MOLECULAR AND CLINICAL CHARACTERISTICS OF TRICARBOXYLIC ACID CYCLE-ASSOCIATED TUMORS

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Academic dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals.

I. Lehtonen HJ, Kiuru M, Ylisaukko-oja SK, Salovaara R, Herva R, Koivisto PA, Vierimaa O, Aittomäki K, Pukkala E, Launonen V, Aaltonen LA. Increased risk of cancer in patients with fumarate hydratase germline mutation. J Med Genet. 2006 43:523-6.

II. Lehtonen HJ, Blanco I, Piulats JM, Herva R, Launonen V, Aaltonen LA. Conventional renal cancer in a patient with fumarate hydratase mutation. Hum Pathol. 2007 38:793-6.

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In addition some unpublished data is presented.

Publication IV was included in the thesis of Sanna Ylisaukko-oja (Defects in Tricarboxylic Acid Cycle Enzymes Fumarate Hydratase and Succinate Dehydrogenase in Cancer, Helsinki 2007)

*Equal contribution

ABBREVIATIONS

ADP/ATP	adenosine di/triphosphate
AE1/AE3	antibody cocktail for cytokeratin recognition
AgNOR	argyrophilic nucleolar organizer region
AKT	murine v-akt thymoma virus homolog (aka protein kinase B, PKB)
APC	adenomatous polyposis coli protein
ARF	$p14^{ARF}$, alternative reading frame of cyclin-dependent kinase inhibitor 2A (CDKN2A) locus
ARNT	aryl hydrocarbon receptor nuclear translocator (aka HIF1β)
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma 2 protein
BER	base excision repair
BHD	Birt-Hogg-Dube syndrome
BNIP3	BCL2/adenovirus E1B 19 kD protein interacting protein
BRCA1/2	breast cancer protein 1 and 2
BWS	Beckwith-Wiedemann syndrome
CBP	CREB binding protein
CD10	common acute lymphocytic leukemia antigen
CDC	collecting duct renal carcinoma
CDK	cyclin dependent kinase
CI	confidence interval
CIN	chromosomal instability
CITED4	CPB/p300 interacting transactivator with ED-rich tail 4
CK7	cytokeratin 7
CLL	chronic lymphocytic leukemia
CLM	cutaneous leiomyoma
CO_2	carbon dioxide
COX-1	cyclooxygenase 1
CRC	colorectal cancer
CSC	cancer stem cell
DNA	deoxyribonucleic acid
cDNA	complementary DNA
E2F1	E2F transcription factor 1
EGFR	epidermal growth factor receptor
EGLN	egg laying nine (aka PHD)
EMA	epithelial membrane antigen
EPAS1	endothelial PAS (per-arnt-sim) domain protein 1 (aka HIF2α)
ERBB2	avian viral v-erb-b2 erythroblastic leukemia homolog 2 (aka Her2 or Neu)
ERK	extracellular signal-regulated kinase
FAD	flavin adenine dinucleotide, in reduced form $FADH_2$
FAP	familial adenomatous polyposis
FH	fumarate hydratase (aka fumarase)
FHv	alternative transcript of FH
FHD	FH deficiency
FIH1	factor inhibiting HIF1
Fum1	yeast fumarase
GFP	green fluorescent protein
GIST	gastrointestinal stromal tumor
GTPase	signal transducer molecule
HGF	hepatocyte growth factor
HIF1	hypoxia inducible factor 1
HIF1α	alpha subunit of HIF1
HIF1β	beta subunit of HIF1 (aka ARNT)
HIF2α	*EPAS1* gene encoded protein
HIF3α	hypoxia inducible factor 3 alpha
HLRCC	hereditary leiomyomatosis and renal cell cancer syndrome
HNPCC	hereditary non-polyposis colorectal cancer syndrome
HPF	high power field
HPGL	hereditary paragangliomatosis syndrome
HPRC	hereditary papillary renal carcinoma syndrome
HPT-JT	hyperparathyroidism-jaw syndrome
HR	homologous recombination
HRE	hypoxia response element
HSE	heat shock response element
HSF	heat shock factor
HSP	heat shock protein
IGF1R	insulin-like growth factor 1 receptor
IHC	immunohistochemistry
ING4	inhibitor of growth 4 protein
IPAS	inhibitory PAS (per-arnt-sim) domain protein (splice variant of HIF3α)
JP	juvenile polyposis
c-JUN	avian v-jun sarcoma virus 17 oncogene homolog

kDa	kilodalton
KIT	feline sarcoma virus v-kit homolog
LOH	loss of heterozygosity
LRS	leaky ribosomal scanning
MAD1	yeast mitotic arrest deficient-like 1 homolog
MAPK	mitogen-activated protein (MAP) kinase
MAX	MYC-associated factor X
MCL	multiple cutaneous and uterine leiomyomatosis syndrome
MDM2	murine double minute 2 homolog
MEK	MAPK/ERK kinase
MEN2	multiple endocrine neoplasia 2
c-MET	mesenchymal epithelial transition factor
MF	mitotic figure
MIB1	antibody against proliferation marker Ki-67
miRNA	microRNA
MLH1	*E. coli* MutL homolog 1
MMP2/7	matrix metalloproteinases 2 and 7
MMR	mismatch repair
MSH2/3/6	*E. coli* MutS homologs 2, 3, and 6
MSI	microsatellite instability
mTOR	mammalian target of rapamycin
c-MYC	avian v-myc viral myelocytomatosis homolog
NAD+	nicotinamide adenine dinucleotide, in reduced form NADH
NADP	nicotineamide adenine dinucleotide phosphate, in reduced form NADPH
NER	nucleotide excision repair
NF1	neurofibromatosis type 1 syndrome, *neurofibromin 1*gene
NFκB	nuclear factor-kappa B
NGF	neuronal growth factor
NHEJ	non-homologous end joining
p53	tumor suppressor protein 53
$p16^{INK4a}$	major product of cyclin-dependent kinase inhibitor 2A (CDKN2A) locus
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PGL	paraganglioma
PGL1/2/3/4	loci of paraganglioma genes 1, 2, 3, and 4
PHD	prolyl hydroxylase-domain protein (aka EGLN)
PHEO	pheochromocytoma
PI3K	phospoinositide 3-kinase
PID	personal identifier
PMS2	yeast post-meiotic segregation homolog 2
PTEN	phosphatase and tensin homolog, deleted on chromosome 10
RAD51	prokaryotic RecA homolog
RAS	rat v-ras sarcoma virus homolog
RB	retinoblastoma, RB protein
RBX1	ring-box 1
RCC	renal cell cancer
RET	rearranged during transfection, oncoprotein
RNA	ribonucleic acid
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
S100	calcium binding protein
SDH	succinate dehydrogenase (including subunits A, B, C, D of which the last three are associated with HPGL)
SIR	standardized incidence ratio
SMAD	*Drosophila* mothers against decapentaplegic homolog
SRC	avian v-src sarcoma virus (Schmidt-Ruppin A-2) homolog
TCA	tricarboxylix acid cycle (aka Kreb's cycle)
TGF-β	transforming growth factor beta
TGF-βR	TGF-β receptor
TMA	tissue microarray
TNM	tumor, lymph node and metastasis based classification of tumors
TS	tuberous sclerosis
TSC1	TS gene 1 (tuberin)
TSC2	TS gene 2 (hamartin)
TSG	tumor suppressor gene
TSP1	thrombospondin 1
UEA-1	*Ulex europaeus* lectin
ULM	uterine leiomyoma
ULMS	uterine leiomyosarcoma
UTR	untranslated region
VEGF	vascular endothelial growth factor
VHL	von Hippel Lindau syndrome, *VHL* tumor suppressor gene
pVHL	VHL protein
WHO	world health organization
WT1	Wilms' tumor suppressor gene
XP	xeroderma pigmentosum

ABSTRACT

Hereditary leiomyomatosis and renal cell cancer (HLRCC) is a tumor predisposition syndrome caused by heterozygous germline mutations in a nuclear gene encoding fumarate hydratase (FH), an enzyme of the mitochondrial tricarboxylic acid cycle (TCA cycle). The syndrome is characterized by cutaneous (CLM) and uterine (ULM) leiomyomas, and renal cell cancer (RCC). Moreover, a few cases of the malignant counterpart of ULM, uterine leiomyosarcoma (ULMS) have been reported in the Finnish HLRCC population. To date, over 130 HLRCC families have been identified throughout the world, but the families with RCC are mainly from North America and Finland. Homozygous germline mutations in *FH* predispose to a severe neurological defect called FH deficiency (FHD). Another tumor predisposition syndrome, hereditary paragangliomatosis (HPGL), is caused by inherited mutations in the genes encoding subunits of succinate dehydrogenase (SDHB/C/D), also an enzyme of the TCA cycle as well as of the mitochondrial electron transport chain. HPGL is associated with paragangliomas and pheochromocytomas. The biallelic inactivation of *FH* and *SDH* in the associated tumors suggests that they operate as tumor suppressor genes. As a consequence of a defective TCA cycle, tumors in these syndromes display an abnormally activated hypoxia-inducible factor 1 (HIF1) pathway causing a stage of "pseudohypoxia". HIF1 is a transcription factor which is normally activated by hypoxia. It drives transcription of genes encoding e.g. angiogenetic and glycolytic factors to promote cells' survival in low oxygen tension but which can also facilitate tumor growth. Recently it has been suggested that hypoxia and HIF1 could be one of the causes of genetic instability, typical for many tumors.

The penetrance of malignant tumors in HLRCC is reduced, and thus the association of other malignancies which are less central for the phenotype may have remained unidentified. Moreover, the actual risk of cancer in the *FH* mutation-positive families has not been determined. To clarify these clinical features of *FH* mutation-associated tumor predisposition, genealogical and cancer data was extensively collected from members of eight Finnish *FH* mutation-positive families, including one FHD family. Altogether 868 individuals were identified. Risk of cancer was determined with the standardized incidence ratio by comparing the number of cancer cases in HLRCC/FHD families with those in the general population. This Finnish Cancer Registry-based analysis revealed increased incidence for RCC (6.5-fold), and for ULMS as defined prior to 2003 (71-fold). In young individuals the risk was even more pronounced. However, many of the ULMSs in the series do not fulfill the most recent and more stringent ULMS diagnosis criteria, but can now be classified as atypical or proliferative ULMs (with a low risk of recurrence). Hence, the risk for ULMS - as defined at present - appears to be lower in HLRCC than originally calculated. The analyses of biallelic inactivation of *FH* in HLRCC/FHD patients' tumors suggested possible association of breast and bladder cancer with the loss of *FH*, though increased incidence of these tumors was not observed in the Cancer Registry risk analysis. In the present study, cancer cases were for the first time detected also in the FHD family.

Typically RCCs in HLRCC are unilateral and display papillary type 2 histology. Occasionally collecting duct carcinomas are also found. A novel type of RCC histology appeared in a young Spanish HLRCC patient case included in this study. The other tumor of this patient's bilateral renal lesions was a conventional (clear cell) type which is distinct from the types previously observed in HLRCC. This discovery suggests *FH* mutation carriers may exist also among young patients with conventional RCC.

Since tumors from *FH* and *SDHB/C/D* mutation carriers display pseudohypoxia associated with stabilized HIF1α (a subunit of HIF1), the recent hypothesis on the link between HIF1/hypoxia and genetic instability was of interest to study in HLRCC and HPGL tumor material. HIF1α stabilization was confirmed with immunohistochemistry to be present in the majority of the studied specimens. However, no repression of MSH2, a protein involved in mismatch repair of DNA, or microsatellite instability (MSI), an indicator of genetic instability, was observed. The findings show that the suggested instability pathway is not activated by pseudohypoxia at least in TCA cycle-deficient tumors.

Recently, a novel *FH* transcript was described in a sequence database. To study putative alternative functions of FH, the variant *FH* transcript (*FHv*) was further characterized. When amplified in full length, *FHv* was found to contain exons 2-10 identical to *FH* and an alternative exon 1b. Thus, FHv lacked the exon 1 encoding the mitochondrial targeting signal. Overexpression analyses showed that localization of FHv was nuclear and cytoplasmic and it was devoid of FH enzyme activity. Moreover, exon 1b was found to be the 5' untranslated region. *FHv* mRNA was ubiquitously expressed in human tissues but at a level hundreds of times lower than *FH*. Subsequently, the endogenous protein could not be reliably confirmed. Interestingly, *FHv* mRNA expression was significantly increased in cell lines treated with hypoxic and heat stress, indicating its possible role in stress-induced apoptosis or survival in unfavorable conditions. However, the function of FHv remains elusive.

1. INTRODUCTION

Any human can contract a cancer or benign neoplasm, but an inherited germline mutation in a certain set of genes can make an individual more susceptible to tumor formation. Thus, a mutant allele in a susceptibility gene causes an increased risk of tumors (benign or malignant). The degree of the risk is influenced by the particular gene and mutation, and other genetic, lifestyle, and environmental factors (Fearon, 1997). The key indicators of tumor heritability include several affected members in a family, young age at tumor onset, tumors arising at multiple sites, and existence of multifocal or bilateral lesions (Greco & Mahon, 2004). Hence, the features of the patient affected by a tumor and the family history are signs that a conscious clinician can utilize in linking the disease to a possible genetic inheritance. To date many susceptibility genes have already been identified, but still some unexplained familial risk of tumor formation exists. The identification of susceptibility genes, and subsequently of the individuals at risk, is an important task considering patient surveillance, possible prevention, and early detection and treatment of a tumor, especially in the case of predisposition to malignancy. Importantly, personal and social pressures followed by knowledge on inherited risk have to be taken into account in predictive testing and genetic counseling (Offit & Thom, 2007).

Although out of all cancer cases only approximately 5-10% is due to an inherited predisposition, the identification of hereditary syndromes has in fact played a significant role in cancer research since the susceptibility genes can be mutated in sporadic tumors as well (Knudson, 2002). However, in order to understand the mechanism by which a mutation in a certain gene leads to tumorigenesis, it is essential to determine the protein product's function and the molecular pathway or pathways where it operates. In general, the tumorigenesis-associated proteins take part in the processes which regulate cell growth, differentiation, or death (Vogelstein & Kinzler, 2004). Finally, knowledge about the function of the "tumor protein" and its interactions with other molecules may provide opportunities to identify possible therapeutic agents.

This work focuses on two rare, recently identified tumor predisposition syndromes, hereditary leiomyomatosis and renal cell cancer (HLRCC) and hereditary paragangliomatosis (HPGL), both of which are characterized by benign and malignant tumors. The susceptibility genes of these syndromes encode proteins of a known function, fumarate hydratase (FH) and succinate dehydrogenase (SDH), respectively, but their contribution to tumorigenesis has not been completely determined. This work has therefore aimed to clarify the molecular pathways in which FH and SDH participate. Since the phenotype of HLRCC is variable, especially related to the penetrance of the malignant tumor types, it was also intended to broaden the clinical definers of the syndrome.

Medical research often requires material from patients, e.g. tumor tissue or blood for DNA extraction. The ethical aspects of human experimentation, including the use of

human tissue material, are regulated by national laws and international declarations such as the Declaration of Helsinki. The main ethical principle in medical research is that the patient's participation in a study is based on an informed decision and consent and that the patient always takes precedence over the interests of science and society. Accordingly, the study presented here was approved by the Helsinki University Central Hospital authorized ethics review committee and all the samples were taken only after obtaining each individual's informed consent or authorization from the National Authority for Medicolegal Affairs.

2. REVIEW OF THE LITERATURE

2.1. Etiology of cancer

2.1.1. Cancer genetics

Cancer includes a variety of different physiological conditions, but the genetic origin of this disease can be used to define its essence. Although many environmental and lifestyle factors as well as viral infections play a role in the genesis of cancer, an aberration in the genetic material is the basal cause or main contributor to the malignant transformation of a cell. The genetic aberrations include changes at the chromosomal level (translocations, whole chromosome arm or chromosome amplifications or deletions), and at the nucleotide level (major and minor amplifications and deletions, and nucleotide substitutions) (Jones & Baylin, 2007). Moreover, epigenetic changes which do not alter the actual DNA sequence can modify DNA. These changes include abnormal chromatin remodeling or methylation, including gene activation by promoter hypomethylation and inactivation by hypermethylation (Feinberg, 2007).

The genes, which in mutated form contribute to tumorigenesis, can basically be grouped by type; oncogenes, tumor suppressor genes (TSG), and DNA repair genes. Mutations in oncogenes usually result in the hyperactivation of the gene or activation of it in abnormal conditions. The oncogenes with their normal activity are also called as proto-oncogenes. In contrast to the gain of function of oncogenes, TSGs lose their function when mutated. Usually the function of TSG is lost only when both the alleles are inactivated (Vogelstein & Kinzler, 2004). However, the tumorigenic effect in the case of haploinsufficient TSGs is attained already by the inactivation of the other allele only (Cook & McCaw, 2000). The DNA repair genes, also termed caretakers, include genes encoding proteins that take care of mismatch repair (MMR), nucleotide excision repair (NER), and base-excision repair (BER). In addition to these subtle error repairing processes, control mechanisms attending to a larger DNA material, for example in the mitotic recombination and chromosomal segregation, can be repressed in cancer cells (Vogelstein & Kinzler, 2004, see also 2.1.2.3). The nature of repair genes is similar to the classic TSGs as both the alleles of these genes usually have to be inactivated to have a physiologic effect, even if some functional defects in the repair system have also been detected in non-neoplastic cells of heterozygous MMR mutation carriers (Alazzouzi et al., 2005).

At present, the number of genes involved in tumorigenesis is estimated at approximately one percent of all human genes (Futreal, 2004). A tumorigenesis associated genetic alteration may occur in a somatic cell or can be present in each cell of an individual due to an inherited germline mutation; the latter resulting in a hereditable tumor predisposition. To date, approximately 30 different high-penetrance genes are linked to hereditary cancer, of which a few are oncogenes and DNA repair genes, but most of them are TSGs. Often, but not always, the same genes are found to underlie the genesis

of corresponding sporadic tumors as well (Knudson, 2002). An explanation for familial clustering of tumors cannot, however always be found from the known genes. The unexplained familial risk can be partly understood by the as yet unidentified high-penetrance (high-risk) cancer genes, but evidence on the association of low-penetrance (low-risk) susceptibility allele variants in cancer predisposition has increasingly emerged (Houlston & Peto, 2004).

