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MOLECULAR BASIS OF HEREDITARY LEIOMYOMATOSIS AND RENAL CELL CANCER (HLRCC)

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Academic dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by Roman numerals I-IV. In addition, some unpublished data will be presented.

I. Launonen V *, Vierimaa O *, Kiuru M *, Isola J, Roth S, Pukkala E, Sistonen P, Herva R, and Aaltonen LA. Inherited susceptibility to uterine leiomyomas and renal cell cancer. *Proceedings of the National Academy of Sciences USA* (2001). 98, 3387-3392.

II. Kiuru M, Launonen V, Hietala M, Aittomäki K, Vierimaa O, Salovaara R, Arola J, Pukkala E, Sistonen P, Herva R, Aaltonen LA. Familial cutaneous leiomyomatosis is a two-hit condition associated with renal cell cancer of characteristic histopathology. *American Journal of Pathology* (2001). 159, 825-829.

III. The Multiple Leiomyoma Consortium.
Group 1: Tomlinson IP, Alam NA *, Rowan AJ *, Barclay E, Jaeger EE, Kelsell D, Leigh I, Gorman P, Lamlum H, Rahman S, Roylance RR, and Olpin S
Group 2: Bevan S *, Barker K, Hearle N, and Houlston RS
Group 3: Kiuru M *, Lehtonen R *, Karhu A, Vilkki S, Laiho P, Eklund C, Vierimaa O, Aittomäki K, Hietala M, Sistonen P, Paetau A, Salovaara R, Herva R, Launonen V, and Aaltonen LA.
Germline mutations in *FH* predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. *Nature Genetics* (2002). 30, 406-410.

IV. Kiuru M *, Lehtonen R *, Arola J, Salovaara R, Järvinen H, Aittomäki K, Sjöberg J, Visakorpi T, Knuutila S, Isola J, Delahunt B, Herva R, Launonen V, Karhu A, and Aaltonen LA. Few *FH* mutations in sporadic counterparts of tumor types observed in hereditary leiomyomatosis and renal cell cancer families. *Cancer Research* (2002). 62, 4554-4557.

* Equal contribution

ABBREVIATIONS

- 1	loss of chromosome 1
+ 1	gain of chromosome 1
1p	short arm of chromosome 1
1q	long arm of chromosome 1
_-KG	_-ketoglutarate
APC	*adenomatous polyposis coli*
AT	ataxia-telangiectasia syndrome
ATM	*ataxia telangiectasia mutated*
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BHD	Birt-Hogg-Dubé syndrome
BLAST	basic local alignment search tool
BLM	Bloom syndrome gene
BMPR1A	*bone morphogenetic protein receptor type 1A*
bp	base pair
BRCA1/2	*breast and ovarian cancer gene –1* and *–2*
CHML	*choroideremia-like*
CSGE	conformation-specific gel electrophoresis
CDK4	*cyclin-dependent kinase 4*
CDKN2	*cyclin-dependent kinase inhibitor 2*
cDNA	complementary deoxyribonucleic acid
CGH	comparative genomic hybridization
CHK2	*checkpoint kinase 2*
CK7	cytokeratin 7
cM	centiMorgan
CNS	central nervous system
C-terminus	carboxyterminus
DAPI	4,6-diamidino-2-phenyindole
DGGE	denaturing gradient gel electrophoresis
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidetriphosphate
EMA	epithelial membrane antigen
EGF	epidermal growth factor
EST	expressed sequence tag
EXO1	*exonuclease 1*
$FADH_2$	flavin adenine dinucleotide, reduced form
FANCA/C/D2/E/F/G	Fanconi anemia genes
FAP	familial adenomatous polyposis syndrome
FH	*fumarate hydratase (fumarase)*

FHIT	*fragile histidine triad*
FISH	fluorescence *in situ* hybridization
G3PDH	*glyceraldehyde-3-phosphate dehydrogenase*
GnRH	gonadotropin-releasing hormone
H&E	hematoxylin-eosin
HIF	hypoxia inducible factor
HLRCC	hereditary leiomyomatosis and renal cell cancer
HMGF1/2	human milk fat globule –1 and –2
HMGIC/Y	genes encoding high mobility group protein isoforms IC and IY
HNPCC	hereditary nonpolyposis colorectal cancer
HPRC	hereditary papillary renal carcinoma
HPT-JT	hyperparathyroidism-jaw tumor syndrome
HREs	hypoxia response elements
IGFI/II	insulin-like growth factor –I and –II
JPS	juvenile polyposis syndrome
K-RAS	gene encoding Harvey sarcoma virus homolog, Kirsten type
kb	kilobase
KMO	*kynurenine 3-monooxygenase*
LKB1	gene encoding a serine-threonine kinase, mutated in PJS
lod	logarithm of odds
LOH	loss of heterozygosity
Mb	megabase
MCL	hereditary multiple cutaneous and uterine leiomyomatosis
MEN1/2	*multiple endocrine neoplasia type 1* and *2*
MET	gene encoding hepatocyte growth factor receptor, mutated in HPRC
MLH1	*human mutator l homolog –1*
MRI	magnetic resonance imaging
MSH2/3/6	*human mutator s homolog –2/–3/–6*
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NCBI	National Center for Biotechnology Information
NF1/2	*neurofibromin –1* and *-2*
nt	nucleotide
N-terminus	aminoterminus
OGDH	oxoglutarate (_-ketoglutarate) dehydrogenase
OPN3	*opsin 3*
p53 (TP53)	*tumor protein 53*
PCR	polymerase chain reaction
PGL	familial paraganglioma
PDGF	platelet-derived growth factor
PMS1/2	*human postmeiotic segregation increased –1* and *-2*
PRKARIA	gene encoding cAMP-dependent protein kinase type I-alpha subunit
PTCH	human homolog of *Drosophila melanogaster patched* gene
PTEN	*phosphatase and tensin homolog*
pter	telomere of short arm of chromosome
qter	telomere of long arm of chromosome

RAD51B	gene homologous to bacterial *RecA* and *Saccharomyces cerevisiae Rad51*
RB	*retinoblastoma*
RCC	renal cell carcinoma
RET	*rearranged during transfection*
RGS7	*regulator of G protein signaling 7*
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SDH	succinate dehydrogenase
SMAD2/4	human homologs of *Drosophila melanogaster Mad* gene
SSCP	single-strand conformation polymorphism
TCAC	tricarboxylic acid cycle
TS	tuberous sclerosis syndrome
TCS1/2	*tuberous sclerosis -1* and *-2*
TGFßRII	*transforming growth factor receptor type II*
TNM	tumor-node-metastasis
TRC8	human gene related to *Drosophila melanogaster patched* gene
TSG	tumor suppressor gene
VHL	von Hippel-Lindau syndrome
VHL	von Hippel-Lindau syndrome gene
v-src	Rous sarcoma virus gene (human homolog *SRC*)
WT1	Wilm's tumor syndrome gene
XPA/B/C/D/E/F/G	xeroderma pigmentosum syndrome genes

ABSTRACT

Much of the present understanding of the development of human cancer has arisen through studies of rare, dominantly inherited cancer syndromes. Investigations of cancer susceptibility have revealed several genes involved in the development of both hereditary and sporadic cancer forms. Furthermore, identification of these genes has provided a means for diagnostic testing of predisposed individuals and subsequent cancer prevention in individuals at risk. Although many cancer syndromes with distinct features have been characterized, novel disease forms no doubt exist. This study describes a previously unrecognized cancer syndrome with predisposition to leiomyomas of the uterus and skin, uterine leiomyosarcoma, and renal cancer (hereditary leiomyomatosis and renal cell cancer, HLRCC).

Hereditary renal cancer has conventionally been divided into two well-characterized syndromes. von Hippel-Lindau disease (VHL), associated with clear cell renal carcinoma and vascular tumors of the retina and central nervous system, is caused by a germline mutation in the *VHL* tumor suppressor gene in 3p25. Hereditary papillary renal cell carcinoma (HPRC), which is associated with multiple bilateral papillary renal carcinomas, is caused by mutations in the *MET* oncogene in 7q21. In addition, renal cancer is associated with other hereditary tumor predisposition conditions such as tuberous sclerosis (TS) and Birt-Hogg-Dubé syndrome (BHD).

Uterine leiomyomas (fibroids, myomas) are the most common tumors in women, with a prevalence of over 25%. They have a major impact on women's health by causing morbidity and infertility and being the leading cause of hysterectomy. Despite the clinical relevance of these lesions, little is known about their molecular background. In rare cases, hereditary predisposition to uterine leiomyomas can be observed together with susceptibility to cutaneous leiomyomas (multiple cutaneous and uterine leiomyomatosis, MCL).

In this study, we identified a novel tumor predisposition condition, HLRCC. Multiple lines of evidence supported this conclusion. First, the tumor spectrum in the disease families, i.e. susceptibility to uterine and/or cutaneous leiomyomas, uterine leiomyosarcoma, and renal cancer, had not been proposed before. Second, the renal cell carcinoma phenotype was unique. The tumors displayed exceptional papillary histology that could also be used to identify additional families. The third and most conclusive evidence was localization of the disease gene to 1q42-q43 by genome-wide search. The linkage was confirmed by detecting wild-type allele loss at this locus in the majority of patients' tumors, which also suggested that the underlying genetic defect would impair the function of a tumor suppressor gene. Furthermore, we found that a family originally diagnosed with MCL displayed similar clinical, histopathological, and molecular features as in HLRCC. These findings, together with a subsequent and independent study reporting linkage to the same chromosomal region in MCL families, demonstrated that MCL and HLRCC are a single disease with a variable phenotype.

Through mutation screening of known and predicted transcripts in the susceptibility locus, we identified the

disease-predisposing gene, *fumarate hydratase* (*fumarase, FH).* *FH* encodes an enzyme component of the mitochondrial tricarboxylic acid (Krebs) cycle, thus playing a key role in cellular energy metabolism. Germline mutations in *FH* were detected in 25 of 42 HLRCC and leiomyomatosis families and included protein-truncating mutations and substitutions or deletions of highly conserved amino acids. Both *FH* alleles were inactivated in almost all patients' tumors. Moreover, FH enzyme activity was reduced in patients' normal tissues and was almost absent in their tumors. Thus, we demonstrated that germline mutations in *FH* underlie HLRCC and that *FH* is a putative tumor suppressor gene.

To investigate the role of *FH* mutations in sporadic tumorigenesis, we performed mutation analyses on a series of sporadic counterparts of tumor types associated with HLRCC. Although mutational inactivation of *FH* appeared to be rare in the studied tumor series, biallelic inactivation of *FH* was detected in a uterine leiomyosarcoma, a cutaneous leiomyoma, and a soft-tissue sarcoma. Whereas the two former tumors originated from a germline mutation, the sarcoma displayed two somatic hits. These findings supported our previous results on the robust biallelic inactivation pattern of *FH* and demonstrated that *FH* could be involved in the development of nonhereditary tumors.

Identification of *FH* in cancer predisposition provides insights into development of leiomyomas, leiomyosarcoma, and renal cell carcinoma. This study is the first to show mutations in a gene encoding a Krebs cycle enzyme in common human tumors. Elucidation of the molecular basis of HLRCC could facilitate diagnosis, detection, and prevention of tumors in the predisposed individuals. Moreover, the findings of this study could shed light on the molecular pathogenesis of the sporadic disease form, thus being relevant for larger populations, including women affected with uterine leiomyomas.

INTRODUCTION

Cancer is one of the leading causes of morbidity and death in developed countries. Up to every third individual develops cancer and every fifth dies from it. Epidemiological studies have shown that environmental and lifestyle factors, such as smoking, diet, certain viruses, radiation, and reproductive and hormonal factors, increase cancer risk (Peto, 2001). At the molecular level, various exogenous and endogenous carcinogens cause damage to the DNA. Accumulation of genetic changes then leads to transformation of normal cells into cancer cells (Nowell, 1976; Hanahan and Weinberg, 2000; Ponder, 2001). Thus, cancer is a disease of the genome.

The great majority of malignancies occur sporadically due to acquired mutations. Although no apparent family history can be detected in these cases, the genotypic profile has nevertheless been proposed to play a role in determining the cancer risk (Ponder, 2001). A small subset of cancers cluster in families. In these familial cancer cases, inherited genetic defects predispose to malignancy. Cancer predisposition can vary from weak to strong depending on the penetrance of the gene (Ponder, 2001). Susceptibility to malignancy is high in hereditary cancer syndromes, which account for approximately 1% of human cancers. These diseases are typically characterized by tumor development at an early age, bilateral disease, multiple primary tumors, and association with other clinical manifestations (Marsh and Zori, 2002).

To date, the genetic basis for over 20 hereditary cancer syndromes has been elucidated (Kinzler and Vogelstein, 2002). Studies on these diseases have provided insights into the mechanisms of cancer development. Furthermore, in clinical practice, diagnostics and prevention of the diseases have been improved through genetic testing and regular screening for tumors in individuals at risk.

Although many cancer syndromes with distinct features have been characterized, some previously unrecognized disease forms are likely to exist. This study describes clinical, histopathological, and molecular features of a novel dominantly inherited tumor predisposition syndrome with susceptibility to uterine and cutaneous leiomyomas and papillary renal cell carcinoma (hereditary leiomyomatosis and renal cell cancer, HLRCC). The findings of this study increase the present knowledge of inherited cancer susceptibility and of the molecular background of some common human tumor types, including leiomyomas, the most prevalent tumors in women.

REVIEW OF THE LITERATURE

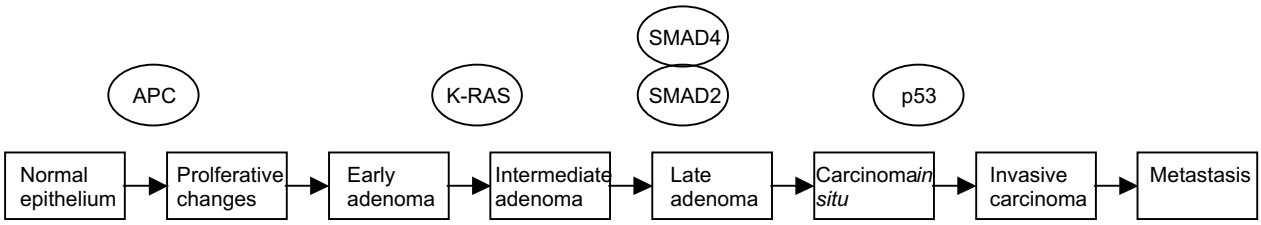
1. CANCER AND GENES

Cancer is characterized by clonal expansion of cells, which become malignant via a series of genetic alterations (Fearon and Vogelstein, 1990). Through accumulation of genetic changes, the cells acquire properties typical of the cancerous phenotype. Hanahan and Weinberg (2000) have proposed six groups of such features: self-sufficiency in signals stimulating growth, insensitivity to negative growth signals, evasion of programmed cell death, capacity for sustained proliferation, angiogenesis, and tissue invasion and metastasis. A widely documented example of the multistep process of tumorigenesis is development of colorectal carcinoma, which is distinguished by well-defined histological stages and referred to as the adenoma-carcinoma sequence (Vogelstein et al., 1988; Fearon and Vogelstein, 1990). Progression from normal epithelium to carcinoma occurs through adenomatous stages and is characterized by accumulation of genetic alterations (Figure 1). The changes include either inactivation of tumor suppressor genes or activation of oncogenes, giving cells a growth advantage over the normal cell population and thus leading to malignancy.

1.1 Oncogenes

Oncogenes are mutated forms of normal cellular components, proto-oncogenes. Mutations in proto-oncogenes enhance their function, providing cells with a growth advantage. Oncogenes were initially discovered in viral genomes (e.g. v-src), as they were able to transduce normal cellular genes to become constitutively active, and thus, induce tumorigenesis (Stehelin et al., 1976; Bishop, 1981). The protein products of proto-oncogenes are highly conserved in evolution and are involved in normal cellular signaling regulating cell growth and differentiation. In human cancer, proto-oncogenes are activated through genetic alterations, such as gain-of-function mutations, gene amplifications, and chromosomal translocations (Bishop, 1991). A single activating mutation in one of the two alleles is sufficient to promote abnormal growth. Thus, oncogenes are dominant at the cellular level. To date, over 100 oncogenes have been identified (Futreal et al., 2001).

Figure 1. A simplistic model of multiple steps in colorectal tumorigenesis (modified from Vogelstein et al., 1988; Fearon and Vogelstein, 1990).



1.2 Tumor suppressor genes

Tumor suppressor genes are negative regulators of cell growth (Marshall, 1991; Weinberg, 1991). The first observations supporting the existence of these genes came from somatic cell studies. Tumor cell growth was demonstrated to be suppressed by fusing nonmalignant cells with cancer cells (Ephrussi et al., 1969; Harris, 1988). Later, the reversion to the tumorigenic phenotype was shown to be associated with loss of specific chromosomal material (Shimizu et al., 1990; Trent et al., 1990).

