Other family studies have further defined the role of the hereditable component in myomata. A study of 97 leiomyomata families showed that the prevalence of uterine fibroids is 2.2-fold higher in families with two or more affected individuals than in controls (Vikhlyaeva *et al.* 1995). Furthermore, in twin-pair studies, the hysterectomy rate in monozygotic twins was 2-fold higher than in dizygotic twins (Treloar *et al.* 1992). An average estimate of familial risk in the first degree relatives of affected probands is approximately 25%, and heritability according to recurrence rate in siblings is 0.26-0.80 (Kurbanova *et al.* 1989a, Kurbanova *et al.* 1989b, Snieder *et al.* 1998, Luoto *et al.* 2000). These findings of a significant genetic factor in uterine myomata predisposition are compatible with the observation of 2-3-fold higher incidence of fibroids in African-American than in Caucasian women (Kjerulff *et al.* 1993, Marshall *et al.* 1997). Clinical and molecular features in familial uterine myomas are different from their sporadic counterparts (Okolo *et al.* 2005). Significantly higher incidence of abdominal swelling, dysmenorrhoea, dyspareunia, and early onset of disease in cases with family history of fibroids compared to those without have been reported. Additionally, patients with a family history of leiomyomas more often have various cancers, mainly in breast, stomach, and oesophagus (Okolo *et al.* 2005).

3. Renal cell cancer (RCC)

Kidney cancer accounts for approximately 2% of all cancer cases worldwide (McLaughlin and Lipworth 2000). Benign papillary adenomas are the most common lesions of the kidney (Van Poppel *et al.* 2000). Renal oncocytoma comprises about 3 to 5% of renal neoplasms in surgical series (Kovacs *et al.* 1997). The third subclass of benign neoplasms includes metaphoric adenomas and adenofibromas. The malignant tumors are almost exclusively renal cell carcinomas originating from the epithelium or collecting tubules of the kidney. Based on morphological features, RCC can be divided into clear cell (conventional, non-papillary), papillary (chromophil), chromophobe, collecting duct, and unclassified subtypes (Kovacs *et al.* 1997, Storkel *et al.* 1997).

Clear cell carcinoma accounts for 70% of malignant renal neoplasms (Storkel *et al.* 1997). The great majority of these tumors are comprised predominantly of cells with clear

cytoplasm. The growth pattern may be solid, trebecular, tubular, or cystic with a characteristic highly branching vascularity pattern (Reuter and Presti 2000).

Papillary renal carcinoma is the second most common malignancy of the renal tubular epithelium and accounts for approximately 10% of cases (Kovacs *et al.* 1997). Papillary RCC is typically divided into two morphologically distinct subgroups (Delahunt and Eble 1997). Papillary type I tumors consist of papillae and tubular structures covered by small cells with pale cytoplasm and characterized by small oval nuclei with inconspicuous nucleoli, frequent glomeruloid papillae, and foamy macrophages in papillary cores. Type 2 tumors consist of papillae covered by large cells with voluminous eosinophilic cytoplasm and characterized by pseudostratified and large nuclei with prominent nucleoli, glomeruloid papillae, and edematous papillae. Type 2 tumors are larger and occur more commonly at younger ages than type I tumors (Delahunt and Eble 1997).

3.1. Familial renal cell cancer (RCC) syndromes

3.1.1. von Hippel-Lindau disease (VHL)

Clear cell carcinomas, pheochromocytomas, retinal hemangioblastomas, and central nervous system tumors are typical tumors in von Hippel-Lindau disease (VHL) (Maher *et al.* 1990). Familial aggregation of retinal angiomas and central nervous system hemangiolipomas was reported by Hippel and Lindau in the beginning of the 20th century (Table 1) (von Hippel 1904, Lindau 1927).

The *VHL* gene was mapped by linkage analysis to chromosome 3p25 in 1988 and identified five years later (Seizinger *et al.* 1988, Latif *et al.* 1993). A chromosomal loss on 3p was first found in sporadic renal cell carcinomas and subsequently in hereditary cases suggesting that one or more tumor suppressor genes relevant in RCC could be located in this region (Zbar *et al.* 1987, Tory *et al.* 1989, Kovacs *et al.* 1991).

In later reports, it has been shown that tumor or cyst development in VHL syndrome is linked to somatic inactivation, through a point mutation or hypermethylation of the promoter region, or loss of the remaining wild type allele of the *VHL* gene (Crossey *et al.* 1994, Herman *et al.* 1994, Shuin *et al.* 1994, Chen *et al.* 1995, Prowse *et al.* 1997). *VHL* consists of three exons and is widely expressed in both fetal and adult tissues (Iliopoulos *et al.* 1995, Richards *et al.* 1996).

More than 300 different *VHL* mutations have been included in the Human Gene Mutation Database so far. The large amount of mutation data has made reasonable genotype-phenotype comparisons possible (Stenson *et al.* 2003). In VHL patients approximately 20 to 30% of *VHL* mutations are whole or partial gene deletions, and 23 to 27% are nonsense or frameshift type of changes (Zbar *et al.* 1996, Stolle *et al.* 1998).

***Table 1**. Most common familial cancer syndromes associated with renal cell cancer (RCC).*

Syndrome	Gene, locus	Clinical Manifestation	Animal Model
VHL von Hippel-Lindau	***VHL*** (3p25) Tumor suppressor	Clear cell carcinoma Pheochromocytoma Retinal hemangiolipoma Central nervous system tumors	**Mouse:** Hepatic hemangioma Angiogenesis
HPRC Hereditary papillary renal cell cancer	***MET*** (7q31-q34) Protooncogene	Papillary renal carcinoma	**Mouse:** Metastasized mammary tumors
TSC Tuberous sclerosis complex	***TSC1*** (1q34) and **TSC2** (16p13) Tumor suppressor	Polycystic disease Oncocytomas RCC (mostly clear cell) Skin lesions Central nervous system tumors Mental disorders	**Rat (Eker rat):** RCC Uterine leiomyomas **Mouse:** RCC Liver hemangiomas
BHD Birt-Hogg-Dude syndrome	***BHD*** (17p11.2) Tumor suppressor	Benign skin tumors Pneumothorax, lung cysts RCC (mostly chromophobe/oncocytic)	**Rat (Nihon rat):** RCC **Dog (German Shepherd dog):** RCC Uterine leiomyomas Skin nodules
HLRCC/MCUL Hereditary leiomyomatosis and renal cell cancer/ Multiple cutaneous and uterine leiomyomata	Study I (1q42-q43)	Uterine and/or skin leiomyomas RCC (mostly papillary type 2) Uterine leiomyosarcoma	No animal models

These mutations are characteristic for subtype 1 VHL with high risk of renal cell carcinoma and absence of pheochromocytoma, whereas families at high risk of developing pheochromocytoma (type 2) harbor almost exclusively *VHL* missense mutations (Zbar *et al.* 1996, Chen *et al.* 1996, Maher *et al.* 1996).

Biallelic VHL inactivation is common in both sporadic hemangioblastomas and sporadic clear cell renal carcinomas. *VHL* is somatically mutated in approximately 33 to 57%, and hypermethylated in up to 20% of sporadic RCCs, and is restricted to the clear cell subtype of tumors (Foster *et al.* 1994, Gnarra *et al.* 1994, Herman *et al.* 1994, Shuin *et al.* 1994, Whaley *et al.* 1994) Similarly, *VHL* mutations have been detected in 30 to 50% of sporadic hemangioblastomas, but rarely in sporadic pheochromocytomas (Hofstra *et al.* 1996, Bender *et al.* 2000, Dannenberg *et al.* 2003). Studies examining a variety of other sporadic tumors have shown that somatic *VHL* mutations are rare in tumor types that are not observed in the VHL syndrome (Whaley *et al.* 1994, Foster *et al.* 1995).

The VHL protein is part of an ubiquitin ligase complex that degrades hypoxia-inducible factor 1 alpha (HIF1α) in the presence of oxygen. In low oxygen conditions or when *VHL* is mutated, stabilization of HIF1α leads to angiogenesis, increased proliferation and decreased apoptosis (Carmeliet *et al.* 1998, Maxwell *et al.* 1999). Angiogenesis, in many cases particularly through HIF1α regulation, has been suggested to be involved directly or indirectly in most oncogenic or tumor suppressor pathways (Vogelstein and Kinzler 2004). In mice, targeted inactivation of Vhl has been shown to cause accelerated angiogenesis in many organs and vascular tumors in liver (Table 1) (Haase *et al.* 2001, Ma *et al.* 2003).

3.1.2. Chromosome 3 translocations

In a subset of families the occurrence of RCCs co-segregates with the presence of constitutional chromosome 3 translocations. Such phenomena can be employed to identify novel candidate genes at or near the translocation break-points for hereditary RCC and other cancers. Although co-occurrence of somatic *VHL* mutations with balanced translocations has been reported in the majority of cases, not all the familial RCCs have been shown to carry a *VHL* change (Bonne *et al.* 2004).

A family with 10 RCC cases with a chromosome 3 balanced translocation was published by Cohen et al. in 1979 (Cohen *et al.* 1979). This cytogenetic rearrangement t(3;8)(p14;q24) produced a fusion of *fragile histidine triad (FHIT)* and *translocation in renal carcinoma on chromosome 8 (TRC8)* genes (Ohta *et al.* 1996, Gemmill *et al.* 1998). Interestingly, in Drosophila TRC8 was shown to directly interact with VHL and inhibit growth when over-expressed (Gemmill *et al.* 2002). However, evidence that *FHIT* is causally related to renal or other malignancies has remained controversial (Gemmill *et al.* 1998).

Two other break-point spanning genes, *disrupted in renal carcinoma 2 and 3* (*DIRC2* and *DIRC3*) have been localized based on a constitutional translocation t(2;3)(q35;q21) resulting in the formation of a DIRC3-HSPBAP1 fusion transcript (Bodmer *et al.* 2002, Bodmer *et al.* 2003). Heat-shock 27-kd protein-associated protein 1 (HSPBAP1) has been found to interact with HSP27, one of the small heat shock proteins, which are suggested to play essential role in stress responses and cancer (Bodmer *et al.* 2003, Mosser and Morimoto 2004). Interestingly, both of these fusion products are transcribed from the derivative chromosome that is lost during tumor development (Gemmill *et al.* 1998, Bodmer *et al.* 2003).

In addition to *FHIT* and *HSPAP1*, some other translocation-associated genes, such as *LSAMP* coding limbic system-associated membrane protein and *NORE1* coding RAS associated domain family protein 5, have been proposed to play a role in cellular stress signaling and thereby probably increase the susceptibility to somatic loss of the translocation derivative 3 chromosomes (Chen *et al.* 2003). At present, many translocation-associated genes potentially contributing to tumorigenesis have not been identified.

Multiple evidence, including familial and non-familial cases, has shown that chromosome 3 translocations are a risk factor for RCC (Kovacs *et al.* 1989, Schmidt *et al.* 1995, Bodmer *et al.* 1998, Gemmill *et al.* 1998, Koolen *et al.* 1998, Kanayama *et al.* 2001, Bodmer *et al.* 2002, Melendez *et al.* 2003, Van Erp *et al.* 2003, Rodriguez-Perales *et al.* 2004). In conjunction with allele segregation analysis, frequent loss of derivative chromosome 3 has been detected. In addition, novel inactivating mutations, including different mutations from the same patient's tumors, have been found in *VHL* so far (Schmidt *et al.* 1995, Bodmer *et al.* 1998, Kanayama *et al.* 2001). Based on these findings, a three-step model of RCC development has been proposed: 1) non-homologous chromatid exchange resulting in balanced chromosome 3 translocation, 2) loss of derivative chromosome 3 containing the p arm segment, and 3) somatic mutations in *VHL* or potentially also in other genes in the region (Bodmer *et al.* 2002a, Bodmer *et al.* 2002b, Pavlovich and Schmidt 2004). However, novel combinatorial molecular and cytogenetic analyses reveal complex genetic alterations in non-papillary RCC (Strefford *et al.* 2005).

3.1.3. Hereditary papillary renal carcinoma (HRPC)

Hereditary papillary renal carcinoma (HPRC) is a rare dominantly inherited cancer syndrome with reduced penetrance (Zbar *et al.* 1995). Malignant papillary renal carcinomas were first characterized by trisomy of chromosomes 7, 16, or 17 and, in men, by loss of the Y chromosome (Kovacs 1993). Later, the predisposing gene was mapped to 7q31-q34, and germline mutations in a tyrosine kinase domain of *MET* protooncogene were identified in affected members of HPRC families and in a subset of sporadic papillary renal carcinomas (Table 1) (Schmidt *et al.* 1997). Overexpression and duplication of the *MET* oncogene was suggested to be crucial step in the pathogenic pathway of papillary renal tumors (Fischer *et al.* 1998). *MET* mutants also exhibited increased levels of tyrosine phosphorylation and enhanced kinase activity when compared with wild-type MET (Jeffers *et al.* 1997). Invasive and metastatic properties of MET mutants were demonstrated as well in cell lines as in mice (Giordano *et al.* 1997, Jeffers *et al.* 1998). In transgenic mice, activated Met induces predominantly metastatic mammary adenocarcinoma (Liang *et al.* 1996, Jeffers *et al.* 1998).

The *MET* protooncogene encodes a transmembrane tyrosine kinase receptor for a hepatocyte growth factor, and drives a cellular program known as invasive growth. Aberrant invasive growth is known to be associated with invasion, metastasis, and neoplastic transformation of cells (Park *et al.* 1987, Bottaro *et al.* 1991, Comoglio and Trusolino 2002). The oncogenic potential of MET, especially through overexpression of the protein, has been reported in numerous human cancers (Di Renzo *et al.* 1991, Natali *et al.* 1996). The overexpression of the MET receptor in hypoxia is probably activated by hypoxia inducible transcription factors HIF1α and AP-1. Recently, molecular genetic evidence linking MET oncogenic activity and homeostasis has been reported (Boccaccio *et al.* 2005).

3.1.4. Tuberous sclerosis complex (TSC)

Tuberous sclerosis complex (TSC) is an autosomal dominant multi-system disorder with characterized by seizures, mental retardation, autism, epilepsy, and tumors in the brain, retina, heart, kidney, and skin (Table 1) (Roach *et al.* 1998). In adults, complications arising from renal, lung, heart, and brain pathology are the most significant cause of death (Shepherd *et al.* 1991). Epithelial renal manifestations of TSC include polycystic disease, oncocytomas, and RCC (Bjornsson *et al.* 1996, Henske 2005). Angiolipomas originate from mesenchymal cells, and are the most common renal lesion in TSC. Most angiolipomas are benign, although they are responsible for the most severe clinical symptoms because of their abnormal vascularity which can lead to spontaneous life-threatening bleeding (Iliopoulos and Eng 2000). A specific feature in TSC, different from for example VHL and MET related renal carcinomas, is the substantial heterogeneity in renal manifestations, including clear cell, papillary, and chromophobe carcinomas (Bjornsson *et al.* 1996, Al-Saleem *et al.* 1998).

The majority of TCS patients exhibit cutaneous signs or lesions (Webb *et al.* 1996, Sun *et al.* 2005). Although 80% of the most prevalent skin lesions, hypomelanotic macules and forehead fibrous plaques are non-sympomatic, they are important in the early diagnosis of the disease. The rest of the skin manifestations are symptomatic and need clinical treatment (Webb *et al.* 1996).