2.1.2. Cancer pathways

Depending on the tumor type, accumulation of a variable number of genetic alterations in the cancer genes finally leads to the malignant transformation of a cell and the formation of cancer. The process may occur in the cells of normal tissue but cancer can also generate from a pre-existing benign lesion. The capabilities that a transforming cell attains from the mutations may be summarized as the six hallmarks of cancer, as introduced by Hanahan and Weinberg: 1) limitless replicative potential, 2) self-sufficiency in growth signals, 3) insensitivity to anti-growth factors, 4) sustained angiogenesis, 5) evading apoptosis, and 6) capability of tissue invasion and metastasis (Hanahan & Weinberg, 2000). Genetic instability has been considered as one of the common hallmarks of cancer cells as well, though the requirement of increased mutation rate to acquire the sufficient number of pathogenic mutations for cancer formation has not been established (Tomlinson et al., 1996). Although the number of pathways associated with these characteristics is limited, they are complexly regulated and thus the number of genes as mutational targets is high. In the following sections some of the pathways and some of the key players in the cellular events related to cancer are presented.

2.1.2.1. Examples of aberrant signaling pathways in cancer

RB PATHWAY: Stated simply, activation of oncogenes and inactivation of TSGs stimulates the cell birth (cell cycle) and maintenance, or inhibit the cell cycle arrest and apoptosis. A well-known tumor suppressor, RB (retinoblastoma protein), operates in a number of processes that are linked to cancer including the cell cycle. Indeed, one of the main pathways of cell cycle regulation involved in cancer is the $p16^{INK4a}$ - cyclinD-CDK4 - RB pathway. In the cell cycle, the regulation of the transition from the resting stage (G0 or G1) to the synthesis (S) phase is particularly important. In the "RB pathway", RB acts as a transcriptional inhibitor of genes that are required for DNA synthesis. RB function can, however, be disrupted for example by mutational inactivation or phosphorylation by cyclinD-dependent kinases. Cyclin-dependent kinases (CDK) are mediators of cell cycle transitions along with their regulatory cyclin subunits (including types D and E). CyclinD-dependent kinases normally act only in response to mitogens, but when over-expressed (e.g. through amplification) they abnormally repress RB's growth-inhibiting function leading to uncontrolled enter to active cell cycle and increased proliferation. Moreover, loss of $p16^{INK4a}$, an inhibitor of cyclinD-dependent kinases, leads to a similar RB repressive effect (Sherr, 2000, Classon & Harlow, 2002).

In addition to the ability to block the cell cycle S-phase, RB has a variety of other functions. It is believed to regulate differentiation, responses to DNA damage and provide protection against apoptosis. The tumor suppressing mechanisms of RB are not fully characterized but alterations in these functions may also be involved in tumorigenesis (Classon & Harlow, 2002). The importance of RB emerged already in 1971, when the comparison studies on the kinetics of retinoblastoma eye tumor formation in familial and sporadic cases established the basis for the requirement of biallelic inactivation of TSGs. (Knudson, 1971). Moreover, *RB* was the first cloned TSG. At present it is estimated that almost all human cancers display defective RB function, either caused by *RB* mutations, blockade of RB by certain viral proteins, or alterations in its upstream regulators (Weinberg, 1995).

p53 PATHWAY: To maintain genetic integrity, mutations in the DNA are aimed to be corrected by the cellular repair mechanisms, and if a cell has undergone irremediable errors, it is normally programmed to die. These processes are regulated by another important tumor suppressor, p53. When a cell is under normal physiological conditions, p53 seems to have very little implication, but when disturbed by the abnormal activation of oncogenes, DNA damage, chromosomal aberrations, and hypoxia among others, p53 becomes active. The p53 response, i.e. triggering pathways that take care of the damage or eliminate the faulty cell, prevents the expansion of cells with a malignant potential (Oren, 2003). The regulatory effects of normal p53 comprise the transcriptional activation of its target genes and inactivation of antiapoptotic genes, as well as of other, transcription-independent activities (Oren, 2003). For example, p53 operates in a cell cycle regulatory pathway with ARF and MDM2. The tumor suppressor *ARF* (*$p14^{ARF}$*), which is able to recruit the growth arrest- or apoptosis-inducing p53, operates in the downstream of RB. ARF is activated by abnormal mitogenic signals, such as over-expression of oncoproteins c-MYC, RAS, and E2F1. The illegitimate signaling cascades are blocked by ARF as it antagonizes MDM2, the negative regulator of p53. Hence, this pathway may be disrupted by inactivating mutations in *p53* and *ARF*, or aberrant activation of *MDM2* (Sherr, 2000).

Naturally, several other factors also play a role in the cell proliferation and apoptosis signaling system. For example, members of the B-cell lymphoma 2 family (BCL2) (e.g. the antiapoptotic BCL2 and one of the target genes of p53, the proapoptotic BAX) under stress conditions "decide" whether to initiate the apoptosis program (e.g. activation of caspases) or not (Cerroni & Kerl et al., 1994, Yin et a., 1997, Adams, 2003). *p53* itself is somatically mutated in approximately half of all human cancers and many more display another genetic alteration leading to the repression of p53 mediated pathways (Royds & Iacopetta, 2006). However, it is notable that in certain mutant forms, p53 not only loses its tumor suppressing capabilities, but has also been shown to gain some oncogenic properties (Kastan & Berkovich, 2007). Inherited mutations in *p53* cause LI-FRAUMENI syndrome which is characterized by a large spectrum of early-onset tumors (Royds & Iacopetta, 2006).

RTK (AKT-mTOR) PATHWAY: Mediating growth-promoting extracellular signals to the cellular machineries is one event linked to cancer. Receptor tyrosine kinases (RTKs) are type I transmembrane proteins, of which cytoplasmic domain's catalytic activity is switched on upon a ligand binding (Hunter, 2000). For example, this signaling cascade can be abnormally activated by mutations in oncogenes encoding RTKs, such as *KIT*, *c-MET*, *RET*, and *ERBB2* in gastrointestinal stromal tumors (GIST), kidney, thyroid, and breast and ovarian cancer, respectively (Vogelstein & Kinzler, 2004). RTK activation by a growth factor or by an activating mutation leads to induction of downstream signal-transduction elements such as the oncoproteins GTPase RAS and the lipid kinase phospoinositide 3-kinase (PI3K). Subsequently, the activating signal is mediated, for example, to the oncogenic mitogen-activated protein kinase (MAPK) or AKT (PKB) family of threonine/serine kinases. In close association with the AKT (and also MAPK) pathway is the threonine/serine kinase mTOR (mammalian target of rapamycin) pathway which is negatively regulated by the complex of the tumor suppressors tuberin (*TSC2*) and hamartin (*TSC1*). Active AKT in turn operates as an inhibitor of TSC2 (Shaw & Cantley, 2006). Hence, in addition to the mutational activation of RTKs, also inactivating mutations in *TSC1*, *TSC2*, and PTEN (the antagonist of PI3K function), or an activating mutation in *RAS* leads to hyperactivation of the AKT-mTOR pathway. *TSC1*, *TSC2*, and *PTEN*, among some other genes, are linked e.g. to a group of familial neuro-cutaneous disorders (phakomatoses) (see also 2.2.2). In general, the AKT-mTOR pathway is associated with increased growth, resistance to apoptosis, and cellular metabolism (Hay, 2005).

TGF-β PATHWAY: Signal transduction through the cell membrane is mediated also by transmembrane threonine/serine kinases, such as transforming growth factor β (TGF-β) receptors 1 and 2 (TGF-βR1, TGF-βR2), which are activated by TGF-β cytokines. These signals activate SMAD proteins which are subsequently translocated to the nucleus leading to activation and repression of hundreds of genes related to diverse cellular processes such as proliferation, invasion, metastasis, apoptosis, immune surveillance, and angiogenesis (Kang at al., 2003, Shi & Massague, 2003, Jakolew, 2006). Because TGF-β generally has a negative effect on cell growth, repression of the TGF-β - SMAD pathway contributes to tumorigenesis (Shi & Massague, 2003). For example, inactivation of *TGF-βR1*, *TGF-βR2* and *SMAD4* has been associated with colorectal and ovarian cancer as well as hereditary JUVENILE POLYPOSIS (JP), respectively (Jakolew, 2006).

WNT PATHWAY: Wnt signaling cascade promotes the expression of genes affecting, for example, proliferation, differentiation, and invasion, such as *c-MYC*, *cyclinD1* (*CCND1*), and *MMP7*, respectively. The Wnt pathway is especially stimulated in colorectal cancer (CRC) for example by activating mutations in β-catenin which after association with the T-cell factor/lymphoid enhancing factor (TCF/LEF) operates as the transcription activator of the above mentioned target genes. However, the majority of sporadic CRCs as well as patients with FAMILIAL ADENOMATOUS POLYPOSIS (FAP) harbor inactivating mutations in the tumor suppressor *adenomatous polyposis coli* (*APC*) which is part of the β-catenin degradation complex. *APC* mutations have been suggested to cause chromosomal instability (CIN) as well (Giles et al., 2003).

2.1.2.2. Hypoxia-inducible factor 1 (HIF1)

Some cancer-associated proteins take part in several pathways and create a cross-talk between them. It appears that many TSG and oncogene pathways are linked directly or indirectly to the HIF1 pathway (Vogelstein & Kinzler, 2004). The hypoxia-inducible factor 1 (HIF1) is the master regulator of oxygen homeostasis and it has been shown to drive transcription of up to 100 cell type-specific target genes (Semenza, 2003, Kim & Kaelin, 2006). The HIF1 heterodimer is comprised of HIF1β (ARNT) and HIF1α subunits, the latter one being highly regulated. The synthesis of HIF1α is induced mainly by growth factor signals through PI3K and MAPK pathways (Fukuda et al., 2002) (Table 1). In normoxic conditions, the HIF1 function is, however, inhibited by targeting HIF1α protein to degradation. Oxygen-dependent prolyl hydroxylase-domain proteins (PHD1/EGLN2, PHD2/EGLN1, and PHD3/EGLN3) hydroxylate Pro-402 and Pro-564 of HIF1α for von Hippel Lindau protein (pVHL) recognition. pVHL is a component of the E3 ubiquitin-protein ligase complex along with elongin B, elongin C, Cullin 2, and Rbx1. Ubiquitylated HIF1α is subsequently degraded by the proteasome (Maxwell et al., 1999). HIF1α is also regulated in an oxygen-dependent manner by factor-inhibiting HIF1 (FIH1), which prevents HIF1α interaction with its co-activators p300 and CBP (Mahon et al., 2001, Hewitson et al., 2002, Lando et al., 2002a & 2002b). As HIF1 responds to lowered oxygen (by becoming stabilized and associated with its co-activators), the genes that are activated through HIF1 binding to the target sequence (hypoxia response element, HRE) encode most of all angiogenic factors (e.g. vascular endothelial growth factor, VEGF), glucose transporters (e.g. glucose transporter 1, GLUT1), glycolytic enzymes (e.g. lactate dehydrogenase A, LDHA), survival factors (e.g. transforming growth factor α, TGFα), and invasion factors (e.g. matrix metalloproteinase 2, MMP2), among others (Semenza, 2003).

Another oxygen homeostasis regulating protein, controlled by PHDs and pVHL-mediated degradation, is HIF2α (encoded by *EPAS1*). HIF2α dimerizes also with HIF1β to drive transcription of a distinct set (overlapping partly with HIF1α) of genes, thus having somewhat differing effects to HIF1α (Semenza, 2003). The function of the third HIFα subunit, HIF3α, is less studied. A splice variant of HIF3α encodes an inhibitory PAS domain protein (IPAS), the expression of which is regulated by HIF1α providing a negative regulatory feedback for HIF1α (Makino et al., 2007).

As a maintainer of the oxygen homeostasis, HIFs, especially HIF1α, are highly expressed in many human cancers due to the requirement for oxygenation of an increased number of cells within a tumor. Moreover, underlying genetic alterations have been observed to be in association with abnormal expression or degradation of HIF1α (Table 1). For example, an activating alteration in the AKT pathway can lead to enhanced translation of HIF1α (Zhong et al., 2000). AKT induces mTOR which regulates the assembly of translation initiation complexes, and especially translation of growth regulators, such as HIF1α (Shaw & Cantley, 2006). The transcriptional activity of HIF1 has also been shown to be regulated through AKT-mTOR pathway. Activated AKT inhibits the function of TSC1/TSC2 complex enabling positive signal transduction to mTOR/Raptor complex

which interacts with HIF1α. This interaction enables the binding of HIF1α to its co-activators CBP and p300 (Land & Tee, 2007).

However, the most marked genetic alteration causing overacive HIF1α (and HIF2α) signaling is the loss of tumor suppressor *VHL* in clear cell renal cancers. The disease-associated mutations in *VHL* disable its association with HIF1α or with the elongins of the ubiquitin complex. When the ubiquitylation and degradation of HIF1α in normoxia is failed, HIF1 and consequently its target genes become abnormally activated (Maxwell et al., 1999, Zhong et al., 1999, see also 2.2.2.). Evidently, the target genes of HIF1 are factors that can facilitate tumor growth, for example by neovascularization, metabolic adaptation, and apoptosis resistance. Furthermore, HIFα overexpression in tumors is associated with treatment failure and increased mortality in many (but not all) cancer types (Semenza, 2003). However, it is not yet established whether HIF1 activation is involved in the actual transformation process.

Table 1. Examples of increased HIF1α due to genetic alterations (modified from Semenza, 2003)

Alteration	*Mechanism of HIF1α induction*	*Reference*
pVHL loss of function	Decreased degradation	Maxwell et al., 1999
p53 loss of function	Decreased degradation Decreased inhibition of HIF1 transcriptional activity	Ravi et al., 2000, Blagosklonny et al., 1998
FH loss of function	Decreased degradation	Isaacs et al., 2005, Pollard et al., 2005b
SDHB/C/D loss of function	Decreased degradation	Selak et al., 2005, , Pollard et al., 2005b
PI3K-AKT-mTOR signaling*	Increased synthesis Increased transcriptional activity	Zhong et al., 2000, Zundel et al., 2000, Laughner et al., 2001, Fukuda et al., 2002, Land & Tee, 2007
MEK-ERK (MAPK) signaling**	Increased synthesis	Liu et al., 2002, Fukuda et al., 2002 & 2003
SRC gain of function	Increased synthesis	Jiang et al., 1997
ARF loss of function	Decreased inhibition of HIF1 transcriptional activity	Fatyol & Szalay, 2001
ING4 loss of function	Decreased inhibition of HIF1 transcriptional activity	Ozer et al., 2005
CITED4 aberrant cellular distribution	Decreased inhibition of HIF1 transcriptional activity	Fox et al., 2004
BCL2 overexpression	Decreased degradation (?)	Iervolino et al., 2002

*Can be activated for example due to the PTEN loss of function, EGFR signaling (e.g. ERBB2 gain of function), and IGF1R signaling. **Can be activated for example due to increased IGF1R, EGFR and PGE2 signaling.

2.1.2.3. Genetic instability

The breakdown of genetic integrity seems to be a substantial component in tumorigenesis as it advances the capability of cancer cells to acquire useful (e.g. growth-promoting or antiapoptotic) characteristics. These advances may be obtained for example by down-regulating cellular repair mechanisms. Especially typical for many cancer tumors is an abnormal karyotype which is defined with changes in chromosome structure and number. This type of chromosomal instability (CIN) is associated with telomerase and mitotic checkpoint maintenance, though its origin is not completely understood. Moreover, in a central role in preventing chromosomal aberrations are proteins operating in the repair of double-strand DNA breaks occurring in the DNA replication. These repair mechanisms include homologous recombination (HR) and non-homologous end joining (NHEJ). (Jefford & Irminger-Finger, 2006) For example, cells deficient for *BRCA1* and *BRCA2*, which operate in the S-phase checkpoint signaling of cell cycle, display chromosomal instability due to defective HR (Heinen et al., 2002, Chen et al., 2008). Thus, a defective double-strand DNA break repair may explain the increased risk of breast and ovarian cancer in patients with mutated germline alleles of *BRCA1* and *BRCA2* (Heinen et al., 2002).

One of the most commonly known cancer-associated genetic instability pathway is MMR. The primary function of MMR is to eliminate single-base mismatches and insertion-deletion loops occurring during replication. The human MMR system comprises proteins taking care of mismatch recognition, including MSH2 which dimerizes with MSH6 or MSH3 depending on the type of error. A heterodimer of MLH1 and PMS2 coordinates the mismatch recognition complex and the other proteins involved in DNA strand discrimination, excision, and resynthesis (Buermyer et al., 1999, Peltomäki, 2003). Inactivating germline mutations in *MLH1*, *MSH2*, *MSH6*, or *PMS2* underlie HEREDITARY NON-POLYPOSIS COLORECTAL CANCER (HNPCC) (Liu et al., 1996, Nyström-Lahti et al., 1996, Wijnen et al., 1999). Moreover, many sporadic tumors of the HNPCC spectrum (colorectum, endometrium) as well as some other sporadic tumors have been shown to harbor an MMR gene mutation or promoter hypermethylation (Peltomäki, 2003). The consequence of defective MMR is a genome-wide microsatellite instability (MSI) which refers to mismatch errors that occur easily in repeat sequences, such as microsatellites, although also take place in the coding regions of genes (Jefford & Irminger-Finger, 2006). The defective MMR system is likely to contribute to tumorigenesis by failing to repair errors in cancer genes. Recently, tumorigenesis due to dysfunctional MMR has been shown to be associated with defective apoptosis pathways as well (Chao & Lipkin, 2006).

NER and BER are repair mechanisms, which are responsible for detecting and eliminating nucleotides with an altered chemical structure generated for example by a mutagenic agent. For example, inherited mutations in certain genes operating in NER cause XERODERMA PIGMENTOSUM (XP) characterized by a decreased ability to repair DNA mutations caused by ultraviolet exposure, such as sun light. Therefore, the XP patients are susceptible to melanoma and other skin cancers (Norgauer et al., 2003).