The leading evidence for the presence of tumor suppressor genes came through studies on the childhood eye tumor retinoblastoma. Knudson (1971) suggested that two mutational events were required for retinoblastoma development. In hereditary cases, one mutation was inherited via the germline of an individual and the second mutation occurred at the somatic level. In the nonhereditary forms of the disease, by contrast, the two events were somatic, and thus, less likely to occur. The two genetic events in the Knudson's model were subsequently shown to represent biallelic inactivation of a tumor suppressor gene. This was demonstrated by cloning of the *retinoblastoma* (*RB*) gene and detecting loss-of-function mutations in both *RB* alleles in retinoblastomas (Lee et al., 1987).

In addition to *RB*, the two-hit inactivation pattern has been demonstrated in other tumor suppressor genes as well. Whereas the first hit is either an inherited or a somatic mutation in a given tumor suppressor gene, the second hit is often a gross chromosomal mechanism, resulting in hemi- or homozygosity of the chromosomal region containing the respective gene, i.e. loss of heterozygosity (LOH). Such chromosomal events include deletion, gene conversion, nondisjunctional chromosome loss, and mitotic recombination. LOH can be detected by comparing the genotypes of the tumor and the corresponding normal tissue at a given polymorphic marker (Devilee et al., 2001). In addition to genetic changes, epigenetic mechanisms, such as transcriptional silencing through promoter hypermethylation, can lead to functional inactivation of tumor suppressor gene alleles (Jones and Baylin, 2002).

1.1.1 Gatekeepers, caretakers, landscapers

Approximately 30 tumor suppressor genes have been identified thus far (Futreal et al., 2001). Due to the growing number of these genes and knowledge about the functional roles of the gene products, they have been further defined and classified. Tumor suppressors have been suggested to include 'genes that sustain loss-of-function mutation in the development of cancer' (Haber and Harlow, 1997). This broad definition includes all such genes regardless of their presumed function and thus takes into account the obscurity of the mechanisms by which inactivation of a tumor suppressor gene actually leads to tumor development.

Genes harboring loss-of-function mutations can be classified as 'gatekeepers' or 'caretakers' depending on their putative role in the cellular processes (Kinzler and Vogelstein, 1997). Gatekeepers are thought to directly regulate tumor growth by inhibiting proliferation or promoting cell death. In the multistep process of cancer development, each cell type presumably has certain gatekeepers whose inactivation is rate-limiting for tumor initiation (Kinzler and Vogelstein, 1997). According to Knudson's model, cells undergo neoplastic transformation when both copies of a given gene are inactivated in the target tissue. For example, *RB*, *VHL*, *NF1*, and *APC* have been proposed to be gatekeepers of tumor growth in the retina, kidney, Schwann cells,

and colon, respectively (Kinzler and Vogelstein, 1997).

In contrast to gatekeepers, caretakers inhibit tumor growth indirectly by maintaining genomic integrity (Kinzler and Vogelstein, 1997). Mutations in caretaker genes increase genetic instability, leading to an elevated mutation rate in all genes, including tumor suppressor genes and oncogenes. Thus, cancer development is accelerated. Caretakers include genes involved in DNA repair and replication, such as mismatch repair genes and *BRCA1* and *BRCA2* genes mutated in the well-known hereditary cancer syndromes hereditary non-polyposis colorectal cancer (HNPCC) and familial breast and ovarian cancer, respectively (see section 1.3 and Table 1).

A third category of tumor suppressors, 'landscapers', has also been proposed (Kinzler and Vogelstein, 1998). Defects in landscapers are speculated to contribute to neoplastic transformation by generating an abnormal stromal environment, and thus, abnormal intercellular signaling. The landscaper effect has been suggested to occur in juvenile polyposis syndrome (JPS) (Kinzler and Vogelstein, 1998). According to the theory, increased colon cancer risk in JPS results from the effects of abundant stroma on epithelial cells in hamartomatous colonic polyps.

1.3 Genes for hereditary cancer

Studies on rare hereditary cancer syndromes have revealed several genes predisposing to cancer (Table 1). Most hereditary cancer syndromes are caused by germline mutations in tumor suppressor genes. In rare cases, however, inherited mutations in proto-oncogenes have been shown to cause susceptibility to cancer. These genes include *MET*, *RET*, and *CDK4*, causing hereditary papillary renal cell carcinoma (HPRC), multiple endocrine neoplasia type 2, and familial melanoma, respectively. Although most hereditary cancer syndromes are dominantly inherited, a few autosomal recessive diseases exist, such as ataxia-teleangiectasia, Bloom's syndrome, Fanconi anemia, and xeroderma pigmentosum.

Mutations in some of the cancer-susceptibility genes have been shown to be key features not only in hereditary but also in sporadic cancers. Examples of such genes are *APC* and *VHL*, which cause familial adenomatous polyposis (FAP) and the hereditary renal cancer syndrome von Hippel-Lindau disease (VHL), respectively. Mutations in *APC* are present in 85-90% of colorectal cancers (Kinzler and Vogelstein, 1996). Loss of *VHL* can be detected in almost all clear cell renal carcinomas. Over half of them display biallelic inactivation of *VHL* through loss of one allele and somatic mutation or promoter hypermethylation of the other allele (Gnarra et al., 1994; Herman et al., 1994).

Studies on cancer-susceptibility genes have provided insights into tumorigenic mechanisms. In addition to the biallelic inactivation of a tumor suppressor gene, other phenomenons, including epigenetic silencing (Jones and Baylin, 2002) and haploinsufficiency (Quon and Berns, 2001), have been shown to contribute to tumorigenesis. Genes such as *p53*, *p27Kip1*, and *LKB1* have been suggested to be haploinsufficient for tumor suppression, i.e. losing only one allele is sufficient for tumor initiation

Table 1. Some genes predisposing to hereditary cancer syndromes (Modified from Marsh and Zori, 2002).

Syndrome	**Gene and location**	**Proposed category of the gene**	**Tumor spectrum**	**Protein function**
Ataxia-teleangiectasia	*ATM* (11q22-q23)	Caretaker	Breast cancer, leukemia, lymphoma	Protein kinase Maintenance of genomic integrity
Bannayan-Riley-Ruvalcaba syndrome	*PTEN* (10q23.3)	Gatekeeper	Intestinal hamartomas, breast and thyroid cancers	Phosphatase inhibiting PI3K-Akt pathway Regulation of cell cycle, apoptosis, angiogenesis
Bloom's syndrome	*BLM* (15q26.1)	Caretaker	Leukemia, lymphoma, multiple carcinomas	DNA helicase Maintenance of genomic integrity
Carney complex	*PRKR1A* (17q22-q24)	TSG	Cardiac myxomas, other myxomas, endocrine tumors, melanotic schwannomas	Protein kinase A regulatory subunit Role in the cAMP pathway
Cowden syndrome	*PTEN* (10q23.3)	Gatekeeper	Intestinal hamartomas, breast and thyroid cancers, skin tumors	Phosphatase inhibiting PI3K-Akt pathway Regulation of cell cycle, apoptosis, angiogenesis
Familial adenomatous polyposis	*APC* (5q21)	Gatekeeper	Adenomatous polyps of the colorectum, colorectal, small intestinal, and gastric cancers, osteomas, desmoid tumors, medulloblastoma	Sequestering ß-catenin in cytoplasm Regulation of cell proliferation, migration, adhesion, chromosomal stability
Familial breast and ovarian cancer	*BRCA1* (17q21), *BRCA2* (13q12.3)	Caretaker	Breast and ovarian cancers, *BRCA2* also in prostate and male breast cancers	Maintenance of genomic integrity (involved in DNA repair)
Familial gastric cancer	*E-cadherin* (16q22)	Gatekeeper	Gastric cancer	Interaction with ß-catenin and regulation of cell adhesion
Familial melanoma	*CDKN2* (*p14, p16, p19ARF*) (9p21) CDK4 *(12q14)*	Gatekeeper Proto-oncogene	Melanoma, pancreatic, bladder, and esophageal cancers, leukemia Melanoma	Inhibition of cyclin-dependent kinases and blocking of cell cycle (p14, p16) Regulation of p53 through MDM2 (p19ARF) Promotion of cell cycle progression
Familial paraganglioma and pheochromo-cytoma	*SDHD* (11q23), *SDHC* (1q21), *SDHB* (1p36.1-p35)	TSG	Paragangliomas, pheochromo-cytomas	Mitochondrial complex II subunits: iron sulfur protein (SDHB), integral membrane proteins (SDHC, SDHD) Involved in the Krebs cycle and the electron transport chain
Fanconi anemia	*FANCA* (16q24.3), *FANCC* (9q22.3), *FANCD2* (3p25.3), *FANCE* (6p22-p21), *FANCF* (11p15), *FANCG* (9p13), *BRCA2* (13q12)	Caretaker	Leukemia, squamous cell carcinoma	Maintenance of genomic integrity
Hereditary nonpolyposis colorectal cancer	*MSH2* (2p16), *MLH1* (3p21), *PMS1* (2q32), *PMS2* (7p22), *MHS3* (5q11-q12), *MSH6* (2p16)	Caretaker	Colorectal, endometrial, stomach, ovarian, hepatobiliary, and urinary tract cancers, glioblastoma	DNA mismatch repair

Hereditary papillary renal cell carcinoma	*MET* (7q31)	Proto-oncogene	Papillary renal cell carcinoma	Receptor tyrosine kinase
Juvenile polyposis	*SMAD4* (18q21), *BMPR1A* (10q22)	Gatekeeper, putative landscaper (*SMAD4*)	Intestinal juvenile polyps, cancer risk elevated	Signal transductor in TGFß pathway (*SMAD4*) Member of TGFß-receptor superfamily, upstream from the SMAD pathway (*BMPR1A*)
Li-Fraumeni	*p53* (17p13) *CHK2* (22q12.1)	Gatekeeper	Sarcomas, breast cancer, brain tumors, leukemia, adrenocortical tumors	Transcription factor, regulation of apoptosis, cell cycle, angiogenesis (*p53*) Checkpoint kinase, involved in DNA damage response (*CHK2*)
Multiple endocrine neoplasia type 1	*MEN1* (11q13)	Unknown	Pancreatic islet cell tumors, pituitary adenomas, parathyroid hyperplasia,	Interaction with the AP-1 transcription factor JunD and repression of JunD-activated transcription
Multiple endocrine neoplasia type 2	*RET* (10q11)	Proto-oncogene	Medullary thyroid carcinoma, pheochromocytoma, parathyroid hyperplasia	Receptor tyrosine kinase
Neurofib-romatosis type 1 and type 2	*NF1* (17q11), *NF2* (22q12)	Gatekeeper	Neurofibromas, neurofibrosarcomas, glioma, astrocytoma, meningeomas, schwannomas	Regulation of Ras signaling
Neurofib-romatosis type 2	*NF2* (22q12)	Gatekeeper	Neurofibromas, neurofibrosarcomas, glioma, astrocytoma, meningeomas, schwannomas	Maintenance of cytoskeleton, suppressor of cell adhesion, motility, and spreading
Nevoid basal cell carcinoma syndrome	*PTCH* (9q22.3)	Gatekeeper	Basal cell skin carcinoma, medulloblastoma	Transmembrane receptor in sonic hedgehog pathway
Peutz-Jeghers syndrome	*LKB1* (19p13.3)	Gatekeeper	Intestinal hamartomas, small intestinal, gastric, colorectal, pancreatic, breast, and ovarian cancers, testicular tumors	Serine/threonine kinase Regulation of cell cycle
Retinoblastoma	*RB1* (13q14)	Gatekeeper	Retinoblastoma, osteosarcoma	Cell cycle control
Tuberous sclerosis	*TSC1* (9q34), *TSC2* (16p13)	Gatekeeper	Hamartomas, renal cell carcinoma, angiomyolipoma, astrocytoma	Regulation of cell proliferation, growth, adhesion
Werner syndrome	*RECQL2* (8p12-p11)	Caretaker	Sarcomas, meningeomas, thyroid cancer, melanoma	DNA helicase and exonuclease Maintenance of genomic integrity
Wilms tumor	*WT1* (11p13)	Gatekeeper	Nephroblastoma (embryonal childhood tumor in kidney)	Zinc finger transcription factor

von Hippel-Lindau syndrome	*VHL* (3p25)	Gatekeeper	Clear cell renal carcinoma, pheochromocytomas, retinal angiomas, hemangioblastomas	Component of ubiquitin-ligase complex Degradation of HIF-1_ transcription factor Involved in regulation of cell cycle, energy metabolism, adhesion, angiogenesis
Xeroderma pigmentosum	*XPA* (9q22), *XPB* (2q21), *XPC* (3p25), *XPD* (19q13), *XPE* (11p12), *XPF* (16p13) *XPG* (13q32)	Caretaker	Melanoma, basal cell carcinoma	DNA excision repair

(Fero et al., 1998; Venkatachalam et al., 1998; Rossi et al., 2002). However, the biallelic inactivation of a tumor suppressor gene has been proposed to contribute to tumor progression and more severe tumor susceptibility (Quon and Berns, 2001; Rossi et al., 2002). The degree of haploinsufficiency may vary among tumor suppressor genes, ranging from no apparent effect to weak or strong effects (Cook and McCaw, 2000; Quon and Berns, 2001).

1.4 The Human Genome Project

The Human Genome Project was launched over a decade ago to create publicly available high-quality sequences of genomes of human and key model organisms (Human Genome Project Information Website, 2002). Within the last decade, the program has rapidly progressed from generation of genetic (Weissenbach et al., 1992) and physical (McPherson et al., 2001) maps to production of a draft sequence of the human genome (Lander et al., 2001). Based on preliminary analysis of the draft genome sequence, the human genome is estimated to contain 30 000-40 000 protein-coding genes, which is only about twice as many as in a worm or a fly (Lander et al., 2001). In addition to the public effort, a private company, Celera Genomics, has reported a nearly complete sequence of the human genome (Venter et al., 2001). The final phase of the Human Genome Project aims to produce a complete sequence of the human genome by 2003.

After the Human Genome Project achieves its primary goal of the production of a publicly available sequence of the human genome, several other objectives remain to be met. One of the most important of these is to further analyze and interpret the raw sequence data to understand its biological significance (Stein, 2001). The process of genome annotation includes identifying genes and their regulatory regions, completing the map of human genome sequence variation, classifying and characterizing proteins, and relating the genome to biological processes. The rapidly expanding human genome data pool also requires concurrent examination and evaluation of ethical, legal, and social implications.

One of the key applications of human genome research has been identification of disease genes by positional cloning, i.e. based on their chromosomal location. Previously, this method included such tedious and time-consuming steps as generation of genetic markers, obtaining genomic sequences by chromosomal walking, and direct sequencing. Through progression of the Human Genome Project, the process has evolved into identification of candidate genes *in silico* and subsequent mutation screening of these sequences, thus

facilitating the search for disease-predisposing genes.

2. CARCINOMA OF THE KIDNEY

Cancer of the kidney accounts for approximately 2% of cancer cases worldwide (McLaughlin and Lipworth, 2000). Although it is less frequent than other urologic cancers, namely prostate and bladder carcinomas, it is the most malignant one, causing death in over 35% of affected individuals (Finnish Cancer Registry, 1999; Van Poppel et al., 2000). Renal cancer incidence has been increasing over the past decades in North America and Northern Europe (McLaughlin and Lipworth, 2000), including Finland (Finnish Cancer Registry, 1999) (see Table 2 for cancer incidence in Finland). Renal cell carcinoma most commonly occurs between the ages of 50 and 70 years, and males are affected twice as often as females. Smoking, obesity, and occupational exposures are associated with increased risk of renal cancer (McLaughlin and Lipworth, 2000). Hereditary predisposition is estimated to account for up to 4% of all cases (Linehan et al., 1995).

2.1 Histopathological and molecular features of renal neoplasms

Renal neoplasms consist of distinguishable entities with characteristic morphological and molecular features (Kovacs et al., 1997; Störkel et al., 1997; Reuter and Presti, 2000) (Table 3). The most common tumors of the kidney are benign papillary adenomas, which are discovered in up to 20% of autopsies in adults (Van Poppel et al., 2000). Almost all of the malignant neoplasms are renal cell carcinomas originating from the epithelium of proximal renal tubules and collecting tubules. These include clear cell (conventional), papillary, and chromophobe renal cell carcinoma, and collecting duct carcinoma. Wilm's tumor, a pediatric cancer of the kidney, is composed of blastemal, stromal, and epithelial components, and is usually excluded from classifications of renal tumors (Linehan et al., 2002).

Traditionally, staging and grading of tumors have been used to evaluate cancer prognosis. The staging scheme for renal tumors is based on the tumor-node-metastasis (TNM) classification, and the most widely used grading system is that proposed by Fuhrman et al. (1982). The Fuhrman classification divides tumors into four grades based on nuclear size, nuclear membrane irregularity, and nucleolar prominence. The prognostic value of the grading and staging systems varies between different histological subtypes (Medeiros et al., 1997; Reuter and Presti, 2000). Thus, subgrouping of tumors is necessary. This has been accomplished not only by evaluating tumor histology but also by cytogenetic and molecular profiling.