Two genetic loci of *TSC* have been identified: *TSC1*, which maps to 9q34, and *TSC2*, which maps to 16p13.3 (Consortium 1993, van Slegtenhorst *et al.* 1997). Large mutation analysis studies have addressed the differences between phenotypes in TSC1 and TSC2 (Jones *et al.* 1999). Mutations of *TSC2* occur more frequently and their clinical symptoms in central nervous system and renal manifestations are more severe than of those *TSC1*. The TSC genes undergo LOH in the majority of lesions, strongly suggesting their role as tumor suppressors (Green *et al.* 1994a, Green *et al.* 1994b).

The Eker rat is the first autosomal dominant inherited rodent model for RCC (Eker *et al.* 1981). In addition to renal manifestations, Eker rats also develop leiomyomas and leiomyosarcomas of the uterus, mostly from smooth muscle of the cervix (Everitt *et al.* 1995). The predisposing defect underlying this phenotype was shown to be a mutation in the T*sc2* tumor suppressor gene, which was inactivated by a large inherited deletion and LOH in the T*sc2* locus (Yeung *et al.* 1994, Kobayashi *et al.* 1995).

In contrast to human TCS patients, Eker rats develop multifocal, bilateral RCC with 100% incidence (Table 1) (Everitt *et al.* 1992). Although these tumors differ from the clear cell type renal lesions frequently observed in human TSC, they share many similarities, like tubular cell origin and high level of vascularization, with their human counterparts.

Eker rat leiomyomas are histologically similar to their human counterparts, and occur at the high frequency of over 60% in females (Everitt *et al.* 1995). Eker leiomyomas have been shown to exhibit a 50% incidence of loss of the wild-type *Tsc2* allele and an almost uniform loss of tuberin protein expression, implicating loss of function of the *Tsc2* gene in these tumors (Hunter *et al.* 2002). Similarly to humans, hormonal factors play an

important role in the development of leiomyomas of Eker rats. Eker leiomyomas and cell lines derived from them express both estrogen and progesterone receptors and respond to estrogen stimulation (Howe *et al.* 1995a, Howe *et al.* 1995b).

Conventional *Tsc1* and *Tsc2* knock-out mice have also been generated to further understand the molecular mechanisms of TSC (Kobayashi *et al.* 1999, Onda *et al.* 1999, Kobayashi *et al.* 2001). Heterozygous mice developed renal and liver tumors whereas homozygous animals died during the embryonic stage. The finding that *Tsc2* heterozygous mice develop renal tumors at younger age than *Tsc1* animals is concordant with the observations that patients with *TSC2* mutations have more severe disease compared to *TSC1* mutation carriers (Dabora *et al.* 2001).

Genetic and biochemical analyses have pointed out that the proteins hamartin and tuberin, the products of the *TSC1* and *TSC2* genes, respectively, form a heterodimer in intact cells (van Slegtenhorst *et al.* 1998). Pathological mutations in TSC disrupt this interaction, demonstrating that hamartin and tuberin act as a complex in tumor suppression (Hodges *et al.* 2001). Genetic studies in mammalian systems and Drosophila have shown that the hamartin/tuberin complex functions in inhibition of cell growth and cellular proliferation by downregulating the mTOR (mammalian target for rapamycin) signaling network (Hino *et al.* 2001, Potter *et al.* 2001, Tapon *et al.* 2001, Kwiatkowski 2003). mTOR controls a large and diverge set of growth-related signaling pathways, including activation of translation initiation in response to amino acids (i.e. nutrients) and growth factors, actin cytoskeleton organization, membrane traffic and protein degradation, protein kinace C signaling, ribosome biogenesis, and transcription (reviewed by Schmelzle and Hall 2000).

3.1.5. Birt-Hogg-Dudé syndrome (BHD)

Birt-Hogg-Dudé syndrome (BHD) is an autosomal dominant disease characterized by benign skin tumors, hair follicle hamartomas, and is associated with the development of pneumothorax, lung cysts, and renal tumors (Table 1) (Birt *et al.* 1977, Toro *et al.* 1999, Schmidt *et al.* 2001). A spectrum of different types of renal tumors are detected in BHD patients including chromophobe, oncocytoma, clear cell, and papillary type lesions, the most prevalent being chromophobe/ oncocytoma hybrid (Pavlovich *et al.* 2002). Renal manifestations are usually bilateral and have been shown to arise in 15-30% of BHD patients. Protein-truncating mutations were recently found in a novel gene called *folliculin* (*FLCN*) encoding a highly conserved protein with a frequency of 44% in BHD families (Nickerson *et al.* 2002).

While no uterine leiomyomas have been reported in human BHD patients, a canine model for BHD develops skin lesions, uterine leiomyoma, and RCC histologically similar to human BHD (Moe and Lium 1997). A disease locus was mapped to a small region on canine chromosome 5, and associated mutations were later found in the homologue of the human *BHD* gene (Jonasdottir *et al.* 2000, Lingaas *et al.* 2003).

Moreover, the Nihon rat, a rodent model for BHD, displays a dominantly inherited predisposition to RCC tumors with 100% incidence, predominantly with clear cell

histology (Okimoto *et al.* 2000). Nihon rat RCC susceptibility is associated with a truncating insertion in the rat *BHD* gene, and LOH of the wild type allele in tumors (Okimoto *et al.* 2004).

3.1.6. Hereditary leiomyomatosis and renal cell cancer (HLRCC)

The association of cutaneous leiomyomas, uterine leiomyosarcoma, and metastasized renal cell cancer was described for the first time in 1973, although no cancer syndrome behind it was proposed (Reed *et al.* 1973). The HLRCC syndrome was described in 2001 (Launonen *et al. 2001*). The tumor predisposition in HLRCC was suggested to be dominantly inherited including susceptibility to cutaneous and uterine leiomyomas with high penetrance, and leiomyosarcoma and renal cell cancer with reduced penetrance (Table 1). Three reported Finnish HLRCC families included 26 affected individuals (Kiuru *et al.* 2001, Launonen *et al.* 2001). All except one male exhibited skin lesions (83%), cutaneous nodules or leiomyomas, and 14 out of 20 (70%) females were diagnosed with uterine leiomyomas. In addition to typical lesions, four patients have been reported to have leiomyomas with atypia (Kiuru *et al.* 2001, Launonen *et al.* 2001). Two cases with uterine leiomyosarcoma were identified; one of them had also been diagnosed with breast cancer. The most common malignant lesion was renal cell cancer, which was diagnosed in seven (27%) HLRCC family members at a relatively young age (33 to 48 years).

To prove that HLRCC was a new cancer syndrome, Launonen *et al.* (2001) excluded the possibility of already known cancer genes as the cause of the disease. VHL and tuberous TSC were excluded because of the lack of characteristic renal and extra-renal features in HLRCC. Especially crucial was the morphological picture of the renal cancers in HLRCC because it was unique, displaying rare clear cell type 2 histology (Kiuru *et al.* 2001, Launonen *et al.* 2001). In type 2 renal cell carcinoma cytoplasm is abundant, nuclei are large, and very large inclusion-like nucleoli can be seen. In contrast to HLRCC, papillary renal carcinomas with *MET* mutations display type 1 histology (Lubensky *et al.* 1999). In addition, the *MET* region in chromosome 7q was excluded by linkage analysis on this locus with a logarithm of odds (LOD) score of < -2 (Launonen *et al.* 2001).

Launonen *et al.* (2001) performed a genome wide linkage analysis in the two Finnish HLRCC families and succeeded in localizing the predisposing gene to chromosome 1q42-q44 (Launonen *et al.* 2001). The telomeric region of the long arm of chromosome 1 was the only region showing suggestive evidence of linkage, and after an analysis with additional markers in the target region, the highest LOD score of 4.11 was achieved at microsatellite marker D1S2811. Additional evidence of the linkage in 1q42-q44 was obtained from the analysis of the third Finnish HLRCC family (Kiuru *et al.* 2001).

LOH in tumors has been widely used as a predictor of location and function of tumor suppressor genes (Thiagalingam *et al.* 2002). Finnish researchers studied five renal cell cancers, three uterine leiomyomas, and seven cutaneos leiomyomas from HLRCC family members, and detected LOH at 1q42-q44 in all tumors except one renal cancer (Kiuru *et al.* 2001, Launonen *et al.* 2001). In order to evaluate the specificity of the observed

chromosomal losses, 26 nonsyndromic uterine leiomyomas and 7 nonsyndromic cutaneous leiomyomas were analyzed for LOH in the HLRCC locus with informative microsatellite markers. Losses of the wild-type chromosome were detected only in one of these tumors, confirming that LOH seen in HLRCC lesions is specific for the disease (Kiuru *et al.* 2001). According to linkage, haplotype, and LOH data, the HLRCC predisposing locus was mapped to an approximately 14 cM region between markers D1S517 and D1S404 (Kiuru *et al.* 2001). The localization of a tumor suppressor gene underlying the HLRCC syndrome in this region was strongly suggested.

3.1.7. Multiple cutaneous and uterine leiomyomata (MCUL)

At the same time, but independently from the Finnish studies of the HLRCC locus, a genome-wide screen on 11 UK families with Multiple cutaneous and uterine leiomyomata (MCUL) found suggestive evidence of linkage (a maximum two-point parametric LOD score 1.84 at marker D1S157) to the q telomere of chromosome 1 (Alam *et al.* 2001). The families consisted of 54 affected individuals, most with multiple myomas of varying size. The initial genome screen detected a maximum LOD score of 1.84 at marker D1S547 in 1q42.3-q43.8 regions with two-point analysis. With additional typing of microsatellite markers in this region, a multipoint LOD score of 5.4 was obtained between markers D1S2785 and D1S547/D1S404. After haplotype analysis, Alam *et al.* (2001) placed the predisposing gene for MCUL within a 14 cM interval between markers D1S517 and D1S2842. The loss of the wild type allele at the MCUL locus in two analyzed leiomyomas led UK researchers to argue that the predisposing gene is a tumor suppressor.

Evidence that the gene underlying MCUL syndrome is dominantly inherited with high penetrance was additionally provided. Of 33 females with the disease-associated haplotype, 29 (88%) had skin and/or uterine leiomyomas. Of 16 males with the disease haplotype, 13 had developed skin leiomyomata (Alam *et al.* 2001).

4. Other familial cancers potentially associated to hereditary leiomyomatosis and renal cell cancer (HLRCC)

4.1. Familial prostate cancer

Hereditary prostate cancer (HPRCA) is a genetically complex disease involving multiple susceptibility genes and a strong environmental component. Segregation analyses in high-risk prostate cancer families support a model of autosomal dominant inheritance with multiple rare high penetrance genes, particularly in early onset subgroups (Gronberg *et al.* 1997, Schaid *et al.* 1998). One of the potential HPRCA loci has been mapped to

chromosome 1q42.2-q43, named the putative predisposing gene for cancer of prostate (*PCAP*), spanning a 20 cM region (Berthon *et al.* 1998, Berry *et al.* 2000). The highest multipoint LOD score of 3.31 at the *PCAP* locus at marker D1S2785 was detected in the subset of early onset prostate cancer families (Berthon *et al.* 1998). LOH in prostate tumors at 1q42.2-43 further supported the linkage evidence and indicated that the causative gene may act as a tumor suppressor (Berthon *et al.* 1998). Linkage to the *PCAP* locus has remained unconfirmed in some data sets (Gibbs *et al.* 1999, Whittemore *et al.* 1999). These and other studies propose a heterogeneous background for HPRCA. Linkage analyses have indicated that several other chromosomal regions may be involved in inherited prostate cancer: a first putative hereditary prostate cancer locus (*HPC1*) at 1q24-q25, prostate and brain cancer susceptibility locus (*CAPB*) at 1p36, hereditary prostate cancer X linked locus (*HPCX*) at Xq27-q28, hereditary prostate cancer locus at chromosome 20q13 (*HPC20)*, and a putative prostate cancer gene *HPC2/ELAC2* at 17p (Smith *et al.* 1996, Xu *et al.* 1998, Gibbs *et al.* 1999, Berry *et al.* 2000, Schleutker *et al.* 2000, Tavtigian *et al.* 2001).

A few genes have been reported to be mutated in HPRCA, none of them representing a high penetrance major susceptibility gene: *ELAC2*, r*ibonucleaseL* (*RNASEL*) at the *HPC1* locus, *5α reductase type II* gene (*SRD5A2*), and *cytochrome P450c17* (*CYP17*) (Lunn *et al.* 1999, Jaffe *et al.* 2000, Rokman *et al.* 2001, Xu *et al.* 2001, Carpten *et al.* 2002, Rennert *et al.* 2002, Rokman *et al.* 2002, Wang *et al.* 2002).

4.2. Familial breast cancer

The susceptibility genes for familial breast cancer are still largely unknown. The major known high-penetrance predisposition genes *BRCA1* and *BRCA2* account for a majority of families with multiple cases of early-onset breast cancer and ovarian cancer, but only a small fraction of familial breast cancer without these characteristics (Vahteristo *et al.* 2001, Ford *et al.* 1998). Genetic linkage studies have suggested breast cancer loci on chromosomes 2q32, 6q25, 8p21, and 13q22 (Kainu *et al.* 2000, Huusko *et al.* 2004, Seitz *et al.* 1997, Zuppan *et al.* 1991). So far, the putative susceptibility genes in these regions have not been identified.

The rationale for linking breast cancer to HLRCC is that three Finnish patients in two HLRCC families had been diagnosed with breast cancer (Kiuru *et al.* 2001, Launonen *et al.* 2001). The first breast cancer detected in an HLRCC patient displayed lobular histology, a histology which comprises only 15% of unselected breast carcinomas (Launonen *et al.* 2001).

AIMS OF THE STUDY

1. To localize and identify the gene predisposing to the HLRCC syndrome
2. To investigate the role of the HLRCC predisposition gene in prostate and breast cancer
3. To investigate the role of the HLRCC gene mutations in nonsyndromic tumor types associated with HLRCC
4. To investigate the role of the gene underlying HLRCC in non-HLRCC associated nonsyndromic tumorigenesis

MATERIALS AND METHODS

1. Familial samples

1.1. HLRCC/ MCUL Families (I)

Three Finnish HLRCC families were included in the gene localization, identification and mutation detection study. The biggest Finnish family, FAM-1, included four individuals with early-onset RCC and 17 individuals with leiomyomas of the uterus and/or skin (Launonen *et al.* 2001). The second family, FAM-II, included two siblings diagnosed with renal cell carcinoma at the age of 32 and 26 and one individual with uterine leiomyomas at the age of 41 (Launonen *et al.* 2001). The third family included seven individuals with multiple cutaneous myomatosis, three individuals with uterine leiomyomatosis and one individual with renal cell cancer (Kiuru *et al.* 2001). Additionally, 39 UK families displaying a typical MCL phenotype with leiomyomata of the skin and uterus were included in the study through collaboration (Alam *et al.* 2001). DNA from 42 probands, from 22 families, including 85 affected individuals was used in fine-mapping of the predisposing gene. Informed consent was obtained from the patients. This study was approved by the authorized ethics review committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa (HUS).