As these data indicate, tumorigenesis related to the inactivation of proteins operating in the repair processes are somewhat different from the other cancer-associated pathways since repression of the genes taking care of the DNA stability results in a higher mutation rate in other genes also. However, it is notable that genetic material can be altered with no essential implication on tumorigenesis. Especially MMR-deficient tumors may harbor a large number of so-called passenger mutations which have no effect on tumor growth (Futreal et al., 2004).

2.1.2.4. Link between hypoxia and genetic instability

Recently, some of the repair genes were also linked to the hypoxia/HIF1α pathway. It has long been known that the hypoxic microenvironment is associated with DNA base substitutions, deletions, and amplifications (Rice et al., 1986, Reynolds et al., 1996, Li et al., 2001). Moreover, hypoxia is associated with tumor progression and resistance to therapies (Brizel et al., 1996, Hockel et al., 1996). Theories on the underlying cause of the hypoxia induced genetic instability include the possibility that the cells under hypoxia are more exposed to DNA damage or that the repair systems are hindered (Mihaylova et al., 2003). The latter theory has been justified with the proposal that in hypoxia, only the mechanisms essential for that condition, such as glycolysis, are supported, but, for example, cell proliferation and DNA repair would be down-regulated (Koshiji et al., 2005).

Lately, several studies aiming to determine the pathways related to the hypoxia-induced instability have been assessed. Evidence for and against the suppression of NER in hypoxia has been presented, thus its implication in hypoxia-associated instability remains to be clarified (Yuan, 2000, Bindra et al., 2004 & 2005a). The role of double-strand break repair has also been a matter of discussion (Huang et al., 2006). Currently it seems that genes operating in homologous recombination (HR), including *RAD51* and *BRCA1,* are down-regulated in hypoxia and functional evidence on a reduced capacity to perform HR has also been given (Bindra et al., 2004, 2005a & 2005b, Meng et al., 2005, Huang et al., 2006).

At present there is compatible evidence from studies on hypoxia treated cell lines showing defects in the MMR genes *MLH1* and *MSH2* (Mihaylova et al., 2003, Koshiji et al., 2005, Bindra & Glazer, 2007a). As a functional consequence of defective MMR in hypoxic cells has been observed mutations in a reporter construct as well as MSI in an endogenous microsatellite locus (Mihaylova et al., 2003, Koshiji et al., 2005). In one study, in addition to hypoxia, hypoglycemia resulted in the repression of MSH2, which was suggested to lead to an increased mutation rate of the oncogene *k-RAS* (Shahrzad et al., 2005). However, the mechanism by which MMR is repressed in hypoxia has been under debate. Koshiji and co-workers have suggested the hypoxic effect is mediated by HIF1α, which displaces the transcriptional activator c-MYC from the MMR gene (*MSH2*) promoter (Koshiji et al., 2005). In contrast, Mihaylova and co-workers, and Bindra and Glazer have shown down-regulation of MMR also in HIF1α-deficient cells, suggesting a

HIF1α-independent mechanism (Mihaylova et al., 2003, Bindra & Glazer, 2007a). The hypoxia-induced, HIF1α-independent regulatory system was based on the replacement of the activating c-MYC-MAX on the *MLH1/MSH2* promoter by the repressive MAD1-MAX and MNT-MAX complexes (Bindra & Glazer, 2007a). Moreover, although the HR gene *BRCA1* expression was originally reported to be regulated by HIF1α (Koshiji et al., 2004), recent evidence supports *BRCA1* and *RAD51* rather to be regulated by the repressive E2F transcription factor (Meng et al., 2005, Bindra et al., 2005a, Bindra & Glazer, 2007b).

2.1.3. MicroRNAs

The importance of small RNA molecules in cancer formation only started to emerge a few years ago. MicroRNAs (miRNAs) are a large class of short non-coding RNAs that are present in plants, animals, and DNA viruses. miRNAs are transcribed from genes as long primary transcripts that are processed enzymatically to a single-stranded RNA of ~22 nucleotides in length. At present, approximately 500 human miRNA genes have been identified. The function of miRNAs is not fully understood but they have a role in growth, development, homeostasis, and disease. miRNAs have been shown to regulate also several cancer-associated genes controlling cell cycle, apoptosis, cell migration and angiogenesis. The mechanism by which miRNAs negatively regulate gene expression is suggested to result from the pairing of miRNA to its target sequence, which in turn causes the mRNA degradation or inhibition of translation of the target gene (Blenkiron & Miska, 2007).

The first evidence of a link between miRNA and human cancer came from the observation that two miRNA genes, *mir-15* and *mir-16*, located on the chromosomal region that was commonly deleted in chronic lymphocytic leukemia (CLL). Moreover, expression of these miRNAs was often reduced in CLL (Calin et al., 2002). Subsequently, several other cancer-associated miRNAs have been identified, also by utilizing miRNA microarray systems (Lu et al., 2005). It is now emerging that a certain common set of miRNAs is deregulated in many different tumor types. Since both TSGs and oncogenes have been shown to be targets of miRNAs, miRNAs can have oncogenic or TSG properties. Known targets of miRNA regulation include tumor suppressors *RB* and *TGFβ-R2*, and oncogenes *BCL2* and *RAS*. *mir-17-92* polycistron, named as "OncomiR-1", is one miRNA with a highly established oncogenic potential (Blenkiron & Miska, 2007). Interestingly, some miRNAs, such as miR-21, seem to act as a TSG or oncogene depending on the cell type (Meng et al., 2006, Si et al., 2007). The type of control by which miRNAs are regulated is not completely understood. The transcription of OncomiR-1 seems to be directly regulated by the oncogene c-MYC, but with other miRNAs, regulation by histone acetylation and DNA methylation as well as post-transcriptional mechanisms has been proposed (Pasquinelli et al., 2000, Calin & Croce, 2006, O'Donnel et al., 2005, Saito et al., 2006). Recently, p53 was shown to control the expression of specific miRNAs (Raver-Shapira & Oren, 2007). As a potential therapeutics method against the oncogenic miRNAs has been tested cell-permeable 2-*O*-methyl

oligoribonucleotides, “antagomirs” which have already given promising results in mouse studies (Krützfeldt et al., 2005).

2.1.4. Cancer stem cells

In a multicellular organism, embryonic development is based on the structured division, differentiation, placing, and elimination of stem cells. Stem cells have also a role in adult tissues in the maintenance of cell homeostasis during the continuous turnover of cells and in the case of injury (Bapat, 2007). A normal stem cell is characterized by its self-renewal capacity that is maintained by symmetric and asymmetric cell divisions. In symmetric cell division both daughter cells retain the same phenotype and in asymmetric division a stem and a more differentiated daughter cell are produced. Stem cells are typically multipotent, i.e. capable of differentiating into different cell phenotypes (Morrison & Kimble, 2006).

Most tumors are comprised of a heterogenous population of cancer cells which is seen, for example, as variability in the stages of differentiation and tumorigenic (clonogenic) potential in *in vivo* and *in vitro* assays. The differences between cells within a tumor can in part be explained by environmental differences as well as by continuing mutagenesis. However, observations on this variability in the characteristcs of neoplastic cells within a tumor led a few years ago to the hypothesis that tumors could bear a small, specific population of cancer cells with similar capabilities to stem cells. These cancer stem cells (CSC) were hypothesized to possibly represent the tumorigenic population and to be responsible for the actual tumor cell mass expansion (Reya et al., 2001). The idea of CSCs has been subsequently supported by the fact that many pathways involved in the maintenance of normal stem cells are often altered in cancer, including Wnt, β-catenin, PTEN, TGF-β, Hedgehog, Notch and Bmi-1 regulated pathways. Moreover, stem cells with ability to unlimited cell divisions could be considered as prone targets for transforming events (Polyak & Hahn, 2005).

The present definition of a CSC as proposed by the American Association of Cancer Research (AACR) workshop on cancer stem cells (2006) is “a small subset of cancer cells within a cancer that constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and to cause heterogenous lineages of cancer cells that comprise a tumor” (Clarke et al., 2006). However, the central dilemma yet to be untangled is how the combination of stemness and tumorigenic potential is acquired. The tumors may derive from stem cells with self-renewal properties which subsequently acquire epigenetic and genetic changes essential for tumorigenity, or alternatively, the tumor stem cells could be proliferative progenitors that acquire the self-renewal capacity (Clarke et al., 2006). The term stem cell has also been considered misleading as the quantity of the stemness is somewhat questionable (Hill & Perris, 2007). Recently, also the names tumor-initiating cell and cancer-initiating cell have been used to describe CSCs (Phillips et al., 2006a, Neuzil et al., 2007). Importantly, the identification of CSCs has led to the possibility to rethink the way of treating tumors. As conventional therapies concentrate on targeting the rapidly dividing cells, the stem cells putatively refractory to

those approaches will probably require completely different methods (Clarke et al., 2006). However, if the CSCs are the only population with a tumorigenic potential within a tumor, the elimination of only them would lead to tumor degeneration and the restriction of disease recurrence (Reya et al., 2001).

2.2. Hereditary renal cancer

Cancer of the kidney accounts for two percent of total human cancers. Approximately 90% of all malignancies of the kidney are renal cell carcinomas (RCC), tumors arising from the epithelium of the renal tubules. Overall RCC is the twelfth most common cancer in men and seventeenth most common in women. Smoking and obesity are the major lifestyle contributors to kidney cancer formation. According to the morphological features of the tumors, RCCs can be classified into subtypes including conventional (clear cell), papillary (type 1 and 2), chromophobe, and collecting duct carcinoma. In addition, benign tumors such as oncocytoma arise from the renal epithelium (WHO Classification of Tumors 2004; Tumours of the Urinary System and Male Genital Organs, Cohen & McGovern, 2005). The staging of renal cancers is based on the TNM system (I-IV) which includes evaluation of tumor size and site, spread to regional lymph nodes, and metastasis to other organs. This staging, as well as the four-tiered Fuhrman classification based on the size and regularity of the nuclei and prominence of the nucleoli, are utilized in the prognosis of the disease. A quarter of the RCC patients present a locally invasive or metastatic disease, and the aggressive growth is associated especially with the clear cell and collecting duct tumors (Reuter, 2006). As in the case of many other tumors, increased risk of renal neoplasia can be inherited. A clear hereditary predisposition to kidney tumors, RCC or other renal lesions underlies less than four percent of the cases. As typical for genetic predisposition, the inherited renal neoplasms are often multifocal and bilateral, and are diagnosed at an earlier age than in general (WHO Classification of Tumors 2004; Tumours of the Urinary System and Male Genital Organs). The genesis of sporadic kidney cancer seems not to be affected by hereditable factors (Lichtenstein et al., 2000).

2.2.1. Hereditary leiomyomatosis and renal cell cancer (HLRCC)

2.2.1.1. Clinical features of HLRCC

HEREDITARY LEIOMYOMATOSIS AND RENAL CELL CANCER (HLRCC, OMIM 605839) is a dominantly inherited tumor predisposition syndrome which was originally discovered by a clinical geneticist through the distinct phenotype clustering in one Finnish family. The clinical manifestations in the family included cutaneous (CLM) and uterine leiomyomas (ULM), renal cell cancer (RCC), and uterine leiomyosarcoma (ULMS) (Launonen et al., 2001). Later on, heterozygous germline mutations in the *fumarate hydratase* (*FH*) gene (located on chromosome 1q43) were found to underlie this syndrome (Tomlinson et al., 2002). To date, more than 130 HLRCC families have been identified throughout the world. Mostly the families are from North America, the UK, and Finland

but a few families have been detected outside North America and Europe, for example in Tunisia and Australia (Tomlinson et al., 2002, Kiuru et al., 2002, Alam et al, 2003, Toro et al., 2003, Martinez-Mir et al., 2003, Alam et al., 2005a, Chan et al., 2005, Chuang et al., 2005, Kim, 2005, Varol et al., 2006, Badeloe et al., 2006, Chuang et al., 2006, Wei et al., 2006, Refae et al., 2007). The population prevalence of allelic variants of *FH* is not known (Alam et al., 2005a).

Leiomyomatosis is the most prominent phenotype of HLRCC, and CLMs and ULMs are seen in all of the HLRCC families. Related to the reduced penetrance of the malignant tumor types in association with the *FH* germline mutations, the initial designation for the syndrome, MULTIPLE CUTANEOUS AND UTERINE LEIOMYOMATOSIS (MCL, OMIM 150800), is sometimes used alongside HLRCC. Leiomyomas are benign smooth muscle tumors. The cutaneous tumors are thought to originate from the pilo arrector muscles of the hair follicle (Alam et al., 2005b). The number and size of the skin lesions in HLRCC patients vary from a single minor tumor to hundreds of lesions which may be many centimeters in diameter. The lesions can be painful and sensitive to temperature changes and pressure. The penetrance of the skin leiomyomas in HLRCC is very high. In a study by Alam and co-workers, all the male carriers were affected with CLM by the age of 35, and 76% of the female carriers by the age of 45 years (Alam et al., 2005b). Thus, existence of multiple CLMs is suggested to be a strong indicator of an underlying *FH* mutation.

ULMs (fibroids) can develop anywhere in the myometrium. Most commonly they locate within the myometrial wall (intramural ULM), but also close to the endometrium (submucosal) or serosa (subserosal). Occasionally they can be found in the cervix, broad ligament, and ovary. In general, hormonal factors and ethnic background influence the formation of these tumors (Robboy et al., 2000). Approximately 80% of female *FH* mutation carriers are affected by ULMs by the age of 45 (Alam et al., 2005b). According to serial hysterectomy specimens, up to 77% of females in the general population seem also to be affected by ULMs but clinically they are apparent only in approximately 25% of females because the tumors can be asymptomatic (Cramer & Patel, 1990). However, when causing symptoms, i.e. menorrhagia, pain, and reproductive problems, ULMs can be a significant health issue. In contrast to the cases in the general population, association of gynecological symptoms in HLRCC patients has been reported in up to 90% of the cases. Moreover, hysterectomy on *FH* mutation carriers is commonly performed already before the age of 30 years, whereas in the general population hysterectomies consequent on ULMs are usually conducted on females who are over 40 years of age. The early onset and severity of the clinical symptoms have also effect on family planning decisions (Toro et al., 2003, Alam et al., 2005b, Hodge & Morton, 2007).

ULMS is the malignant counterpart of ULM and comprises 1% of all uterine malignancies. ULMSs localized to the uterus cause similar symptoms as ULMs, though rapid increase in size of the uterus may be indicative for ULMS (WHO Classification of Tumors 2003; Tumors of the Breast and Female Genital Organs). ULMSs usually arise *de novo* and only rarely appear to form from pre-existing ULM (Hart, 2002). These tumors are highly malignant and in most cases they recur within two years from the primary

diagnosis and tumor removal (WHO Classification of Tumors 2003; Tumors of the Breast and Female Genital Organs). Altogether six cases of exceptionally early-onset ULMS have been reported to date in the context of *FH* mutation, all of which in the Finnish population (Launonen et al., 2001, Kiuru et al., 2005, Ylisaukko-oja et al., 2007a). Some of the other features of ULMS and challenges in the differential diagnosis between ULM and ULMS will be discussed in section 6.2.2.

The RCCs associated with HLRCC mainly display features of papillary type 2 histology, large cells with abundant eosinophilic cytoplasm, large nuclei, and prominent inclusion-like eosinophilic nucleoli. Moreover, the tumors are typically solid, unilateral, and very aggressive already as small lesions (Merino et al., 2007). Altogether, approximately a fifth of HLRCC families have cases of RCC, most of which, however, are in Finland and North America. The prevalence of HLRCC-associated RCC is generally speaking very low as to date only 56 cases have been reported world-wide (Launonen et al., 2001, Tomlinson et al., 2002, Alam et al, 2003, Toro et al., 2003, Alam et al., 2005b, Kiuru et al., 2005, Chan et al., 2005, Badeloe et al., 2006, Chuang et al., 2005 & 2006, Wei et al., 2006, Refae et al., 2007). As common in hereditary tumor predisposition, the age of RCC onset in HLRCC is younger than on average with the median diagnosis age of 44 years (Pithupakorn & Toro, 2006; http://www.geneclinics.org). The youngest RCC case that has been reported in HLRCC was 16 years of age (Alam et al., 2005b).

2.2.1.2. Fumarate hydratase (FH)

Mitochondria are cell organelles formed of matrix and inner and outer membrane. These organelles have a central role in energy (adenosine triphosphate, ATP) production since the oxygen-dependent tricarboxylic acid cycle (TCA cycle) takes place in the mitochondrial matrix. In addition, mitochondria are involved in a range of other processes, such as cell signaling, cellular differentiation, apoptosis, and control of the cell cycle and cell growth (McBride et al., 2006). A mitochondrion has its own genome in a circular DNA, but many mitochondrial proteins are encoded by nuclear genes (Anderson et al., 1981).

FH, the predisposing gene of HLRCC, encodes an enzyme of the mitochondrial TCA cycle (see also 2.4.). The human *FH* gene is located on the chromosomal region 1q43, and it generates a transcript of 1.5 kb. *FH* consists of 10 exons of which the first encodes a localization signal that targets FH to mitochondria (Tolley & Graig, 1975, Edwards & Hopkinson, 1979a & 1979b, Kinsella & Doonan, 1986). At least in the case of yeast fumarate hydratase (Fum1), it seems that the protein import to mitochondria is not post-translational but exceptionally the translocation and translation of the protein is physically connected (Karniely et al., 2006, Yogev et al., 2007). The active form of FH is a tetramer and is comprised of four identical subunits. FH is also found in the cytosol, where it takes part in the urea cycle. The mitochondrial and cytosolic forms are encoded by the same gene but the mechanism for the dual distribution of human FH has not been defined. In the yeast a part of the protein is translocated back to the cytosol from the mitochondrial outer membrane after the removal of the mitochondrial localization signal

(Stein et al., 1994, Sass et al., 2001 & 2003). One suggestion related to the retrograde movement involves the rapid folding of Fum1 into an import-incompetent stage, which compels the processed protein back to the cytosol (Sass et al., 2003). On the other hand, the retrograde movement of both the human and yeast fumarase might result from the poor mitochondrial targeting sequence (Singh & Gupta, 2006).