Clear cell carcinoma is the prevailing histologic subtype of renal carcinomas, comprising 70-75% of these tumors (Kovacs et al., 1997; Störkel et al., 1997). Histologically, clear cell carcinomas exhibit either an exclusively solid or acinar growth pattern, or a mixture of cystic, pseudopapillary, and tubular structures. Tumors are usually highly vascularized (Reuter and Presti, 2000). Tumor cells are cuboidal or polygonal in shape and have predominantly clear cytoplasm. Cells with eosinophilic or granular cytoplasm can also be observed and are often associated

Table 2. Prevalent cancer types in Finland (Finnish Cancer Registry, 1999).

Males	N (%)	Females	N (%)
Prostate	3112 (29.2)	Breast	3578 (31.7)
Lung, trachea	1473 (13.8)	Colon	738 (6.5)
Colon	598 (5.6)	Corpus uteri	695 (6.2)
Bladder, ureter, urethra	593 (5.6)	Ovary	522 (4.6)
Stomach	445 (4.2)	Lung, trachea	498 (4.4)
Rectum, rectosigmoid	440 (4.1)	Skin, nonmelanoma	453 (4.0)
Kidney	417 (3.9)	Nervous system	442 (3.9)
Skin, nonmelanoma	357 (3.4)	Rectum, rectosigmoid	425 (3.8)
Nervous system	330 (3.1)	Stomach	380 (3.4)
Melanoma of the skin	323 (3.0)	Pancreas	342 (3.0)
Pancreas	315 (3.0)	Non-Hodgkin's lymphomas	333 (3.0)
Non-Hodgkin's lymphomas	300 (2.8)	Melanoma of the skin	317 (2.8)
Leukemia	216 (2.0)	Kidney	299 (2.6)
Total	10643 (100.0)	Total	11286 (100.0)

with higher nuclear grade.

Genetically, clear cell carcinomas are characterized by losses of 3p (Kovacs, 1993) and defects in *VHL* at 3p25 (Table 3), the gene whose germline mutations cause the most common hereditary renal cancer syndrome, von Hippel-Lindau disease (see section 2.2.1). In a study on *VHL* involvement in sporadic renal cell carcinomas, loss of one *VHL* allele was observed in as many as 98% of clear cell carcinomas and mutational inactivation of the other *VHL* allele in 57% of cases (Gnarra et al., 1994). Promoter hypermethylation of *VHL* was later detected in 4 of the 7 tumors that had not displayed mutations in the earlier study (Herman et al., 1994). Inactivation of *VHL* has been shown to be specifically associated with clear cell carcinoma (Foster et al., 1994; Kenck et al., 1996).

Papillary renal cell carcinoma accounts for about 10-15% of renal carcinomas (Kovacs et al., 1997). The growth pattern of these lesions is characterized by papillary fronds containing a fibrovascular core and lined by neoplastic epithelial cells. In addition, tumors usually have small areas with tubular, tubulo-papillary, and solid structures. Papillary renal cell carcinomas can be further divided into type 1 and type 2 tumors, as proposed by Delahunt and Eble (1997). Type 1 and type 2 histologies correspond to previous divisions of tumors into basofilic and eosinophilic subgroups, respectively (Thoenes et al., 1986; Zambrano et al., 1999). Type 1 tumors consist of small cells with pale cytoplasm and small nuclei with inconspicuous nucleoli and frequent glomeruloid papillae with edema, foamy macrophages, and psammoma bodies. In contrast, type 2 tumor cells are larger, often have abundant cytoplasm and a tendency towards pseudostratification, and contain large nuclei with prominent nucleoli. Nuclear grade, growth kinetics, and survival rates have been found to differ between type 1 and 2 tumors (Delahunt et al., 2001).

Approximately 80% of papillary renal cell carcinomas have been observed to contain chromosomal gains. Characteristic changes include gains of chromosomes 7 and 17, and loss of chromosome Y in men (Meloni et al., 1992; Kovacs, 1993). These changes have also been detected in benign papillary adenomas, suggesting the possibility of an adenoma-carcinoma sequence in development of papillary renal tumors (Kovacs, 1993). Chromosome 7 harbors the *MET* oncogene, which is the predisposing gene for hereditary papillary

renal cell carcinoma (HPRC) (see section 2.3.1). In addition to frequent gains of the *MET* locus in sporadic papillary tumors, mutations in *MET* have been observed in approximately 10% of these lesions (Schmidt et al., 1997, 1999) (Table 3). In addition, other chromosomal abnormalities, such as somatic trans-location involving chromosomes 1 and X, have been detected in some papillary tumors (de Jong et al., 1986; Tomlinson et al., 1991; Ohjimi et al., 1993; Sidhar et al., 1996). In recent studies, some genetic differences between type 1 and 2 papillary renal cell carcinomas have been reported: gains of 7p and 17p and allelic imbalance on 17q were more frequent in type 1 than type 2 tumors, whereas allelic imbalance on 9p was present only in type 2 tumors (Jiang et al. 1998; Sanders et al., 2002).

Chromophobe renal cell carcinomas account for 6-11% of renal carcinomas (Reuter and Presti, 2000). Typically, tumors display a solid or mixed growth pattern and cells with either pale reticular or eosinophilic granular cytoplasm (Kovacs et al., 1997). The former group may be difficult to distinguish from clear cell carcinoma and the latter from oncocytoma. Oncocytomas are benign tumors that are approximately as common as chromophobe renal cell carcinomas. They are well-circumscribed lesions that contain nests and tubules of epithelial cells. Cells are round or polygonal and have abundant eosinophilic cytoplasm, round nuclei with uniform contours and prominent nucleoli, and high content of mitochondria (Erlandson et al., 1997; Reuter and Presti, 2000).

Due to different clinical behavior of chromophobe carcinoma and onco-cytoma, their diagnostic separation is important. The most typical features of chromophobe tumors, diffuse staining with Hale's colloidal iron and numerous cytoplasmic microvesicles (Thoenes et al., 1985, 1988) could help in differential diagnosis (Skinnider and Jones, 1999). In addition to histological features, specific genetic changes have been used for characterization although they partly overlap. Chromophobe tumors harbor losses in chromosomes 1, 2, 6, 10, 13, 17, and 21 (Bugert and Kovacs, 1996) (Table 3). Loss of chromosome 1 is also detected in oncocytomas, which are usually heterogenous tumors with various chromosomal alterations (Kovacs et al., 1989; van den Berg et al., 1995; Herbers et al., 1998) (Table 3). In addition, oncocytomas have been shown to contain an increased amount of mitochondrial DNA, reflecting increased mitochondrial density of the lesions (Simonnet et al., 2002), and according to some reports, rearrangements of mitochondrial DNA (Kovacs et al., 1989).

Table 3. Classification of renal epithelial neoplasms (modified from Zambrano et al., 1999 and Reuter and Presti, 2000).

Tumor type	Genetic abnormalities	Other genetic abnormalities
Malignant tumors		
Clear cell (conventional) RCC	- 3p, *VHL* mutations	+ 5q, - 8p, - 9p, - 14q
Papillary RCC	+ 7, + 17, - Y, *MET* mutations	+ 12, + 16, + 20, - 9p, - 11q, - 14q, - 17p, - 21q
Chromophobe RCC	- 1, - Y, - 2, - 6, - 10, - 13, - 17, - 21	
Collecting duct carcinoma	- 1, - 6, - 14, - 15, - 22	- 8, - 13
RCC, unclassified	variable changes	
Benign tumors		
Papillary-tubular adenoma	+ 7, + 17, - Y	
Oncocytoma	no specific changes, - 1, - Y	
Metanephric adenoma	no specific changes, + 7, + 17, - Y (1 reported case)	

Development of high-throughput methods for molecular profiling may provide means for more accurate diagnosis of renal tumors in the future. Based on the comprehensive molecular characterization of these lesions, they could be clustered into subtypes with distinct biological and clinical characteristics. Recent studies have shown that molecular profiles detected by array-based CGH and gene expression analysis are consistent with previous clinical, histological, and molecular understanding of these tumors. These methods could be valuable in evaluation of disease prognosis and in differentiation of tumors with highly similar morphology (Moch et al., 1999; Boer et al., 2001; Takahashi et al., 2001; Young et al., 2001; Wilhelm et al., 2002).

2.2 Familial clear cell carcinoma

2.2.1 von Hippel-Lindau disease

von Hippel-Lindau disease is the most common hereditary renal cancer syndrome. It is characterized by predisposition to vascular tumors of the retina and central nervous system (CNS), clear cell renal carcinoma, and pheochromocytomas. The earliest reports on the hereditary nature of retinal angiomas and CNS hemangioblastomas are from the beginning of the 20th century (von Hippel, 1904; Lindau, 1927). More complete characterization of the disease was reported later (Melmon and Rosen, 1964).

The most common and earliest features of VHL are CNS hemangio-blastomas and retinal angiomas, occurring in approximately 60% of VHL patients. They are diagnosed on average at ages 30 and 25 years, respectively (Maddock et al., 1996). Hemangio-blastomas are usually located in the cerebellar hemispheres, spinal cord, and brain stem. Due to their space-occupying nature, they cause severe morbidity and are the predominant cause of death in VHL patients together with renal cell carcinoma (Maddock et al., 1996). Renal cell carcinoma occurs in approximately 25-45% of VHL patients and arises between the ages of 25 and 58 years (Maddock et al., 1996; Linehan et al., 2002). Typically, tumors are multiple and bilateral and display clear cell histology. Pheochromocytomas, which are catecholamine-producing tumors arising most often in the adrenal medulla, are diagnosed in about 15% of patients, and they tend to cluster in some families. In addition, VHL patients may develop renal and pancreatic cysts, cystadenomas or islet cell tumors of the pancreas, endolymphatic sac tumors, and papillary cystadenomas of the epididymis. Based on the occurrence of renal cancer and pheochromocytoma, VHL has been classified as VHL1 without pheochromo-cytoma, VHL2A with pheochromocytoma and with low risk of renal cancer, VHL2B with pheochromo-cytoma and high risk of renal cancer, and VHL2C with pheo-chromocytoma only (without any other benign or malignant tumors).

The predisposing gene for VHL was first mapped to 3p25-p26 (Seizinger et al., 1988) and then positionally cloned (Latif et al., 1993). The *VHL* gene is comprised of three exons and encodes two transcripts. Characteristic germline mutations have later been identified for the different cancer phenotypes. Missense mutations associate with pheochromo-cytoma (VHL2A, VHL2B, VHL2C), whereas deletions and truncating mutations are linked with renal cell carinoma (VHL1) (Crossey et al., 1994b; Chen et al., 1995; Zbar et al., 1996).

Evidence for *VHL* being a tumor suppressor gene was found in molecular genetic analyses of tumors from VHL patients displaying two inactivated alleles of the gene (Crossey et al., 1994a) and in subsequent functional analyses (Iliopoulos et

al., 1995). The VHL protein is involved in regulation of cellular responses to hypoxia by targeting the hypoxia inducible factor 1_ (HIF-1_) to proteosome-mediated degradation in the presence of oxygen (Iliopoulos et al., 1996; Maxwell et al., 1999). HIF-1_ is a subunit of heterodimeric transcription factor HIF-1, which binds to hypoxia response elements (HREs) and activates the expression of several genes including proangiogenic factors and glycolytic enzymes. Mutations in VHL prevent ubiquitylation and degradation of HIF-1_, resulting in accumulation of HIF-1_ and continuous activation of hypoxia response genes. VHL has also been implicated in control of the cell cycle exit (Pause et al., 1998), fibronectin assembly, and extracellular matrix formation (Ohh et al., 1998).

2.2.2 Familial clear cell carcinoma with constitutional translocation

A family segregating clear cell renal carcinoma and a balanced translocation t(3;8)(p14;q24) was described over twenty years ago (Cohen et al., 1979). The genes located in the translocation breakpoints, *FHIT* and *TRC8*, were later isolated (Ohta et al., 1996; Gemmill et al., 1998), but their role in development of renal cell carcinoma in this family has remained controversial. Tumors from several families with different chromosome 3 constitutional translocations have later been analyzed and an alternative mechanism involving *VHL* inactivation has been suggested. Namely, tumors displayed loss of the derivative chromosome harboring the *VHL* gene and harbored mutations in the remaining *VHL* allele (Schmidt et al., 1995; Bodmer et al., 1998; Kanayama et al., 2001).

2.3 Familial papillary renal cell carcinoma

2.3.1 Hereditary papillary renal cell carcinoma

Hereditary papillary renal cell carcinoma (HPRC) is a rare dominantly inherited condition characterized by predisposition to multiple bilateral papillary renal cell tumors (Zbar et al., 1994, 1995). The carcinomas exhibit type 1 papillary histology with small cells, low-grade basophilic nuclei (mainly Fuhrman grades 1-2), and inconspicuous amphophilic cytoplasm and nucleoli (Lubensky et al., 1999). In the disease families, some individuals have also been affected with nonrenal malignancies, but any association with predisposition to renal cell carcinoma has remained obscure (Zbar et al., 1995).

The disease gene was mapped to 7q31-q34 by linkage analysis in the HPRC families, and germline mutations were identified in the tyrosine-kinase domain of the *MET* proto-oncogene (Schmidt et al., 1997). Trisomy of chromosome 7 had previously been observed in sporadic papillary renal cell carcinomas (Kovacs, 1993) and renal cell carcinomas from members of HPRC families (Schmidt et al., 1997). The mutant allele was subsequently demonstrated to be duplicated and overexpressed in the tumors (Fischer et al., 1998). *MET* encodes a tyrosine-kinase receptor whose ligand is the hepatocyte growth/scatter factor (HGF) (Bottaro et al., 1991). Cells expressing mutant MET were shown to display enhanced kinase activity, be tumorigenic in nude mice, and introduce invasive and metastatic properties in cell lines and mice (Giordano et al., 1997; Jeffers et al., 1997; Jeffers et al., 1998).

2.3.2 Familial papillary renal cell carcinoma associated with papillary thyroid carcinoma

Occurrence of papillary thyroid cancer, nodular thyroid disease, and tumors of the

kidney has been reported in one large family (Malchoff et al., 2000). Renal neoplasms included one papillary renal cell carcinoma, multifocal papillary adenomas, and renal oncocytoma. After exclusion of the *MET* gene, the condition was mapped to 1q21.

2.4 Other familial renal tumor syndromes

2.4.1 Tuberous sclerosis

Tuberous sclerosis (TS) is a dominantly inherited disease characterized by multiple hamartomas in different organs. The clinical manifestations, including learning disability, epilepsy and behavioral problems, are caused by CNS lesions. TS is genetically heterogeneous disease with two main predisposing genes, *TSC1* at 9q34 and *TSC*2 at 16p13 (Consortium TECTS, 1993; van Slegtenhorst et al., 1997). The most common renal manifestations are angiomyolipomas, which despite their benign nature, can cause severe morbidity due to hemorrhage or compression of healthy renal tissue (Iliopoulos and Eng, 2000). On rare occasions, malignant renal tumors can be detected. These are most often clear cell carcinomas, although other histological types have also been observed (Sampson et al., 1995; Sampson, 1996). Renal carcinoma is more frequent in the animal model of the disease, the rat with germline mutations in *Tsc2* (Ecker rat). These animals also develop tumors of the pituitary, spleen, and uterus. Thus, the phenotype differs from the human one (Yeung et al., 1994; Everitt et al., 1995; Kobayashi et al., 1995).

2.4.2 Birt-Hogg-Dubé syndrome

Birt-Hogg-Dubé syndrome (BHD) is characterized by three types of cutaneous lesions, i.e. fibrofolliculomas, trichodiscomas, and acrochordons (Takahashi et al., 2002). Renal lesions in BHD include variable histologic subtypes such as oncocytomas, chromophobe and papillary renal cell carcinomas, and clear cell carcinomas (Toro et al., 1999). The gene for BHD was localized to 17p11 (Schmidt et al., 2001), and recently, the disease gene was cloned (Nickerson et al., 2002). The gene sequence predicts a novel protein that is highly conserved in evolution but has no homology to any known proteins.

2.4.3 Other hereditary conditions associated with renal neoplasms

Renal cell carcinoma may also be associated with hereditary nonpolyposis colorectal cancer (HNPCC) and hyperparathyroidism-jaw tumor syndrome (HPT-JT).

In HNPCC, which is caused by germline mutations in the mismatch repair genes, the most common manifestations are colorectal and endometrial cancers. Affected individuals also have an increased risk of transitional cell cancer of the renal pelvis and ureters, and other malignancies, such as cancers of the stomach, small intestine, ovaries, and hepatobiliary tract (Lynch and Lynch, 2000).

HPT-JT is an autosomal dominant disorder typified by primary hyperparathyreoidism caused by parathyroid adenomas. The disease is associated with ossifying fibroma of the jaw and different types of renal lesions including benign cysts, hamartomas, Wilm's tumor, and bilateral papillary carcinoma (Haven et al., 2000). HPT-JT is linked to 1q21-q31 (Szabo et al., 1995).