1.2. Finnish prostate cancer families (II)

Finnish prostate cancer family samples were available from University of Tampere through collaboration. The sample collection has been described previously by Schleutker *et al.* (2000). Altogether 70 blood samples for DNA isolation from the youngest affected subjects, whose samples were available, of each of the 70 Finnish families were obtained for mutation analysis. At least one inclusion criterion had to be fulfilled: (1) prostate cancer in three generations, (2) three or more first degree relatives with prostate cancer, or (3) two subjects with prostate cancer diagnosed under the age of 60. The average age of diagnosis was 62.2 years (range 44-79) and the number of affected subjects per family was 3.2 (range 2-6). Two of the patients had kidney cancer, but their leiomyoma status was unknown. No 1q42.2-43 linkage data were available.

1.3. US prostate cancer families (II)

A total of 152 prostate cancer families including 522 genotyped men were previously ascertained through the Mayo Clinic radical prostatectomy database (Gibbs *et al.* 1999). The research protocol and informed consent forms were approved by the Mayo Clinic Institutional Review Board. Nineteen prostate cancer families for *fumarase* (*FH)* mutation screening were selected on the basis of positive linkage information over *PCAP* locus. Two individuals from each family were screened for *FH* mutations. The average age of onset in the families was 64.89 years (range 54.75-69.67 years) and the average number of affected subjects in each family was 3.36 (range 2-6). Within these 38 individuals, kidney cancer was observed in six different kindreds in nine subjects altogether. Four of these cases also had prostate cancer. The average age at diagnosis of the kidney cancer cases was 58.55 years.

1.4. Breast cancer families (III)

DNA samples extracted from blood from 85 *breast cancers genes 1* or *2* (*BRCA1* or *BRCA2)* mutation-negative breast cancer patients were included in this study. Samples were collected at the Helsinki University Central Hospital (Vehmanen *et al.* 1997, Eerola *et al.* 2000, Vahteristo *et al.* 2001a, Vahteristo *et al.* 2001b). 75 cases were selected based on family or personal history of renal cell cancer, sarcoma, uterine cancer (including uterine sarcoma), lobular breast cancer, prostate cancer, or multiple myeloma. 26 of these families had one such malignancy, 32 families had two, 12 families had three, and five families had four or more such cancers. In addition, 10 breast cancer patients not fulfilling the above-defined cancer history were included in this study because the experiments were performed on a 96-well plate format and space allowed inclusion of these samples. These 10 patients had at least one first-degree relative affected with breast or other cancer, or were themselves affected with a second cancer.

2. Control individuals (I, IV)

To investigate the population frequency of a putative Finnish founder mutation (I) and an *FH* allele detected in the germline of a uterine leiomyoma patient (IV) 448 and 134 blood samples from anonymous control individuals, respectively, were obtained from the Finnish Red Cross, Helsinki. Over 150 population based control samples were used to investigate the population frequency of detected UK *FH* mutations (I).

3. Tumor samples

3.1 Tumors from HLRCC family members (I)

Tumors from HLRCC/MCL family members were analyzed for allelic loss in 1q42.3-q43. Paraffin-embedded tissue blocks, hematoxylin/eosin-stained slides, and pathologists' gross reports were obtained for histopathological analyses from the Departments of Pathology at Oulu University Hospital, Helsinki University Central Hospital, and Turku University Hospital (Kiuru *et al.* 2001, Launonen *et al.* 2001). Tumors included seven renal cell carcinomas, seven uterine leiomyomas, seven cutaneous leiomyomas, and one uterine leiomyosarcoma. Additionally, these tumors were analyzed for LOH or somatic mutations in the *FH* gene.

3.2. Nonsyndromic tumor samples (IV, V)

In the study IV, 200 nonsyndromic tumors from 194 individuals with no known family history of cancer were studied for *FH* mutations. The study focusing on tumor types observed in HLRCC syndrome (IV) included 10 cutaneous leiomyomas, 41 uterine leiomyomas 52 renal cell carcinomas, 18 uterine leiomyosarcomas, 35 other sarcomas, 29 prostate carcinomas, and 15 lobular breast carcinomas. The histological subtypes of renal cell cancers included 9 papillary type tumors, 40 clear cell carcinomas, and three other tumor types. Five of the renal cell carcinomas displayed the rare papillary type II histology associated with HLRCC. Sarcomas included 2 extra-uterine leiomyosarcomas, 9 liposarcomas, 5 histiocytomas, 4 osteosarcomas, 2 chondrosarcomas, 1 fibrosarcoma, 1 Edwing's sarcoma, and 11 sarcomas of undefined histology.

In the study V evaluating the role of FH in nonsyndromic uterine leiomyomas, 153 lesions from 46 individuals were first analyzed for allelic loss at the *FH* locus, and when displaying LOH, screened for *FH* mutations (V).

To further evaluate the role of *FH* inactivation in nonsyndromic tumorigenesis (V) a series of 299 malignant tumors representing 10 different malignant tumor types were analyzed for *FH* mutations. Samples included 52 papillary thyroid carcinomas, 60 ovarian tumors, 44 nonlobular breast carcinomas, 14 testicular carcinomas of germ cell origin, 34 lung carcinomas, 23 colorectal cancers, 15 cutaneous melanomas, 18 adrenal pheochromocytomas, 25 glioblastomas, and 14 head and neck squamous cell carcinomas.

4. DNA and RNA extraction, purification, and cDNA synthesis (I, II, III, IV, V)

DNA from blood samples, cell lines, and fresh-frozen patient tissue samples was extracted according to the nonenzymatic method of Lahiri and Nurnberger (1991). DNA extraction from paraffin-embedded tissue samples was performed using protocol described by Kannio *et al.* (1996). The product was purified using the NucleoSpin PCR purification kit (Macherey-Nagel, Duren, Germany). Total cellular RNA (tRNA) was extracted using the RNeasy kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The extracted total RNA (tRNA) was used for cDNA synthesis in a reverse transcriptase polymerase chain reaction (RT-PCR) according to the standard protocol (Promega, Madison, WI, USA).

5. Microsatellite marker analysis (I, IV, V)

5.1. Fine-mapping at chromosome 1q

To map more finely the gene for HLRCC/MCUL, we obtained DNA from 42 individuals, including 22 linkage informative families. In addition to microsatellite repeats from public databases, we identified 28 microsatellite repeats at the HLRCC locus to attempt to restrict the target region (Table 2). Repeats were identified by text and Basic Local Alignment Search Tool (BLAST http://www.ncbi.nlm.nih.gov/BLAST/) searches of the draft genome BAC clone sequences (Sanger Center Institute, 2001). Primers for PCR were designed using the Primer3 server (http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi) (Rozen and Skaletsky 2000). PCR amplicons were run on an Applied Biosystems PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and fragment sizes analyzed by the Genescan 3.1 and Genotyper 2.5.2 software (Applied Biosystems). The most likely haplotypes assuming a minimum number of recombinations were manually constructed in the target region.

5.2. Loss of heterozygosity (LOH) analysis (I, IV, V)

LOH analysis with 40 microsatellite markers, 12 from the commercial set (ABI medium density Linkage Mapping Set-MD10 (Applied Biosystems)) and 28 novel repeats, was performed on the available tumors from the HLRCC families to narrow down the target region of the predisposing gene to HLRCC (I) (Table 2).

To study LOH in nonsyndromic uterine leiomyomas (V), genomic sequence covering the *FH* locus and the flanking sequence derived from the Ensemble Human Genome Browser

(http://www.ensembl.org) was scanned for novel microsatellite repeats using the Tandem Repeats Finder software (Benson 1999). We identified two microsatellite repeats, flanking the *FH* locus. The markers FH-C and FH-T were located 2.8 Mb centromeric and 0.3 Mb telomeric respectively from the *FH* locus. Primers for PCR for the repeats were as follows: FH-C (centromeric) forward primer 5'-AGCAATGATGGTTTCTCTCTCA-3'; FH-C reverse primer 5'-CAGCACTAGCAGAATATGTGTAA-3'; FH-T (telomeric) forward primer 5'-CCTTACCATTGCTCCCAAGA-3'; FH-T reverse primer 5'-ACCTTCATCCCTGTCCTGTG-3'. PCR amplicons were run on an Applied Biosystems PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and fragment sizes analyzed by the Genescan 3.1 and Genotyper 2.5.2 software (Applied Biosystems). LOH was visually estimated from ABI PRISM chromatographs.

***Table 2**. Summary of the methods used in this study.*

Special Aims	**Method**	**Reference**
Fine-mapping of a target region/regions and localization of candidate susceptibility genes	Additional genotyping of linked families/additional families with microsatellite markers LOH[1] in tumors	Study I
Identification of the susceptibility gene and causative mutations	Mutation detection with sequencing FISH[2] LOH and somatic mutations in tumors	Study I
Evaluation of population frequencies of detected changes	Sequencing DHPLC[3] SSCP[4]	Study I Study IV
Functional consequences of the detected mutations	Enzyme activity assay	Study I
Evaluation of the role of the predisposing gene in other familial cancers	Mutation detection with sequencing and DHPLC LOH analysis in tumors of other familial cancers	Study II Study III
Evaluation of the role of the predisposing gene in sporadic tumorigenesis	Mutation detection by sequencing and DHPLC LOH analysis in nonsyndromic tumors	Study IV Study V

[1] Loss of heterozygosity
[2] Fluorescence in situ hybridization
[3] Denaturing high-performance liquid chromatograph
[4] Single-stranded conformational polymorphism

6. Agarose gel electrophoresis (I, II, III, IV, V)

Specificity and intensity of all PCRs were verified using gel electrophoresis. 4 µl of a PCR product was mixed with 2 µl of loading buffer (95% of formamide, 20 mM of EDTA, 0.05% of xylene cyanol, and 0.05% of bromophenol blue). The mixture was run on a 2.5% SeaKem LE Agarose gel (Cambrex Bio Science Rocland Inc., Rockland, ME, USA) in 1 x TBE buffer at 150 V for approximately 30 min. The bands were visualized under ultra violet light with ethidium bromide.

7. Identification of transcripts on 1q42-q43 (I)

Sequences used in gene predictions were derived from the chromosome 1 physical map published by the Sanger Center Institute. The nucleid acid identification program called NIX, provided by the UK Human Genome Mapping Project Resource Center (UK), was used to identify ESTs and known putative novel and predicted genes in the target region. NIX is a genome annotation package which runs multiple gene prediction and comparative sequence analyses programs simulataneously (Jones *et al.* 2002). Target sequences were analyzed on both directions in overlapping segments of 20-500 kb. Exon-intron structures of the candidate sequences were extracted from NIX results web pages and verified by Blast search. Additionally, the NCBI Entrez Genome Map Viewer (http://www.ncbi.nlm.nih.gov/entrez/) and Ensembl Human Genome Server were used to find known genes in the target region. Most of the predicted transcripts were verified by RT-PCR amplification of human total mRNA (RNeasy kit, Qiagen).

8. Fluorescence *in situ* hybridization (FISH) (I)

Due to non-mendelian inheritance detected in two UK families and observations that RCC exists only in the Finnish HLRCC families, fluorescence *in situ* hybridization analysis (FISH) was carried out to detect putative large germline deletions or rearrangements at the HLRCC locus (Table 2). Metaphase spreads of lymphoblast cell lines from probands from two Finnish families and two UK families were prepared by standard protocols. BAC clones (from RP11-467120 to RP11-211A7 from the Sanger Center map) in the target region were fluorescently labaled with Fluoro Green, Fluoro Red, and Cy5sUTP by nick translation (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and hybridized overnight to metaphase spreads under competitive conditions. Slides were counterstained with 4,6-diamidino-2-phenylindole. Images were displayed using a cooled charge-coupled camera (Photometrics, Huntington Beach, CA, USA) attached to Axioplan 2 Imaging Microscope and SmartCapture 2 software (Applied Imaging, San Jose, CA, USA). This analysis was carried out in the laboratory of Dr. Ian Tomlinson.

9. Mutation detection

9.1. Single-stranded conformational polymorphism (SSCP) (I)

SSCP analysis was used to evaluate the Finnish population frequency of a putative Finnish *FH* founder mutation using DNA samples from 448 anonymous blood donors using MDE solution (FMC BioProducts) (Table 2). PCR products were run on 0.6xMDE gels, at 4W for 20 hours in 0.6xTBE running buffer. SSCP gels were silver-stained according to standard protocols.

9.2. Denaturing high-performance liquid chromatograph (DHPLC) (II, III, IV, V)

FH mutation screening by DHPLC was performed on 38 US familial prostate cancer patients from 19 prostate cancer families (II), 85 Finnish breast cancer patients from 85 breast cancer families (III), 267 malignant tumors from 267 Finnish patients (V), and 5 uterine leiomyomas from 5 unrelated individuals displaying LOH in their myomas at the *FH* locus (V) (Table 2). DHPLC was also used to examine the population frequency of a *FH* change detected in the germline of a patient with uterine leiomyosarcoma (IV). DNA from healthy 134 controls was included.

All 10 *FH* exons and the flanking intronic sequences were amplified in 50 µl reaction volumes consisting of 50 ng genomic DNA, 0.7xPlatinum PCR Buffer (Invitrogen, Carlsbad, CA, USA), 200 µM of each dNTP (Finnzymes, Espoo, Finland), 0.3 µM of both primers, and 1.25 U of DNA polymerase Platinum Taq (Invitrogen), 0.60 U of Titanium Taq (Clontech, Palo Alto, CA, USA), and 0.60 U of AmpliTaq Gold (Applied Biosystems). The mixture of the three enzymes was used to obtain high fidelity, sensitivity, and specificity. The hot start PCR cycling conditions were as follows: 94°C for 12 min, followed by 35 cycles of denaturing for 30 s, varying annealing temperatures for 30 s, elongation at 72°C for 45 s, and final extension at 72°C for 10 min. The denaturing temperature was lowered from 94°C to 89°C after 10 cycles. Reactions were carried out in a PTC-200 Peltier Thermal Cycler (MJ Research).

Samples from two subjects were pooled before they were denatured at 95°C for three min and reannealed by reducing the temperature 0.5°C/30 s for 45 min. DHPLC heteroduplex analyses were performed using an automated HPLC instrumentation with an Agilent 2G experimental dsDNA 2.1x75 mm 3.5-micron column (Agilent Technologies, Palo Alto, CA, USA). Samples from pools displaying aberrant chromatographs were reanalyzed individually.

The melting temperatures for each amplicon were obtained using the algorithm at the Stanford DHPLC Melt program web page (http://insertion.stanford.edu/melt1.html). The optimal melting temperatures were experimentally evaluated according to the protocol

described by Xiao and Oefner (2001) and tested with known polymorphisms. The analytical acetonitrile gradient was composed by mixing Helix BufferPak A for DHPLC and 55-75% B (Varian Analytical Instruments, Walnut Creek, CA, USA) at a flow rate of 0.4 ml/min. Single-base substitutions and small insertions or deletions can be detected with DHPLC by different retention times of homo- and heteroduplex double-stranded PCR amplicons under partial denaturation. The sensitivity and specificity of DHPLC have been shown to vary between 94% and 100% when compared to direct sequencing (Xiao and Oefner 2001).

9.3. DNA and cDNA sequencing (I, II, III, IV, V)

To identify the predisposing gene, mutation screening of 6 known genes, *regulator of G protein signaling 7* (*RGS7), FH, kynurenine 3-monooxygenase (KMO), opsin 3 (OPN3), CHM-like* (*CHML),* and *exonuclease 1* (*EXO1*), and 41 other candidate sequences, including predicted genes and ESTs, was performed. Exons and the flanking intronic sequences were analyzed by genomic sequencing of genomic DNA of probands from three Finnish and 39 UK families (I) (Table 2).