The mutation spectrum of *FH* includes 89 different *FH* mutations identified to date. Of these changes, the majority are missense (>50%) or nonsense (~10%) mutations, and frameshift (~20%) mutations caused by either small insertions or deletions. A few splice site mutations and inframe deletions or insertions of one to two amino acids, one exon 7 duplication, and two whole gene deletions have also been found. The mutations lie throughout the gene, an exception being exon 1 (Tomlinson et al., 2002, Kiuru et al., 2002, Alam et al, 2003, Toro et al., 2003, Martinez-Mir et al., 2003, Alam et al., 2005c, Chan et al., 2005, Chuang et al., 2005, Kim, 2005, Varol et al., 2006, Badeloe et al., 2006, Chuang et al., 2006, Wei et al., 2006, Refae et al., 2007, Campione et al., 2007, Ylisaukko-oja, 2007). Since the malignant tumors of HLRCC have been observed only in certain populations and families, attempts have been made to determine the influence of the mutation type and site to the phenotype, yet no clear correlation has been found. *FH* is considered to be a TSG as the majority of the associated tumors are found with biallelic inactivation of *FH*. Mostly the tumors display loss of the wild-type allele, but point mutations as second hits have also been observed (Launonen et al., 2001, Kiuru et al., 2002).

FH DEFICIENCY (FHD, MIM 606812), is an autosomal recessive condition caused by homozygous (or compound heterozygous) germline mutations in the *FH* gene and was originally described in 1986 by Zinn and co-workers (Zinn et al., 1986). The clinical outcome of the patients includes psychomotor retardation, muscular hypotonia, microcephaly, and occasionally also seizures and dysmorphic facial fetures (Kerrigan et al., 2000, Deschauer et al., 2006). Fumarate excretion in the urine is one clear indicator of FHD. Patients of FHD usually die at a few months of age but some survive nearly their first decade (Bourgeron et al., 1994). FH enzyme activity in the heterozygous parents' tissues is around 50% and in the patients' very low, varying from 0.2% to 23% compared to control samples (Zinn et al., 1986, Petrova-Benedict et al., 1987, Gellera et al., 1990, Remes et al., 1992, Bourgeron et al., 1994, Deschauer et al., 2006). Due to the FH defect, fumarate has been shown to accumulate in cells and inhibit SDH, aspartase, adenosuccinate lyase, and arginosuccinase. Hence, inhibition of many other enzymatic pathways in addition to the oxidative phosphorylation has been suggested as having an important role in the pathophysiology of FHD (Deschauer et al., 2006). Altogether 18 distinct *FH* mutations have been reported in FHD families (Gellera et al., 1990, Bourgeron et al., 1994, Rustin et al., 1997, Coughlin et al., 1998, Alam et al., 2003, Remes et al., 2004, Loeffen et al., 2005, Pollard et al., 2005b, Maradin et al., 2006, Phillips et al., 2006b, Zeng et al., 2006, Deschauer et al., 2006, Ylisaukko-oja, 2007). Of these 18 mutations six are associated also with HLRCC (tumorigenesis of leiomyomas, RCC or ovarian cystadenoma). They are located at four different exons and include five point mutations (R58X, K187R, R190H, R190C and H275Y) and one amino acid insertion (435insK) (Toro et al., 2003, Wei et al., 2006, Ylisaukko-oja et al., 2006b). The *FH*

mutations in FHD are usually of the missense type, and it has been postulated that the biallelic truncating mutations are likely to be embryonic lethal (Tomlinson et al., 2002, Alam et al., 2003). Since the mutation spectrum overlaps with FHD and HLRCC, it is surprising that only a couple of CLM cases have been reported in heterozygous *FH* mutation carriers in FHD families (Tomlinson et al., 2002, Maradin et al., 2006).

2.2.2. Other hereditary forms of renal cancer

The most common inherited RCC syndrome is the VON HIPPEL LINDAU disease. The syndrome is associated with hemangioblastomas of the central nervous system and retina, RCC, renal cysts, pheochromocytoma (PHEO, see also 2.3), as well as with some other benign and malignant tumors. RCC is found in 24-45% of VHL cases. Of the four phenotypic subclasses of VHL (1, 2A, 2B, 2C), RCC is present in type 1, type 2B, and sometimes in 2A. The tumors display clear cell histology and are often bilateral and multifocal (Lonser et al., 2003). This autosomal dominant disease is caused by mutations in the tumor suppressor *VHL*. *VHL* is commonly associated with the genesis of sporadic RCCs as well, since LOH of 3p (location of *VHL*) has been observed in 95% of sporadic clear cell tumors. Inactivation of the other allele by mutation or hypermethylation is seen in over half of the sporadic cases (Gnarra et al., 1994). Since pVHL acts as a negative regulator of HIFα proteins (HIF1α and HIF2α), inactive pVHL allows abnormal activation of the HIF pathways (Seitzinger et al., 1988, Latif et al., 1993, Maxwell et al., 1999, see also 2.1.2.2.). Of note, HIF activation takes place already in the early lesions (dysplastic and cystic lesions) in the VHL patients' kidneys so that HIF1α accumulation is switched to HIF2α accumulation when the degree of dysplasia increases. This would suggest that HIF2α has a more oncogenic role in the setting of VHL-defective RCC. The additional genetic changes probably required for the transformation of the preneoplastic cysts have, however, not yet been determined (Mandriota et al., 2002, Kim & Kaelin, 2006). Since not all types of *VHL* mutations cause RCC (as in the case of type 2C VHL with PHEOs only) and lead to activation of HIF, pVHL's role in other cellular processes (e.g. extracellular-matrix deposition and turnover, epithelial differentiation), possibly distinct from HIF mediated effects, may be involved specifically in the formation of PHEOs (Clifford et al., 2001, Kim & Kaelin, 2003). In addition, pVHL has an important part in stabilizing p53 in response to genotoxic stress (Roe et al., 2006).

HEREDITARY PAPILLARY RENAL CARCINOMA (HPRC) is dominantly inherited disorder characterized by RCCs of type 1 papillary growth pattern, small cells, low Fuhrman grade (1-2), and inconspicuous cytoplasm and nucleoli (Zbar et al., 1994, Lubensky et al., 1999). The tumors are bilateral and multiple, and even up to 3000 microscopic tumors have been found from an affected kidney (Ornstein et al., 2000). The predisposition is transmitted by germline mutations in the *c-MET* oncogene located on chromosome 7q (Schmidt et al., 1997). c-MET is a tyrosine kinase and it is located on the cell surface as a receptor for the hepatocyte growth factor (HGF). Activation of c-MET by its ligand HGF or through a mutation results in the initiation of multiple signal transduction cascades involved in proliferation, migration, and morphogenesis (see also 2.1.2.1.). HPRC tumorigenesis appears to involve the gain of function of the mutant c-MET as the tumors

from HPRC patients and from the mutant *c-Met* mouse model harbor the duplication of the chromosome containing the mutant allele (Zhuang et al., 1998, Graveel et al., 2005). In sporadic papillary tumors, *c-MET* mutations are observed quite rarely, indicating differential molecular pathways in the genesis of hereditary and sporadic papillary RCC (Schmidt et al., 1999).

TUBEROUS SCLEROSIS (TS) is characterized by multiple hamartomas in several organs. Those in the central nervous system may result in a learning disability, seizures, and behavioral problems. Associated renal lesions include angiomyolipomas, renal and perirenal cysts, RCCs, and oncocytomas (Lendway & Marshall, 2003). The two predisposing genes of this dominantly inherited syndrome, *TSC1* (on 9q) and *TSC2* (on 16p), encode peptides for a protein dimer acting as a negative regulator of the cancer-associated PI3K-AKT-mTOR pathway (European Chromosome 16 TS Consortium 1993, van Slegtenghorst et al., 1997, Hay, 2005, Astrinidis & Henske, 2005, see also 2.1.2.1.). Interestingly, the first ever naturally occurring hereditary cancer in an animal model was the "Eker rat" (Eker et al, 1981), which was later on identified with a germline mutation in *Tsc2* (Hino et al., 1994, Yeung et al., 1994). The phenotype of the rat includes RCC, hemangiomas/hemangiosarcomas of the spleen, ULM/ULMS, and pituitary adenomas (Hino et al., 2003).

BIRT-HOGG-DUBE (BHD) syndrome is an autosomal dominant disease, initially characterized by different types of cutaneous lesions (Birt et al., 1977). Predisposition to RCC was later connected to BHD (Roth et al., 1993, Toro et al., 1999). The features of the renal tumors in BHD are variable. The tumors are mainly oncocytomas, but chromophobe, and clear cell type tumors are found as well (Pavlovich et al., 2002). The predisposing gene (*BHD*) is located on 17p and encodes folliculin protein (Nickerson et al., 2002). The actual function of the protein is not known but it is suggested to operate as TSG (Vocke et al., 2005).

COWDEN SYNDROME (CS) is an autosomal dominant disorder characterized by multiple hamartomas, commonly as mucocutaneous manifestations. Moreover, benign and malignant tumors of the breast, thyroid, and endometrium are commonly observed. As well as several other types of lesions, the patients are occasionally observed with RCC or ULMs. The predisposition to CS is caused by mutations in the tumor suppressor *PTEN*, encoding a lipid and protein phosphatase affecting on cell growth, spread, and apoptosis. These functions are mediated by its regulatory role in the PI3K-AKT and MAPK pathways as well as in focal adhesion (Gustafson et al., 2007). Recently, PTEN has been suggested to regulate certain double-strand DNA break repair proteins and thus have also a role in maintaining chromosomal integrity (Shen et al., 2007). Gene and protein loss of PTEN have also been observed in sporadic RCCs as well as in many other sporadic cancers (Brenner et al., 2002, Velickovic et al., 2002, Shen et al., 2007).

HYPERPARATHYROIDISM-JAW (HPT-JT) patients typically manifest ossifying fibroma of the jaw, parathyroid adenomas, benign cysts and hamartomas of the kidney, Wilms' tumor, and papillary RCC (Szabo et al., 1995). Heterozygous germline mutations in the

predisposing gene *HPRT2* (on 1q) are found in over half of HPT-JT patients, and genetic evidence indicates it acts as TSG (Carpten et al., 2002). *HPRT2* is mutated also in a fraction of sporadic parathyroid and renal tumors. The gene product of *HPRT2* is parafibromin, a component of the RNA polymerase II associated factor. The function of parafibromin is still unclear but at least certain mutations result in the defective suppression of cyclinD1 and consequent abnormal cell proliferation (Zhao et al., 2007).

Other familial inheritance of RCC includes families with a TRANSLOCATION OF 3p (Bonne et al., 2004), and HNPCC. HNPCC-associated uroepithelial cancers results from inactivation of the MMR genes, of which MSH2 seems to be mostly associated with extra-colonic tumors (Vasen, 2005, see also 2.1.2.3.). An underlying *SDHB* mutation has been observed in three RCC cases (two solid, and one clear cell) in the context of HEREDITARY PARAGANGLIOMATOSIS (Vanharanta et al., 2004, see also 2.3.) BECKWITH-WIEDEMANN SYNDROME (BWS), of which susceptibility gene is located on 11p15.5, is an overgrowth syndrome occasionally associated with Wilms' tumor (Smith et al., 2007). These hereditary RCC-associated syndromes are summarized in Table 2. In addition, there is a group of familial non-syndromic clear cell carcinomas which is not linked to any of the above-mentioned syndromes (Woodward, 2004).

2.3. Hereditary pheochromocytoma and paraganglioma

Paraganglia are aggregations of cells originated from the embryological neural crest and located throughout the body in the vascular and neuronal adventitia. The tumors arising from these neuronal cells include paragangliomas (PGL) and pheochromocytomas (PHEO). When the tumor is originated from the symphatetic nervous system and it secretes catecholamines, the tumor is referred to as PHEO. These tumors are most commonly located in the adrenal medulla (a modified ganglia), but also extra-adrenal PHEOs exist (also called symphatetic PGLs). Head and neck PGLs are derived from the parasymphatetic ganglia, and they commonly arise from the carotid body (an oxygen sensing organ). PGLs, like normal paraganglia, comprise type II (sustentacular) and type I (chief) cells, of which the latter ones represent the neoplastic population. The nomenclature of these lesions is somewhat confusing and often tumors of head and neck are named PGLs while tumors below neck are termed PHEOs (van Schlothorst et al., 1998, Maher & Eng, 2002, Jiménez et al., 2006, Martin et al., 2007).

PGLs/PHEOs are rare, estimated to comprise 0.01% of human tumors (Martin et al., 2007). These neoplasms are mainly benign but a malignant potential (defined by metastasis) is seen in 6-19% of PGLs/PHEOs of different sites (Myssiorek, 2001). A familial clustering of PGLs was observed for the first time in 1964 in two siblings (Kroll et al., 1964). The mapping of the predisposing gene for HEREDITARY PARAGANGLIOMATOSIS (HPGL) started in the Netherlands, which led to the identification of locus on 11q23, named *PGL1*. Later the locus was found to contain the gene of *succinate dehydrogenase subunit D* (*SDHD*), and germline mutations in the gene to explain head and neck PGLs in a subset of the studied PGL families (Baysal et al., 2000). Families

harboring a germline *SDHD* mutation were observed to display a phenotype with PGLs only or combined with PHEOs. Moreover, families with PHEOs only were found (Maher & Eng, 2002) (Table 2). The PGL/PHEO phenotype, caused by an *SDHD* mutation, is shown only in paternal transmission, suggesting maternal imprinting of the gene (van der Mey et al., 1989, Baysal et al., 2000). However, according to biallelic expression of *SDHD* in certain tissues and the consistent loss of the wild-type (maternal) allele in the associated tumors, it seems that the maternal allele is actually expressed in the normal paraganglia. Indeed, recent studies have shown that *SDHD* PGLs display an entire loss of maternal chromosome 11. The tumorigenesis is now suggested to involve the loss of the wild-type *SDHD* allele as well as the loss of an unknown paternally imprinted gene located on chromosome 11p. This transmission mode would explain the only paternally expressed phenotype (Hensen et al., 2004).

SDHD is part of the SDH enzyme, comprised of four subunits SDHA/B/C/D, which operates in the TCA cycle as well as in the respiratory chain as a part of the mitochondrial complex II. In the TCA cycle, SDH catalyzes the oxidation of succinate to fumarate. In addition to *SDHD*, other subunits of SHD have been linked to hereditary paragangliomatosis. The *PGL3* locus on 1q21 and *PLG4* on 1p36 contain genes encoding SDHC and SDHB, respectively. Association of these genes with familial PGLs (*SDHC*) and both PGLs and PHEOs (*SDHB*) has been observed in a few families (Niemann & Muller, 2000, Astuti et al., 2001). Moreover, a putative *PGL2* locus on 11q13 is linked to a large Dutch PGL family but no susceptibility gene has been identified yet (Mariman et al., 1995) (Table 2). *SDHB* and *SDHC* are TSGs (as *SDHD*) and display somatic loss of the wild-type allele in the tumor (Gottlieb & Tomlinson, 2005). Specifically *SDHB* mutations have been linked to the malignant outcome of the tumors since 34-71% of the *SDHB* carriers are found with a metastatic disease (Gimenez-Roqueplo et al., 2003, Neumann et al., 2004, Amar et al., 2007). Recently malignant tumors have been observed in *SDHD* cases too (Havekes et al., 2007, Timmers et al., 2007). In contrast to the association of *SDHB/C/D* with tumorigenesis, homozygous germline mutations in the fourth SDH subunit, *SDHA*, result in degenerative disorders such as an infantile encephalomyelopathy called Leigh syndrome and a disorder with late-onset optic atrophy, ataxia, and myopathy (Bourgeron et al., 1995, Birch-Machin et al., 2000).

PHEOs are also components of other familial cancer syndromes including VHL, MULTIPLE ENDOCRINE NEOPLASIA 2 (MEN2), and rarely NEUROFIBROMATOSIS TYPE I (NF1) (Pacak et al., 2001) (Table 2). In the VHL syndrome, caused by mutations in the tumor suppressor *VHL,* PHEOs are associated with type 2 (2A, 2B, 2C) disease comprising 10-15% of the VHL cases. (Maher & Eng, 2002, Lonser et al., 2003, see also 2.2.2.). A mutation in the oncogene *RET* underlies 95% of MEN2A and MEN2B cases, the clinical subtypes of MEN2 (Eng et al., 1996). PHEOs are present in approximately half of MEN2 patients. Another endocrine tumor type observed in MEN2B is medullary thyroid carcinoma and MEN2A patients are additionally affected by parathyroid hyperplasia (Maher & Eng, 2002). NF1, associated with germline mutations in *NF1* (*neurofibromin 1*), is classically characterized by “café au lait” spots, cutaneous neurofibroma, and tumors of the nervous system (Kramer et al., 2007).