3. UTERINE SMOOTH MUSCLE TUMORS

3.1 Uterine leiomyomas

3.1.1 Clinical and histopathological features

Uterine leiomyomas (myomas or fibroids) are the most common tumors in premenopausal women. They are clinically evident in 25% of women (Zaloudek and Norris, 1994), but by careful pathological examination of surgical specimens, they can be diagnosed in up to 77% of women (Cramer and Patel, 1990). Although leiomyomas are benign tumors, they have a major impact on women's health by causing numerous symptoms, being associated with infertility, and being the leading cause of hysterectomy.

Leiomyomas arise from the uterine smooth muscle cells or their progenitors in submucosal, intramural, and subserosal locations. Leiomyomas are roughly spherical, well-circumscribed lesions with a firm fibrous appearance. Histologically, they are composed of interlacing bundles of elongated, eosinophilic smooth muscle cells surrounded by well-vascularized connective tissue (Crow, 1998; Robboy et al., 2000). Typically, tumor cells have cigar-shaped nuclei, and mitoses are rarely detected in the lesions. In addition to the usual appearance of leiomyomas, a number of variant forms exist. Cellular leiomyomas have a higher density of smooth muscle cells, and atypical leiomyomas are characterized by cells with pleomorphic nuclei and often giant cells. Epithelioid leiomyomas are composed of polygonal rather than spindle cells, and lipoleiomyomas contain a variable amount of adipose tissue. In addition, some leiomyomas may harbor degenerative changes such as hyalinization, calcification, and red degeneration. Calcification is often detected postmenopausally, whereas red degeneration commonly occurs during pregnancy. In rare cases, leiomyomas may have unusual growth patterns. These entities include intravascular leiomyomatosis, benign metastasizing leiomyoma, disseminated peritoneal leiomyomatosis, lymphangioleiomyoma-tosis, and diffuse leiomyomatosis (Robboy et al., 2000).

Leiomyomas cause variable symptoms, which are mainly dependent on the location and size of the lesions. Abnormal or prolonged bleeding is commonly caused by submucosal leiomyomas, acute pain can indicate torsion of a pedunculated tumor, and urinary symptoms are usually the result of anterior and constipation by posterior lesions. During pregnancy uterine leiomyomas typically enlarge and are associated with complications such as miscarriage, placental abruption, and premature labor (Stewart, 2001).

The diagnosis of uterine leiomyomas is generally based on clinical pelvic examination and transvaginal ultrasonography. Ultrasonography is as good as magnetic resonance imaging (MRI) in detection of leiomyomas, but MRI may provide advantages in determining tumor location (Dueholm et al., 2002). The treatment of leiomyomas depends on their size and location, the presenting symptoms, and the age and reproductive desires of the patient. Surgical procedures, including myomectomy and hysterectomy, are widely used. For patients who desire future pregnancies myomectomy is an option, but recurrent myomas often develop after the procedure. Due to risk of complications, such as infections, hemorrhage, and bowel or ureteric injury in patients undergoing hysterectomy (Takamizawa et al., 1998), new surgical (hysteroscopic removal, endometrial ablation, arterial embolization) and nonsurgical (GnRH agonists or antisteroid compounds) methods have been developed (Stewart, 2001; Walker et al., 2002).

3.1.2 Etiology and pathogenesis

The etiology and pathogenesis of uterine leiomyomas are poorly understood. However, estrogen and progesterone play a role in their development; leiomyomas are not seen before puberty, they increase in size during pregnancy, and shrink postmenopausally and after treatment with GnRH agonists (Morton, 1998). Leiomyomas have been observed to display increased expression of both estrogen and progesterone receptors. This is proposed to lead to increased end-organ sensitivity to these hormones (Brandon et al., 1993, 1995). Progesterone has been suggested to be one of the major factors contributing to leiomyoma growth; when progesterone levels are high, as in the luteal phase of the menstrual cycle, mitotic activity in leiomyomas is elevated (Kawaguchi et al., 1989). Similarly, leiomyomas tend to enlarge during pregnancy when progesterone levels are increased.

In addition to ovarian steroid hormones, several growth factors have been implicated in the pathogenesis of leiomyomas. Expression of either growth factors or their receptors has been observed to be higher in leiomyomas than in corresponding normal myometrium (Andersen, 1998). Factors involved include insulin-like growth factors (IGF-I, IGF-II), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and their receptors. In a recent cDNA array analysis of uterine leiomyomas, *IGF-II* was one of the overexpressed genes in tumors as compared with normal myometrium (Tsibris et al., 2002). This and other studies have also demonstrated elevated mRNA levels of components in the Wnt signaling pathway, including frizzled and secreted frizzled related protein 1 (sFRP1) (Fukuhara et al., 2002; Tsibris et al., 2002). Aberrations in the Wnt signaling pathway have previously been implicated in the pathology of human neoplasms, including colorectal carcinoma (Taipale and Beachy, 2001).

Genetic predisposition to uterine leiomyomas has been proposed based on case reports of disease families (Vikhlyaeva et al., 1995), but inherited genetic defects have not been identified. Compared with malignant neoplasms, leiomyomas appear to display fewer chromosomal aberrations. However, about 40% of these tumors harbor chromosomal alterations. The most frequent changes include translocation involving chromosomes 12 and 14, rearrangements in 6p, and deletion of 7q (Nilbert et al., 1990a; Morton, 1998; Ligon and Morton, 2000). In addition, chromosome 1 alterations, losses on chromosome 4, and gains on chromosomes 9, 12, 14, and 19 have been observed, among others (Packenham et al., 1997; Levy et al., 2000). Studies on the genes located at the breakpoints in chromosomes 12, 14, and 6 have led to identification of genes *HMGIC* at 12q15 and *HMGIY* at 6p21. The gene products belong to a family of high-mobility-group proteins which play a role in transcription regulation and chromatin organization. Alterations of *HMGIC* and *HMGIY* have been observed in several tumor types of mesenchymal origin including leiomyomas (Schoenmakers et al., 1995; Kazmierczak et al., 1998; Mine et al., 2001). In chromosome 14, the *RAD51B* gene has been identified and suggested to play a role in tumorigenesis and convey tissue-specificity in leiomyomas with translocation t(12;14) (Schoenmakers et al., 1999). *RAD51B* belongs to a family of genes highly similar to bacterial *RecA* and *S. cerevisiae Rad51*, which are known to be involved in homologous recombination and repair. Observations of 7q losses detected by cytogenetic analyses have been supported by several studies on allelic loss by microsatellite markers (Ishwad et al., 1995, 1997; Vanni et al., 1997, 1999; van der Heijden et al., 1998; Mao et al., 1999). In a

genome-wide microsatellite marker analysis of allelic loss, 7q was the only locus displaying frequent LOH (Mao et al., 1999).

3.2 Uterine leiomyosarcoma

Leiomyosarcoma is a malignant mesenchymal neoplasm with smooth muscle differentiation. Tumors typically display spindle cells with blunt-ended nuclei and eosinophilic cytoplasm and stain positive for _-smooth muscle actin and desmin. Leiomyosarcoma can occur in a variety of anatomical sites, including the retroperitoneum, skin, extremities, and uterus (Enzinger and Weiss, 1995). Uterine leiomyosarcoma comprises approximately one-fourth of uterine sarcomas, which constitute 3% of uterine malignancies. Other uterine sarcomas include endo-metrial stromal sarcoma, malignant mixed müllerian tumor, and müllerian adeno-sarcoma (Rosai, 1996). Uterine leiomyo-sarcomas usually occur during menopause in women over the age of 40 (Barbieri and Andersen, 1992) and are aggressive tumors with a five-year survival rate of 40% (Kumar et al., 1997).

Typically, gross appearance of leiomyosarcomas differs from that of uterine leiomyomas. Leiomyosarcomas invade the adjacent myometrium, are not demarcated from normal tissue, and contain areas with necroses and hemorrhages. Histologically, the tumors are densely cellular and display cells with a variable degree of differentiation. Diagnostic difficulties may be encountered when benign leiomyomas display unusual features resembling leiomyosarcoma (Wilkinson and Rollason, 2001). Features utilized in differential diagnosis include mitotic activity, nuclear atypia, coagulative necrosis, degree of cellularity and differentiation, presence of giant cells, and invasion to blood vessels and adjacent myometrium (Robboy et al., 2000). Previously, the value of mitotic activity was emphasized, and the threshold for diagnosis of leiomyosarcoma was set at 10 mitoses per 10 high-power-field. However, by taking into account all relevant features, tumors that are clearly benign or malignant as well as those in the intermediate group can be classified more accurately.

In contrast to leiomyomas, leiomyosarcomas are characterized by a wide range of chromosomal anomalies. These involve chromosomes 1, 3, 6, 7, 10, 11, 17, and 19 (Nilbert et al., 1990; Iliszko et al., 1998). By CGH analyses, many alterations, such as gains of chromosomes 1, 8, 10, 17, and Xp, and losses of 1p, 10q, 13q, 14q, and 22q, have been detected (Packenham et al., 1997; Levy et al., 2000). Due to overlap in morphologic phenotypes between uterine leiomyo-sarcomas and leiomyomas, the hypothesis of leiomyosarcoma arising from pre-existing leiomyoma has been raised. Some estimates propose that 0.1% of benign uterine leiomyomas would progress to malignancy (Morton, 1998). However, evidence for the progression could not be demonstrated by CGH and LOH analyses as no specific changes common to both tumor types were apparent (Packenham et al., 1997; Quade et al., 1999; Levy et al., 2000).

3.3 Hereditary multiple cutaneous and uterine leiomyomatosis (MCL)

Multiple cutaneous and uterine leiomyomatosis (MCL) is an autosomal dominant disorder characterized by development of leiomyomata of the skin and the uterus (fibroids) in affected individuals. Leiomyomas of the skin are rare benign tumors thought to originate from the arrectores pilorum muscle of the hair follicle (Virchow, 1854). They are smooth, erythematous nodules of 0.2-2 cm in diameter and usually appear on the face, back, and extensor surfaces of the

extremities. They are often painful and sensitive to cold and touch (Spencer and Amonette, 1996). Histologically, these lesions, also called piloleiomyomas, are composed of interlacing bundles of smooth muscle cells and are sometimes difficult to identify (Spencer and Amonette, 1996). These tumors appear to be equally common in men and women (Raj et al., 1997). Although estrogen and progesterone have been implicated in the development of uterine and some extrauterine smooth muscle tumors, leiomyomas of the skin have not been reported to express elevated levels of estrogen or progesterone receptors (McGinley et al., 1997).

The first report on inherited susceptibility to cutaneous leiomyomas was based on a family with several affected individuals (Kloepfer et al., 1958). An association with uterine leiomyomas in affected women was later proposed (Mezzadra, 1965; Reed et al., 1973; Jolliffe, 1978; Engelke and Christophers, 1979; Thyresson and Su, 1981; Garcia Muret et al., 1988; Fernandez-Pugnaire and Delgado-Florencio, 1995; Vellanki et al., 1996). An autosomal dominant inheritance of the condition was suggested by examination of pedigrees, but details on the molecular basis of the disease remained unclear. The only proposed susceptibility locus for MCL, 18p11, was based on observations of multiple cutaneous leiomyomata in a woman with 9p trisomy/18pter monosomy (Fryns et al., 1985).

AIMS OF THE STUDY

1. To characterize the main clinical features of hereditary leiomyomatosis and renal cell cancer (HLRCC) families.
2. To characterize the main histopathological and molecular features of HLRCC tumors.
3. To localize the gene predisposing to HLRCC.
4. To identify the gene predisposing to HLRCC and the mutations causing the disease.
5. To investigate the role of the *HLRCC* gene in sporadic tumor types associated with the HLRCC phenotype.

MATERIALS AND METHODS

1. PATIENTS AND TUMOR SAMPLES

1.1 Families and clinical documentation (I, II)

The proband (FAM-1/IV-7; see Figure 1 in Study I) of a large kindred (FAM-1) was given genetic counseling at the Department of Clinical Genetics, Oulu University Hospital, Oulu, because her mother (FAM-1/III-9) and one of her sisters (FAM-1/IV-4) had been affected with renal cell carcinoma at the relatively early ages of 48 and 44 years, respectively. To obtain a documented disease history of the family, data were collected by patient interviews, examination of patient records, and identification of the proband's relatives from the official population registries followed by their tumor status verification through patient records and the Finnish Cancer Registry. The Cancer Registry covers the cancer cases in the Finnish population almost completely and with good accuracy (Kyllönen et al., 1987; Teppo et al., 1994). Altogether four individuals in FAM-1 were diagnosed with early-onset renal cell carcinoma. The family also included seventeen individuals with leiomyomas of the uterus and/or skin, and two with uterine leiomyosarcoma.

To identify additional families with a phenotype similar to FAM-1, a search of renal cancer patients between the ages of 15 and 35 years was carried out utilizing the Cancer Registry data. As a result of the search, histology of tumor slides from 27 patients was examined to identify lesions with the same distinct features as in carcinomas from FAM-1. This effort led to recognition of the second family (FAM-2; see Figure 1 in Study I), which included two siblings diagnosed with renal cell carcinoma at the ages of 32 (FAM-2/II-2) and 26 (FAM-2/II-3) and one with multiple uterine leiomyomas at the age of 41 (FAM-2/II-1).

The third family (FAM-3; see Figure 1 in Study II) was originally diagnosed with multiple cutaneous and uterine leiomyomatosis (MCL) at the Department of Clinical Genetics at Turku University Hospital, Turku. Detailed disease history of the family was derived through patient interviews, and investigation of patient records, population registries, and Cancer Registry data. The family included seven individuals with cutaneous leiomyomas and three with leiomyomas of the uterus.

In addition to the three Finnish families, 39 families from the UK were included in the study (III) through collaboration with Drs. Ian Tomlinson (Imperial Cancer Research Fund, London, UK) and Richard Houlston (Institute of Cancer Research, Sutton, Surrey, UK). These families had a typical MCL phenotype with leiomyomata of the uterus and skin (Alam et al., 2001). Predisposition to renal cancer was not observed.

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 Cancer-free control individuals (IV)

Blood samples from anonymous cancer-free individuals were obtained from the Finnish Red Cross, Helsinki. DNA from the control individuals was used in Study IV to examine

the Finnish population frequencies of two germline mutations observed in the *FH* gene.

1.3 Tumor samples (I, II, III, IV, unpublished data)

Tumors from HLRCC patients were analyzed for LOH (I, II, unpublished data) and/or mutations in the *FH* gene (III, unpublished data). Tumors included seven renal cell carcinomas (from patients FAM-1/III-7, FAM-1/III-9, FAM-1/IV-4, FAM-1/IV-11, FAM-2/II-2, FAM-2/II-3, FAM-3/II-4), seven uterine leiomyomas (from patients FAM-1/III-1, FAM-1/III-3, FAM-1/III-4, FAM-1/III-7, FAM-2/II-1, FAM-3/II-4), seven cutaneous leiomyomas (from patient FAM-3/III-5), and one uterine leiomyosarcoma (from patient FAM-1/III-13).

Sporadic tumors from individuals with no known family history of cancer were also studied for LOH at the *HLRCC* locus (II, unpublished data) and mutations in the *FH* gene (IV). Samples included 10 cutaneous leiomyomas (II, IV), 41 uterine leiomyomas (26 in Study II and 41 in Study IV), 58 renal cell carcinomas (IV, an unpublished study), 18 uterine leiomyosarcomas (IV, an unpublished study), 35 sarcomas (IV), 29 prostate carcinomas (IV), and 15 lobular breast carcinomas (IV). Renal cell carcinomas included 40 clear cell carcinomas, 4 papillary type 1 tumors, and 11 papillary type 2 tumors, and 3 other tumors. Sarcomas included 2 extrauterine leiomyosarcomas, 5 malignant fibrous histiocytomas, 2 chondrosarcomas, 1 fibrosarcoma, 9 liposarcomas, 4 osteosarcomas, 1 Ewing's sarcoma, and 11 sarcomas of undefined histology.

1.4 DNA and RNA extraction (I, II, III, IV)

DNA from blood samples and cell lines of HLRCC patients, fresh-frozen tumor samples, and blood samples of control individuals was extracted using standard procedures (Lahiri and Nurnberger, 1991). Informed consent was obtained from all patients. DNA extraction from the paraffin-embedded tumor and normal tissues was carried out using deparaffinization with xylene, washing with ethanol, treatment with proteinase K, and extraction with phenol and chloroform (Kannio et al., 1996). Examination of histology and dissection of tumor samples were performed to confirm the histology and to obtain the maximum amount of tumor tissue for subsequent analyses of the tumor DNA. Total cellular RNA from two cancer-free control individuals and fresh-frozen normal myometrium was extracted using the RNeasy kit (Qiagen).