Genomic sequencing was used in 70 Finnish prostate cancer families (II), and in 14 sporadic testicular tumors in a study of sporadic counterparts of tumor types observed in HLRCC (IV), Sequencing was used also for specifying and confirming the changes detected by DHPLC (II, III, IV, V).

Sequencing was carried out in the sequencing core facility at the University of Helsinki, Haartman Institute (Helsinki, Finland). PCR mixtures and conditions were the same as in the DHPLC analysis (see section 8.2.). PCR products were purified using the NucleoSpin PCR purification kit (Matcherey-Nagel, Duren, Germany) according to the manufacturer's instructions. Direct sequencing of the PCR products was performed using the BigDye3 termination chemistry (Applied Biosystems) and an ABI 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions, and the sequences were analyzed with the Sequencing Analysis 3.0 software (Applied Biosystems).

Pheochromocytomas were screened by sequencing of cDNA, because only the corresponding RNA was available (IV). Primers for the 5' fragment of *FH* were CTCCCTCAGCACCATGTACC (forward) and CCACTTTTGCAGCAACCTTT (reverse); for 3' fragment CTTGGGCAGGAATTTAGTGG (forward) and GCAGTTTCCTTTCAAACTTATCC (reverse). PCR reactions were performed in a 50 µl reaction volume containing 200 ng cDNA, 1xPCR buffer (Applied Biosystems), 300 µmol/L each dNTP (Finnzymes), 1.25 of µmol/L of both primers, and 2.5 U of AmpliTaqGOLD polymerase (Applied Biosystems). $MgCl_2$ concentrations were 2.8 mmol/L for the 5' fragment and 1.4 mmol/L for the 3' fragment. The following cycling conditions were used for the 5' fragment: 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 56°C for 1 minute, and elongation at 72°C for 1 minute, and final extension at 72°C for 10 minutes. Equivalent conditions for

the 3' fragment were used despite the annealing temperature, which was decreased from 60°C to 57°C at 0.5°C per cycle, and from 57°C to 56°C after five cycles.

The corresponding cDNA of leiomyoma P4M3 was analyzed to detect a splicing defect on the mRNA level (V). Primers for cDNA amplification were as follows: forward primer, TGGTATGGCAGACTGGATCA; reverse primer, CACCACGCAGTTTTCTGTA. PCR reactions and conditions were slightly different from those for pheochromocytoma 5' fragment amplification in $MgCl_2$ concentration (4.2 mmol/L), annealing temperature (57°C), and number of denaturing/annealing/elongation cycles. In order to separate differentially-spliced mRNA variants, P4M3 and the corresponding normal sample were run on a 1% agarose standard low melting gel (Bio-Rad Laboratories, Herculer, CA, USA) at 100 V for 60 min (V). The separated bands were cut from the gel. PCR products were purified using the QIAquick PCR purification kit 250 (Qiagen) according to the manufacturer's gel extraction protocol. The purified products were reamplified using the same protocol as in the original cDNA PCR to get sufficient amount of template for sequencing reactions. The sequencing protocol for cDNA was the same as that for genomic sequencing.

10. Fumarate hydratase enzyme activity assay (I)

In order to study the functional relevance of *FH* mutations, enzyme activity of FH was assayed in lymhoblastoid cell lines from affected individuals and spouse controls and in leiomyomata according to the protocol described by Hatch *et al.* (1978) (Table 2). The assay is based on coupling of malate production by FH to nicotinamide adenine dinucleotide (NADP+) reduction via the malic enzyme. The fumarase enzyme present in the cells/ tissue catalyzes the conversion of fumarate to malate. Reduction of NADP+ to NADPH occurs via the malic enzyme in the conversion of malate to pyruvate. The formation of NADPH is measured by absorbance detection. The assay starts with fumarate (final concentration 10 nM), lymphoblast sonicate, 25 mM HEPES-KOH pH 7.5, 0.27 nM NADP+, 4 mM $MgCl_2$, 5 mM potassium phosphate, and 0.2 units/ml of purified malic enzyme (EC $_{1.1.1.40}$, Sigma, St Louis, MO, USA). These measurements were carried out in the laboratory of Dr. Simon Olpin (Sheffield Children's Hospital, Sheffield, UK).

11. Splice site prediction (III)

The potential effect of intronic and silent changes to splicing was predicted by computational methods using NetGene2 splice site prediction web server (http://genome.cbs.dtu.dk/services/NetGene2/).

RESULTS

1. *Fumarase (FH)* is the predisposing gene in the hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome (I)

Because of similarities detected in phenotypes and overlapping linkage regions, it was evident that HLRCC and MCUL might actually be one and the same syndrome. In order to further narrow down the target region of the HLRCC/MCUL predisposing gene, additional genotyping in three Finnish and 19 UK families was performed. In addition to microsatellite markers in public databases, we identified 28 new polymorphic tandem repeats between markers D1S517 and D1S2842/D1S404, which was the minimal disease locus previously defined by Alam *et al*. (2001) and Launonen *et al.* (2001). Haplotype constructions pointed out critical recombinations in two large families, FAM-1 from Finland and family n:o 307 from the UK. The disease locus was placed on the 1.6 Mb region between clones BAC RP11-25B4 (centromeric border) and BAC RP11-553N16 (telomeric border). The target area could not been further narrowed down by LOH analysis of tumors.

The co-occurrence of leiomyomas and renal cell cancer in three Finnish families and non-mendelian inheritance in two UK families suggested the possibility of large germline deletions in the target region. Deletions of 2.4 Mb and 1.9 Mb were detected in two UK families (207 and 713, respectively) but not in any of the Finnish families in FISH analyses. The data matched closely the minimal region defined in microsatellite mapping.

In the minimal region, 6 known genes, 17 putative predicted new genes, and 42 ESTs were identified. Most of the predicted genes and ESTs (14 and 32, respectively) recognized had no homology to any known gene or protein. The known genes included *RGS7, FH, KMO, OPN3, CHML* and *EXO1*). The majority of the candidate sequences in the minimal region were analyzed for mutations, but putative pathogenic changes were found only in *fumarase (fumarate hydratase, FH*) located in the BAC clones RP11-409K12 and RP11-527D7 (Table 3). The *FH* gene encodes a mitochondrial FH protein which is a key enzyme in energy metabolism, catalyzing fumarate to malate in the Krebs cycle, i.e. the tricarboxylic acid cycle (TCAC) (Figure 2). The *FH* gene has been believed to additionally encode a shorter cytosolic form. The cytosolic isoform of FH is involved in the urea cycle and amino acid synthesis.

FH consists of 10 protein-coding exons, spanning over 22 kb in the human genomic sequence. The first exon encodes the mitochondrial signal peptide (named exon 0) of 44 amino acids, and the other nine the mature mitochondrial protein of 466 amino acids. The numbering of the exons, amino acid residues, and nucleotide positions presented in this study is based on the mature cytosolic isoform of *FH* (NM_000143.2) (Figure 3).

***Table 3**. Fumarase (FH) mutations detected in this study in hereditary leiomyomatosis and renal cell cancer (HLRCC)/multiple cutaneous and uterine leiomyomata (MCUL) (Study I) and in nonsyndromic counterparts of tumor types observed in HLRCC (Studies IV and V).*

Family or tissue	Origin	Mutation	Exon	Study
HLRCC[1] (UK)	Germline	Whole gene deletion	0-9	I
HLRCC (UK)	Germline	Whole gene deletion	0-9	I
HLRCC (UK)	Germline	Gln4Stop	1	I
Sarcoma of a limb, (FIN[2])	Somatic (+LOH[3])	Arg8Glu	1	IV
HLRCC (UK)	Germline	58delC	2	I
RCC[4], HLRCC FAM-2 (FIN)	Somatic (2nd hit)	121delTG	2	I
HLRCC (UK)	Germline	Arg58Stop	2	I
HLRCC (UK)	Germline	Arg58Stop	2	I
HLRCC (UK)	Germline	Arg58Stop	2	I
HLRCC (UK)	Germline	Asn64Thr	2	I
HLRCC (UK)	Germline	Asn64Thr	2	I
HLRCC (UK)	Germline	Asn64Thr	2	I
HLRCC (UK)	Germline	Asn64Thr	2	I
HLRCC (UK)	Germline	Asn64Thr	2	I
HLRCC (UK)	Germline	Asn64Thr	2	I
HLRCC (UK)	Germline	Ala74Pro	2	I
HLRCC (UK)	Germline	His137Arg	3	I
HLRCC (UK)	Germline	Gln142Arg	3	I
ULMS[5] (FIN)	Germline	His153Arg	4	I
HLRCC FAM-1 (FIN)	Germline	541delAG	4	I
HLRCC FAM-2 (FIN)	Germline	541delAG	4	I
Cutaneous leiomyoma (FIN)	Germline	541delAG	4	IV
HLRCC (UK)	Germline	Lys187Arg	4	I
HLRCC (UK)	Germline	Lys187del	4	I
HLRCC (UK)	Germline	Arg190His	4	I
HLRCC (UK)	Germline	IVS4-15T>G	4	I
Uterine leiomyoma (FIN)	Somatic (+LOH)	Ala196Thr	4	V
Uterine leiomyoma (FIN)	Somatic (+LOH)	IVS4 + 3A>G	4	V
HLRCC (UK)	Germline	Gly239Val	5	I
ULMS (FIN)	Somatic (2nd hit)	Leu240Stop	5	IV
RCC, HLRCC FAM-1 (FIN)	Somatic (2nd hit)	Met285Arg	6	I
HLRCC FAM-3 (FIN)	Germline	Arg300Stop	6	I
Cutaneous leiomyoma (FIN)	Somatic (2nd hit)	Arg300Stop	6	IV
HLRCC (UK)	Germline	1220delG	8	I

[1] Hereditary leiomyomatosis and renal cell cancer
[2] Finnish origin
[3] Loss of heterozygosity
[4] Renal cell cancer
[5] Uterine leiomyosarcoma

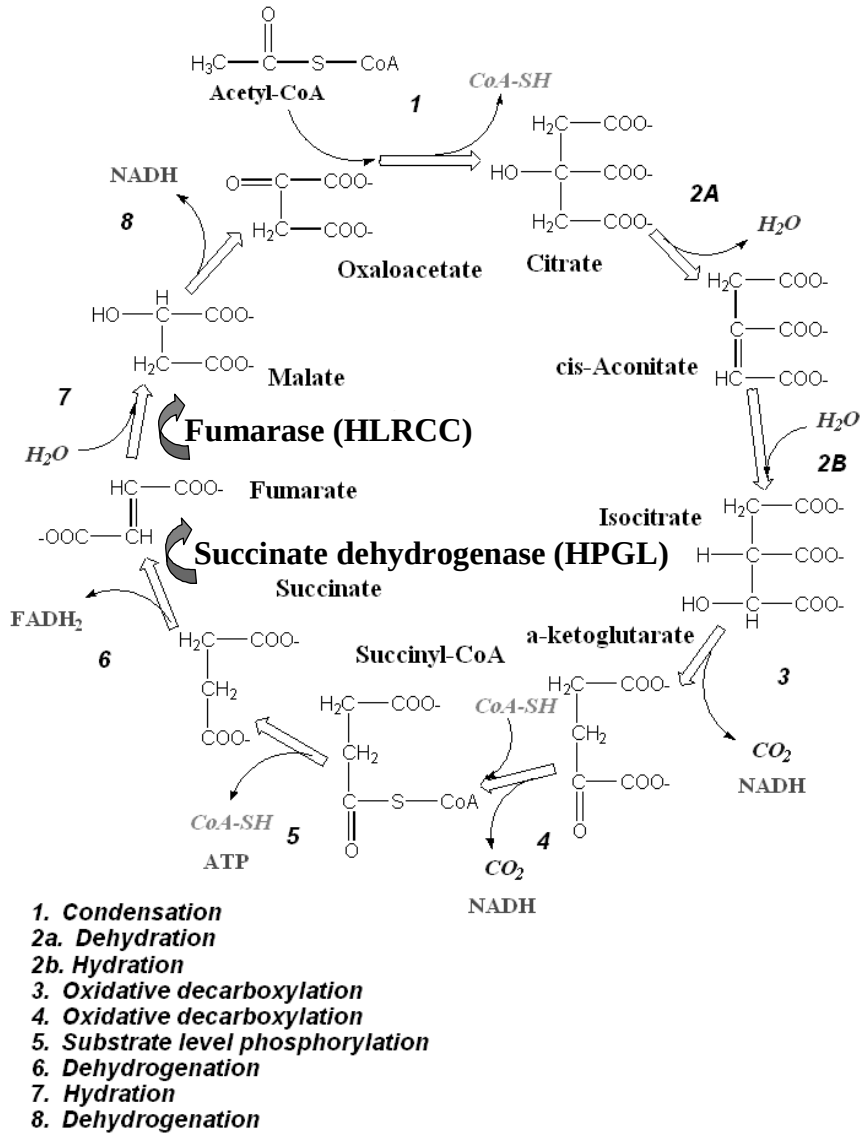

Figure 2*. Tricarboxylic acid cycle (TCAC) and tumorigenesis. TCAC is the final common route for the oxidation of amino acids, fatty acids, and carbohydrates. Dysfunction of two TCAC enzymes has been reported to be involved in human tumogenesis so far. Biallelic inactivation of fumarase (FH) has been observed in most of the tumors in hereditary leiomyomatosis and renal cell cancer (HLRCC). Defects in three of the four components of succinate dehydrogenase (SDH) complex have been identified to predispose to hereditary head and neck paraganglioma (HPGL) and pheochromocytoma.*

FH mutations were found in 25 individuals, covering all three Finnish families and 19 out of 39 UK families (Table 4). Sequence alterations were found in 7 out of 10 exons and included 10 protein truncating changes (nonsense and frameshift mutations), 2 large germline deletions, 12 single amino acid substitutions, and 1 whole gene deletion (Table 3). The missense mutations were targeted in highly conserved amino acids and none of the changes were reported as known polymorphisms in public databases nor were found in 150 healthy population controls.

Table 4. *Fumarase (FH) mutation positive hereditary leiomyomatosis and renal cell cancer (HLRCC) families and the number of malignancies.*

Country of origin	Mutation positive families	Families studied	Families (patients) with renal cell carcinoma	Families (patients) with leiomyosarcoma of the uterus	References (mutations detected)
Finland	6	6	5 (12)	2 (4)	Study I Lehtonen *et al.* (2005)
UK	38	42	1 (1)		Study I Alam *et al.* (2003) Alam et al. (2005)
USA	52	56	18 (38)		Toro *et al.* (2003) Wei *et al.* (2005)
Tunisia, Ethiopia, Greece, Puerto Rico Australia, Iran	16	16			Martinez-Mir *et al.* (2003) Chuang *et al.* (2005)
Poland	1	1	1 (1)		Chan *et al.* (2005)
TOTAL	**113**	**121**	**25 (52)**	**2 (4)**	

Two of the Finnish families, FAM-1 and FAM-2, shared a deletion of 2 base pairs at nucleotide 543 (of the mature mitochondrial protein), six UK families carried the same missense change Asn64Thr, and three UK families shared the nonsense mutation Arg58Stop. Common ancestry was not known for any of the families sharing the same mutation. In the third Finnish family, FAM-3, a truncating mutation Arg300Stop was detected (Table 3).