Table 2. Hereditary tumor syndromes associated with renal lesions, paragangliomas, and pheochromocytomas

Tumor	*Syndrome*	*Tumor type*	*Gene*	*Central functions*
Renal tumor	von Hippel Lindau (types 1, 2A, 2B)	clear cell RCC	*VHL*	ubiquitin ligation; TSG
	Hereditary papillary renal cell carcinoma	papillary type 1 RCC	*c-MET*	receptor tyrosine kinase; oncogene
	Hereditary leiomyomatosis and renal cell cancer	papillary type 2, collecting duct, clear cell (study II) RCC, oncocytoma	*FH*	metabolism (TCA cycle, urea cycle); TSG
	Hereditary paragangliomatosis	clear cell, solid RCC	*SDHB*	metabolism (TCA cycle, electron transport chain); TSG
	Tuberous sclerosis	angiomyolipoma, renal and perirenal cyst, RCC, oncocytoma	*TSC1, TSC2*	regulator of GTPase Rheb in mTOR pathway; TSG
	Birt-Hogg-Dube	oncocytoma,chromophobe, clear cell, papillary RCC	*BHD*	not known; TSG
	Cowden	RCC (not specified)	*PTEN*	lipid and protein phosphatase, DNA repair; TSG
	Hyperparathyroidism-jaw	hamartoma, cyst, Wilms' tumor, papillary RCC	*HPRT2*	transcription elongation, RNA processing; TSG
	Translocation of 3p	clear cell RCC	not identified	-
	Hereditary non-polyposis colorectal cancer	uroepitelial carcinoma	*MLH1/MSH2 /MSH6/PMS2*	mismatch repair; TSG
	Beckwith-Wiedemann syndrome	Wilms tumor	not identified	-
PGL	Hereditary paragangliomatosis		*SDHC*	metabolism (TCA cycle, electron transport chain); TSG
	Hereditary paragangliomatosis		*PGL2* locus	-
PHEO	von Hippel Lindau (types 2A, 2B, 2C)		*VHL*	ubiquitin ligation; TSG
	Multiple endocrine neoplasia 2		*RET*	receptor tyrosin kinase; oncogene
	Neurofibromatosis type 1		*NF1*	regulator of GTPases (e.g. RAS); TSG
PGL/ PHEO	Hereditary paragangliomatosis		*SDHD*	metabolism (TCA cycle, electron transport chain); TSG
	Hereditary paragangliomatosis		*SDHB*	metabolism (TCA cycle, electron transport chain); TSG

RCC: renal cell carcinoma; PGL, paraganglioma; PHEO, pheochromocytoma; TSG, tumor suppressor gene

In the analyses of apparently sporadic PHEOs, an underlying germline mutation predisposing to PGL/PHEO (*SDH*, *VHL*, *RET*) is found in 10-24% of cases (Neumann et al., 2002, Gottlieb & Tomlinson, 2005, Jimenez et al., 2006, Martin et al., 2007). The role of intragenic somatic mutations in these genes in genesis of true sporadic tumors is, however, minor (Eng et al., 1995, Hofstra et al., 1996, Gimm et al., 2000).

2.4. Tricarboxylic acid cycle defect and tumorigenesis

In the presence of oxygen, the aerobic step in the energy production, cellular respiration, takes place. Pyruvate formed in cytosolic glycolysis, fatty acids, and some amino acids are oxidized to yield two-carbon fragments in the form of acetyl-coenzyme-A (Acetyl-CoA). These acetyl groups then enter the tricarboxylic acid cycle (TCA cycle) which enzymatically oxidizes them to CO_2. In brief, the TCA cycle encompasses the following steps: Acetyl-CoA is condensed by oxaloacetate and citrate synthase to citrate. Aconitase catalyzes the conversion of citrate to isocitrate, which is oxidized to α-ketoglutarate by isocitrate dehydrogenase (yielding CO_2). The α-ketoglutarate is dehydrogenated and decarboxylated to succinyl-CoA (and CO_2) by α-ketoglutarate dehydrogenase. It reacts with ADP and P_i to form succinate and ATP (the only one formed straight from the TCA cycle) which is catalyzed by succinyl-CoA synthase. Succinate is then oxidized to fumarate by SDH. Fumarate is hydrated by FH to L-malate, which is oxidized by L-malate dehydrogenase to regenerate oxaloacetate. The energy released from these oxidation reactions is conserved in the reduced electron carriers NADH and $FADH_2$ (Stryer, 1999, Nelson & Cox, 2000) (Figure 1).

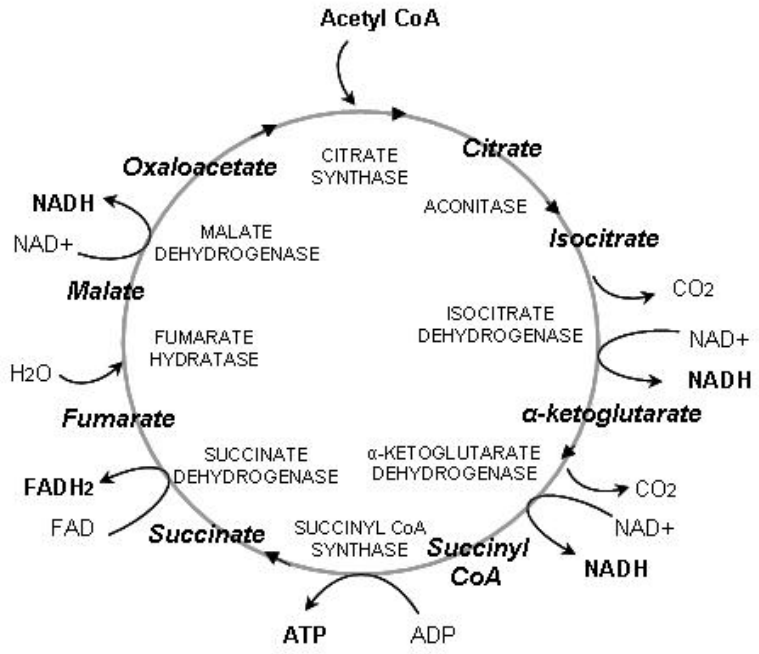

Figure 1. The tricarboxylic acid cycle. In the first stage of cellular respiration Acetyl-CoA is formed e.g. by oxidation of pyruvate obtained from glycolysis of glucose. In the second step, Acetyl-CoA is oxidated in the TCA cycle, which yields CO_2, an ATP, and reduced electron carriers NADH and $FADH_2$. NADH and $FADH_2$ proceed into the third stage, electron transfer chain in which the flow of electrons drives the production of ATP. In this respiration process altogether 32 ATPs are formed from one glucose molecule. In addition to energy (ATP) production, TCA cycle provides precursors (such as α-ketoglutarate and oxaloacetate) for many biosynthetic pathways (Nelson & Cox, 2000).

Subsequently the electron carriers, NADH and $FADH_2$ are themselves oxidized, releasing protons and electrons. In the next step, in the electron transfer chain on the mitochondrial inner membrane, the free electrons are transferred to O_2. In the course of the electron transfer, a large amount of energy is produced by the oxidative phosphorylation of ADP to ATP (Stryer, 1999, Nelson & Cox, 2000).

HLRCC and HPGL are tumor predisposition syndromes caused by mutations in genes encoding the TCA cycle enzymes FH and SDH (subunits SDHB/C/D). As well as the TCA cycle, SDH operates in the electron transport chain. It is interesting that defects in energy production seem to contribute to tumorigenesis rather than cause cell death. Several hypotheses and also evidence of the relation of the TCA cycle to resistance to apoptosis, increased redox stress, and activation of HIF1 have been put forward (Gottlieb & Tomlinson, 2005). The apoptosis-related theories have been based on the central role of mitochondria in apoptotic processes. Subsequently, the defect in the mitochondrial TCA cycle has been suggested to generate apoptosis-resistant cells including a theory based on the up-regulation of glycolytic pathways as a consequence of the down-regulation of oxidative phosphorylation (Newmeyer & Ferguson-Miller, 2003, Gottlieb & Tomlinson, 2005). Glycolytic enzymes have, among other functions, the capability to block apoptosis (Kim & Dang, 2005). On the other hand, SDH, SDHC particularly, has been implicated in cell line studies to potentially act as an apoptosis regulator (Albayrak et al., 2003, Ishii et al., 2005, Gottlieb & Tomlinson, 2005). Curiously, in these studies the acute response to the down-regulation of SDH was induction of apoptosis, whereas the chronically SDH-deprived cells became tumorigenic (Albayrak et al., 2003, Ishii et al., 2005). However, it remains elusive whether the effects seen in the cell line studies are meaningful in SDH-deficient tumors. Moreover, mutations in the subunit C are not that common. The apoptosis response was shown to be triggered by reactive oxygen species (ROS), generated due to the defective SDH (Albayrak et al., 2003, Ishii et al., 2005). Additionally, ROS were suggested to increase mutagenesis and thereby drive the tumorigenic progress (Ishii et al., 2005). However, ROS production has not been observed in all the studies on SDH- or FH-deficient cells and thus its role HLRCC and HPGL tumorigenesis remains unclear (Selak et al., 2005, Pollard et al., 2005b).

Evidently, activation of HIF1 is one of the consequences of the TCA cycle defect. The physiological function of HIF1 is to orchestrate cells' adaptation and survival in low oxygen conditions by inducing oxygen-independent glycolysis as an energy source and also vessel growth. It was first indicated in SDH- and then in FH-deficient tumors that the defective function of these enzymes leads to accumulation of their substrates, succinate and fumarate, respectively. These in turn were shown to inhibit PHD (prolyl hydroxylase-domain) enzymes under normoxic conditions triggering a stage of "pseudohypoxia". In pseudohypoxic cells, the normal targeting of HIF1α subunit to degradation is hindered and activation of HIF1 target genes is enabled (Selak et al., 2005, Isaacs et al., 2005, Pollard et al., 2005b, see also 2.1.2.2) (Table 1, Figure 2). For example, FH-deficient ULMs have been shown to express increased levels of HIF1 targets such as glycolytic enzymes and angiogenic factors (e.g. *VEGF*) as well as display increased microvessel density (Pollard et al., 2005a & 2006, Vanharanta et al., 2006, Catherino et al.,

2007). Also overexpression of HIF2α has been observed in the context of FH and SDH down-regulation, but to a lesser extent than that of HIF1α (Pollard et al., 2006 & 2007). In contrast, since fumarate and succinate do not seem to repress FIH1, the inhibitor of the transcriptional activity of HIF1, *FH* and *SDH* mutations are not likely to influence on the transcription of HIF1 target genes by altering FIH1 function (Koivunen et al., 2007).

The role of the TCA cycle intermediates as inhibitors of PDHs is well established and fumarate especially has been shown to efficiently prevent the function of all the PHDs (Koivunen et al., 2007). Moreover, there is a clear association between HIFs and the features that HLRCC and HPGL tumors display, such as high vascularization, but whether up-regulation of HIFs is sufficient to cause the actual tumorigenesis is still under investigation. The PHD catalyzed proline hydroxylation of HIF1α/HIF2α (HIFs) takes place through a reaction of conversion of molecular oxygen and α-ketoglutarate to CO_2 and succinate. Interestingly, α-ketoglutarate supplied to cells with excessive fumarate or succinate has been shown to compete for the PHD interaction and to restore the normal PHD function. This provides one potential basis for therapeutical aspects (MacKenzie et al., 2007). However, it is notable that the possible role of inhibition of dioxygenases other than the PHDs in TCA cycle-associated tumorigenesis has not yet been evaluated (Koivunen et al., 2007, Pollard et al., 2007).

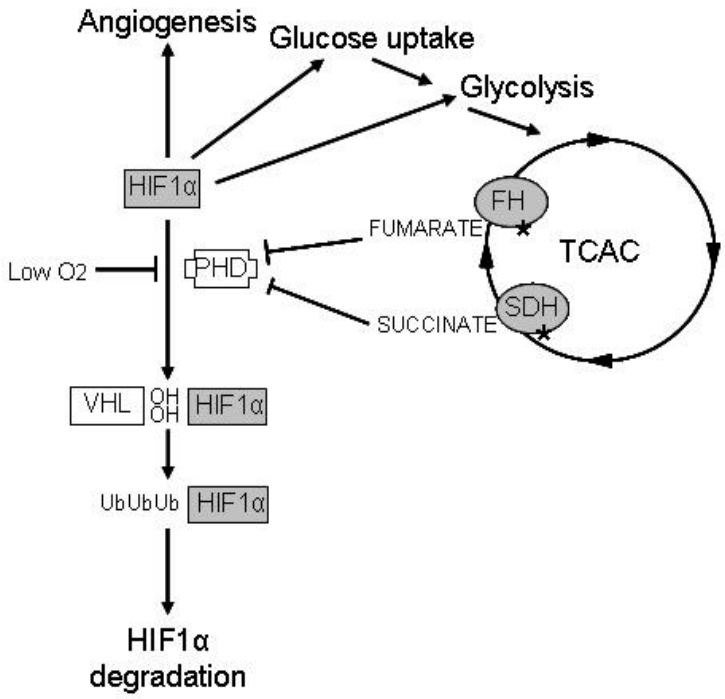

Figure 2. Activation of the HIF1 pathway due to defective SDH and FH. Inactivating mutations in *SDH* and *FH* (marked with asterisks) cause accumulation of their substrates, succinate and fumarate, respectively. These inhibit similarly to low oxygen the oxygen-dependent function HIF1α prolyl hydroxylase-domain (PHD) proteins. Non-hydroxylated HIF1α is able to enter the nucleus, dimerize with HIF1β, and activate the transcription of factors facilitating tumor growth. In contrast, normally hydroxylated (OH) HIF1α is recognized by the tumor suppressor pVHL, which targets it to ubiquitylation (Ub) and degradation. HIF2α is similarly activated by pseudo-hypoxia, although to a lower extent than HIF1α (Semenza, 2003, Selak et al., 2005, Isaacs et al., 2005, Pollard et al., 2005b, Figure modified from Esteban & Maxwell, 2005).

Separate from the HIF pathway, inherited mutations in the genes (*SDH*, *VHL*, *RET*, *NF1*, see also 2.2.2.) predisposing to PGLs/PHEOs were recently shown to share a common pathway leading to tumorigenesis. PGLs/PHEOs originate from neural crest cells, which during embryonic development undergo c-JUN dependent apoptosis when neuronal growth factor (NGF) becomes limited. Mutant forms of these genes seem to result in failure of the normal apoptosis. Hence, the survived neural crest cells have been suggested to have tumorigenic potential and act as neoplastic progenitors for PGL/PHEO (Lee et al., 2005, Maxwell, 2005).

3. AIMS OF THE STUDY

1) To elucidate the clinical characteristics of hereditary leiomyomatosis and renal cell cancer (HLRCC)

– by specifying the risk of cancer, the tumor spectrum and tumor characteristics of HLRCC

2) To elucidate the molecular characteristics of HLRCC and hereditary paragangliomatosis (HPGL)

– by studying the putative link between HIF1α and mismatch repair in HLRCC and HPGL tumors

– by characterizing the novel alternative transcript of FH (FHv)

4. MATERIALS AND METHODS

4.1. Sample material

4.1.1. Finnish HLRCC families and patients (I-IV)

Finnish hereditary leiomyomatosis and renal cell cancer (HLRCC) families found so far include seven families that have been identified through a benign (leiomyomatosis) or malignant (renal cell cancer; RCC or uterine leiomyosarcoma; ULMS) phenotype. In addition, one FH deficiency (FHD) family has been reported in Finland (Table 3).

In study I, the eight Finnish *FH* mutation-positive families were extended and prospected for cancer cases as described in section 4.2. The *FH* mutation status was analyzed from all the available individuals, and subsequently all the available tumors from the *FH* mutation carriers were studied for biallelic inactivation of *FH*. Varying sets of these tumors were then used in the subsequent approaches (studies II, III, IV). In addition to the tumors identified through the search in study I, uterine leiomyomas (ULM) as described previously (Launonen et al. 2001, Kiuru et al., 2001, Tomlinson et al. 2001, Kiuru et al., 2002, Vanharanta et al., 2006) were utilized in studies III and IV.

Table 3. Finnish HLRCC families

Family	*Phenotype exploited in family identification*	*Germline mutation**	*Reference*
FAM1	RCC	E181fs**	Launonen et al., 2001
FAM2	RCC	E181fs**	Launonen et al., 2001
FAM3	Leiomyomas	R300X	Tomlinson et al., 2002
FAM4	ULMS	H153R	Study I
FAM5	RCC	H153R	Study I
FAM6	ULMS	H153R	Kiuru et al., 2002
FAM7	Leiomyomas	E181fs**	Kiuru et al., 2002
FAM8	FHD	Q333P	Remes et al., 1992 & 2004

RCC; renal cell cancer, ULMS; uterine leiomyosarcoma

*Site of the mutation in the *FH* sequence is calculated starting from the last codon of exon 1 (encoding the signal peptide) (Tomlinson et al., 2001). **Corresponding mutation name 541delAG used in the original publications. The frameshift results in a premature stop codon and truncated protein product.

4.1.2. Spanish HLRCC patient case (II)

A germline *FH* mutation analysis of the Spanish RCC patient and the patient's mother was conducted from blood-extracted DNA. DNA from the patient's two paraffin-embedded renal tumors was used for *FH* and *VHL* mutation analyses and for 3p LOH analysis. RCC tissue sections were analyzed for histological and immunohistochemical

(IHC) characteristics. The patient's two uterine leiomyomas also were histologically evaluated. Three RCCs from FAM1 were exploited as controls in the IHC.

4.1.3. Samples exploited in the analysis of HIF1α and mismatch repair (III)

4.1.3.1. HLRCC samples

Paraffin-embedded HLRCC tumors from Finnish *FH* mutation carriers including 11 RCCs, 12 ULMs, one ULMS, and as a control, four non-familial ULMs were analyzed in studying the relation between HIF1α and MSH2 protein expression. Microsatellite instability (MSI) was analyzed from DNA of the respective RCCs (n=10), ULMs (n=8), and ULMS (n=1) as well as from another four HLRCC-associated ULMs. Corresponding normal DNA was used as a control when available.

4.1.3.2. HPGL samples

A tissue microarray slide (TMA) containing 17 PHEOs and 100 PGLs was analyzed similarly as the HLRCC samples. The 117 tumors comprised 63 *SDHB/C/D* mutation-positive (62 PGLs and 1 PHEO), 9 other familial (*NF, VHL*, *RET*, *PGL2*) and 45 non-familial tumors. All the samples in the array were represented as triplicates. Seventeen PGL tumor DNA samples (9 *SDHD*, 1 *PGL2*, and 7 non-familial) corresponding to the array samples as well as 10 additional samples (9 *SDHD* and 1 *PGL2*) were used for MSI analysis. If available, corresponding normal DNA was used as a control.

4.1.4. Samples exploited in the analysis of the alternative transcript of FH (FHv) (IV)

4.1.4.1. Mutation analysis of FH exon 1b

A total of 139 human DNA samples were screened for *FH* exon 1b mutations including 21 cell lines (12 RCC, 4 prostate cancer, 5 sarcoma cell lines), 99 non-syndromic HLRCC-associated tumors (39 ULMs, 43 RCCs, and 17 ULMSs), 9 HLRCC patient-derived tumors without detected second hits in *FH* (study I, Vanharanta et al., 2006), and 10 blood samples from *FH* mutation-negative individuals (6 cases with early-onset RCC, and 4 cases with RCC or leiomyomatosis).