2. HISTOPATHOLOGICAL EVALUATION (I, II)

Paraffin-embedded tissue blocks, hematoxylin/eosin (H&E) –stained slides, and pathologists' gross reports for the seven renal cell carcinomas (from patients FAM-1/III-7, FAM-1/III-9, FAM-1/IV-4, FAM-1/IV-11, FAM-2/II-2, FAM-2/II-3, FAM-3/II-4) were obtained for histopathological analyses from the Departments of Pathology at Oulu University Hospital, Helsinki University Central Hospital, and Turku University Hospital. The tumors were classified according to the terms of the Armed Forces Institute of Pathology classification (Murphy et al., 1994), and the tumor histopathology was compared with criteria for papillary renal cell carcinoma proposed by Delahunt and Eble (1997). H&E staining and immunohisto-chemistry for epithelial membrane antigen (EMA), vimentin, cytokeratin 7 (CK7), and human milk fat globule (HMFG1 and HMFG2) were performed on all tumors.

In addition to renal tumors, paraffin-embedded tissue blocks and/or H&E slides from the following tumors were obtained for histopathological re-evaluation: from FAM-1, two uterine leiomyomas (from patients FAM-1/III-12 and FAM-1/III-13), two cutaneous leiomyomas (from patients FAM-1/III-12 and FAM-1/III-13), metastases (ovaries, omentum, para-aortic and clavicular lymph nodes) of a cancer of unknown origin (from patient FAM-1/III-5), one breast cancer (from patient FAM-1/III-13), and one bladder cancer (from patient FAM-1/III-10); from FAM-3, seven cutaneous leiomyomas (from patient FAM-3/III-5) and two uterine tumors (from patient FAM-3/I-1).

3. COMPARATIVE GENOMIC HYBRIDIZATION (CGH) (UNPUBLISHED DATA)

Comparative genomic hybridization (CGH) was performed on seven renal cell carcinomas from families FAM-1, FAM-2, and FAM-3 to investigate the genetic profile of these tumors. Standard methods described by Kallioniemi et al. (1992) were used. The analysis was performed in the laboratory of Professor Jorma Isola (Institute of Medical Technology, Tampere, Finland).

4. MAPPING OF THE DISEASE LOCUS

4.1 Microsatellite marker analysis (I, II, III)

To exclude *MET* and *VHL* as predisposing genes for the phenotype seen in FAM-1, genotyping of four renal cell carcinoma patients from FAM-1 was carried out by microsatellite markers spanning the respective loci. After demonstrating negative linkage at these loci, a genome-wide search with Perkin-Elmer Applied Biosystems PRISM Linkage Mapping Set MD-10 (P/N 450067, 10 cM density) containing 370 markers was performed in FAM-1. PCR amplicons were run on an Applied Biosystems PRISM 377 DNA sequencer (Applied Biosystems) and data analyzed by using Genescan 3.1 software (Applied Biosystems). To confirm the results of the genome-wide search, additional genotyping was carried out at the most positive locus. Twelve markers located in 1q42-q44 were analyzed in FAM-1, FAM-2, and FAM-3. To reduce the target area, markers from the commercial sets and 28 novel short tandem repeats were used for genotyping in Study III.

4.2 Linkage analysis and haplotype construction (I, II, III)

All linkage analyses were performed by using the GENEHUNTER (Kruglyak et al., 1996) and FASTLINK (Cottingham et al., 1993) programs. In addition, a modified MLINK program of the Fastlinkage package LMLINK (Rohde et al., 1995) was used to include the LOH data of Study I in the linkage analysis. When analyzing the data obtained from the genome-wide search, the FAM-1 pedigree was divided into two due to its large size to enable multipoint linkage analysis by the GENEHUNTER program. The chromosomal regions displaying lod score 1 or higher were then reanalyzed with the combined pedigree structure. When analyzing the genotyping data from FAM-1 and FAM-2, dominant inheritance for the tumor predisposition phenotype was presumed and penetrance was conservatively estimated to be 0.4. Individuals with cutaneous nodules/ leiomyomas, uterine leiomyomas, and renal cell carcinoma were considered to be affected. Probabilities for phenocopies were set at 0.002 for leiomyomas of the skin, 0.2 for uterine leiomyomas, and 0.00001 for renal cell carcinoma. For subsequent analysis of FAM-3, penetrance was estimated to be 0.8.

The most likely haplotypes assuming the minimum number of recombinations were manually constructed in the chromosomal regions displaying lod score 1 or higher in the genome-wide linkage analysis in FAM-1. Haplotype construction was also carried out when narrowing the final target region.

4.3 Identification of novel short tandem repeats (III)

Short tandem repeats were identified at the *HLRCC* locus by inspection of the draft genome sequence of BAC clones (Sanger Centre Institute, 2001) and by BLAST searches (NCBI Blast, 2001). Primers for PCR were designed utilizing the Primer3 server (Primer3 Server, 2002) to amplify 100-250 bp fragments containing the microsatellite sequences.

4.4 Fluorescence in situ *hybridization (FISH) analysis (III)*

Due to observations on predisposition to renal cancer only in the Finnish HLRCC families and non-mendelian inheritance of microsatellite alleles in two UK pedigrees, fluorescence *in situ* hybridization (FISH) analysis was carried out in the laboratory of Dr. Ian Tomlinson to detect putative large germline deletions. Metaphase spreads of lymphoblast cell lines from probands of Finnish families FAM-1 and FAM-2, and UK families 207 and 713 were prepared by standard protocols. BAC clones in the target region were fluorescently labeled by nick translation with Fluoro Green, Fluoro Red, and Cy5-sUTP (Amersham Phamacia Biotech), and were hybridized overnight to the metaphase spreads under competitive conditions. Slides were washed and counterstained with 4,6-diamidino-2-phenyindole (DAPI). Images were acquired using a cooled charge-coupled camera (Photometrics) attached to a Zeiss Axioplan 2 Imaging Microscope and SmartCapture 2 software (Applied Imaging).

5. LOSS OF HETEROZYGOSITY ANALYSIS (LOH) (I, II, UNPUBLISHED DATA)

LOH analysis by microsatellite markers was performed on the available tumors from the HLRCC families to confirm the linkage of HLRCC to 1q42-q44 (I, II), to narrow the target region containing the *HLRCC* gene (III), and to investigate the characteristics of the *HLRCC* gene (I, II). Seven uterine leiomyomas, seven cutaneous leiomyomas, seven renal cell carcinomas, and one uterine leiomyosarcoma were included in the analyses (I, II, unpublished data). Eighteen polymorphic microsatellite markers spanning the *HLRCC* locus were used to amplify the tumor and normal tissue DNA from HLRCC patients.

LOH analysis was also carried out on sporadic forms of tumor types associated with the HLRCC phenotype to evaluate the involvement of the *HLRCC* locus in sporadic tumorigenesis (unpublished data). Samples genotyped with markers DIS517, DIS547, and D1S423 included 10 cutaneous leiomyomas, 26 uterine leiomyomas, 18 uterine leiomyosarcomas, and 58 renal cell carcinomas with corresponding normal tissues.

Electrophoresis and subsequent data analysis in all LOH analyses were performed as described in section 4.1. Allele loss was scored if the area under an allele peak was reduced to <40% of its original value (Canzian et al., 1996).

6. MUTATION SCREENING

6.1 Identification of known and predicted transcripts (III)

The genomic sequence used in the gene predictions was obtained from the chromosome 1 physical map published by the Sanger Centre Institute (2001). Nucleic acid identification program (NIX, 2001), NCBI Entrez Genome Map Viewer (2001), and Ensembl Human Genome Server (2001) were used to identify known genes, ESTs, and putative novel genes in the target region. Exon-intron boundaries of the candidate sequences were determined by BLAST searches and verification of expression of a gene prediction was performed by RT-PCR from human total mRNA derived from two cancer-free control individuals (Qiagen RNEasy).

6.2 Sequencing (III, IV)

To identify the disease-predisposing gene, mutation screening of 6 known genes (*RGS7*, *FH*, *KMO*, *OPN3*, *CHML*, *EXO1*) and 41 positional candidate sequences was performed by sequencing of genomic DNA of probands from 42 families (3 from Finland and 39 from the UK) (III). In the collaborative groups, mutation analyses were performed by single-strand conformational polymorphism (SSCP) and conformation-specific gel electrophoresis (CSGE). In Study IV, sporadic tumors were analyzed for *FH* mutations by genomic sequencing.

All PCR primers were designed utilizing the Primer3 server (2002) to amplify known or predicted exons and the flanking intronic sequence. PCR reactions were carried out in a 50-_ l reaction volume containing 100 ng of genomic DNA, 1X PCR buffer (Applied Biosystems), 300 _ M of each dNTP (Finnzymes), 1 _ M of forward and reverse primers, and 2.5 units of AmpliTaqGOLD polymerase (Applied Biosystems). For different exons, optimal $MgCl_2$ concentrations and annealing temperatures were between 1.5 mM and 3.0 mM, and 56_ C and 64_ C, respectively. The following general cycling conditions were used: 10 min at 95_ C, then 35 cycles of denaturation at 95_ C for 45 s, annealing at a corresponding temperature for 1 min, elongation at 72_ C for 1 min, and final extension at 72_ C for 10 min. The PCR product (5 _ l) was run on an agarose gel to verify the specificity of the reaction, and the rest of the product was purified using the NucleoSpin PCR purification kit (Macherey-Nagel). Direct sequencing of PCR products was performed using BigDye3 termination chemistry (Applied Biosystems) with the ABI 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. All sequence changes were verified by reamplification of the corresponding PCR fragment and sequencing of both DNA strands.

6.3 Reverse transcriptase polymerase chain reaction (RT-PCR) and mRNA expression analysis (III)

Expression levels of *FH* in human tissues (smooth muscle, brain, heart, kidney, liver, lung, pancreas, placenta, skeletal muscle, colon, ovary, peripheral blood leukocyte, prostate, small intestine, spleen, testis, thymus) were measured by using RT-PCR. mRNA from smooth muscle was obtained from the normal myometrium of a hysterectomized patient (Qiagen RNEasy), and cDNA was synthesized by reverse transcription reaction (Promega). cDNA from other tissues was provided by Clontech. *FH* cDNA representing exons one to five was amplified by PCR. *G3PDH* gene was used as a control gene to compare cDNA amplification levels. Samples were run on agarose gel.

6.4 Single-strand conformational polymorphism (SSCP) (IV)

SSCP analysis was performed to investigate the Finnish population frequency of a putative Finnish founder mutation in *FH* using DNA samples from 448 anonymous cancer-free blood donors. The analysis was performed utilizing MDE gel solution (FMC BioProducts). PCR products were run on 0.6xMDE gels, 4W for 20 hours. The running buffer was 0.6xTBE. SSCP gels were silver-stained according to standard procedures.

6.5 Denaturing high-performance liquid chromatography (DHPLC) (IV)

To examine the population frequency of an *FH* allele detected in the germline of a patient with uterine leiomyosarcoma, DNA from 134 controls was used. The amplicons for the DHPLC analysis were generated in 50 _ l PCR reactions consisting of 50 ng of genomic DNA, 0.7X Platinum PCR Buffer (Invitrogen) 200 _ M of each dNTP (Finnzymes), 0.3 _ M of forward and reverse primers, 1.4 mM of $MgCl_2$, and DNA polymerases Platinum Taq (1.25 units; Invitrogen), Titanium Taq (0.6 units; Clontech), and AmpliTaq Gold (0.6 units; Applied Biosystems). The hot-start PCR cycling conditions were as follows: 94_ C for 12 min, then 35 cycles of denaturing for 30 s, 58_ C for 30 s and 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. Samples were denatured at 95_ C for 5 min and reannealed by gradually cooling down 1_ C/min for 50 min. DHPLC heteroduplex analysis was performed at 55_ C using automated HPLC instrumentation with an Eclipse dsDNA analytical column (Agilent Technologies). The analytical gradient was created by mixing buffer A (100 mM triethylammonium acetate and 0.1 mM EDTA) and 57.4-71.9% buffer B (100 nM triethylammonium acetate, 0.1 mM EDTA and 25% acetonitrile) at a flow rate of 0.4 ml/min.

7. ACTIVITY ASSAY FOR FUMARATE HYDRATASE (III)

Fumarate hydratase enzyme activity was measured in lymphoblastoid cell lines of leiomyomatosis patients and spouse controls from UK families and in patients' leiomyomas as described by Hatch (1978). These measurements were carried out in the laboratory of Dr. Simon Olpin (Sheffield Children's Hospital, Sheffield, UK). Later, the assay was also set up in our laboratory. The assay is based on coupling of malate production by FH to nicotinamide adenine dinucleotide (NADP+) reduction via the malic enzyme. Malate is formed from fumarate by fumarate hydratase present in the tissue/cell sonicate. Reduction of NADP+ to NADPH occurs via the malic enzyme in the conversion of malate to pyruvate. The increase in absorbance at 340 nm due to formation of NADPH is then measured. The linked assay starts with fumarate (final concentration 10 mM), tissue or lymphoblast sonicate, 25 mM HEPES-KOH pH 7.5, 0.27 nM NADP+, 4 mM MgCl2, 5 mM potassium phosphate, and 0.2 units/ml of purified malic enzyme (EC 1.1.1.40, Sigma).

RESULTS

1. CLINICAL FEATURES OF HEREDITARY LEIOMYOMATOSIS AND RENAL CELL CANCER (HLRCC) (I, II)

The tumor predisposition in FAM-1, including susceptibility to uterine and cutaneous leiomyomas, renal cell carcinoma and uterine leiomyosarcoma, appeared dominantly inherited (see Table 4 for summary of the features of affected individuals). The most common manifestation was multiple leiomyomas of the uterus, which was diagnosed in 12 women in FAM-1 between the ages of 25 and 48 years. Two patients had been affected with and operated on for malignant uterine leiomyosarcoma at the ages of 35 (FAM-1/III-9) and 40 (FAM-1/III-13). In addition, a third patient (FAM-1/III-7) was suspected of being affected with uterine leiomyosarcoma based on ultrasonography, but due to her general condition, an operation was not performed, and thus, the diagnosis remained unconfirmed. Eight individuals, including both men and women, had lesions on their skin. Two of these were available for histopathological analysis and were verified as leiomyomas. Renal cell carcinoma occurred in four individuals between the ages of 33 and 48. All the patients had metastasized disease at the time of diagnosis. They underwent nephrectomy and received adjuvant treatment. Three of the patients died 5 to 18 months after diagnosis, and the fourth patient (FAM-1/IV-4) died after six years due to relapse of the cancer. In addition, patient FAM-1/III-10 had been diagnosed with bladder cancer at the age of 65, and the patient with uterine leiomyosarcoma (FAM-1/III-13) had been affected with breast cancer and multiple myeloma at the age of 61.

FAM-2 was identified by examining tumor histology of young renal cancer patients. One tumor in this series displayed a similar histology to the tumors from FAM-1. The patient (FAM-2/II-2) had been 33 years old at the time of diagnosis and had a brother (FAM-2/II-3) with renal cell carcinoma diagnosed at age 26. Both patients died within a year after cancer diagnosis. The family included two other siblings. One (FAM-2/II-1) had been operated on at the age of 41 due to multiple uterine leiomyomas, and recently, the other (FAM-2/II-4) was diagnosed with the same condition. The father of the family (FAM-2/I-1) had been diagnosed with prostate cancer at age 65.

FAM-3 included seven individuals with cutaneous nodules. Lesions of three patients were histologically verified as leiomyomas. The proband (FAM-3/III-5) had hundreds of leiomyomas on the skin of his left arm, trunk, and left leg, some of which were painful and were surgically removed. The tumors were first observed when the patient was 10 years old and a cast covering his left arm from shoulder to wrist was removed. Two individuals of FAM-3 were affected with leiomyomas of the uterus at the ages of 23 (FAM-3/II-2) and 34 (FAM-3/II-4). In addition, one family member (FAM-3/I-1) reported that she had been operated on at the age of 27 due to suspicion of uterine malignancy. Data from the patient records and the Cancer Registry revealed that patient FAM-3/I-1 was diagnosed with uterine leiomyosarcoma at age 27, patient FAM-

Table 4. Phenotypes of all affected individuals in FAM-1, FAM-2, and FAM-3.