Biallelic inactivation of *FH* was observed in 28 out of 32 (88%) tumors studied. Allelic loss was detected in most cases, in 21 out of 25 leiomyomas and in 5 out of 7 papillary renal cell cancers. The remaining two renal cancers displayed somatic second hits, a 2 base pair deletion at codon 41 and a missense change Met285Arg.

To confirm the hypothesis that an absence or reduction of FH through biallelic inactivation is the cause of tumorigenesis in HLRCC/MCUL, FH enzyme activity was measured. The FH enzyme activity assay showed a significant decrease of enzyme activity in tumor samples (range 198-438 pmol/mg/min) compared to control values (459-1104 pmol/mg/min). Lower FH enzyme activity in individuals with missense mutations compared to individuals with whole gene deletion or truncation of the protein was observed.

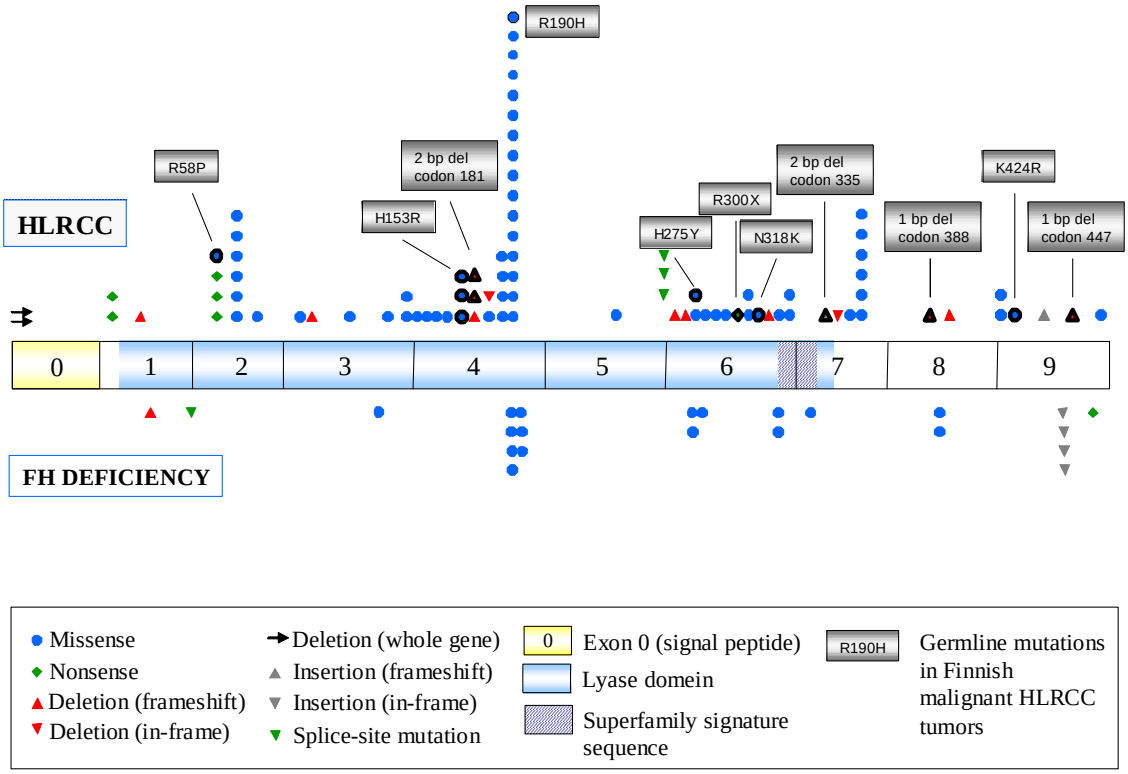

Figure 3*. Structure of the fumarase (FH) gene and the known mutations in hereditary leiomyomatosis and renal cell cancer (HLRCC), nonsyndromic tumors, and fumarase deficiency (FHD). FH consists of 10 exons. Exon 0 is the mitochondrial signal peptide. Mutations in HLRCC and FHD overlap. No genotype-phenotype correlation has been detected in HLRCC. The numbering of the exons, amino acid residues, and nucleotide positions are based on the mature cytosolic isoform of FH (NM_000143.2). Figure updated from Kiuru and Launonen (2004).*

2. Fumarase (FH) in familial prostate cancer (II)

The occurrence of prostate cancer in a *FH* mutation carrier in one HLRCC family and a frequent occurrence of kidney cancer (nine cases in 19 families) among US HPRCA families showing linkage to 1q42.2-43 supported the possible role of FH defects in prostate cancer predisposition (Launonen *et al.* 2001). No germline mutations in the coding sequence or within the conserved splice site regions of *FH* was detected in the series of 108 prostate cancer patients from 89 families. One unique base pair substitution T>C was observed in the 3‘-untranslated region (UTR), nine base-pairs after the termination codon of the gene. A silent polymorphism 798G>A was observed in five samples in exon 7, and there was one heterozygous substitution C>T 11 base-pairs upstream from the translation initiation codon.

3. Fumarase (FH) in familial breast cancer (III)

In this study, 85 Finnish breast cancer cases for *FH* mutations were analyzed. The rationale of the work was occurrence of breast cancers cases in Finnish HLRCC patients carrying a germline *FH* mutation (Launonen *et al.* 2001). Mutation analysis revealed no disease-causing changes. Three previously detected polymorphisms (unpublished data) were found: 798 G>A (Pro266Pro) (two individuals, one heterozygous and one homozygous), IVS2+61T>A (one individual), and IVS3+32A>G (one individual). The changes were not predicted to have an effect on splicing tested *in silico* by the NetGene2 program. Most (75 out of 85, 88%) of the studied cases were selected based on positive family or personal history for tumors seen in HLRCC.

4. The role of fumarase (FH) in nonsyndromic tumorigenesis

4.1. *Fumarase* (*FH*) mutations in tumor types observed in HLRCC (IV)

To clarify the role of FH in nonsyndromic tumorigenesis, tumor types associated with HLRCC/MCUL were analyzed for *FH* mutations. The extended series of 200 tumors for *FH* mutation screening included 10 cutaneous leiomyomas, 41 uterine leiomyomas, 52 renal cell carcinomas, 53 sarcomas, 29 prostate carcinomas, and 15 lobular breast carcinomas (Table 5).

In the nonsyndromic tumors, the frequency of *FH* mutations was very low, with mutations detected only in 3 out of 200 (1.5%) lesions. In the leiomyosarcoma from patient LS10, a missense mutation His153Arg in exon 4 and a nonsense mutation Leu240Stop in exon 5 were detected (Table 3). The His153Arg change was found also in the germline, suggesting the familial origin of the case. The change occurred in the evolutionarily conserved region of *FH*. The change was not reported as a known polymorphism nor was detected in a panel of 268 control chromosomes. Patient LS10 was first diagnosed with uterine leiomyomatosis, and despite two myomectomies, the malignant uterine leiomyosarcoma was detected two years later. The young age of onset (32 years) was compatible with predisposition to hereditary cancer syndrome.

Table 5. *Nonsyndromic tumor types analyzed for fumarase (FH) defect and observed mutations. Somatic mutations detected jointly with germline changes are shown in parentheses.*

Tumor Type	Number of Cases	Germ-line Muta-tions	Somatic Muta-tions	Other Findings	Reference
HLRCC[1] associated tumors					
Uterine leiomyomas	41				Study IV
	129			5% LOH[2] at FH[3] locus	Barker *et al*. (2002)
	153		2	3.3% LOH at FH locus	Study V
	45	1			Barker *et al.* (2005)
Cutaneous leiomyomas	10	1	(1)		Study IV
Uterine leiomyosarcomas	18	1	(1)		Study IV
	26			54% AI[4] at 1q42	Barker *et al.* (2002)
	67	1			Ylisaukko-oja *et al.* (2006)
	9				Barker *et al.* (2005)
Other sarcomas	35		1		Study IV
Renal cell cancer	52				Study IV
Other tumors	44				Study IV
	15				Matyakhina *et al.* (2005)
Non-HLRCC associated tumors	299				Study V
TOTAL	**943**	**4**	**3 (+2)**		

[1] Hereditary leiomyomatosis and renal cell cancer
[2] Loss of heterozygosity
[3] *Fumarase*
[4] Allelic imbalance

In the cutaneous leiomyoma from patient IL10, a 2 base pair deletion 541delAG in exon 4 was detected, in addition to the tumor, in the germ line of the patient (Table 3). This mutation was found previously in two Finnish HLRCC families, nevertheless no common ancestry is known. The 541delAG was not present in the 448 Finnish healthy controls. Additionally, a somatic nonsense mutation Arg300Stop was found in exon 6.

The only biallelic inactivation of *FH* purely at the somatic level was found in a soft-tissue sarcoma. The sequence change Arg8Glu in exon 1 targeted an amino acid conserved in yeast and *Escherichia coli*. The tumor displayed also a loss of the wild type *FH* allele. At the time of the diagnosis, the patient was 48 years old and had a metastasized soft-tissue sarcoma in her right lower limb.

4.2. Fumarase (FH) in nonsyndromic uterine leiomyomas (V)

4.2.1. LOH analysis

LOH at the *FH* locus was analyzed by two polymorphic microsatellite markers in the set of 166 unselected uterine leiomyomas from 51 individuals. The markers were located on either side of the *FH* locus. 153 leiomyomas from 46 individuals were informative for at least one of the markers. The presence or absence of LOH was visually evaluated based on the different heights of the allele peaks. Allelic loss was observed in five myomas (5 of 153, 3.3% of myomas), derived from five different patients (5 of 46, 11% of patients). One of these lesions was informative with both markers and showed LOH at both marker loci, whereas the other four were informative at one marker, two with the centromeric and two with the telomeric microsatellite repeat.

4.2.2. *Fumarase* (*FH)* mutation screening

Five leiomyomas showing LOH were subjected to *FH* mutation screening by DHPLC to detect the putative second alteration in the remaining allele. Two myomas, P32M1 and P4M3, showed aberrant chromatographs in exon 4. To confirm the finding and define the actual mutations, all five myomas were reanalyzed by sequencing of exon 4. Three lesions were mutation-negative, and two displayed a *FH* mutation. The three myomas where a mutation in exon 4 was not found were sequenced for the rest of the coding sequence. The result of this analysis was also negative, compatible with the results from the DHPLC analysis.

Myoma P32M1 harbored a missense change Ala196Thr (586G>A) in exon 4 (Table 3 and 5). The altered amino acid is completely conserved among species from humans to *Escherichia coli*. This change was not found in six other leiomyomas from patient P32. Allelic imbalance was not present at the Ala196Thr mutation site, suggesting that the deletion observed in the marker analysis was centromeric. Myoma P4M3 harbored a splice

site change IVS4 + 3A>G, which is predicted to result in deletion of exon 4. Loss of the remaining wild type allele at the mutation site was also seen. The additional two myomas from patient P4 were mutation-negative in sequencing of exon 4. Genomic normal tissue DNA samples from these individuals were sequenced for all *FH* exons but no alterations were detected, confirming the somatic origin of the mutations and nonsyndromic nature of the cases.

The predicted consequence of the splice site change, deletion of exon 4, was confirmed by cDNA amplification and sequencing. Histopathological evaluation of the *FH* mutation-positive lesions confirmed that they were typical leiomyomas without any distinct features such as atypia (data not shown).

4.3. *Fumarase* (*FH*) mutations in non-HLRCC associated sporadic tumors (V)

In the *FH* mutation analysis of this large series of non-HLRCC associated, nonsyndromic (apparently sporadic) neoplasias, including 10 malignant tumor types, were successfully examined (success rate 94%), but no coding region mutations or splice site changes were identified (Table 5). Probable polymorphisms detected were as follows: silent change 798G>A (10 tumors), and three intronic alterations IVS2-21A>T (six tumors), IVS3 + 32A>G (one tumor), and IVS2 + 61T>A (one tumor). The heterozygous substitution from C to T 11 base-pairs upstream from the translation initiation codon was observed in one papillary thyroid carcinoma. The corresponding germline change was detected in a familial prostate cancer patient, and therefore seems to be an infrequent polymorphism (II).

DISCUSSION

1. Localization and identification of the predisposing gene for hereditary leiomyomatosis and renal cell cancer (HLRCC) (I)

In the genome wide linkage study of two Finnish families, Launonen *et al.* (2001) demonstrated that the susceptibility gene for HLRCC is likely located on chromosome 1q42-q44. Independently in UK, linkage and haplotype analysis of 11 families segregating MCUL showed evidence for linkage to the same region on chromosome 1q42-q43 (Alam *et al.* 2001). LOH analysis in tumors from HLRCC family members at 1q42-q43 was compatible with the linkage data and confirmed the findings. Detection of wild-type allele deletions at 1q42-44 in most tumors of both Finnish kindreds studied also suggested that the underlying genetic defect is associated with a tumor suppressor function (Kiuru *et al.* 2001, Launonen *et al.* 2001).

In this study, the predisposing gene for the HLRCC syndrome was localized and identified. The critical step in the gene identification effort was that the minimal target region could be narrowed down to 1.6 Mb by combining marker data from Finnish and UK families. *FH* was the only gene out of 6 known genes and over 50 transcripts in the minimal target region carrying putative pathogenic changes (I).

In this study, it was further confirmed that the tumors from HLRCC/MCUL family members followed the classical Knudson's model of tumor suppressor inactivation (Knudson 1971). All seven papillary renal cell cancers and 21 out of 25 leiomyomas studied showed inactivation of the wild type allele, in most cases through allelic loss (I). This finding was further confirmed by an FH enzyme activity assay which showed a significant decrease of enzyme activity in tumor samples compared to control values. Lower FH enzyme activity in individuals with missense mutations compared to the individuals with whole gene deletion or truncation of the protein indicates susceptibility to a dominant-negative effect (I). This phenomenon is logical given the fact that the functioning enzyme is a homotetramer. In subsequent studies it has come evident that the FH enzyme activity assay provides comprehensive sensitivity and specificity in detecting pathogenic *FH* alterations (Alam *et al.* 2003, Wei *et al.* 2005).

These data strongly suggested that *FH* is a predisposing gene for HLRCC/MCUL and acts as a tumor suppressor.

1.1. *Fumarase* (*FH*) gene

The human *FH* gene consists of 10 exons encoding 510 amino acids (Figure 3). The first exon (exon 0) encodes a mitochondrial localization signal peptide (Edwards and Hopkinson 1979a, Edwards and Hopkinson 1979b). FH is highly conserved in evolution,

with 66% identity shared between the human and *S. cervisiae* FH proteins. The lyase domain of human FH includes the catalytic site of the protein (Figure 2). The superfamily signature peptide is conserved between proteins including FH, aspartase, adenylosuccinate lyase, arginosuccinate lyase, and δ-crystallin (Estevez *et al.* 2002).