4.1.4.2. Expression analysis of FH exon 1b

FH exon 1b expression was analyzed in fetal (skeletal muscle, lung, brain, kidney, liver, heart, spleen, thymus) and adult (skeletal muscle, lung, brain, kidney, liver, heart, placenta, pancreas) human tissue cDNA (Human MTC™ Panel 1 and Human Fetal MTC™ Panel, BD Biosciences Clontech, Palo Alto, CA). Moreover, expression of 1b was analyzed in 15 cell lines (see 4.7.1.).

4.2. Genealogy and cancer data collection (I)

To broaden the knowledge about the tumor spectrum of HLRCC, particularly about malignant tumors, an extended search for family and cancer data was performed on the eight Finnish *FH*-mutation-positive families (see 4.1.1., Table 3). The genealogical data of the families were collected through church parish registries and the Population Register Centre. Population registration in Finland began in the 16th century and in 1967 each resident in Finland was given a personal identifier (PID) enabling rapid tracking of family members. Family branches observed to be *FH* mutation-negative were not further followed and were therefore not included in the study. Cancer history of the identified individuals was verified from the Finnish Cancer Registry and death certificates. Cancer data was subsequently utilized in the collection of tumors for *FH* mutation analysis and for calculating the incidence of cancer in HLRCC families compared to the general population. Existence of other lesions (mainly kidney cysts and adrenal gland tumors) was examined from patient reports if available. The data collection process is illustrated in Figure 3.

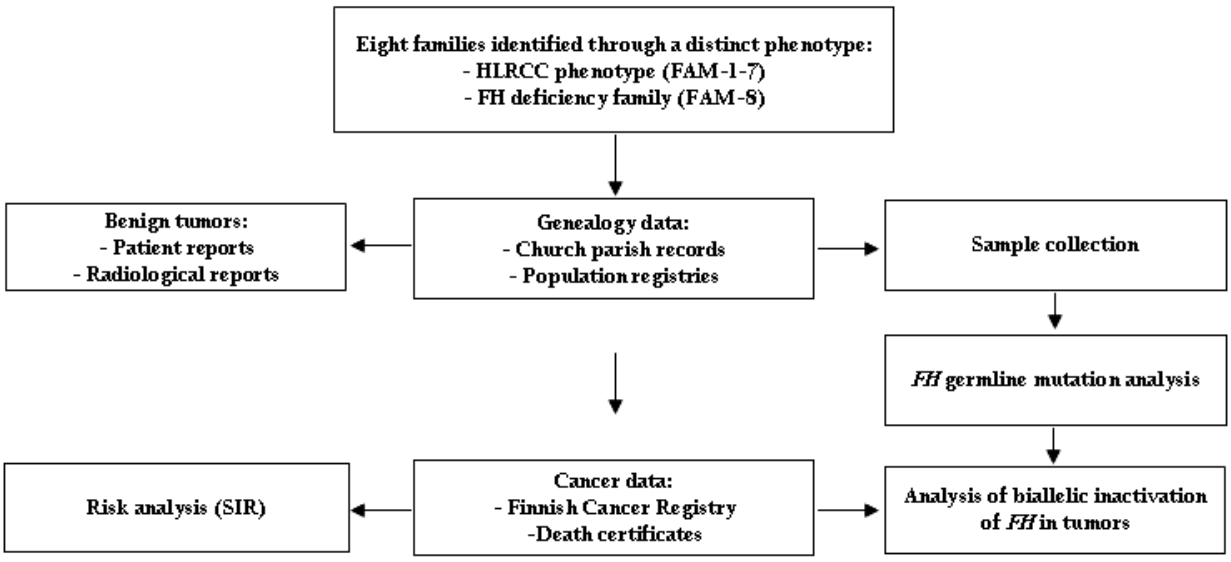

Figure 3. Process of the genealogy, cancer data, and sample collection of Finnish *FH* mutation-positive families. DNA samples for *FH* germline mutation analysis were obtained in addition to blood extracted lymphocytes also from paraffin embedded tumor tissue.

4.3. Statistical analyses (I)

The cancer risk in *FH* mutation-positive families compared to the general population was calculated with the standardized incidence ratio (SIR). The family members were followed up for cancer incidence by a personal identifier-based (PID) automatic record linkage with the Finnish Cancer Registry. The follow-up started on January 1st 1953 and ended on December 31st 2002 or at a person's death or date of emigration. Individuals deceased before 1967 with no PID were excluded from the Cancer Registry search. To

avoid the ascertainment bias inherent in the family identification, the lowest possible estimate of the cancer risk was calculated. This was done by excluding cancers in all the index patients (five cancers in eight patients) and the other patients with RCC or ULMS (seven cancers in six patients) used for the identification of the families. Moreover, to avoid selection bias, only completely traced generations were included in the analysis. No selection according to mutation status was made. The total number of family members included in SIR analyses was 256. The SIRs were calculated by dividing the observed numbers of cancer cases by expected numbers. The expected numbers were based on person-years at risk and population-based gender-, age-, and calendar periodic-specific incidence rates in the general population. Exact 95% confidence intervals (CI) were defined assuming that numbers of observed cases followed the Poisson distribution. Comparison of the age at cancer diagnosis between *FH* mutation carriers and non-carriers were calculated by Fisher's exact test.

4.4. Genetic analyses

4.4.1. Mutation analysis (I-IV)

Mutation analyses were performed by direct sequencing. Standard procedures were used for the extraction of DNA from normal (lymphocyte/tissue) or tumor samples. Polymerase chain reaction (PCR) with AmpliTaq Gold® DNA polymerase (Applied Biosystems Foster City, CA, USA) was used for amplification of DNA fragments. Next, the excess of deoxynucleotides was purified by ExoSAP-IT enzyme mix (USB Corporation, Cleveland, Ohio, USA) and the fragments were analyzed using BigDye 3.1 chemistry and ABI3730xl DNA Analyzer (Applied Biosystems). In studies I and II, in order to identify *FH* mutation carriers and LOH in tumors, *FH* exons 1-10 were analyzed. *FH* primer sequences are described by Kiuru and co-workers (2002). The *FH* exon 1b mutation status was analyzed with 1b specific primers (study IV) and *VHL* with three exons in order to exclude the contribution of the VHL defect in the tumorigenesis of the Spanish RCC case (study II). *FH* 1b and *VHL* primer sequences are described in Table 4.

Table 4. Sequencing primers of *FHv* exon 1b and *VHL*

Purpose	*Study*	*F sequence 5'-3'*	*R sequence 5'-3'*
FH ex 1b	IV	GGCTGTCAGAGAGGGTCCTA	TACGGGGGAAACCATAGTCA
VHL ex 1 (1st fragment)	II	CGCGAAGACTACGGAGGT	GGCCTCCATCTCCTCCTC
VHL ex 1 (2nd fragment)	II	GAGTACGGCCCTGAAGAAGA	CGGTAGAGGGGCTTCAGAC
VHL ex 2	II	AGCCAGGACGGTCTTGATCTC	GGCAAAAATTGAGAACTGGGC
VHL ex 3	II	ACAGGTAGTTGTTGGCAAAGC	CCTAAACATCACAATGCCTAG

4.4.2. MSI analysis (II, III)

Fluorescently labeled microsatellite marker primers (D3S1038 and D3S3659) were used to analyze possible loss of *VHL* in chromosomal location 3p in two RCCs of the Spanish patient (study II). The Bethesda panel (BAT25, BAT26, D2S123, D5S346 and D17S250), a marker set recommended for MSI analysis by the National Cancer Institute workshop on microsatellite instability in 1997 (Boland et al., 1998), was used in studying the link between HIF1α and MSI (study III). The amplified fragments were assessed with ABI3730xl DNA Analyzer (Applied Biosystems) and the results were analyzed with GeneMapper 4.0 (Applied Biosystems).

4.5. Expression analyses

4.5.1. Quantitative real-time PCR (IV)

RNA from cell lines was collected from 6-well plates and isolated with RNeasy mini purification columns (Qiagen, Heiden Germany). Reverse transcription of RNA to cDNA was performed with M-MLV enzyme (Promega, Madison, WI, USA).

A quantitative real-time PCR with a TaqMan chemistry and GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) was used in the analysis of *FH* (exon 1) and *FHv* (exon 1b) expression in human tissues and cell lines (see 4.1.4.2. and 4.7.1). All primer and probe combinations were designed to span exon-exon junctions using a Primer Express 3.0 (Applied Biosystems). The human housekeeping genes *phosphoglycerokinase* or *beta-actin* were used in normalization of the relative mRNA copy numbers within each sample (ABI PRISM 5700 Sequence Detection System User's Manual, Applied Biosystems).

4.5.2. Immunoblotting (IV)

To study the translation initiation site of *FHv*, HEK293 cells were transfected with various FHv-GFP constructs (see 4.6.) and after 24-hour incubation, proteins were extracted with an M-PER cell lysis reagent (Pierce, Rockford, IL, USA). A spectrophotometric bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA) method was used to determine the protein concentration of the samples. Twenty-five µg of protein were loaded in a precast 10% Tris-HCl polyacrylamide gel (BioRad, Hercules, CA, USA) and detected with an FH antibody (dilution 1:500; Nordic Immunology, Tillburg, The Neatherlands) and an ECL Plus™ Western blotting system (Amersham Biosciences, UK Ltd, Buckinghamshire, UK).

4.5.3. Immunohistochemistry (II, III)

The renal tumors of the Spanish patient (study II) were characterized by immunohistochemical stainings of FH (dilution 1:1000; Nordic Immunology, Tillburg, The Neatherlands), vimentin (1:1500; Dako, Glostrup, Denmark), CD10 (1:50; Novocastra, Newcastle, UK), EMA (1:500; Dako), S-100 (1:2000; Dako), and CK8 (1:100; Enzo Life Sciences, Farmingdale, NY), AE1/AE3 (1:700; Dako) and CK7 (1:300; Novocastra). FH was detected with a Powervision+ detection kit (Immunovision, Springdale, AR, USA) and the other proteins were detected with an EnVision™ detection system (Dako, Copenhagen, Denmark). The staining result was scored as negative (-), light (-/+) or positive (+).

HIF1α and MSH2 protein expression was analyzed in HLRCC and HPGL tumors (see 4.1.3.) in study III. HIF1α was detected utilizing a monoclonal HIF1α antibody (dilution 1:100, clone 54, BD Biosciences, Franklin Lakes, NJ, USA) and a Powervision+ detection kit (Immunovision). Nuclear HIF1α staining was scored as 0, 1, 2, and 3 describing a negative staining, positive staining in 1-10% (weak), 10-50% (moderate), or >50% (high) of the cells in the specimen, respectively. A specimen from an ischemic bowel displaying a high HIF1α protein expression was used as a positive control. MSH2 staining (1:600, Santa Cruz Biotechnology Inc, CA, USA) in HLRCC tumors was assayed in a LabVision autostainer™ (Labvision Freemont, CA, USA). The TMA slide with HPGL tumors was stained for MSH2 (1:75, clone GB12, Calbiochem, Cambridge, MA, USA) in a Dako Techmate 500+ autostainer. In both sample sets the detection was performed with an EnVision™ detection system (Dako). The MSH2 staining was scored as negative (-) or positive (+).

The tissue sections used for the IHC were derived from paraffin-embedded specimens and were of four to five µm in thickness. In all the applications, diaminobenzidine (DAB) was used as a chromogen and haematoxylin as a counterstain.

4.6. Constructs (IV)

The cDNA of *FH* and *FHv* was cloned into a pCI-neo Mammalian Expression Vector (Promega Corporation, Madison, WI, US) and a pEGFP-N3 vector (BD Biosciencies Clontech, Mountain View, CA, US). The cloning primers are listed in Table 5. For identification of the translation initiation codon of *FHv*, several constructs with different putative initiation sites were created in the basic FHv-pEGFP vector ($FHv_{ctg1ccc,}$-GFP, $FHv_{ctg2ccc,}$-GFP, $FHv_{ctg1atg,}$-GFP, $FHv_{ctg2atg}$-GFP, $FHv_{atg1ccc}$-GFP, $FHv_{atg2ccc}$-GFP, and $FHv_{atg3ccc}$-GFP) by a site-directed mutagenesis (QuickChange® Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA). In addition, a construct without exon 1 or 1b and with an additional ATG at the beginning of the exon 2 was created ($FH_{no\text{-}signal}$-GFP) (see study IV, Figure 4a). All the constructs were verified by direct sequencing.

Table 5. Cloning primers for cDNA constructs

Purpose	*F sequence 5'-3'*	*R sequence 5'-3'*
FHv to pcI-neo vector	TTTCTCGAGGCCGCCCTGAACTGTGGTTTGTTTC*	TTTGTCGACTCACTTTGGACCCAGCATG
FHv to pEGFP-N3 vector	TTTCTCGAGGCCGCCCTGAACTGTGGTTTGTTTC*	TTTGGATCCCTTTGGACCCAGCATGTCCTT
FH to pEGFP-N3 vector**	TTTGAATTCAGCACCATGTACCGAGCACTT	TTTGGATCCCTTTGGACCCAGCATGTCCTT
$FH_{no\text{-}signal}$ to pEGFP vector	TTTGAATTCGCTGGAATGGCAAGCCAAAATTC	TTTGGATCCCTTTGGACCCAGCATGTCCTT

*With the primers the *FHv* sequence starts from the first CTG in exon 1b (see also Figure 5). ***FH* cDNA inserted into the pCi-neo vector was digested out from the pEGFP vector.

4.7. Cell culture (IV)

4.7.1. Cell lines

HEK293 (human embryonic kidney cells; CRL-1573, American Type of Culture Collection, ATCC), HeLa (cervical cancer cells; CCL-2, ATCC), and SK-UT-1B (uterine leiomyosarcoma cells; HTB-115, ATCC) were utilized in studying the characteristics of FHv. In addition, 12 more cell lines were studied for FHv expression in stress conditions including ATCC lines SV7tert, SK-LMS-1, A704, LNCaP, DU-145, DLD-1, RKO, SW-480, HCT-116, SK-UT-1, MES-SA, and line LS174T from European Collection of Cell Cultures (ECACC). These cells originated from ULMS, RCC, prostate cancer, colorectal cancer, or from a normal kidney.

4.7.2. Culture conditions (normal and stress conditions)

Cell lines were cultured in medias recommended by ATCC or EACC, and were supplemented with penicillin-streptomycin (100 U/ml and 0.1 mg/ml) (Sigma-Aldrich) and 10% FBS (5% FBS for HEK293). Normal culture conditions were set as 37ºC, 5% CO_2, and 21% O_2.

Various stress conditions including glucose or serum deprivation, hydrogen peroxide exposure, hypoxia and heat shock were used to test induction of *FHv*. The glucose and serum deprivation was performed by culturing cells in DMEM without glucose and either with low (0.1%) or without FBS for 24 and 48 hours. In hydrogen peroxide exposure, cells were incubated with 200 µM H_2O_2 for 30 minutes. After the exposure, incubation was continued for 24 and 48 hours in normal media. Hypoxia (1% O_2, 5% CO_2, 94% N_2) was generated in a hypoxia incubator (Invivo$_2$ Hypoxia Workstation 400, Ruskinn Technology Ltd, Leeds, UK), in which cells were cultured for 24, 48, 72, and 96 hours. The heat stress was created at a temperature of 42°C in a regular culture incubator for 0.5, 1, and 2 hours.

4.8. Transfection and immunofluorescence (IV)

To monitor FH and FHv protein distribution in a cell, HEK293 cells in approximately 70% confluence were transfected with GFP-constructs (FH-GFP, FHv-GFP, $FHv_{atg1ccc}$-GFP, $FHv_{atg2ccc}$-GFP, $FHv_{atg3ccc}$-GFP) (see 4.6.) using a FuGENE 6 reagent (Roche Applied Science, Indianapolis, IN). After 24 hours from transfection, the cells were fed with a MitoTracker (100 pmol/ml, MitoTracker Red CMXRos; Molecular Probes/Invitrogen, Carlsbad, CA, USA) and fixed with 4% paraformaldehyde. The immunodetection of the endogenous FH protein was performed on fixed cells with a porcine FH antibody (1:100; Nordic Immunology, Tilburg, Netherlands) and a FITC-conjugated goat anti-rabbit IgG. Nuclei were visualized with a Hoechst (33258) stain (Sigma-Aldrich). The subcellular distribution of the proteins was monitored using a Leica TCS SP1 confocal microscope or Axioplan upright epifluorescence microscope.

4.9. FH enzyme activity assay (IV)

To measure the enzyme activity of FHv, HEK293 cells were transfected with FHv-pCi-neo and for comparison with a FH-pCi-neo construct. The method, originally described by Hatch in 1978, is based on the detection of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced in a reaction containing fumarate, FH from the cell sample under investigation, malate dehydrogenase (MDH), and oxidized nicotinamide adenine dinucleotide phosphate (NADP). The NADPH in the assay mix is produced along with oxaloacetate through the following reaction:

$$\text{FUMARATE} \xrightarrow{\text{FH}} \text{MALATE} \xrightarrow[\text{NADP}]{\text{MDH}} \text{NADPH (+ oxaloacetate)}$$

In short, fumarate mixed with FH produces malate, which is oxidated into oxaloacetate by malate dehydrogenase (MDH) simultaneously reducing NADH to NADPH. The production of NADPH is detected spectrophotometrically (A_{340}), and the FH activity is quantified as the amount of NADP consumed per minute per mg of total protein in the sample.

4.10. *In silico* analyses (IV)

Following electronic databases and web-based tools were utilized in the analyses of the alternative transcript of *FH* (*FHv*) (Table 6).