Individual[1]	Sex	Skin lesions	Uterine tumors	RCC	Other neoplasms
FAM-1/II-1	F	Cutaneous nodules	No	No	No
FAM-1/II-3	F	No	Leiomyomas (40)	No	No
FAM-1/II-4	M	Cutaneous nodules	-	No	No
FAM-1/III-1	F	No	Leiomyomas (44)	No	No
FAM-1/III-2	F	No	Leiomyomas (48)	No	No
FAM-1/III-3	F	No	Leiomyomas (40)	No	No
FAM-1/III-4	F	No	Leiomyomas (34)	No	No
FAM-1/III-5	F	No	Leiomyomas (37)	No	Primary site unknown (42)
FAM-1/III-7	F	No	Leiomyomas (39)	Yes (39)	No
FAM-1/III-9	F	Cutaneous nodules	Leiomyosarcoma (35)	Yes (48)	No
FAM-1/III-12	F	Leiomyomas	Leiomyomas (39)	No	No
FAM-1/III-13	F	Leiomyomas	Leiomyomas, leiomyosarcoma (40)	No	Breast carcinoma (61), multiple myeloma (61)
FAM-1/III-14	M	Cutaneous nodules	-	No	No
FAM-1/IV-4	F	No	Leiomyomas (29)	Yes (42)	No
FAM-1/IV-11	F	Cutaneous nodules	No	Yes (33)	
FAM-1/IV-14	F	No	Leiomyomas (25)	No	No
FAM-1/IV-16	F	Leiomyomas	Leiomyomas (33)	No	No
FAM-2/I-1	M	No	-	No	Prostate cancer (65)
FAM-2/II-1	F	No	Leiomyomas (41)	No	No
FAM-2/II-2	F	No	No	Yes (33)	No
FAM-2/II-3	M	No	-	Yes (26)	No
FAM-2/II-4	F	No	Leiomyomas (40)	No	No
FAM-3/I-1	F	Leiomyomas (31)	Leiomyomas (typical&symplastic) (27)	No	No
FAM-3/I-3	M	Cutaneous nodules	-	No	No
FAM-3/II-2	F	Cutaneous nodules	Leiomyomas (23)	No	No
FAM-3/II-4	F	Cutaneous nodules	Leiomyomas (34)	Yes (35)	No
FAM-3/II-5	M	Leiomyomas (35)	-	No	No
FAM-3/III-1	F	Leiomyomas (28)	No	No	No
FAM-3/III-5	M	Leiomyomas (10)	-	No	No

[1] All listed individuals segregated a disease-associated haplotype and were later shown to be mutation carriers. The three individuals in generation I in FAM-1 (FAM-1/II-1, FAM-1/II-3, FAM-1/II-4) are obligate carriers. One unaffected male in FAM-1 also displayed a linked haplotype. Age at diagnosis, if known, is in parentheses.

3/I-2 with metastasized cancer of unknown primary site at age 61, and patient FAM-3/II-4 with renal cell carcinoma at age 35.

2. HISTOPATHOLOGICAL FEATURES OF TUMORS FROM HLRCC PATIENTS (I, II)

All seven renal cell carcinomas (from patients FAM-1/III-7, FAM-1/III-9, FAM-1/IV-4, FAM-1/IV-11, FAM-2/II-2, FAM-2/II-3, FAM-3/II-4) were unilateral, solitary lesions. The tumor diameter varied from 5 to 22 cm. Six of seven cases were metastasized at the time of diagnosis, and only the patient without metastases survived. Tumors mainly displayed a papillary growth pattern, but some of them also contained areas with tubulo-papillary, solid, and cystic structures, which are common to papillary tumors (Reuter and Presti, 2000). The tumor cells had a large amphophilic cytoplasm, large nuclei, and inclusion-like eosinophilic nucleoli. The Fuhrman nuclear grade was 3 to 4. Mitoses were moderate, and some round cell infiltrates and apoptotic cell groups were detected in all lesions. No psammoma bodies or stromal macrophages were present. Necroses and hemorrhages

occurred in some tumors. The histological picture of the carcinomas was unique; it was distinct from papillary type 1 renal cell carcinomas but had some resemblance to type 2 carcinoma epithelium (Delahunt and Eble, 1997). In immunohistochemical stainings, all five tumors available for analysis were negative for CK7 and four tumors were positive for vimentin. Three tumors were positive for EMA and HMFG2, and two of these also for HMFG1. CK7 has been considered to be a positive marker for papillary tumors, yet further studies have demonstrated that mainly papillary type 1 tumors express it (Delahunt and Eble, 1997). Vimentin expression has been found to vary in papillary carcinomas (Delahunt and Eble, 1997).

Nine skin lesions examined from three patients (FAM-1/III-12, FAM-1/III-13, FAM-3/III-5) were typical piloleiomyomas with bundles of smooth muscle cells. Smooth muscle origin was confirmed in two tumors by positive immunohistochemistry for desmin and _-SMA.

Uterine tumors from patients FAM-1/III-12 and FAM-1/III-13 were typical benign leiomyomas devoid of nuclear atypia, increased mitotic index, or necrosis. One uterine tumor from patient FAM-3/I-1 turned out to be an atypical leiomyoma characterized by cells with pleomorphic nuclei. In the patient records and the Cancer Registry, the tumor had been reported to be a malignant uterine leiomyosarcoma. The other tumor from the patient was a benign leiomyoma.

Histology of the metastases from lymph nodes, ovaries, and omentum from the cancer of unknown origin (patient FAM-1/III-5) was also re-examined. The metastases displayed small epithelial cells without clear organization but suggestive of papillary pattern. Cells were well-defined with a large cytoplasm, round and sometimes multiple nuclei, and partly eosinophilic enhanced nucleoli. However, the origin of the primary tumor could not be determined. The other two malignancies available for analyses were a lobular infiltrating carcinoma (from patient FAM-1/III-13) and a III grade transitional cell bladder carcinoma (from patient FAM-1/III-10).

3. MOLECULAR FEATURES OF RENAL CELL CARCINOMAS FROM HLRCC PATIENTS (UNPUBLISHED DATA)

To evaluate the cytogenetic profile of seven renal cell carcinomas from HLRCC families, comparative genomic hybridization (CGH) analysis was carried out. In CGH, the tumors displayed a strikingly similar molecular profile (Table 5). Of the seven tumors, five harbored loss of genetic material in 4q21-q33

Table 5. Alterations in HLRCC renal cell carcinomas in CGH.

Patient	Alterations in tumor
FAM-1/III-7	- 4q, - 13q12-q31, + 16, + 17, and + 19
FAM-1/III-9	+ 2q34-qter, - 3p14-q27, + 7, + 9q33-qter, + 17q23-qter, + 19p
FAM-1/IV-4	+ 8, - 13q21-q31, - 14q12-q24, + 17, - 18, + 19, + 20q13-qter, - X
FAM-1/IV-11	- 4q, - 12pter-q22, - 13q13-q31, + 16, + 17q, - 18, + 19, + 20q, - X
FAM-2/II-2	- 4q, - 5q12-q34, + 9q32-qter, + 17
FAM-3/II-3	- 4q13-q33, - 9p, - 14q, + 16p, + 18p, - Xp11.2-p22.1
FAM-3/III-4	- 2p16-qter, - 4q21-qter, - 13q12-q31, + 17, - 18q, + 20, - X

and four in 13q14-q27 and in Xp, and six tumors displayed gains of genetic material in 17q23-qter.

4. LOCALIZATION OF THE *HLRCC* GENE (I, II)

First, *MET* and *VHL* were excluded by negative linkage (lod score < -2) at the corresponding loci in renal cell carcinoma patients from FAM-1. Then, a genome-wide analysis was carried out in FAM-1. After haplotype construction of chromosomal regions displaying lod score 1 or higher, only the telomeric end of the chromosome 1 long arm showed substantial positivity. To confirm this, 12 microsatellite markers at 1q42-q44 were genotyped, which resulted in the maximum lod score of 4.11 at marker D1S2811. This was more than 10 times higher than the level of significance for simple inherited conditions (lod score 3). Using the same set of markers, affected individuals in FAM-2 and FAM-3 were shown to share the 1q42-q44 region.

To further ensure the linkage to 1q42-q44, LOH analysis was performed on the available tumors from affected individuals. Loss of the wild-type allele in tumor DNA compared with normal tissue DNA was seen in 18 of 22 cases: 6 of 7 uterine leiomyomas, 7 of 7 cutaneous leiomyomas, and 5 of 7 renal cell cancers. The uterine leiomyosarcoma did not display allele loss. When the LOH data on four renal cell carcinomas and one uterine leiomyoma from FAM-1 were incorporated into the linkage data, the lod score at marker D1S2811 was 5.31. These findings supported the linkage to 1q42-q44 and proposed that inactivating mutations in the *HLRCC* gene would cause the tumor predisposition phenotype.

5. IDENTIFICATION OF THE DISEASE-PREDISPOSING GENE *FUMARATE HYDRATASE* (*FUMARASE*, *FH*) (III)

To reduce the target area from approximately 14 cM defined by the genome-wide searches (I) (Alam et al., 2001), additional genotyping of individuals from the Finnish families and the UK families was performed.

In addition to markers from the commercial sets, 28 novel short tandem repeats were recognized and used for genotyping. Critical recombinations were identified in FAM-1 and UK family no. 307. The disease gene was thus placed between the clones BAC RPII-25B4 (centromeric end), and RPII-553N16 (telomeric end) comprising a region of 1.6 Mb. The minimal region matched the data on deletions of 2.4 Mb and 1.9 Mb detected by FISH analysis in the two UK families (nos 207 and 713), respectively. By examination of allele loss in tumors, the target region could not be narrowed further.

In the target region, 6 known genes and over 50 predicted genes or transcripts were identified (Table 6).

Putative pathogenic sequence changes were only detected in *fumarate hydratase* (*fumarase, FH*). *FH* is located in the BAC clones RP11-409K12 and RPII-527D7. It encodes an enzyme component of the mitochondrial tricarboxylic acid (Krebs) cycle, thus playing a key role in cellular energy metabolism. Mutations detected in *FH* are depicted in Table 7. Mutations were found in all three Finnish families and in 19 of 39 families from the UK. Sequence changes included protein-truncating changes (nonsense and frameshift mutations) (10/25), large germline deletions (2/25), and substitutions (12/25) or deletions (1/25) of highly conserved amino acids. The missense mutations were targeted at highly conserved amino acids and were not reported as known

polymorphisms. All the mutations segregated with the disease phenotype and none of them were detected in a panel of 150 control individuals. FAM-1 and FAM-2 shared the 2-bp deletion at nt 541, although no common ancestry was known. In FAM-3, a premature termination codon, Arg300X, was detected. Six UK families carried the same change Asn64Thr and three families the nonsense mutation Arg58X.

Biallelic inactivation of *FH* was observed in 21 of 22 HLRCC tumors; allelic loss was detected in 18 of 22 tumors (see section 4) and somatic mutations were observed in three tumors. One renal cell carcinoma displayed missense mutation Met285Arg and the other carcinoma a 2-bp deletion at codon 41 (Table 7). The uterine leiomyosarcoma had acquired amino acid change Glu319Asp (Table 7).

The data on germline mutations and biallelic inactivation of *FH* in patients' tumors suggested that absent or reduced function of FH could be the cause of tumorigenesis in HLRCC. To confirm this, FH enzyme activity was measured in some affected individuals. All patients with *FH* germline mutation had decreased activity compared with control individuals. Consistent with these findings and with data from the molecular genetic changes in patients' tumors, leiomyomas from affected individuals had nearly undetectable enzyme activity.

By RT-PCR, FH was shown to be expressed at approximately similar levels in a wide variety of human tissues.

6. ROLE OF *FH* IN TUMORIGENESIS OF SPORADIC COUNTERPARTS OF TUMOR TYPES OBSERVED IN HLRCC FAMILIES (IV, UNPUBLISHED DATA)

Before identification of the disease-predisposing gene, the role of the *HLRCC* locus in development of sporadic tumors was assessed by LOH analysis of tumor

Table 6. Candidate sequences identified in the target region.

Sequence type	Protein function	Number of sequences identified
Known genes		6
RGS7 (*regulator of G protein signaling 7*)	Modulates G protein-dependent signaling pathways via GTPase-activating domain	
FH (*fumarate hydratase*)	Enzyme operating in the mitochondrial tricarboxylic acid cycle	
KMO (*kynurenine 3-monooxygenase*)	Enzyme of the kynurenine pathway from tryptophan to quinolinic acid	
OPN3 (*opsin 3*)	G-protein-coupled receptor Similar to photoreceptors involved in phototransduction	
CHML (*choroideremia-like*)	Supports geranylgeranylation of most Rab proteins	
EXO1 (*exonuclease 1*)	5' to 3' exonuclease activity and Rnase H activity Similar to the S. cerevisiae protein Exo1 which interacts with Msh2 and is involved in mismatch repair and recombination	
ESTs		
With homology		9
Without homology		32
Gene predictions		
With homology		3
Without homology		14

types associated with HLRCC. Analysis was carried out on 10 cutaneous leiomyomas, 26 uterine leiomyomas, 18 uterine leiomyosarcomas, and 58 renal cell carcinomas (II, unpublished data). Allele loss was detected in 1 of 24 informative uterine leiomyomas, 5 of 17 informative

Table 7. FH mutations detected in Studies III and IV. Some of the mutations have been detected in multiple families. Mutations reported to cause FH deficiency, which is a recessive disease characterized by severe neurological impairment, are also included (see Discussion section 2.3).

Mutation	Disease/origin	Figure of FH[1]	Reference
Whole gene deletion	MCL/HLRCC		III
Whole gene deletion	MCL/HLRCC		III
Gln4X	MCL/HLRCC		III
Arg8Glu (s[2])	Sarcoma		IV
58delC	MCL/HLRCC		III
66del74	FH deficiency		Coughlin et al., 1998
121delTG (s)	RCC[3] (FAM-2)		III
Arg58X	MCL/HLRCC		III
Arg58X	MCL/HLRCC		III
Arg58X	MCL/HLRCC		III
Asn64Thr	MCL/HLRCC		III
Asn64Thr	MCL/HLRCC		III
Asn64Thr	MCL/HLRCC		III
Asn64Thr	MCL/HLRCC		III
Asn64Thr	MCL/HLRCC		III
Asn64Thr	MCL/HLRCC		III
Ala74Pro	MCL/HLRCC		III
His137Arg	MCL/HLRCC		III
Gln142Arg	MCL/HLRCC		III
His153Arg	LMS[4] (uterus)		IV
541delAG	MCL/HLRCC		III
541delAG	MCL/HLRCC		III
541delAG	LM[5] (skin)		IV
Lys187Arg	MCL/HLRCC		III
Lys187Arg	FH deficiency		Coughlin et al., 1998
Lys187del	MCL/HLRCC		III
Arg190His	MCL/HLRCC		III
Arg190His	FH deficiency		Alam et al., unpublished data
Arg190Cys	FH deficiency		Rustin et al., 1997
IVS4-15T->G	MCL/HLRCC		III
Gly239Val	MCL/HLRCC		III
Leu240X (s)	LMS (uterus)		IV
Ala265Thr	FH deficiency		Coughlin et al., 1998
Phe269Cys	FH deficiency		Coughlin et al., 1998
Met285Arg (s)	RCC (FAM-1)		III
Arg300X	MCL/HLRCC		III
Arg300X (s)	LM (skin)		IV
Glu319Gln	FH deficiency		Bourgeron et al., 1994
Glu319Asp (s)	LMS (uterus) (FAM-1)		unpublished data
Asp383Val	FH deficiency		Coughlin et al., 1998
1220delG	MCL/HLRCC		III
1302insAAA	FH deficiency		Coughlin et al., 1998
Trp458X	FH deficiency		Coughlin et al., 1998

[1] black vertical line depicts mutation site and white bar the truncated or absent protein product; [2] somatic mutation; rena renal cell carcinoma; [4] leiomyosarcoma; [5] leiomyoma

uterine leiomyosarcomas, and 7 of 57 informative renal cell carcinomas. One renal tumor displaying LOH was a papillary type 2 tumor.

Tumors analyzed for FH mutations included 10 cutaneous leiomyomas, 41 uterine leiomyomas, 52 renal cell carcinomas, 53 sarcomas, 29 prostate carcinomas, and 15 lobular breast carcinomas. Most of the tumors analyzed for LOH were included in *FH* mutation screening, but sequencing was not performed on six papillary type 2 renal cell carcinomas due to unsuccessful PCR. One of these tumors had displayed LOH at the *FH* locus.

Mutations were detected in one uterine leiomyosarcoma, one cutaneous leiomyoma, and one soft-tissue sarcoma. The uterine leiomyosarcoma harbored changes Leu240X and His153Arg. The missense mutation His153Arg was present in the patient's germline and was not observed in 134 control individuals. Compatible with hereditary cancer susceptibility, the patient was only 32 years old at the time of leiomyosarcoma diagnosis.

Similarly, in addition to somatic change Arg300X in the tumor, germline mutation 541delAG was detected in the patient with cutaneous leiomyoma. The same 2-bp deletion had segregated in FAM-1 and FAM-2, and therefore, the Finnish population frequency of this putative founder mutation was measured. The allele was not present in 448 cancer-free controls. Patient records revealed that the individual had been operated on several times due to skin leiomyomata but had no history of malignancy.

Biallelic inactivation of *FH* was also detected in the soft-tissue sarcoma, but in this case, both events, missense mutation Arg8Glu and loss of the wild-type *FH* allele, occurred at the somatic level. Compatible with tumor sequencing, the tumor had displayed loss at the *FH* locus in previous CGH analysis (Knuutila, pers. comm.). The patient was 48 years old at the time of diagnosis of metastasized soft-tissue sarcoma in her right lower limb.