Mitochondrial FH is a key enzyme in energy metabolism, catalyzing fumarate to malate in the Krebs cycle, i.e. the tricarboxylic acid cycle (TCAC) (Figure 2) (Krebs and Johnson 1937). TCAC is the final common metabolic pathway for the oxidation of amino acids, fatty acids and carbohydrates under aerobic conditions generating high-energy molecules, ATPs (Stryer 1995, Rustin *et al.* 1997). In addition to the mitochondrial peptide, a cytosolic isoform of FH also exists. The cytosolic isoform of FH is involved in the urea cycle and amino acid synthesis. In humans, two FH isoforms have been shown to be encoded by a single gene (Tolley and Craig 1975, Edwards and Hopkinson 1979b). Studies in rat suggested that mitochondrial and cytosolic fumarases are synthesized from one species of mRNA by two alternative translational initiation codons (Suzuki *et al.* 1989, Tuboi *et al.* 1990, Suzuki *et al.* 1992). According to yeast studies, there is only one translation product (Stein *et al.* 1994). After signal peptide cleavage by mitochondrial matrix peptidase, a subset of FH will be recruited back to the cytosol (Sass *et al.* 2001). It was further shown in yeast that proper and rapid folding of the protein is the driving force for the recruitment of processed FH back to cytosol (Sass *et al.* 2003).

1.2. Fumarase deficiency (FHD)

Mutations in *FH*, in a homozygous or compound heterozygous form, were originally found in a metabolic disease FH deficiency (FHD, OMIM 136850) characterized by neurological impairment and encephalopathy (Zinn *et al.* 1986, Bourgeron *et al.* 1994, Deschauer *et al.* 2006). FH enzyme activity is absent or significantly reduced in all FHD patients' tissues due to homozygous germline mutations of the gene, and these patients usually die at the age of few months (Rustin *et al.* 1997). Although the decrease in the enzyme activity in FHD patients is similar compared to the HLRCC tumors, no leiomyomata has been reported in FHD, definitely because of the very short life span of these patients. FH enzyme activities of heterozygous parents of FHD patients are typically 30 to 60% of those of healthy controls (Gellera *et al.* 1990, Bourgeron *et al.* 1994).

2. Fumarase (FH) in hereditary leiomyomatosis and renal cell cancer (HLRCC) (I)

2.1. Clinical features

Leiomyomas of the skin and uterus were the most prevalent lesions in the HLRCC families (I). In Finnish HLRCC families these tumors were found in 87% (26 out of 30) of individuals segregating germline mutations in *FH*. Nevertheless, occurrence of RCC was limited to the three Finnish HLRCC families with low penetrance (7 out of 30 Finnish patients; 23%), renal lesions were crucial in the characterization of the syndrome in Finland. Even though the phenotypes are congruent in many respects, the considerably high frequency of renal cancers among Finns and absence among UK MCUL families could not be explained by different mutation spectrums (Figure 3). Much more data will be needed to determine if tumor spectrums vary between populations or if the differences are due to selection bias. Finnish HLRCC families were collected and primarily characterized by RCC, more precisely according to it's papillary type II histology, whereas UK families were identified merely based on multiple skin and uterine leiomyomas. Currently 112 HLRCC families segregating *FH* mutations have been identified world-wide (Table 4) (I) (Alam *et al.* 2001, Kiuru *et al.* 2001, Launonen *et al.* 2001, Alam *et al.* 2003, Martinez-Mir *et al.* 2003, Toro *et al.* 2003, Kiuru and Launonen 2004, Alam *et al.* 2005, Chan *et al.* 2005, Chuang *et al.* 2005, Lehtonen *et al.* 2005, Wei *et al.* 2005). Although there is no population-based information of the prevalence of HLRCC, it has been estimated to be very low (Kiuru and Launonen 2004).

The penetrance of cutaneous and/or uterine leiomyomas is high, up to nearly 100%, in all populations (I) (Toro *et al.* 2003, Alam *et al.* 2005, Chuang *et al.* 2005, Wei *et al.* 2005). In HLRCC patients, uterine leiomyomas cause more severe symptoms than in the general population. HLRCC myomas are more numerous and larger, appear at younger age, and result more often in earlier hysterectomy than other myomas (Alam *et al.* 2005, Toro *et al.* 2003). In the UK study of MCUL, 87% of women with uterine fibroids also had skin leiomyomas (Alam *et al.* 2005a, Alam *et al.* 2005b). Cutaneous leiomyomas are benign tumors composed of interlacing bundles of smooth muscle cells with centrally located blunt-ended nucleus. The size of most skin myomas of HLRCC patients varies between 2 and 20 mm and they diverge greatly in appearance (Alam *et al.* 2005). Cutaneous leiomyomatosis in HLRCC typically has early onset, ranging from 10 to 47 years (Toro *et al.* 2003, Alam *et al.* 2005). Penetrance estimates for skin leiomyomas are 100% in men and 76% in women. The higher number of skin myomas in men than in women suggests a putative role of hormonal regulation in these lesions (Alam *et al.* 2003).

In addition to benign lesions, malignant RCC and ULMS are present in a subset of HLRCC families. Currently, 52 patients with RCC have been identified in 24 HLRCC families, originating predominantly from Finland and the US (24 out of 112 families, 22%) (Table 4) (Launonen *et al.* 2001, Kiuru *et al.* 2001, Alam *et al.* 2003, Wei *et al.* 2005, Kiuru and Launonen 2004, Toro *et al.* 2003, Chan *et al.* 2005). Our original findings

suggested striking differences in renal cancer prevalence between Finland and the UK (I). Only one metastasized collecting duct carcinoma case diagnosed at age 16 years was reported in the UK compared to 12 RCC cases in Finland (Alam *et al.* 2003, Lehtonen *et al.* 2005) (I). The currently available data still supports the original finding that the prevalence of kidney cancer is higher in Finland and the US than in other populations (Table 4). A key feature in these RCC tumors is their histopathology, which was crucial in identification of the syndrome (Launonen *et al.* 2001). HLRCC renal tumors typically display rare papillary type 2 histology, but altogether two collecting duct renal carcinomas have also been described (Launonen *et al.* 2001, Alam *et al.* 2003, Wei *et al.* 2005). The age of onset is low (median 40.5 years in Finland) and the progression of the disease is aggressive (Launonen *et al.* 2001, Toro *et al.* 2003, Lehtonen *et al.* 2005).

ULMS is a rare malignant mesenchymal tumor. At present, four patients with leiomyosarcoma in two HLRCC families have been identified. Interestingly, leiomyosarcoma cases are restricted to the Finnish population (Kiuru *et al.* 2001, Launonen *et al.* 2001, Lehtonen *et al.* 2005). The age of diagnosis of ULMS has varied from 30 to 39 years, whereas in the general population they typically occur in old age (Kiuru *et al.* 2001, Launonen *et al.* 2001, Lehtonen *et al.* 2005).

In order to study the cancer risk and mutation spectrum in Finnish HLRCC families, comprehensive and unbiased data has been obtained from 868 individuals included in the *FH* mutation-positive families (Lehtonen *et al.* 2005). The risk analysis provided statistically significant results with 6.5-fold increased risk for RCC and 71-fold increased risk in ULMS compared to the general population. More evidence for the crucial role of FH was obtained from LOH analysis of the tumor samples available from *FH* mutation carriers. Somatic inactivation was observed in 83% (10/12) of renal cell tumors and in 100% (3/3) of uterine leiomyosarcomas (Lehtonen *et al.* 2005). The results further confirmed our original finding that malignant lesions, predominantly renal cell carcinoma and uterine leiomyosarcoma, are significant components of HLRCC tumor spectrum.

Other tumor types have also been detected in HLRCC families: breast carcinoma, prostate cancer, hematological malignancies, leiomyosarcoma of the skin, bladder cancer, brain tumor, and angiolipomata of kidney (Launonen *et al.* 2001, Toro *et al.* 2003, Alam *et al.* 2005, Wei *et al.* 2005). Lehtonen *et al.* (2005) provided evidence that other malignancies may also be promoted by loss of FH. All four breast and one bladder carcinomas studied displayed loss of the wild type allele at the *FH* locus.

Recently, the massive macronodular adrenocortical disease (MMAD) has been added to the list of HLRCC phenotypes (Matyakhina *et al.* 2005). An inactivating *FH* mutation was found in a patient with atypical Cushing syndrome (CS, OMIM 219080) due to MMAD with adrenocorticotrophic hormone (ACTH)-independent and bilateral hyperplasia of the adrenal glands. MMAD is a rare heterogenous disorder of unknown etiology associated with CS. ACTH-independent CS is caused by adrenocortical tumors or hyperplasias. CS may also be part of a complex including cutaneous and cardiac myxomas, spotty pigmentation of the skin, and hypertension (Teding van Berkhout *et al.* 1986, Teding van Berkhout *et al.* 1989, Torpy *et al.* 2002).

2.2. Germline mutations of *fumarase* (*FH*) in hereditary leiomyomatosis and renal cell cancer (HLRCC)

In the predisposing gene identification study (I), *FH* mutations segregating in HLRCC families included whole gene deletions 8% (2/25), truncating mutations 40% (10/25), or substitutions and deletions 52% (13/25) of conserved amino acids (Table 3). This mutation data did not display any logical support for genotype-phenotype correlation. Although Finnish changes were protein truncating mutations and could potentially lead to more severe symptoms (a 2 bp deletion and a nonsense mutation), no malignancies were detected in UK families with truncating changes including whole gene deletions, two different nonsense mutations, and a 1 bp deletion causing a frame shift (I). In these families mutations tended to occur towards the amino terminus of the gene and some clustering of mutations could be detected in exons 2 and 4. Mutations Arg58Stop and Asn64Thr were detected in three and six UK families, respectively, indicating the location of a putative mutation hot spot. Additionally, five of the mutations were detected between codons 181 and 190, representing the third possible hot spot. Two of these changes were found in the Finnish HLRCC families. In none of these cases could the possibility of founder mutations be excluded because of lack of sufficient haplotype information (I).

Mutation analyses of additional HLRCC families have offered more detailed information about *FH* mutations and their distribution (Figure 3) (Kiuru *et al.* 2001, Launonen *et al.* 2001, Alam *et al.* 2003, Martinez-Mir *et al.* 2003, Toro *et al.* 2003, Chan *et al.* 2005, Chuang *et al.* 2005, Lehtonen *et al.* 2005, Wei *et al.* 2005). Single base pair substitutions (78%, 87/112) represent the great majority of the mutations reported in HLRCC families. Only 14% of the substitutions are nonsense changes creating a stop codon. *FH* missense mutations have been recently found to be clustered to fully conserved amino acids, functional substrate-binding sites, or in the sub-unit interaction regions (Alam *et al.* 2005). No apparent phenotypic differences can be detected between mutation types (Figure 3). *FH* mutations have been detected in totally over 90% of published HLRCC/MCUL families (112 out of 120) reflecting homogenous genetic background of this syndrome (Table 4).

In *FH*, exons 4 and 6 seem to be mutation hot spots although mutations extend along the whole gene. Thirty-five percent of *FH* germline mutations are located in exon 4, and almost half of these in codon 190. The second most common change Arg58Stop, originally found in the UK, has been reported also in HLRCC families from other populations (Figure 3) (Chuang *et al.* 2005, Wei *et al.* 2005). Increasingly accumulating evidence for the non-existence of genotype-phenotype association comes from the finding that both of these hot-spot mutations have been detected in families with and without RCC (Wei *et al.* 2005). Due to lack of haplotypes of key individuals, it remains unsolved if some of these mutations represent either an actual hot spot or a founder mutation (Kiuru and Launonen 2004, Wei *et al.* 2005).

The reduction of FH enzyme activity (50%) in parents of FHD patients is very similar than activity in the germline of heterozygous *FH* mutation carriers in HLRCC families (Bourgeron *et al.* 1994) (I). Surprisingly, leiomyomas or other lesions characteristic for

HLRCC have not been reported previously in heterozygous parents or siblings of FHD patients. After careful examination, one heterozygous parent of FHD patients was observed to have cutaneous leiomyomas (I). This family, previously described by Gellera (1990), segregates a different missense change in the same codon (190) as one of the UK MCUL families (Arg190Cys and Arg190His, respectively) (I). Other genotypic similarities, such as Arg190His mutations, have also been observed in both FHD and HLRCC, therefore the mutation spectrum seems to overlap (Figure 3) (Kiuru and Launonen 2004). In two other families where clinical examination of HLRCC-related neoplasias was executed, no signs of leiomyomatosis nor RCC was detected (Remes *et al.* 2004, Deschauer *et al.* 2006). In the first family the clinical examinations of the father and mother of these patients were made at the ages of 36 and 33 years, and for the second family at 46 and 52 years. FH enzyme activity in their fibroblasts was close to the expected 50% of the normal value. Furthermore, Alam *et al.* (2003) reported an FHD family where neither of the heterozygous parents had developed HLRCC-associated lesions. This outcome is extremely confusing considering the almost complete penetrance of myomatosis and early onset of symptoms in HLRCC. The median age of diagnosis in HLRCC leiomyomas is 25 (skin) to 30 (uterine) years, and 36 (Finnish population) to 44 years (North American patients) in RCC. Although overlap in the mutation spectrum and clinical features of FHD and HLRCC families exists, genetic and molecular mechanisms behind observed discrepancies remains unknown.

3. The role of fumarase (FH) in other familial cancers

3.1. Familial prostate cancer (II)

It was hypothesized that FH defects may be associated not only with HLRCC, but also with prostate cancer predisposition. An *FH* mutation carrier in one HLRCC family had had prostate cancer. Kidney cancer cases were unexpectedly frequent among US HPRCA families showing linkage to 1q42.2-q43. The fact that the genetic locus harboring *FH* had been associated with hereditary prostate cancer made *FH* an attractive positional candidate for a prostate cancer predisposition gene. The combination of functional and linkage data indicates that mutations in *FH* might underlie HPRCA in a subset of families. An extensive effort to screen for FH defects in HPRCA was performed to test the hypothesis that *FH* mutations may predispose to prostate cancer.

Analyses of 89 high-risk HPRCA families, of which 19 showed suggestive evidence of linkage to 1q42.2-q43, was performed for *FH* mutations. The absence of coding region or splice site aberrations in this data set strongly suggests that *FH* is not a predisposing gene for hereditary prostate cancer. These conclusions were strongly supported by negative results from *FH* mutation screening of 160 affected individuals from 77 UK prostate cancer families (Bevan *et al.* 2003).

Neoplastic formation of the prostate could in many ways be linked to TCAC. The product of the *FH* gene acts as a key enzyme in mitochondrial energy metabolism in the citric acid cycle. In normal prostate cells, the oxidation of citrate is inhibited by aconitase, which leads to the accumulation of citrate. Transformation from citrate producing benign cells to citrate oxidizing malignant cells appears to be a key event in prostate carcinogenesis (Costello and Franklin 2000). Mitochondrial aconitase (m-aconitase, *ACO-2*) catalyzes the first step leading to the oxidation of citrate via TCAC. In malignant prostate cells, citrate production, a characteristic phenomenon of normal prostate epithelial cells, is shifted towards citrate oxidation which is activated by m-aconitase overexpression regulated by testosterone and prolactin (Costello and Franklin 2000). Reaction between m-aconitase and superoxide generates free hydroxyl radicals that may enhance mitochondrial oxidative damage (Vasquez-Vivar *et al.* 2000). Citrate serves as an oxidizable intermediate in TCAC and as a precursor for lipogenesis, which is accelerated in malignant cells possibly owing to increased ATP production (Costello and Franklin 2000).

3.2. Familial breast cancer (III)

An indication of the contribution of *FH* to mammary gland tumors was that three Finnish patients with *FH* mutations in two HLRCC families had been diagnosed with breast cancer (Kiuru *et al.* 2001). The first breast cancer detected in an HLRCC patient displayed lobular histology, a histology which comprises 15% of unselected breast carcinomas (Launonen *et al.* 2001).