Table 6. Electronic tools utilized in the study of alternative *FH* transcript (*FHv*)

Purpose	*Tools*	*Website*	*References*
Search for variant transcripts	AceView gene database	http://ncbi.nih.gov/IEB/Research/Acembly/index.html	-
Promoter and transcription binding site analyses	Genomatix tools: Eldorado,GEMS Launcher,Gene2 Promoter, MatInspector	http://www.genomatix.de	-
Conservation of genomic sequences between species	ERC Browser NCBI Ensembl	http://ercbrowser.dcode.org http://www.ncbi.nlm.nih.gov/dbEST/index.html http://www.ensembl.org/index.html	Ovcharenko, et al., 2004 Boguski et al., 1993 -
CpG islands prediction	Webgene	http://www.itb.cnr.it/sun/webgene	-
Prediction of the 5' end of transcripts	Cage cap database Transcription start site database Variant transcript databases	http://gerg01.gsc.riken.jp/cage/hg17prmtr http://fantom3.gsc.riken.jp http://dbtss.hgc.jp http://statgen.ncsu.edu/asg/index.php http://www.ebi.ac.uk/asd	Carninci, 2005 Suzuki et al., 2004 Leipzig et al., 2004 Stamm et al., 2006 Thanaraj et al., 2004
Pepide mass predictor	Pepcalc	http://www.ionsource.com/programs/PEPCALC.HTM	-

5. RESULTS

5.1. Novel clinical features of HLRCC

5.1.1. The tumor spectrum and cancer risk in Finnish HLRCC families (I)

An extended search for the family members and their cancer status was performed for the seven Finnish HLRCC and one FHD families (Table 3). Altogether 868 family members were identified (Appendix 1). A set of members of the eight families (n=256) was included in the cancer risk analysis determined by a standardized incidence ratio (SIR). The comparison of the cancer cases in the families and in the general Finnish population elicited a higher incidence of renal cell cancer (RCC) and uterine leiomyosarcoma (ULMS). The incidence of RCC was 6.5-fold (95% CI: 2.1 to 15.0) and 71-fold (95% CI: 8.6 to 260) for ULMS. SIR for common cancer types (breast, prostate, lung, and colorectal carcinoma) and hematopoietic and lymphoid malignancies was also calculated but no higher incidence was observed in the HLRCC/FHD families.

DNA for the *FH* mutation analysis was available from 98 members of the eight families and 54 were found as *FH* mutation positive. Of these, 26 were diagnosed with cancer (Table 5, Appendix 1). The number of cancer cases was particularly high in young age groups since 14 cases (43%) were diagnosed at less than 44 years of age. In comparison, in the mutation-negative individuals, all but one (15/16, 94%) cancer cases were diagnosed at the age of 45 or later. The result, however, was significant (p=0.009) only if the probands' cancers were included in the statistical comparison. As also seen in the SIR analysis, the most prominent tumor types in the *FH* mutation carriers were RCC (n=12) and ULMS (n=5). In addition, several cases of breast cancer, and hematopoietic and lymphoid malignancies were observed. Four *FH* mutation-positive individuals had more than one malignancy. Two of them had ULMS and breast cancer, one had ULMS and RCC, and one had RCC and basal cell carcinoma. Strikingly, the other ULMS patient with breast cancer was also affected with multiple myeloma and non-Hodgkin's lymphoma. Tumors including RCCs (n=12), ULMSs (n=3), breast cancers (n=3), and single cases of bladder cancer, non-Hodgkin's lymphoma, Hodgkin's lymphoma, chronic lymphatic leukemia, esophageal cancer, and basal cell cancer were analyzed for inactivation of the wild-type *FH* allele. The analyses showed somatic inactivation of the wild-type *FH* allele in the majority of the analyzed RCCs (10/12, 83%), all the ULMSs (3/3), breast cancers (3/3), and bladder cancer (1/1) (Table 5).

The radiological reports on the pelvic area, available from 33 individuals, revealed existence of some benign tumors. Kidney cysts and adrenal gland adenomas were observed in 42% (14/33) and 12% (4/33) of these individuals, respectively. One liver hemangioma was detected as well. Moreover, reports about four atypical uterine leiomyoma cases and one patient operated frequently for benign fibrocystic lesions of the breast were found.

Table 5. Cancer cases in *FH* mutation carriers

Cancer	*n*	*Age at diagnosis (median)*	*Somatic second hit detected/analyzed*
RCC	12*	26, 32, 33, 35, 36, 39, 42, 48, 49, 68, 71, 90 (40.5)	10/12**
ULMS	5*	27, 30, 32, 35, 39 (32)	3/3**
Breast cancer	4*	50, 53, 55, 61 (52.5)	3/3
Bladder	1	71	1/1
Non-Hodgkin's lymphoma	1	63	0/1
Hodgkin's lymphoma	1*	24	0/1
Chronic lymphatic leukemia	1	48	0/1
Esophagus cancer	1	53	0/1
Basal cell cancer	2	70, 83	0/1
Multiple myeloma	1*	61	NA
Prostate cancer	1*	63	NA
Liver/bile duct cancer***	1	82	NA
Unknown origin	1	42	NA

NA, not analyzed. *These lesions, except one of the breast cancers were previously reported by Launonen et al. (2001) and Kiuru et al. (2002 & 2005). **Seven RCCs and two ULMSs were analyzed for *FH* (1q42-q44) LOH by Launonen et al. (2001), Kiuru et al. (2001), and Tomlinson et al. (2002). ***Obligate mutation carrier.

5.1.2. Conventional renal cell cancer in HLRCC (II)

The Spanish RCC case was originally admitted to hospital because of unusual uterine bleeding. This 23-year-old female patient was then diagnosed by ultrasound to have a myomatous uterus and incidentally also a bilateral renal mass. A computed tomography (CT) scan confirmed the lesions suggestive of RCC. The lesions were subsequently removed, the right-side tumor via a partial and the left-side tumor via a radical nephrectomy. The right-side tumor was 2.3cm in diameter and it showed cystic, papillary and trabecular areas. It displayed papillary morphology, abundant eosinophilic cytoplasm, and was classified Fuhrman grade 2 with medium-sized nuclei and small nucleoli. The left-side tumor was 13cm in diameter and showed solid, tubular, and cystic areas and it displayed conventional (clear) cell type histology with a moderate cytoplasm. The Fuhrman grade was 2 with small nuclei and inconspicuous nucleoli. The IHC profile of the tumors was compared with three RCCs from the Finnish HLRCC FAM-1. EMA, S-100, vimentin, CK7, and AE1/AE3 stained similarly in the Spanish tumors and in the FAM1 tumors (EMA, however, negative in one FAM1 tumor). As an exception, the Spanish RCCs were positive in CD10 and CK8 staining. In addition, the right-side tumor differed from others being negative for AE1/AE3 and positive for FH. The positive staining for vimentin and CD10 differentiated the clear cell tumor from chromophobe RCC, which also displays a clear cytoplasm. Two ULMs were later removed by myomectomy. These tumors were of 8.5 and 2.8 cm in diameter and displayed nuclear atypia and a low mitotic index.

The patient and her mother were screened for *FH* mutations and were found to carry a novel missense mutation N330S (AAC>AGC), which results in the change of the amino acid asparagine into serine. The search for inactivation of the wild-type *FH* allele in the tumors revealed loss of the gene in the left-side conventional renal tumor but not in the

right-side papillary tumor. Since mutations in the *VHL* gene on chromosome 3p lay commonly under clear cell tumors, the left-side tumor was analyzed for mutations in *VHL* and for LOH in 3p. No alterations at the *VHL* locus were observed.

5.2. Characteristics of *FH* and *SDH*

5.2.1. HIF1α and mismatch repair in FH and SDH tumors (III)

HIF1α stabilization in the HLRCC tumors (11 RCCs, 12 ULMs, and 1 ULMS) as well as in the 63 PGLs/PHEOs with an *SDHB/C/D* mutation (on the TMA) was studied with IHC. In the HLRCC samples, HIF1α was present in a moderate or high proportion of the tumor cells (scores 2 and 3, respectively) in 67% (16/24) of RCC and ULM/ULMS specimens (Table 7). In general, the staining was mainly nuclear but some cytoplasmic positivity was observed in a few specimens. The normal renal glomeruli and tubular epithelium stained with a low intensity and in some of the specimens a weak positive staining was observed in the normal myometrium. The PGLs with an *SDHB/C/D* mutation showed in 79% of the cases (48/61) moderate or high levels of HIF1α. One (1/62) PGL could not be analyzed. The non-familial PGLs (n=35) showed a similar HIF1α stabilization in 71% (25/35) of the samples. Of note, all the PHEOs (17/17) displayed no or low HIF1α expression (Table 7).

Table 7. HIF1α immunohistochemistry

Tissue	*No. of samples*	*Scoring 0*	*1*	*2*	*3*	*no result*
HLRCC tumors						
RCC	11	1*/11	2/11	1/11	7/11	
ULM	12	1/12	3/12	2/12	6/12	
ULMS	1		1/1			
Total (%)	*24*	*2/24 (8.0)*	*6/24 (25)*	*3/24 (12.5)*	*13/24 (54)*	
Non-familial ULM (%)	4		1/4 (25)	2/4 (50)	1/4 (25)	
Pheochromocytomas						
SDHD	1		1			
NF	2		2			
VHL	1		1			
RET	3		3			
Total (%)	*7*	*0/7 (0)*	*7/7 (100)*			
Non-familial** (%)	10	2/10 (20.0)	8/10 (80.0)			
Paragangliomas						
SDHB	2				2	
SDHC	1			1		
SDHD***	59	3	10	16	29	1
Total (%)	*62*	*3/62 (4.8)*	*10/62 (16.1)*	*17/62 (27.4)*	*31/62 (50.0)*	*1/62 (1.6)*
PGL2**** (%)	3		2/3 (66.7)		1/3 (33.3)	
Non-familial** (%)	35	4/35 (11.4)	6/35 (17.1)	11/35 (31.4)	14/35 (40)	

HIF1α staining scoring: 0 = negative, 1 = 1-10% of cells positive, 2 = 10-50%, 3 = >50%. *Necrotic tissue. **No SDHB/C/D mutation/not analyzed. ***Including one tumor originating from the Zuckerkandl's organ. ****Tumors linked to the putative *PGL2* gene locus (Maher and Eng, 2002).

MSH2 protein expression by IHC was analyzed from the same set of samples. The HLRCC samples and the samples on the TMA were analyzed with 96%, and 85% success, respectively. In the remainder of the PGLs/PHEOs, absence of tumor tissue in the specimen, negative staining in both normal and tumor tissue, and detaching of the specimen were used as exclusion criteria. The negative staining in the normal tissue probably resulted from technical issues or a low proliferation rate, typical for this tissue. All the analyzable specimens showed normal positive MSH2 staining in the tumor.

Microsatellite instability in the HLRCC tumors (10 RCCs, 12 ULMs, and 1 ULMS) as well as in 27 PGLs (18 *SDHD*, 2 *PGL2*, and 7 non-familial) was studied with the Bethesda panel microsatellite markers with an 88% success rate. All these tumors were microsatellite stable.

5.2.2. Characteristics of the alternative transcript of FH (FHv) (IV)

5.2.2.1. Structure and mutation status of the FHv transcript

An AceView gene database (http://ncbi.nih.gov/IEB/Research/Acembly/index.html) first released a 414 bp *FH* sequence (GenBank accession no. AI971779.1) containing exon 2, a part of exon 3, and an upstream sequence (exon 1b) that mapped the intronic region between exons 1 and 2. When the transcript, named *FHv*, was amplified, it was in full length found to contain exon 1b, which replaces exon 1 encoding the mitochondrial signal peptide of *FH*, and exons 2 to 10 identical to *FH*.

In silico methods were utilized to search the *FH* variant sequence for a 5' extension of exon 1b, CpG island, core promoter, evolutionary conserved transcription factor binding sites such as heat or hypoxia response elements, or nuclear localization signal with negative results. A transcript including exon 1b was not found in other species, but a similar kind of variant lacking the first exon encoding the mitochondrial signal peptide was found in another gene of TCA cycle, *SDHB* (http://vega.sanger.ac.uk/index.html).

A set of human DNA samples (n=139) was screened for mutations in exon 1b. The mutation analysis revealed no alterations in exon 1b or its flanking sequences.

5.2.2.2. Increased expression of FHv by prolonged hypoxia and heat shock

FHv was shown to be widely expressed in fetal and adult human tissues (tissue cDNA panels). However, the expression of *FHv* was clearly lower compared to mitochondrial *FH*, the average difference being approximately 300-fold.

The effect of different stress conditions on the expression of *FHv* was studied with HEK293 and HeLa cells. The glucose and serum deprivation, H_2O_2 treatment, or hypoxia (1% O_2) for up to 48 hours had no detectable effect on endogenous *FHv* or *FH* mRNA expression levels. However, more prolonged (72 to 96 hours) hypoxia somewhat increased *FHv* expression. Therefore, 13 additional cell lines were analyzed in long-term

hypoxia. A similar trend was observed in approximately half of the other cell lines studied. Especially one cell line, a uterine leiomyosarcoma line HTB115, displayed a clear (four-fold) increase in *FHv* mRNA levels after 72-96 hours exposure compared to the untreated control cells. Subsequently, HTB115 cells were analyzed for *FHv* response at a temperature of 42°C. The heat shock of 0.5, 1, and 2 hours resulted in a 3-, 6-, and 9-fold increase in *FHv* expression, respectively (Figure 4). A two-fold and three-fold increase after two hours of exposure was observed in HEK293 and CRL2461 cells, the other cell lines analyzed. For reference, *FH* mRNA levels sustained approximately at a basal level during hypoxia and heat shock.

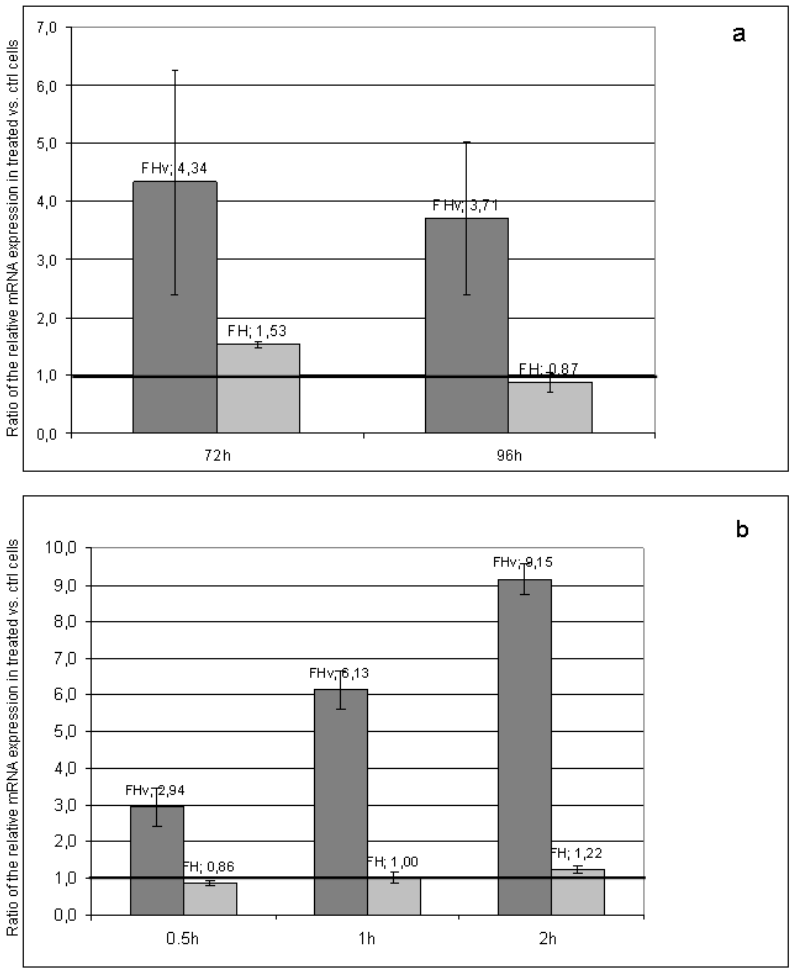

Figure 4. Relative (*beta-actin* normalized) mRNA expression of *FHv* (dark bars) and *FH* (light bars) in HTB115 cells after hypoxia (1% O_2) and heat shock (42°C). The results are shown as a ratio of the expression of stress treated cells and non-treated control cells (the ratio of 1 is obtained if there is no difference between treated and control samples); a) *FHv* mRNA expression increased 4-fold during 72-96 hours exposure in hypoxia when compared to the control cells; b) In heat stress, *FHv* expression increased during two hours exposure up to 9-fold. No significant changes were observed in the expression of *FH* in either condition. Since the expression level of *FHv* in general is much lower than of *FH* (in HTB115 cells over 100 times), the expression levels of *FHv* and *FH* were analyzed individually and thus are not in the figure comparable with each other.

5.2.2.3. Translation, subcellular distribution, and activity of FHv

To determine the *FHv* translation initiation site, different *FHv* cDNA constructs (pEGFP-based) were expressed in HEK293 cells. FHv-GFP resulted in three protein products in a Western blot, of which the middle one was the faintest. Because exon 1b does not contain any ATG, two in frame CTGs (in exon 1b) and three ATGs (two in exon 2 and one in exon 3) were considered as putative translation initiation sites (Figure 5). First, both the

CTGs were separately mutated to CCC (constructs $FHv_{ctg1ccc}$-GFP and $FHv_{ctg2ccc}$-GFP) and then to ATG ($FHv_{ctg1atg}$-GFP and $FHv_{ctg2atg}$-GFP) in order to respectively inhibit or enhance possible translation. With this setup, none of the three bands vanished after the mutation of the CTGs to CCCs. Moreover, when the CTGs were mutated to ATGs, additional bands with a higher molecular weight appeared. Therefore, the possibility of translation of *FHv* from 1b was excluded. Subsequently, when the three ATGs in exons 2 and 3 were separately mutated to CCCs ($FHv_{atg1ccc}$-GFP, $FHv_{atg2ccc}$-GFP, and $FHv_{atg3ccc}$-GFP), each of the three bands vanished one by one indicating the translation of *FHv* to be initiated from these codons. The reading frame thus remains the same as that of *FH*.