DISCUSSION

1. HLRCC PHENOTYPE

1.1 Hereditary predisposition to uterine and cutaneous leiomyomas, uterine leiomyosarcoma, and renal cell carcinoma

The initial step of the study was recognition of inherited susceptibility to renal cell carcinoma and leiomyomas in a large kindred (FAM-1). Compatible with hereditary tumor predisposition, the family included several individuals with multiple tumors at an early age: four with renal cell carcinoma, seventeen with leiomyomas of the uterus and/or the skin, and two with uterine leiomyosarcoma. The tumor spectrum indicated a novel syndrome, as inherited susceptibility to renal cell carcinoma and uterine leiomyomas (HLRCC) had not been previously suggested. In our literature searches, we were able to find one publication of two leiomyomatosis kindreds from 1973 that included a description of a 20-year-old patient with uterine leiomyosarcoma and metastasized renal cancer (Reed et al., 1973). The patient and two of her four siblings had leiomyomas of the skin, and the mother had uterine leiomyomas. That the phenotype of the patient highly resembled the features in FAM-1 and that such a case was found among the limited leimyomatosis literature supported the notion of combined predisposition to renal cancer and leiomyomata.

The exceptional features of the renal cell carcinomas in FAM-1 further confirmed the hypothesis of a previously unrecognized syndrome. The lesions were solitary and the disease appeared aggressive, as all but one patient had metastasized disease at the time of diagnosis and died soon after between the ages of 27 and 50 years. These features did not correspond to the well-documented familial renal cancer syndromes with *VHL* or *MET* germline mutations (Lamiell et al., 1989; Zbar, 1995; Zbar and Lerman, 1998; Walther et al., 1999; Iliopoulos and Eng, 2000). Furthermore, the renal tumors in FAM-1 shared exceptional papillary histology. The tumors displayed distinct features with papillary growth pattern and large cells containing abundant cytoplasm, large nuclei with high nuclear grade (Fuhrman grade 3-4), and prominent nucleoli. The neoplastic epithelium did not correspond to the histology in papillary tumors with *MET* mutations, which display features of type 1 papillary renal cell carcinoma epithelium (Lubensky et al., 1999), but had some resemblance to type 2 epithelium (Delahunt and Eble, 1997). Moreover, that the unique histopathology could be used as a tool to identify additional families, spoke for a novel condition.

Adult renal tumors with similar histology usually share distinct genetic profiles (Störkel et al., 1997; Takahashi et al., 2002). Compatible with these previous observations, the tumors from FAM-1 and the additional families displayed a similar cytogenetic profile in CGH analysis. The most common changes in the tumors included losses of 13q, 4q, and Xp, and gains of 17q. These alterations are found in sporadic papillary renal cell carcinomas (Jiang et al., 1998). However, gains of chromosome 7, the most typical change in papillary tumors with *MET* mutations (Schmidt et al., 1997) and more often

detected in papillary type 1 than type 2 tumors (Jiang et al., 1998), were not common in the HLRCC carcinomas. Thus, not only the histological but also the molecular features of HLRCC tumors were distinct.

The most conclusive evidence for HLRCC being a novel syndrome was the localization of the disease-predisposing gene to 1q42-q43 by a genome-wide search. Detection of wild-type allele loss in patients' tumors, including renal cell carcinomas and leiomyomas, further confirmed the linkage and suggested that inactivation of the disease gene would be the cause of HLRCC.

A hereditary syndrome predisposing to multiple uterine and/or cutaneous leiomyomas has previously been recognized (multiple cutaneous and uterine leiomyomatosis, MCL) and FAM-3 had in fact originally been diagnosed with this disease. However, over the course of the study, the family turned out to include an individual with early-onset renal cell carcinoma. Histological features of the lesion were similar to those seen in HLRCC families FAM-1 and FAM-2, suggesting that affected family members were also predisposed to renal cell carcinoma. Moreover, the molecular features in these families were alike: FAM-3 was compatible with linkage to the *HLRCC* locus and tumors from FAM-3 harbored deletions targeted at the wild-type chromosome. These findings demonstrated that HLRCC and MCL are a single disease with a variable phenotype. This conclusion was further supported by a subsequent and independent work reporting linkage to the same locus in leiomyomatosis families from the UK (Alam et al., 2001).

1.2 Penetrance and manifestations of HLRCC

Leiomyomas of the skin and/or the uterus were the predominant finding in the HLRCC families. These tumors occurred in 87% (26/30) of individuals with germline mutation in the disease-predisposing gene, which was identified as *fumarate hydratase (FH)* (III) (see section 2). Penetrance of leiomyomas was complete in FAM-1 and FAM-3, but appeared to be 40% (2/5) in FAM-2. Renal cell carcinoma occurred in only 23% (7/30) of individuals [FAM-1 22% (4/18), FAM-2 40% (2/5), FAM-3 14% (1/7)] but at an early age, the age at diagnosis varying from 26 to 48 years (mean 32 years). In addition, only 1 of the 39 leiomyomatosis families from the UK appears to include an individual affected with renal cell carcinoma (Tomlinson I., pers. comm.).

The interfamilial phenotypic variability may be due to differences in the first selection criterion used in family identification. This explanation seems reasonable because renal cancer appeared to be rare in UK families, which were identified based on the occurrence of leiomyomata. Moreover, renal cancer was not observed in families of the two patients with germline mutations in *FH*, who had been detected among apparently sporadic cases of uterine leiomyosarcoma and cutaneous leiomyomas (IV). Some of the phenotypic variation could also be explained by allelic heterogeneity in the *FH* gene. As the germline mutations of only three HLRCC families are currently known, additional mutation data are required to shed light on this issue. In addition to family ascertainment and allelic heterogeneity, either environmental or genetic modifiers could play a role in clinical expression of HLRCC, especially since intrafamilial differences in the occurrence of renal cell carcinoma and the number of leiomyomas were observed.

Genetic modifiers have previously been implicated in a few hereditary syndromes. For example, germline *APC*

mutations can predispose to hereditary colon cancer syndromes with striking phenotypic variation, such as FAP characterized by development of hundreds or thousands of colonic polyps and attenuated polyposis coli by only a few polyps. In mice carrying a mutant mouse *Apc* gene [Multiple Intestinal neoplasia (Min) mice], a modifying locus *Mom-1 (Modifier of Min-1)* was identified (Dietrich et al., 1993). *Mom-1* locus was shown to control about 50% of genetic variation in tumor number in the mice. Another example is higher risk for ovarian cancer in *BRCA1* carriers with rare alleles of *HRAS-1* VNTR (Phelan et al., 1996). Modifier effects in VHL have also been proposed based on differences in the presence and number of ocular tumors within families (Webster et al., 1998). Recently, an association between a certain allele of *cyclin D1* and risk of multiple retinal angiomas and CNS hemagioblastomas in VHL patients was observed (Zatyka et al., 2002). Complete characterization of the natural history of HLRCC and utilization of animal models in investigation of environmental and genetic modifiers will add to our knowledge of the penetrance and variability of clinical phenotype in HLRCC.

In addition to variability in clinical expression, a tissue-specific cancer spectrum is characteristic for hereditary cancer syndromes. One explanation for this puzzling observation could be alternative functions of the gene product in different tissues (Fearon, 1997). Although determination of the HLRCC tumor spectrum requires further study, the disease apparently affects renal tissue and smooth muscles of the uterus and the skin. The kidney, uterus, and arrectores pilorum muscle, the origin of cutaneous leiomyomas, all develop from the mesoderm during embryogenesis (Carlson, 1999). Thus, the tissue manifestations in HLRCC could reflect a common embryological origin of proliferating cells in the different tumors. Some resemblance to HLRCC phenotype can be found in the animal model for tuberous sclerosis (TS) (Ecker rat) and in the Birt-Hogg-Dubé syndrome (BHD). The Ecker rat harbors a germline mutation in the rat *Tsc2* gene and develops both renal cell carcinoma and uterine leiomyomas (Yeung et al., 1994; Everitt et al., 1995; Kobayashi et al., 1995; Hino et al., 1999). The animals are also predisposed to sarcomas of the uterus and the spleen and pituitary tumors. BHD is characterized by benign tumors of the skin and is associated with renal neoplasms, which display variable histology including the papillary subtype (Toro et al., 1999). Interestingly, the cutaneous tumors in BHD arise in the hair follicle, which is connected to the arrectores pilorum muscle. It remains to be elucidated whether some overlap exists between the pathways of tumorigenesis in HLRCC, the animal model for TS, and BHD that could explain the similarities of tissue manifestations in these diseases. Investigations on the putative tissue-specific functions of FH, the protein product of the disease gene, could also shed light on the HLRCC phenotype.

2. FUMARATE HYDRATASE (FH) IN TUMORIGENESIS

2.1 Mutations in FH cause tumor development

Through identification of critical recombinants in the disease families and screening of known and predicted transcripts at the *HLRCC* locus in 1q42-q43, the disease gene, *fumarate hydratase* (*fumarase*, *FH)*, was identified. FH operates in the mitochondrial tricarboxylic acid cycle (Krebs cycle), playing a key role in cellular energy metabolism. Germline mutations in *FH* were detected in 25 of 42 families and

included protein-truncating mutations (10/25), large germline deletions (2/25), and substitutions (12/25) or deletions (1/25) of highly conserved amino acids. Both *FH* alleles were inactivated in almost all patients' tumors through a germline mutation in one allele and loss of or a somatic mutation in the other allele (21 of 22 tumors from Finnish families). Thus, tumors followed Knudson's model of tumor suppressor gene inactivation (Knudson, 1971). Furthermore, FH enzyme activity was reduced in patients' normal tissues and nearly absent in their tumors. These findings indicate that germline mutations in *FH* predispose to HLRCC and that *FH* is likely to be a tumor suppressor gene.

Further insights into the role of *FH* in tumor development were gained by analyzing sporadic tumors for *FH* mutations and LOH at the *FH* locus. Studies included sporadic forms of tumor types associated with HLRCC. In the series of 194 tumors studied for *FH* mutations, somatic mutations appeared to be rare. Biallelic inactivation of FH was detected in one uterine leiomyosarcoma, one cutaneous leiomyoma and one soft-tissue sarcoma. The two former lesions originated from a germline mutation and the sarcoma had acquired two somatic hits. In the LOH analysis of 112 tumors, 13 tumors displayed allele loss, but none of the tumors harbored *FH* mutations.

Several points can be raised from the findings of the mutation and LOH analyses. First, because two of the three cases with somatic mutation also harbored an inherited mutation, germline mutations in *FH* could possibly be required for efficient tumorigenesis. However, the finding of two somatic hits in one lesion demonstrated that *FH* could be involved in development of nonhereditary tumors. Second, compatible with the previous data (I, II, III, unpublished data), biallelic inactivation of *FH* was detected in all three tumors. The inactivation model of *FH* seems remarkable in that two hits can be found in nearly all tumors. Third, that one uterine leiomyosarcoma arose from a germline mutation further confirmed the association of uterine leiomyosarcoma with the HLRCC phenotype. Fourth, *FH* defects were rare in the studied papillary type 2 renal cell carcinomas. Histologically, all eleven tumors had some resemblance to carcinomas from HLRCC families, but only two lesions were highly similar to them. It is conceivable that papillary type 2 renal cell carcinomas may be divided into further subgroups with distinguishable histopathological and molecular features, and thus, some other genes than *FH* might also contribute to their development. Fifth, none of the tumors displaying LOH harbored *FH* mutations, which could be due to random loss, haploinsufficiency, or some alternative mechanism for gene inactivation. Random loss would not be unlikely because malignant tumors are known to harbor numerous chromosomal abnormalities (Fearon and Vogelstein, 1990; Devilee et al., 2001). Haploinsufficiency has been demonstrated with some other tumor suppressor genes (Fero et al., 1998; Venkatachalam et al., 1998; Cook and McCaw, 2000; Quon and Berns, 2001). Although the robust biallelic inactivation pattern of *FH* in several hereditary and sporadic tumors does not support this phenomenon, haploinsufficiency could still be involved in early development of some of the lesions.

Not all cancer predisposition genes are mutated in corresponding sporadic lesions. The low somatic mutation rate in *BRCA1* and *BRCA2* in sporadic breast and ovarian carcinomas has been thought to implicate the genes only being important for tumorigenesis of familial cancer forms (Takahashi et al., 1995; Lancaster et al., 1996; Teng et al., 1996). *BRCA1* has later been demonstrated to be epigenetically

inactivated in some sporadic tumors, particularly those displaying LOH (Esteller et al., 2000). Epigenetic silencing, especially through promoter hyper-methylation, has been discovered in most human tumor types, and it is estimated to be involved in inactivation of over 50% of tumor suppressor genes in sporadic cancers (Jones and Baylin, 2002). Investigation of the alternative mechanisms of *FH* inactivation will further enlighten the role of *FH* in development of sporadic tumors.

2.2 Characteristics of FH

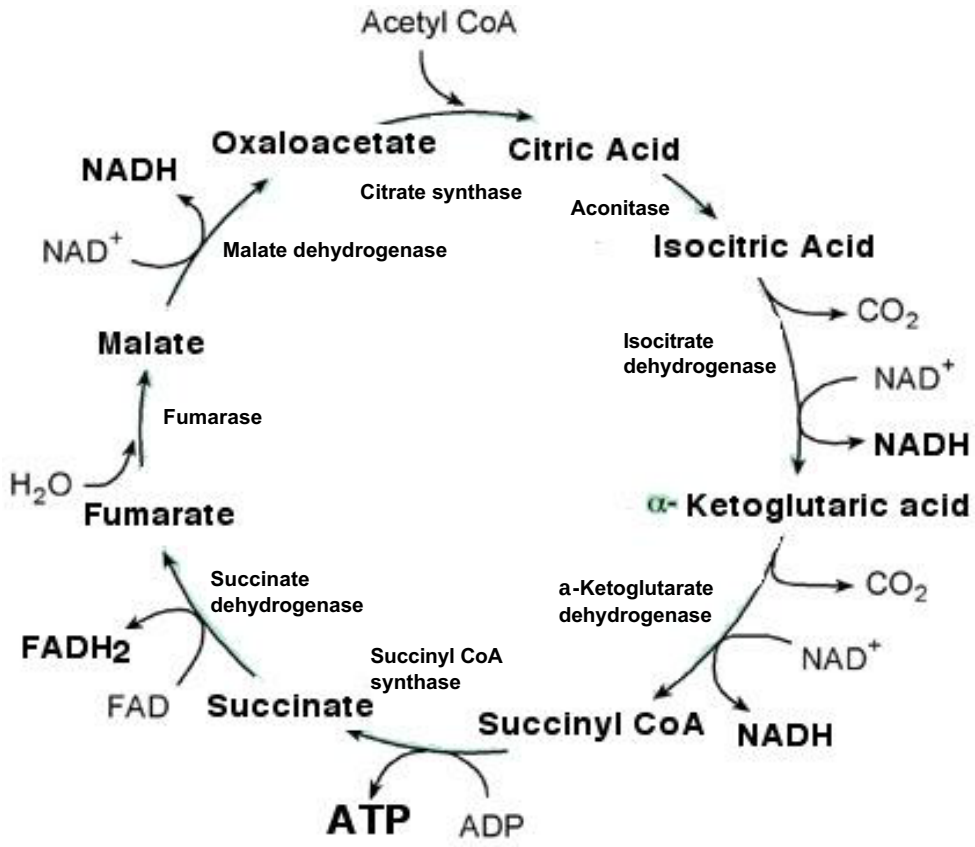
FH consists of 11 exons spanning over 20 kb in 1q42-q43 and giving rise to a transcript of 1.5 kb (Craig et al., 1976; Despoisses et al., 1984; Kinsella and Doonan, 1986). The mature peptide is present both in the mitochondria and the cytosol. The existence of the two isoforms was first demonstrated by detecting differences in the electrophoretic mobility of the protein (Tolley and Craig, 1975; Edwards and Hopkinson, 1979a, 1979b). Studies on human, pig, and rat fumarases have provided evidence that the two isoforms are encoded by a single gene (Tolley and Craig, 1975; Edwards and Hopkinson, 1979a; Edwards and Hopkinson, 1979b; Kobayashi and Tuboi, 1983; O'Hare and Doonan, 1985). Based on yeast studies, the proteins are products of either two different transcripts (Wu and Tzagoloff, 1987), a single transcript with two translation initiation sites (Tuboi et al., 1990; Suzuki et al., 1992), or a single translation product distributed between the cytosol and the mitochondrion (Stein et al., 1994; Sass et al., 2001). Sass et al. (2001) demonstrated the presence of only one fumarase translation product, which is targeted to mitochondria by an amino-terminal signal peptide. The signal is then removed by the mitochondrial processing peptidase and some of the protein molecules are transported back into the cytosol resulting in identical mature cytosolic and mitochondrial peptides. In humans, the signal sequence for mitochondrial targeting is thought to reside in the first exon (GenBank U59309) (GenBank, 2002).