So far, patients with breast cancer have only been detected in the Finnish HLRCC families, in three *FH* mutation-positive females (Kiuru *et al.* 2001, Launonen *et al.* 2001). Interestingly, two of these patients had also been diagnosed with uterine leiomyosarcoma. Breast cancer being the most common cancer type in females, it is conceivable that these cases detected in the Finnish families are actually sporadic ones (Finnish Cancer Registry, http://www.cancerregistry.fi (2004)). In this study, *FH* germline mutations were examined in 85 Finnish patients with breast carcinoma, but no disease-causing mutations were found. These results make it unlikely that *FH* is a major susceptibility gene for breast cancer.

4. Fumarase (FH) defects in nonsyndromic tumors

4.1. *FH* mutations in tumor types associated with HLRCC (IV)

To further evaluate the role of FH in tumorigenesis we analyzed nonsyndromic tumors for FH inactivation. Sporadic lesions, restricted to the tumor types associated with HLRCC, were analyzed for *FH* mutations (Table 5). In this study, only one case of a somatic loss-of-function of *FH* (a missense mutation with LOH) was found in an unspecified soft-tissue sarcoma of a lower limb. Additionally, one out of 10 cutaneous leiomyomas displayed a mutation also in the germline. The 2-bp deletion 541delAG in exon 4 was earlier found in two Finnish HLRCC families, although no common ancestry was known for them (I). The somatic second hit Arg300Stop was also identified (Table 3). The same mutation was detected also in one Finnish HLRCC family (I), which could be explained by this being a mutation hot-spot region. Robust conclusions cannot be drawn about frequency of nonsyndromic cutaneous leiomyomas with an FH defect. That even a single mutation positive case (1/10) was detected in a dataset this small was surprising. More data will be needed to determine the role of FH in cutaneous lesions.

In one case of 18 unselected uterine leiomyosarcomas, a germline *FH* defect coupled with a nonsense somatic mutation was also identified. All three tumors displayed biallelic inactivation of FH by two mutation events, further confirming the biallelic inactivation model of FH tumorigenesis. Surprisingly, no *FH* mutations were found in 52 analyzed RCCs. Although five tumors had some histological resemblance to renal tumors from HLRCC patients, only two shared considerable similarity. Considering the specific histology of the HLRCC related RCC, this collection of nonsyndromic renal tumors was not probably optimal for the detection of *FH* alterations.

Previously, four cases with ULMS segregating *FH* mutations in two Finnish HLRCC families have been identified (Table 4) (Launonen *et al.* 2001, Lehtonen *et al.* 2005). In addition to this effort (IV) in the population-based cohort of early onset nonsyndromic leiomyosarcomas an FH amino acid change was found in 1 out of 67 (1.5%) tumors (Table 5) (Ylisaukko-Oja *et al.* 2006). According to these studies, the prevalence of *FH* germline mutations in nonsyndromic ULMS in Finland is 2.4 % (2/85). *FH* mutations in nonsyndromic ULMS have been examined in four separate studies including altogether 120 lesions but ULMS with *FH* defects have not been reported in any population other than Finns (Table 5) (Barker *et al.* 2002, Barker *et al.* 2005, Ylisaukko-Oja *et al.* 2006) (IV). Altogether, among Finnish *FH* mutation positive women, there are six cases of histologically verified ULMS, as well as two cases with leiomyomas with atypia (Kiuru *et al.* 2001, Launonen *et al.* 2001, Kiuru and Launonen 2004, Ylisaukko-Oja *et al.* 2006). Diagnoses of ULMS were at very young ages, 27 to 39 years, and with atypia at 27 and 36 years. In the general population, ULMSs are rare and typically occur in old age. These findings seem to support the hypothesis that leiomyosarcomas belong to the tumor spectrum of the HLRCC syndrome.

Although germline *FH* mutations in nonsyndromic ULMS are rare, the question about association of benign leiomyomas and malignant leiomyosarcomas has arisen. This is of interest considering the elevated risk of ULMS among *FH* mutation carriers in Finland (Lehtonen *et al.* 2005) Although previous cytogenetic studies have been unable to provide a link between nonsyndromic leiomyomas and leiomyosarcomas, recent microarray expression data has provided evidence that some leiomyosarcomas may arise from leiomyomas (Levy *et al.* 2000). A rare subset of leiomyomas with chromosome 1 deletions was clustered together with leiomyosarcomas (Christacos *et al.* 2003).

Allelic imbalance (AI) has been observed in a UK dataset in 54% of nonsyndromic leiomyosarcomas (Barker *et al.* 2002). Association of FH defects and AI at 1q42 cannot be directly drawn, because random chromosomal aberrations are common in ULMS (Packenham *et al.* 1997, Hu *et al.* 2001). Interestingly, no LOH or somatic mutations were found in the ULMS with a germline mutation in a population-based effort (Ylisaukko-Oja *et al.* 2006). Significantly reduced FH enzyme activity was supported the preassumption of the dominant negative effect and pathogenic nature of missense mutations.

PCR fragments amplified in this study covered only the coding sequence and the flanking intronic sequences of *FH*. Untranslated 5' and 3' regions, distant regulatory regions, and most of the intronic sequences were not analyzed. Like all PCR-based mutation-detection technologies, our mutation analysis method was not capable of detecting large genomic deletions or insertions. Epigenetic alterations, which include DNA methylation and histone modification, are also undetectable by standard DHPLC or genomic sequencing. In many cancer cells, tumor suppressor genes, such as *VHL* and *retinoblastoma* (*RB1)*, are inactivated by hypermethylation of their promoter CG-rich regions designated as CpG islands (Sakai *et al.* 1991, Herman *et al.* 1994). To examine possible epigenetic mechanisms in *FH* inactivation, Barker *et al.* (2005) used immunohistochemistry to study FH protein expression in nonsyndromic ULMS. No changes in FH expression was found in any of the 9 tumors studied.

4.2. Fumarase (FH) in nonsyndromic uterine leiomyomas (V)

It has been demonstrated that germline *FH* mutations predispose to myomas of the uterus and skin with high penetrance in HLRCC families (I). Biallelic inactivation of *FH*, in the majority of cases by LOH, is essential for FH related tumor growth. However, no *FH* mutations were found in our first set of 26 unselected nonsyndromic uterine leiomyomas, based on the previous findings somatic *FH* mutations do occur, though at a low frequency (I, IV). It was hypothesized that if *FH* is associated with the development of nonsyndromic uterine leiomyomas, most mutations should be found in lesions displaying allelic loss at 1q43. To test this hypothesis an extensive set of nonsyndromic 166 uterine myomas was analyzed, first for LOH at the *FH* locus, and in the presence of allelic loss additionally for *FH* mutation status.

In this study, allelic loss at the *FH* locus was detected in five of 153 (3.3%) informative unselected leiomyomas from 46 individuals (10.9%). Somatic *FH* mutations were detected in two out of five cases displaying LOH in the region of the *FH* locus. The wild type A allele seems to be under-represented in the sequence chromatograph of the IVS4 +3 A>G mutation site. This finding together with the LOH data supports our conclusion that FH is inactivated through a somatic point mutation and loss of the second allele, and is a true target of the 1q43 deletions.

A negative germline *FH* mutation analysis of all exons, performed from normal tissue DNA, confirmed the nonsyndromic nature of both of the mutation-positive lesions and excluded the possibility of polymorphism. The *FH* mutation data obtained are quite convincing. The missense mutation affects a conserved amino acid, and changes the amino acid subclass from neutral and hydrophobic to neutral and polar. The splice site change results in deletion of the entire exon 4. Both mutations are located in the *FH* mutation hot spot detected in HLRCC families (Figure 3). Three lesions displayed LOH at the *FH* locus but no mutations were detected. Although random allelic loss is rare in uterine leiomyomas (Mao *et al.* 1999), random deletion of a chromosomal material at 1q cannot be excluded in the three mutation-negative cases. To determine the frequency of loss of FH in uterine myomas, Gross *et al.* (2004) performed a FISH analysis of 11 tumors with 1q rearrangements. Their data, showing absence of one copy of *FH* in nine of 11 myomas, suggested that loss of FH is involved in the development of a subset of nonsyndromic uterine leiomyomas. Furthermore, mosaicism in these tumors indicated that chromosome 1 cytogenetic rearrangements are secondary changes in the pathogenesis of nonsyndromic uterine leiomyomas. Multiple reports of cytogenetic rearrangements, alterations in gene expression, and analysis of candidate genes in uterine leiomyomas have been published in the last few decades, reviewed by Newbold *et al.* (2000). Barker *et al.* (2005) studied FH expression in a series of nonsyndromic 45 uterine leiomyomas, but found no evidence of contribution of epigenetic mechanisms to formation of these lesions.

Our LOH derived study design was based on the existing knowledge that in most of the hereditary cases, biallelic inactivation of FH results from loss of a wild type allele. We have found point mutations in a very small number of tumors. In other studies examining *FH* mutations on unselected sets of uterine leiomyomas, only one germline mutation was detected with loss of the normal allele (Barker *et al.* 2002, Barker *et al.* 2005). Altogether 368 nonsyndromic uterine leiomyomas have been studied for *FH* alterations, but only three mutations have been detected (1 germline and 2 somatic) (Table 5).

This is the first time, to our knowledge, that specific inactivating point mutations in nonsyndromic uterine leiomyomas have been detected in any gene. Although mutational *FH* inactivation in nonsyndromic uterine leiomyomas is uncommon at the tumor level, the frequent co-existence of multiple myomas in patients suggests that the occurrence of *FH*-deficient leiomyomas is not extremely rare. Overall, a minimum of 2.8% (2 of 72) of our Finnish uterine nonsyndromic leiomyoma patients harbored *FH* somatic mutations in at least one of their myomas. This study further confirmed the hypothesis that FH acts as a tumor suppressor.

To investigate the role of FH in sporadic tumorigenesis, 652 lesions, including a series of 353 nonsyndromic counterparts of tumor types associated with HLRCC, were analyzed (IV, V) (Table 5). In this study, altogether five of the tumors displayed biallelic inactivation of FH, three out of them purely on a somatic level. *FH* mutations in nonsyndromic tumors are rare (5 of all 652 lesions, 0.8%) and appear to be limited to the counterparts of tumor types associated with HLRCC (5 out of 353 HLRCC-associated tumor types, 1.5%). Word-wide, seven tumors with an *FH* mutation have been detected in studies including 644 HLRCC associated nonsyndromic lesions (1.1%) (Table 5).

5. Function of tricarboxylic acid cycle (TCAC) genes in tumor predisposition

5.1. Function of *fumarase (FH)* mutations

The recent findings that *FH*, known as a housekeeping–like gene, acts as a predisposing gene in cancer has encouraged efforts to better understand molecular alterations promoted by TCAC defects. Considering the logical correlation of observed FH enzyme activity and tumor predisposition in HLRCC-related tumors, it could be postulated that the effect is mediated through the function of the enzymatically active homotetramer (I).

Evidence that FH defects are mediated through TCAC was given by Kokko *et al.* (2005) who studied the effect of HLRCC-associated *FH* missense mutation and knock-out strains in yeast using a microarray platform. Two previously identified putative pathogenic mutations were analyzed: His153Arg associated with malignant phenotype (RCC and ULMS) and Lys187Arg associated only benign myomas and FHD (Coughlin *et al.* 1998) (I). Generally, transcription of the majority of TCAC enzymes was down-regulated in response to *FH* mutations (Kokko *et al.* 2005). Differences between the mutants could not be detected in FH expression, FH enzyme activity or in regulation of genes responding to *FH* mutations. Additionally, FH knock-out strains showed, in general, expression patterns similar to mutants. According to these findings, modifier gene/genes rather than genotype-phenotype correlation could better explain the heterogeneity in symptoms seen in families segregating different *FH* mutations.

To examine a poorly understood mechanism leading from FH defects to neoplastic pathway in uterine leiomyomas Vanharanta *et al.* (2005) used a genome wide expression microarray approach for showing differences in gene expression in FH-deficient compared to proficient uterine leiomyomas. Both nonsyndromic *FH* mutation-positive myomas, 4M3 and 32M1 (IV), and 7 familial myomas from a patient from a Finnish HLRCC family (I), were compared to 15 fibroids with wild type *FH.* All studied myomas, except two from the familial individual, showed LOH at the *FH* locus reflecting biallelic inactivation of the gene. Among 297 differentially expressed genes, the most interesting up-regulated genes were related to glycolysis, and adaptation to a glycolytic phenotype (acidosis),

which has been activated in many malignant tumors and associated with invasive phenomena (Gatenby and Gillies 2004, Vanharanta *et al.* 2005). Additionally, genes related to apoptosis (*apoptois-antagonizing factor, AATF, CDKN1A*), cell cycle arrest prevention (*transketolase, TK*), muscle development, and call adhesion were shown to be differentially expressed (Rais *et al.* 1999, Kogel *et al.* 2001, Di Padova *et al.* 2003, Vanharanta *et al.* 2005). *FH* mutant myomas also showed increased expression of iron homeostasis and oxidoreduction genes, possibly due to oxidative stress. Generally, published expression data indicated that FH inactivation-derived tumorigenesis is more likely to be related, directly or indirectly, to the pathways linked to TCAC than to other possible, currently unknown, functions of FH.

5.2. Succinate dehydrogenase (SDH) genes in tumorigenesis

FH mutations found in HLRCC made it evident that dysfunction of TCAC plays a general role in tumorigenesis. Just prior to definition of the HLRCC syndrome, the observation that a TCAC component could be involved in tumorigenesis was made by Baysal *et al.* (2000). Germline mutations in subunits of succinate-ubiquinone oxidoreductase, *succinate dehydrogenase subunit D (SDHD*), were observed in patients with hereditary head and neck paraganglioma (HPGL) and pheochromocytoma, and somatic mutations were detected in respective nonsyndromic lesions (Baysal *et al.* 2000, Gimm *et al.* 2000, Astuti *et al.* 2001a, Astuti *et al.* 2001b, Baysal *et al.* 2001, Baysal *et al.* 2002). Subsequently, it was suggested that germline *SDHB* mutations can predispose to early-onset kidney cancers, showing some similarities in the tumor spectra of *FH* and *SDH* mutations (Vanharanta *et al.* 2004).

Paraganglioma and pheochromocytoma are tumors of the autonomic nervous system. The term paraganglioma is used for benign extra-adrenal tumors typically located in the head and neck area, whereas pheochromocytomas are defined according to their adrenal medulla origin (Maher and Eng 2002). The four succinate dehydrogenase subunits, SDHA, SDHB, SDHC, and SDHD, comprise mitochondrial complex II of the electron transport chain and catalyze the oxidation of succinate to fumarate (Figure 3). In the TCAC, fumarate is converted to malate by FH. Although the tumor spectrum in HLRCC and PLG is different, it has been postulated that mutations in *FH* and SDH genes affect same molecular pathways (Gottlieb and Tomlinson 2005). This hypothesis is supported by a yeast study where defects in either *SDH* or *FH* had been shown to produce similar expression patterns (McCammon *et al.* 2003). Similarly to *FH* alterations in nonsyndromic uterine leiomyomas, allelic loss and somatic mutations in *SDHB* and *SDHD* have been reported in nonsyndromic pheochromocytomas (Gimm *et al.* 2000, Astuti *et al.* 2001a).