Crystal structures of bacterial (FumC) and yeast (Fum1) fumarase proteins have been characterized. Being a highly conserved protein in evolution, human FH is 59% and 66% identical to the corresponding proteins of *E. coli* and *S. cerevisiae*, respectively. An even higher level of identity resides within three specific regions: region 1 from His129 to Thr146 (amino acid numbering is based on FumC sequence), region 2 from Val182 to Glu200, and region 3 from Gly317 to Glu331 (Estevez et al., 2002). A sequence motif in the third region characterizes a superfamily of proteins including fumarase, aspartase, argininosuccinate lyase, adenylosuccinate lyase, and crystallin. The enzymatically active form of FH has been shown to operate as a homotetramer (Hill and Teipel, 1971). Each subunit has three domains, D1, D2 and D3; the central domain, D2, consists of five helixes forming a 20-helical core in the tetramer (Weaver et al., 1995, 1998; Estevez et al., 2002). The active site is composed of side chains from three of the four subunits (Weaver et al., 1995, 1997, 1998; Estevez et al., 2002). Mutation Glu315Gln in *E. coli* FumC, which is homologous to naturally occurring human mutation Glu319Gln (see Table 7), was shown to result in a 10-fold reduction in the catalytic activity of the protein (Estevez et al., 2002).

The well-known function of mitochondrial homotetrameric FH is to catalyze the conversion of fumarate to l-malate in the tricarboxylic acid cycle (TCAC) or Krebs cycle (Krebs and Johnson, 1937) (Figure 2). TCAC is the final common route for the oxidation of fuel molecules - amino acids, fatty acids, and carbohydrates.

In this reaction series, free energy is released via biological oxidations of two-carbon molecules derived from primary substrates, with concomitant reduction of coenzymes NADH and $FADH_2$. Reducing equivalents are then fed into the mitochondrial electron transport chain, and generation of high-energy ATP molecules results (Stryer, 1995; Rustin et al., 1997). For each two-carbon compound entering TCAC, 10 molecules of ATP are generated, 9 from the electron transport chain and 1 directly in the cycle. TCAC

Figure 2. The Krebs cycle.



functions only under aerobic conditions due to a constant requirement of NAD+ and FAD, which are produced when NADH and $FADH_2$ transfer their electrons to O_2 in the electron transport chain generating ATP. The cycle integrates into several cellular metabolic pathways by providing intermediates for biosynthetic processes. For example, FH is involved in the processing of intermediates of the urea cycle (Medical Biochemistry Page, 2002). However, the role of the cytosolic isoform of FH is largely unclear.

2.3 FH and other Krebs cycle enzymes in human diseases

Fumarase deficiency, a progressive mitochondrial encephalomyopathy, has been previously described (Zinn et al., 1986). The disease was shown to be a recessive disorder caused by homozygous/ compound heterozygous mutations in *FH* (Bourgeron et al., 1993; Coughlin et al., 1993) (Table 7). This syndrome is an example of the disease group affecting the brain, skeletal muscle, and other organs due to defects in mitochondrial function (DiMauro et al., 1985). Clinically, FH deficiency is characterized by neurological impairment, hypotonia, growth and developmental delay, and fumaric aciduria (Whelan et al., 1983; Zinn et al., 1986; Petrova-Benedict et al., 1987; Gellera et al., 1990; Remes et al., 1992; Bonioli et al., 1998; Kerrigan et al., 2000; Manning et al., 2000). Neurological features include retardation, seizures, and visual impairment. Patients have significantly reduced FH enzyme activity (residual activity varies from 0.5% to 25%) and usually die within a few months of birth (Rustin et al., 1997), although some patients have survived to their second decade (Kerrigan et al., 2000). However, no tumors have previously been reported in the patients or their first-degree relatives. By careful clinical and histopathological examination, we were able to detect leiomyomas of the skin in a mother of one FH deficiency patient (III). Thus, these tumors, which may be difficult to recognize, may have previously gone unnoticed in these mutation carriers. Interestingly, we found that the nature and location of mutations differed between FH deficiency and leiomyomatosis patients (III). In the leiomyomatosis families, mutations occurred significantly more often in the 5'-end of the gene and were more frequently truncating than mutations in FH deficiency patients. One simple explanation to these findings is that truncating N-terminal changes might result in embryonic death when homozygous and thus be underrepresented in FH deficiency. In addition, or alternatively, some mutations causing FH deficiency might not be penetrant for leiomyomatosis.

Defects in two other Krebs cycle enzymes, _-ketoglutarate dehydrogenase (oxoglutarate dehydrogenase, OGDH) and succinate dehydrogenase (SDH), have also been identified to cause mitochondrial encephalomyopathy with neurological defects and muscular involvement (Taylor et al., 1996; Rustin et al., 1997; Rustin, 2002; Rustin and Rotig, 2002). The molecular genetic basis for _-ketoglutarate (_-KG) deficiency has not been reported, but homozygous mutations in *SDHA* have been shown to underlie SDH deficiency (Bourgeron et al., 1995; Parfait et al., 2000). SDH has four subunits and operates in the Krebs cycle and the electron transport chain as a component of the mitochondrial complex II. SDH includes the catalytic domains flavoprotein SDHA and iron sulfur protein SDHB, which are anchored to the inner mitochondrial membrane by the other two subunits SDHC, and SDHD. Interestingly, mutations in subunits *SDHD*, *SDHC* and *SDHB* have been identified to cause hereditary predisposition to endocrine

tumors in hereditary paraganglioma syndrome (PGL) (Baysal et al., 2000; Niemann and Muller, 2000; Astuti et al., 2001b). In PGL, affected individuals develop highly vascularized, benign, and slow-growing tumors of the parasympathetic ganglia referred to as paragangliomas. The most common location for these lesions is the carotid bifurcation. Mutations in *SDHD* and *SDHB* have also been detected in familial (Astuti et al., 2001a, 2001b) and sporadic (Gimm et al., 2000; Astuti et al., 2001b) pheochromocytoma. Typically, hereditary pheochromocytoma is observed in the context of VHL, MEN2, and NF1 syndromes (Marsh and Zori, 2002). Pheochromocytoma is a catecholamine-producing tumor occurring in sympathetic ganglions of the body. Most pheochromocytomas arise in the adrenal medulla, but approximately 10% of the tumors are located in the abdominal and mediastinal sympathetic ganglions and are therefore sometimes called para-gangliomas.

2.4 Putative mechanisms of tumorigenesis in HLRCC

At present, the mechanisms by which mutations in *FH* lead to tumor development remain obscure. However, some explanations can be suggested based on the findings of this study and the previous literature.

First, FH could have unknown functions unrelated to aerobic energy production through the Krebs cycle. This would not be surprising, especially considering the poorly understood functions of the cytosolic FH. Novel roles in cellular processes have been demonstrated for other enzymes of the Krebs cycle. The cytosolic form of malate dehydrogenase was discovered to be involved in the nucleotide translocation pore (Hanss et al., 2002). Yeast mitochondrial isocitrate dehydrogenase was shown to bind to 5' leader sequences of mitochondrial mRNA and suggested to be involved in translation regulation (de Jong et al., 2000). In addition, yeast _-ketoglutarate dehydrogenase was demonstrated to be a bifunctional protein involved not only in the Krebs cycle but also in stabilizing mitochondrial DNA (Kaufman et al., 2000).

Second, a block in energy production through the Krebs cycle could enhance generation of reactive oxygen species (ROS) and lead to an accelerated mutation rate by affecting the balance of the electron transport chain, as these two processes are tightly coupled. ROS, which are primarily produced at mitochondrial electron transport chain complexes I and III (Liu et al., 2002), have been implicated in carcinogenesis, partially due to triggering generation of mutations in nuclear and mitochondrial DNA (Penta et al., 2001). Although the exact role of mitochondrial DNA rearrangements in tumorigenesis is obscure, accumulation of mutations in the nuclear DNA induced by ROS have been suggested to drive tumor development and progression (Jackson and Loeb, 2001). At present, hypermutability of HLRCC-associated tumors is not known. However, CGH, LOH, and p53 mutation analyses on sporadic leiomyomas, the most common tumor type in HLRCC, suggest that these tumors appear not to be unstable genetically (Jeffers et al., 1995; Packenham et al., 1997; Mao et al., 1999).

Third, a defect in cellular energy production could induce growth-promoting signals. Such signals could be metabolic intermediates or ROS, which accumulate due to the defective Krebs cycle and electron transport chain in the mitochondria. This could, for instance, trigger hypoxia response, resulting in various downstream effects promoting tumor growth. The role of hypoxia in cell growth is widely documented (Harris, 2002). Hypoxia response is known

to lead to induced expression of genes involved in an array of processes, such as angiogenesis, glycolysis, growth factor signaling, immortalization, genetic instability, and tissue invasion and metastasis.

Interestingly, hypoxia has been proposed in the development of uterine leiomyomas. Myometrial smooth muscle cells proliferate in the luteal phase of the menstrual cycle, and upon menstruation, blood supply to the myometrium decreases during myometrial contraction, possibly inducing hypoxic state in the myometrial smooth muscle cells. It has been hypothesized that a subset of cells surviving these repeated cycles of proliferation and ischemia could acquire changes, giving them an additional growth advantage and rendering them putative progenitor cells of leiomyomas (Fujii et al., 1999; Fukuhara et al., 2002). Furthermore, early kidney development occurs at an embryological stage, when circulation is poorly developed and oxygen levels are low (Carlson, 1999). Thus, hypoxia could be speculated to promote proliferation of a certain population of cells in the uterus and kidney, leading to organogenesis in embryonic development or tumor formation in the adult.

A key transcription factor mediating hypoxia response is HIF-1. HIF-1 is a heterodimer that consists of the hypoxia response factor HIF-1_ and the nuclear translocator HIF-1ß. When oxygen levels are low, the heterodimer binds to the hypoxia-response elements activating transcription of hypoxia-inducible genes. In the presence of oxygen, HIF-1_ is ubiquitylated by VHL and targeted to proteosomal degradation. Interaction between HIF-1_ and VHL was shown to require covalent modification of HIF-1_ by prolyl hydroxylase (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). Prolyl hydroxylase is active in the presence of oxygen, ferrous iron, and _-ketoglutarate (2-oxoglutarate) (Ryle and Hausinger, 2002). Interestingly, _-ketoglutarate is an intermediate of the mitochondrial TCAC and could be a candidate mediating oxygen sensing. Involvement of mitochondria has previously been proposed in activation of hypoxia response via generation of ROS, leading to accumulation of HIF-1_ (Chandel et al., 2000). However, the role of mitochondria in hypoxia response has not been confirmed (Srinivas et al., 2001).

Activation of hypoxia response via HIF-1_ is the most widely characterized mechanism of tumorigenesis in the hereditary renal cancer syndrome VHL. Mutations in *VHL* prevent ubiquitylation of HIF-1_, resulting in constant activation of hypoxia-response genes. Hypoxia response has also been proposed in SDH defects. Paragangliomas, which develop in individuals with mutations in SDH subunits, occur most often in the carotid body. This organ senses the body oxygen tension and develops paraganglioma-like cellular hyperplasia/anaplasia in individuals exposed to chronic hypoxia (e.g. in people living at high altitudes). At the molecular level, hypoxia response has been demonstrated by detecting elevated levels of expression of angiogenic factors in hereditary and sporadic paragangliomas (Jyung et al., 2000; Gimenez-Roqueplo et al., 2001).

Loss of the *SDHD* locus has been described in pheochromocytomas from VHL patients, in addition to sporadic endocrine tumors of different types (Kytölä et al., 2001, 2002; Lui et al., 2002). Loss at 11q appeared to be specific to pheochromocytomas from VHL patients, as it was not detected in other lesions from the patients (Lui et al., 2002). Although VHL-associated pheochromo-cytomas did not display mutations in *SDHD*, inactivation of the gene could be involved in the tumorigenetic pathway of these lesions.

In conclusion, the tumorigenic mechanisms of *FH* mutations require further elucidation. Some overlap in clinical manifestations and molecular background appears to exist between the tumor predisposition syndromes with mutations in *VHL, SDH*, and *FH* genes. Understanding the differences and similarities between these diseases may shed light on how *FH* mutations lead to tumor development in HLRCC.

3. Clinical relevance and implications for diagnosis, follow-up, and treatment of HLRCC patients

The discovery of HLRCC and the identification of *FH* as the disease-predisposing gene provide the basis for improved diagnosis, prevention, and treatment of tumors in predisposed individuals. Nevertheless, further work is needed to clarify the natural history of HLRCC. In particular, the HLRCC incidence, tumor spectrum, and cancer risk should be evaluated. The optimal methods for prevention of malignancies should also be assessed.

Due to variable manifestations and clinical expression in HLRCC, identification of HLRCC patients requires cooperation between general practitioners and different specialists, including dermatologists, gynecologists, urologists, clinical geneticists, and pathologists. Leiomyomas of the skin could be valuable in identifying individuals with hereditary cancer risk because they are one of the most common manifestations in these patients and are detectable by gross inspection. The role of cutaneous leiomyomas was emphasized by detecting a *FH* germline mutation in one patient with cutaneous leiomyomas out of ten apparently sporadic cases (IV).

After identification of *FH* as the causative gene for HLRCC, direct and accurate diagnosis of affected individuals by genetic testing has been possible. This provides a means to guide individuals at risk to preventive screenings and follow-up to reduce cancer incidence and mortality. Genetic testing has been provided to HLRCC family members and has been accompanied by information on the disease, genetic counseling, and support. So far, we have carried out genetic testing on 18 patients.

Although the HLRCC cancer risk has not been accurately determined, a predisposition to renal cancer and uterine leiomyosarcoma clearly exists in a subset of the leiomyomatosis families. Therefore, regular screening for tumors in individuals at risk to detect these tumors at an early stage is reasonable. The screening methods in HLRCC include abdominal and transvaginal ultrasonography for detection of malignant tumors of the kidney and the uterus, respectively. Notably, without screening, renal cell carcinoma in particular is often diagnosed at a considerably more advanced stage due to its nature of generating symptoms relatively late (Motzer et al., 1996). While the optimal frequency of follow-up needs to be clarified, annual examination, including abdominal and transvaginal ultrasonography, can be recommended because of the safety, accuracy, and clinical applicability of these screening methods (Mihara et al., 1999; Dueholm et al., 2002). As regards uterine manifestations, prophylactic hysterectomy may be an option for an at-risk women beyond child-bearing age.

Genetic testing and preventive screening have a major psychosocial impact on the patient. Thus, genetic counseling and support should always be provided to these patients. Patients should also be assisted in making informed decisions and understanding their disease risk and the implications for cancer prevention. To

benefit from the advances in molecular characterization of HLRCC in the future, collaborative efforts are needed to evaluate the psychosocial influence and usefulness of genetic testing and preventive screening in HLRCC.

CONCLUSIONS AND FUTURE PROSPECTS

This study describes a novel cancer syndrome with predisposition to leiomyomas of the uterus and the skin, uterine leiomyosarcoma, and distinct papillary type 2 renal cell carcinoma (HLRCC). Tumor predisposition in HLRCC is caused by dominantly inherited mutation in *fumarate hydratase* (*fumarase, FH*) in 1q42-q44. *FH* is known to encode an enzyme component of the mitochondrial tricarboxylic acid cycle. *FH* is likely to be a tumor suppressor gene based on the data on inactivating germline mutations segregating in the disease families, biallelic inactivation of *FH* in patients' tumors, reduced FH enzyme activity in patients' normal tissues, and nearly undetectable activity in their tumors.

Mutational inactivation of *FH* appeared to be rare in sporadic forms of tumor types observed in HLRCC. Biallelic inactivation of *FH* was detected in a uterine leiomyosarcoma, a cutaneous leiomyoma, and a soft-tissue sarcoma. Whereas the two former tumors originated from a germline mutation, the soft-tissue sarcoma displayed two somatic hits, demonstrating the involvement of *FH* in the development of nonhereditary tumors. Not all cancer predisposition genes are mutated in the respective sporadic lesions but instead can be inactivated through epigenetic mechanisms. Further studies are needed to reveal the role of alternative mechanisms in *FH* inactivation in sporadic tumorigenesis.

Discovery of *FH* in cancer predisposition clearly adds to our knowledge of tumorigenesis. Notably, this study provides the first evidence of a defect in a Krebs cycle enzyme predisposing to common human tumors. Challenging tasks for the future include determining the role of *FH* inactivation in sporadic tumors, investigating the mechanisms by which *FH* mutations lead to tumorigenesis, characterizing the natural history of HLRCC, and improving the diagnosis, prevention, and treatment of the disease.

FH is the first gene shown to predispose to uterine leiomyomas, one of the predominant human tumor types. This finding and future studies on genetic and epigenetic inactivation of *FH* in sporadic leiomyomas may provide clues to understanding, preventing, and treating the common forms of these lesions.

At present, the mechanisms by which mutation in *FH* results in tumor development remain speculative. For example, FH could have unknown functions unrelated to aerobic energy production. Alternatively, a block in the Krebs cycle and the electron transport chain could enhance generation of ROS, thus accelerating the mutation rate. It is also conceivable that a defect in cellular energy production could induce growth-promoting signals through, for example, the hypoxia pathway. The obscure mechanisms of tumorigenesis caused by *FH* mutations open up a wide field for future research.

Characterization of the natural history of HLRCC is essential. This includes determining HLRCC incidence, tumor spectrum, cancer risk, and optimal methods and intervals for screening of tumors. The growing knowledge of the molecular mechanisms involved could provide tools for optimal diagnosis, prevention, and treatment of hereditary and sporadic forms of the disease.

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