5.3. TCAC defects and apoptosis

Several explanations have been given concerning mechanisms of tumorigenesis caused by mutations in *FH*, including apoptosis and mitochondrial dysfunction, oxidative stress, pseudo-hypoxia, anabolic drive, or unknown function of the cytosolic form of FH (Eng *et al.* 2003, Pollard *et al.* 2003, Kiuru and Launonen 2004, Gottlieb and Tomlinson 2005). Mitochondria have a central role in programmed cell death. In response to upstream signals apoptotic proteins such as cytochrome c are released from the mitochondria and activate the energy-dependent apoptotic protease cascade (Liu *et al.* 1996, Li *et al.* 1997, Earnshaw 1999). The role of SDHC as an apoptotic regulator has been proposed in a number of studies (Albayrak *et al.* 2003, Ishii *et al.* 2005).

5.4. TCAC defects and reactive oxygen species (ROS)

Defects in mitochondrial metabolism have been suggested to promote production of reactive oxygen species (ROS) (Arosio and Levi 2002, Rustin 2002). ROS have been proposed to participate in the etiology of cancer and aging (Oberley and Buettner 1979, Oberley 2002). Overproduction of ROS and changes in cellular energy metabolism had been previously detected in ceSDHC (homologous to human *SDHB*) mutant in *Caeornorhabditis elegans* (Ishii *et al.* 1998, Senoo-Matsuda *et al.* 2001). Ishii *et al.* (2005) tested this phenomenon in mammals using a transgenic cell line with a mutated SDHC gene and detected over-produced superoxide anion (one type of ROS), elevated apoptotic rate, and significantly higher mutation frequency compared to wild type constructs. In contrast to this finding, two other studies observed neither ROS generation or nor a shift to a more oxidized state in SDH- and FH-deficient cell lines (Pollard *et al.* 2005, Selak *et al.* 2005).

5.5. Pseudo-hypoxia model of mitochondrial tumor suppression

Most evidence has been obtained for the pseudo-hypoxia model derived by loss-of-function of mitochondrial tumor suppressor genes. Recently, evidence has been presented that HPLG tumors have activated hypoxia and angiogenic pathways, i.e. vascularization (Gimenez-Roqueplo *et al.* 2001). Pheocromocytomas are also associated with VHL, which is the most common condition predisposing to hereditary RCC (Maddock *et al.* 1996). Pheochromocytomas with *VHL*, *SDHB*, or *SDHD* mutations have been linked together by HIF1α mediated hypoxia pathway signals (Dahia *et al.* 2005). The existence of an autoregulatory loop where HIF1α contributes to decreased SDHB levels, resulting in mitochondrial complex II inhibition, was supported. Mutations in the *VHL* gene cause stabilization of the sub-units of hypoxia-inducible factors (HIF) resulting in activation of pro-angiogenic proteins such as vascular endothelial growth factor (VEGF) (Maxwell *et al.* 1999, Jones *et al.* 2001). In the subsequent studies of Pollard *et al.* (2005), up-

regulation of *VEGF* and *BCL2 interacting protein 3 (BNIP3)*, and down-regulation of *thrombospondin 1 (TSP1)* were found in HLRCC uterine leiomyomas. The *VEGF* promoter contains a hypoxia response element and the protein is an angiogenic activator through induction of cell proliferation and inhibition of apoptosis (Leung *et al.* 1989). Contrary to *VEGF*, *TSP1* suppresses angiogenesis by inducing endothelial cell apoptosis (Nor *et al.* 2000).

It has been previously suggested that succinate, the TCAC intermediate and respiratory chain substrate, is capable of restoring the HIF1α hypoxic induction through electron transport chain or through some metabolic pathway (Agani *et al.* 2000, Agani *et al.* 2002). Using the RNAi technique Selak *et al.* (2005) described a mitochondria-to-cytosol signaling pathway that links mitochondrial defects to tumorigenic processes. Down-regulation of SDH activity caused accumulation of succinate in mitochondria. Succinate is freely transported to cytosol where it is able to inhibit HIF1α prolyl hydroxylases (PHD), which leads to dissociation of VHL from HIF1α.

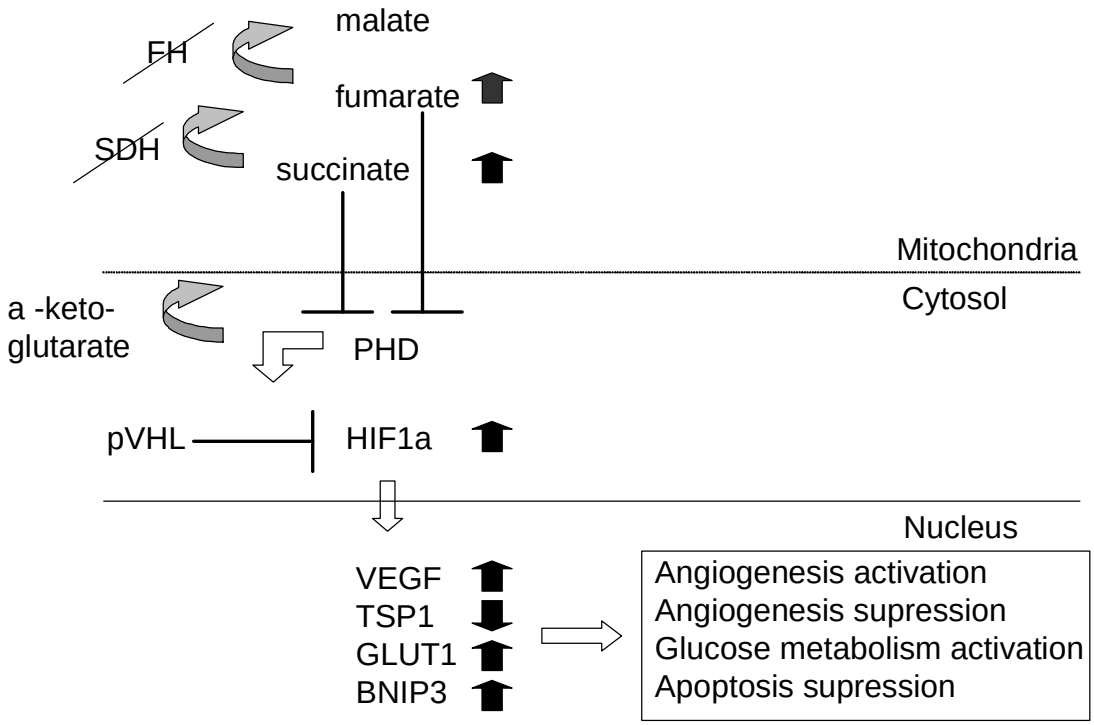

Figure 4. *Molecular effects of TCAC blockage in FH/SDH deficient tumors. Inactivation of FH results in accumulation of fumarate and succinate in mitochondria. The excess amount of these enzymes will be transported to cytosol. Elevated fumarate and succinate inhibit thereby HIF1α hydroxylation leading to decreased binding of pVHL to HIF1α. Increased HIF1α activity results in changes in hypoxia pathway (upregulation of VEGF, BNIP3, and GLUT1, and down-regulation of TSP1). IN SDH deficient tumors, signaling is driven predominantly through accumulation of succinate.*

This event stabilizes HIF1α and leads to activation of the HIF pathway in kidney cells. Pollard *et al.* (2005) confirmed these findings in benign and malignant HLRCC tumors. HIF1α over-expression, moderate in HLRCC myomas and strong in HLRCC clear cell cancer, probably due to accumulation of fumarate and succinate, was demonstrated. Accumulation of succinate and over-expression of HIF1α and VEGF was shown also in SDHB-deficient HPGL tumors.

HIF1α and HIF2α over-expression was observed directly in renal tumor tissues from HLRCC patients by Isaac *et al.* (2005). To test the hypothesis that the HIF over-expression is due to loss of FH, conditions in HLRCC tumors were mimicked in cells using FH/SDH inhibitor, 3-nitropropionic acid (3-NPA). Accumulation of fumarate due to FH inhibition correlated with HIF upregulation in protein but not in the mRNA level. Isaac *et al*. (2005) further demonstrated that an increase in the intracellular fumarate level up-regulates VEGF and GLUT1 in a HIF-dependent manner.

VEGF is a key mediator of angiogenesis in the majority of human tumors, because it facilitates endothelial cell proliferation, promotes cell migration, and inhibits apoptosis (Leung *et al.* 1989, Neufeld *et al.* 1999, Bergers and Benjamin 2003). VEGF expression is induced by a large number of growth factors, hypoxia, or acidosis (Shweiki *et al.* 1992, Fukumura *et al.* 2001, Ferrara 2004). VEGF over-expression has been demonstrated in fumarate-induced cells and in HLRCC myomas (Isaacs *et al.* 2005, Pollard *et al.* 2005). Microvessel density, probably due to response to hypoxia, has been shown to be remarkably higher in HLRCC leiomyomas compared to surrounding myometrium or sporadic, FH proficient myomas (Pollard *et al.* 2005). However, it remains unknown how activation of angiogenic signaling leads to tumor growth through accelerated cell proliferation or decreased cell death.

Most human invasive cancers show significant increases in glucose uptake because of upregulation of glucose transporters, notably GLUT1 and GLUT3, and hexokinases (Mathupala *et al.* 1997, Macheda *et al.* 2005). Glygolysis requires conversion of glucose to pyruvate and then to lactic acid. In normal human cells, glygolysis is inhibited by oxygen leading to oxidation of pyruvate in mitochondria. Increased glycolysis in the presence of oxygen and accumulation of lactate are well known phenomenon of cancers, leading to microenvironmental acidosis (Weinhouse 1976). Main intracellular signals that lead to inappropriate activation of angiogenic pathways in TCAC-defective tumors are summarized in Figure 4.

Hypoxia and energy starvation have been shown to activate multiple key regulators, such as translation eukaryotic initiation factor-2-alpha (eIF2α), eukaryotic elongation factor 2 (eEF2), and mTOR effectors eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP1) and $p70^{S6K}$ kinase, independent of HIF (Liu *et al.* 2006). These effects could be potentially linked to *FH* mutation-derived tumorigenesis through altered energy metabolism and overlapping phenotypes of HLRCC, VHL and TSC.

Mitochondrial involvement in common multifactorial cancers could be also speculated. Significant decreases in the activities of mitochondrial enzymes, including ketoglutarate dehydrogenase (KDH), malate dehydrogenase (MDH), and SDH was detected in benzo(a)pyrene-induced lung tumors in mice (Selvendiran *et al.* 2005).

In conclusion, the molecular mechanisms of FH derived tumorigenesis require more detailed examination. Most evidence has been obtained for the pseudo-hypoxia model of mitochondrial tumor suppressor function. Similarities in clinical symptoms and molecular background between the tumor predisposition syndromes link defects in FH, SDH, VHL, and TSC1/2 to overlapping pathways, probably through HIF signaling.

6. Clinical implications

The accurate diagnosis of HLRCC has become possible with the identification of the *FH* gene mutations causing the syndrome. The recognition of mutation carriers is particularly important because these persons are at risk of developing malignant cancers, especially RCC and ULMS. The renal cancers which are related to the HLRCC syndrome seem to be exceptionally aggressive, and the majority of these patients die because of metastasized disease shortly after diagnosis (Launonen *et al.* 2001). Consequently, regular follow-up of the mutation-positive family members is important. On the other hand, the unnecessary follow-up of mutation-negative members of the family can also be avoided. Due to the aggressiveness of renal lesions in HLRCC, in contrast to other hereditary renal cancer syndromes, immediate radical hysterectomy has been recommended for HLRCC patients with renal tumors (Kiuru *et al.* 2004).

In Finland, genetic testing is very well accepted and the subjects have been satisfied with their decision to take tests (Liljestrom *et al.* 2005). Neither increased anxiety nor other long-term psychological consequences have been detected regarding genetic testing when combined with proper genetic counseling and support among HNPCC patients (Aktan-Collan *et al.* 2001). Therefore, genetic testing of *FH* mutations should be considered also among HLRCC family members.

Discovery of TCAC defects in human tumor predisposition increases our knowledge of molecular mechanisms of tumorigenesis in general. Further insight into the role of basic cellular energy metabolism may help us to better understand complex genetic and environmental interactions in common tumors, like uterine leiomyomas. Differences in mitochondrial function between normal and cancer cells might offer potential for anticancer drug and therapy development.

CONCLUSIONS AND FUTURE PLANS

1. This study describes a novel cancer gene. *Fumarase* (*FH*) mutations predispose, with high penetrance, to dominantly inherited cancer syndrome, HLRCC/MCUL. Biallelic inactivation of FH, in the most cases through LOH, is typically needed for tumor development, suggesting the role of FH as a tumor suppressor.

2. *FH* is unlikely to be a predisposing gene in other studied familial cancers, breast and prostate cancers.

3-4. Data from nonsyndromic tumor studies, including 943 lesions, show that mutations in *FH* are rare outside the HLRCC context. Purely somatic biallelic inactivation of FH has been detected in this study only in two uterine leiomyomas and one soft-tissue sarcoma. Additionally, we have found somatic mutations combined with inherited *FH* alterations in one cutaneous leiomyoma and one uterine leiomyosarcoma. In addition to this study, only two *FH* mutations in nonsyndromic tumors, one uterine leiomyoma and one leiomyosarcoma, both with germline changes, have been found. Studies in apparently sporadic tumors have given further evidence to the proposed Knudson's two-hit model of FH inactivation. This study also shows additional evidence that uterine leiomyosarcoma belongs to the tumor spectrum of HLRCC.

FH mutations appear to be rare in nonsyndromic tumors, and when they exist, seem to be restricted to tumor types associated with the HLRCC syndrome. However, our strategy of using LOH as a preliminary target indicator identifies *FH* as the first gene known to undergo somatic inactivation in nonsyndromic uterine leiomyomas by specific point mutations. This is of great clinical interest, because leiomyomas are the most common tumors of women. To our knowledge, *FH* is the first gene shown to undergo mutational inactivation, in both the somatic and hereditary forms of the disease, in leiomyomata of the uterus.

Much effort will be needed to define the actual molecular mechanism of how FH inactivation leads to accelerated tumor growth, and which factors determine phenotype differences in *FH* mutation carriers and differences in frequencies of malignant tumors in different populations. We believe that a combinatorial effect of *FH* and an unknown modifying gene is needed for predisposition for ULMS and RCC in HLRCC. One of our current main efforts, in addition to FH related functional studies, is to localize and identify the potential modifier gene/genes.

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ELECTRONIC DATABASE INFORMATION

Basic Local Alignment Search Tool BLAST	http://www.ncbi.nlm.nih.gov/BLAST/
Ensemble Human Genome Browser	http://www.ensembl.org
Finnish Cancer Registry	http://www.cancerregistry.fi
Melt program web page	http://insertion.stanford.edu/melt1.html
NCBI Entrez Genome Map Viewer	http://www.ncbi.nlm.nih.gov/entrez/
NetGene2 server	http://genome.cbs.dtu.dk/services/NetGene2/
NIX	Nucleic Acid Identification Program (Human Genome Mapping Project Resource Center, UK)
Primer3 server	http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi
Tumor Gene Database	http://condor.bcm.tmc.edu/oncogene.html