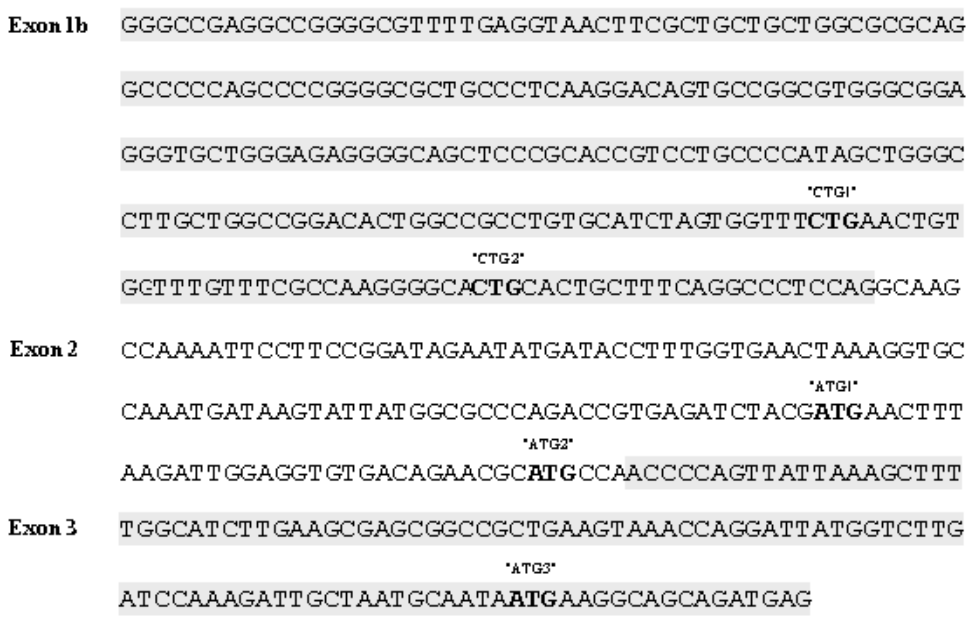

Figure 5. Sequence of *FHv* showing exons 1b, 2 and 3 (exons are distinguished from each other with the gray shading). The putative initiation codons are in bold and named referring to the terms used in the expression constructs. Nine bases upstream of "CTG1" is an inframe stop codon (TAG).

The subcellular distribution of the FHv protein was studied by expressing a GFP-tagged *FHv* construct in cells. FHv-GFP distributed in the nucleus and cytosol. No changes were seen in the localization when the three ATGs in exons 2 and 3 were separately inactivated. For reference, the construct lacking exon 1 was present only in the cytosol. Endogenous FH and FH-GFP localized to the mitochondria. In addition, cytosolic protein was also observed in a subset of cells expressing FH-GFP.

The FH enzyme activity of FHv was determined by an enzyme kinetics assay with a spectrophotometrical detection. The basal level of FH activity of the cells used (HEK293) was 36 nmol/min/mg of protein. Overexpression of FH (from pCi-neo vector) resulted in an increased activity up to 390 nmol/min/mg of protein. However, overexpression of FHv did not have any effect on the activity as it remained at the basal level (36 nmol/min/mg of protein).

6. DISCUSSION

6.1. Cancer risk and tumors associated with HLRCC (I)

Hereditary leiomyomatosis and renal cell cancer (HLRCC) is a tumor predisposition syndrome caused by heterozygous germline mutations in the tumor suppressor *fumarate hydratase*, *FH*. The most prominent feature of HLRCC is the leiomyomatosis of the skin and uterus, and approximately in every fifth family there are cases of renal cell cancer (Launonen et al., 2001, Tomlinson et al., 2002, Toro et al., 2003, Alam et al., 2003 & 2005b, Kiuru et al., 2005, Chan et al., 2005, Badeloe et al., 2006, Chuang et al., 2005 & 2006, Wei et al., 2006, Refae et al., 2007). The majority of RCC cases have been reported in North America and Finland. In the UK, which has several HLRCC families, only two RCC cases have been found. This number may, however, be somewhat biased as many families in the UK have been identified based on the leiomyomatosis phenotype. The malignant counterpart of ULM, uterine leiomyosarcoma (ULMS) in association with germline mutations in *FH* is detected only in the Finnish population (Launonen et al., 2001, Kiuru et al., 2002, Ylisaukko-oja et al., 2006a). Of note, the diagnosis criteria of ULMS have been become more stringent during the recent years. Therefore, in comparison with the calculated risk obtained in study I, a revised, lower risk estimate is presented in section 6.2.2. in the context of the histopathological features of uterine smooth muscle tumors.

Due to the reduced penetrance of the malignant tumors in HLRCC and their concentration on certain families and populations, it is possible that some tumors that are less central in the phenotype may not have been identified. Moreover, the actual risk of cancer has been remained unclear. Hence, we aimed to define the cancer risk, and to identify possible additional malignancies among *FH* mutation carriers utilizing the Finnish HLRCC/FHD families. The resources for hereditary cancer research are exceptional in Finland as clinicians are obliged to report all the cancer cases to the Finnish Cancer Registry, of which coverage and quality have been estimated to be very high (Teppo et al. 1994). Moreover, the population-based verified genealogy data in the Finnish Population Register Centre facilitates research of this type. Through the extensive search for members of the seven Finnish HLRCC families and the FHD kindred, altogether 868 individuals were identified. Of the 98 individuals analyzed for a germline *FH* mutation, 54 were found to be mutation carriers. Cancer was observed in 27 patients, of which four were affected with more than one malignancy.

The cancer risk in HLRCC/FHD family members was assessed from a sample of 256 individuals with the Finnish Cancer Registry based analysis. These kinds of analyses are typically sensitive to the ascertainment criteria of the families and selection bias. This was avoided by excluding all the cancers in the index patients and other individuals utilized in the HLRCC family identification from the analysis and by including only the completely traced generations. The analysis revealed 6.5-fold incidence (SIR, standardized incidence ratio) of RCC (95% CI; 2.1-15.0), being the first calculated estimate for cancer risk in HLRCC. All the 12 patients affected with RCC were *FH*

mutation carriers covering 22% (12/54) of all the individuals observed with a germline *FH* mutation. Typical of a hereditary tumor predisposition is that tumors arise at a young age. The age at RCC diagnosis varied between 26 and 90 years of age (average 47.5, median 40.5), whereas the average diagnosis age for RCC in the Finnish population is 64 in males and 69 in females (Pukkala et al., 2006; Finnish Cancer Registry 1994-2003 statistics). Of note, in the Finnish HLRCC families 75% (9/12) of the RCC cases were found in female mutation carriers. However, when the cases in other populations (24 males and 21 females; Toro et al., 2003, Alam et al, 2003 & 2005b, Chan et al., 2005, Chuang et al., 2005 & 2006, Wei et al., 2006, Badeloe et al., 2006, Refae et al., 2007, study II) are counted, the female distribution evens out to 53% (30/57). In comparison, in the general population RCC is seen with a 1.6 to 2 -fold male predominance (Finnish Cancer Registry; http://www.cancerregistry.fi/statistics, Wallen et al., 2007, Jemal et al., 2007). In the SIR analysis, ULMS was the only other tumor type in addition to RCC that obtained significant risk values. The incidence of ULMS was calculated as 71-fold but as based on a few tumors, the 95% confidence interval was quite broad (95% CI; 8.6-260) (see also 6.2.2.). Altogether five ULMS tumors were observed in the female mutation carriers (5/33, 18%) with diagnosis age between 27 and 39 years (average 33, median 32). The age of onset in these cases is very early, the median age for ULMS reported between 50 and 55 years (Major et al., 1993, Gadducci et al., 1996). The risk estimates for RCC and ULMS were, however, obtained from a high risk population and cannot therefore be directly conveyed to other populations.

All the available tumors from the *FH* mutation carriers were analyzed for biallelic inactivation of the gene. Eighty-three percent (10/12) of the RCCs displayed loss or a point mutation in the wild-type allele. Interestingly, all the breast cancers (3/3) and one bladder cancer (1/1) analyzed were detected with the loss of the wild-type allele indicating that they might result from a germline *FH* mutation. Moreover, according to genomic copy number analyses (array CGH) the loss of 1q (the *FH* locus at 1q43) is not a typical alteration in sporadic breast and bladder cancers (Sandberg, 2002, Miller et al., 2003, Blaveri et al., 2005, Mao et al., 2005, Climent et al., 2007, Chin et al., 2007). One malignant and two benign tumors of the breast and one bladder cancer have also been observed in *FH* mutation carriers in the UK and North America (Alam et al., 2005b, Wei et al., 2006). However, data on the biallelic inactivation of *FH* in these types of tumors have not previously been reported. The age at diagnosis of the breast cancers in the Finnish patients were on average somewhat younger (50, 53, 55, 61; median 52.5, average 55) than in general. On average Finnish breast cancer patients are diagnosed at 61 years of age (Pukkala et al., 2006; Finnish Cancer Registry statistics 1994-2003). Notable is that the diagnosis age of breast cancer has lowered significantly since population screenings by mammography started in 1987 (Pukkala et al., 2006).

In addition, several other tumor types were detected in the Finnish HLRCC patients, for example prostate cancer and hematological and lymphoid malignancies (Table 5, Appendix 1). In the *FH* germline mutation carriers in other populations a case of skin leiomyosarcoma, two adult Leydig cell tumor cases (with biallelic loss of *FH*), a malignant brain tumor, an ovarian cyst and gastric polyp (in one patient), Cushing

syndrome, and cerebral cavernomas have been reported in addition to the classic HLRCC manifestations (Toro et al., 2003, Alam et al., 2005b, Matyakhina et al., 2005, Carvajal-Carmona et al., 2006, Varol et al., 2007, Campione et al., 2007). Some extensive screenings have also been performed to study the role of *FH* in the genesis of different tumor types. The sample sets have included familial and sporadic tumors of the breast, prostate and ovaries (Kiuru 2002 & 2005, Bevan et al., 2003, Lehtonen 2003 & 2004, Ylisaukko-oja et al., 2006b). Moreover, sporadic bladder, testicular, lung, colorectal, and head and neck squamous cell carcinomas as well as melanomas and PHEOs have been evaluated for *FH* mutations (Lehtonen et al., 2004). In these screenings, two patients affected with ovarian cystadenoma were identified with germline *FH* coding region alterations (Ylisaukko-oja et al., 2006b). Otherwise only some *FH* polymorphisms were found. The role of *FH* in the tumorigenesis of sporadic CLM and ULM, and sarcomas including uterine tumors have been studied in several approaches but only a few ULMs and a soft tissue sarcoma of a lower limb have emerged with the somatic inactivation of *FH* (Kiuru et al., 2002, Barker et al., 2002, Lehtonen et al., 2004, Gross et al., 2004, Ylisaukko-oja et al., 2006a). Thus, *FH* mutations occasionally contribute to tumorigenesis of sporadic ULMs but have a limited role in the genesis of tumors other than the HLRCC spectrum. The analyses have, however, incidentally revealed *FH* germline carriers, including probands of the Finnish families FAM6 and FAM7 (with ULMS and CLM, respectively) as well as another patient with ULMS (Kiuru et al., 2002, Ylisaukko-oja et al., 2006a). In the recent ULMS case, however, allelic loss of *FH* in the tumor failed to be proven (Ylisaukko-oja et al., 2006a).

Previously in the heterozygous *FH* mutation carriers in FHD families only three cases of CLM have been reported (Tomlinson, et al., 2002, Maradin, 2006). In our analyses, the bladder cancer and one of the breast cancers with biallelic inactivation of *FH* were found in the FHD family. These results indicate potential for malignancies also in these families, although the most common tumor types (leiomyomas and RCC) were absent. The variety in the phenotypes, between HLRCC and FHD and even within HLRCC families in the issue of RCC, has been widely discussed. Since no clear genotype-phenotype correlation has been observed, the possibility of another genetic factor or factors modifying the morbidity risk has been considered as a strong option but no evidence supporting this has been attained so far.

Cutaneous or uterine leiomyomas were not assessed in this particular approach but some other benign tumors were identified, such as adrenal gland adenomas (n=4) and kidney cysts (n=14). The incidence of adrenal tumors seemed to be higher in HLRCC (found in 12% of the mutation carriers) compared to only 0.5-2% for the population in general (Barzon et al., 2003). Though kidney cysts are generally common, interestingly, the incidence of these lesions was higher in the *FH* mutation carriers than in the general population in regard to individuals under 40 years of age. The incidence was 36% in the mutation carriers vs. 4.6-8.2% (detection method ultrasound or computed tomography, respectively) in the general population (Terada et al., 2002, Carrim & Murchison, 2003). Moreover, some of the Finnish HLRCC patients' RCCs showed cystic features. Recently, kidney-specific *Fh1* knockout mice were shown to develop large and numerous kidney

cysts which finally led to a polyuric renal failure. The cyst formation was suggested to result from an increased cell proliferation due to activation of the hypoxia pathway (Pollard et al., 2007). Since both HLRCC and VHL patients display renal cysts, they have been postulated as premalignant lesions (Mandriota et al., 2002, Pollard et al., 2007, see also 2.2.2.). Consequently, the tumorigenic pathway in an FH-deficient kidney has been suggested to be comprised of a sequence from a simple cyst through a dysplastic cyst to a carcinoma. In this hypothesis, *FH* inactivation is an initiating event, and additional genetic events would take place along with the transformation process. In the *Fh1* knockout mice the lethal renal failure, however, disables the monitoring of possible renal cancer formation (Pollard et al., 2007).

6.2. Histopathological features of HLRCC-associated tumors (I, II, unpublished data)

Neoplasms are assessed with variable methods including evaluation of tumor size and site, as well as possible invasion. Moreover, histopathological classification (i.e. morphological features), as well as genetic determinants and specific protein expression patterns are utilized in the main objectives of tumor classification, the prediction of the clinical behavior of the tumor, prognosis, and responsiveness to therapies.

6.2.1. Renal tumors

Epithelial renal neoplasms include a variety of tumor types with different histopathological and genetic features (see also 2.2.). The distinct morphology of the renal tumors in the first HLRCC family was a central observation when the syndrome was discovered. The tumors were found with a papillary growth pattern, the cells were large with abundant cytoplasm and displayed a high Fuhrman nuclear grade (3 or 4) with large and prominent “owl-eye like” nucleoli (Launonen et al., 2001). Mostly the tumors resembled the type 2 papillary histology described by Delahunt and Eble (1997). These unique characteristics have subsequently been used in the identification of new HLRCC families, as in the case of the Finnish FAM2 (Launonen et al., 2001). Later on, four cases of renal collecting duct carcinomas (CDC) and one oncocytoma were also reported in the context of a germline *FH* mutation (Alam et al., 2003, Toro et al., 2003, Wei et al., 2006). In study II, the tumors of the young Spanish HLRCC patient with a bilateral RCC resembled only partly the previously observed HLRCC-associated RCCs. The right-side tumor had a cystic and papillary structure and large cells with abundant eosinophilic cytoplasm. However, the Fuhrman grade was low (2) and the nuclei were only medium-sized and the nucleoli small. The left-side tumor displayed tubular structure and clear cells with a solid and acinar pattern, and a low Fuhrman grade (2) with small nuclei and inconspicuous nucleoli. Curiously, the more exceptional left-side tumor displayed LOH at the *FH* locus and thus connected the conventional (clear cell) type RCC to a germline *FH* mutation. Biallelic inactivation of *FH* in the right-side tumor could not be detected with conventional sequencing or IHC.

Recently, Merino and co-workers evaluated a set of North American HLRCC renal tumor material for their pathologic features. Of the 40 tumors, the majority displayed a papillary architecture and some showed a tubulo-papillary, tubular, solid, or mixed pattern. In a few cases, clear cell components were seen in association with papillary and solid elements. All the tumors were classified with a high Fuhrman grade. Subsequently, common to all the histological components, the presence of a large nucleus with a prominent eosinophilic nucleolus surrounded with a clear halo was seen (Merino et al., 2007). However, the cells of the both RCCs in the Spanish patient had inconspicuous nucleoli related to a low Fuhrman grade. To exclude the influence of possible underlying *VHL* loss on the tumor phenotype, mutation and LOH analysis at *VHL* locus was performed with negative results. The *FH* germline alteration found in the patient and the patient's mother was a novel missense mutation (N330S). The type or site of a mutation could be postulated to affect on the appearance of a tumor. CDC is also representing a minority type of RCC in HLRCC. However, at least in the case of CDCs, any specific *FH* mutation type does not seem to define the formation of this tumor type (Alam et al., 2003, Toro et al., 2003, Wei et al., 2006). Moreover, of the CDC-associated *FH* mutations, R58X has been seen in the formation of papillary RCCs as well (Wei et al., 2006).

The HLRCC-associated renal tumors are highly aggressive already as small lesions compared to the types in other hereditary syndromes. Thus, it is relevant to find clear identifiers for HLRCC-associated renal tumors and to make pathologists aware of this entity (Grubb et al., 2007). No clear immunophenotype has been established for the HLRCC tumors, but absence of CK7 and UEA-1 expression has been proposed as one alternative for facilitating the differential diagnosis (Merino et al., 2007). In our experience, at least the lack of CK7 expression is common to the Finnish HLRCC renal tumors and also to the Spanish patient's tumors with the divergent morphological appearance. Moreover, utilization of FH IHC in the differentiating diagnosis of early-onset RCC is a potential alternative as seen in our study (II). The aggressive phenotype also elicits the importance of establishing optimal strategies for the resection of the tumors, follow-up, and screening the *FH* mutation carriers (Grubb et al., 2007).

6.2.2. Uterine tumors

Smooth muscle tumors (SMTs) include cutaneous, vascular, peripheral soft tissue, gastrointestinal, and uterine tumors among others (Miettinen & Fetsch, 2006). Uterine smooth muscle tumors (and other SMTs) can be roughly categorized as leiomyomas (ULM, benign) and leiomyosarcomas (ULMS, malignant) based on the locality of the tumor as well as on cytolocigal characteristics: the degree of nuclear atypia (mild, moderate or severe), mitotic activity, and coagulative necrosis. The ordinary types of tumors are quite simply defined. However, the overlapping features in ULMs and ULMSs, the subjectivity of the observations, and sometimes also differences in opinions and limited experience in some rarer cases may cause problems in the differential diagnosis of benign and malignant uterine tumors. Therefore, sometimes, if the clinical behavior of a tumor is truly difficult to predict, a category of tumors with "uncertain malignant potential" can be used (Kempson & Hendrickson, 1999, Hart, 2002).

An ordinary ULM is characterized by whorled bundles of elongated smooth-muscle cells that are closely packed so that the tumor appears to be more cellular. The nuclei of cells are elongated and uniform. Mitoses are absent or sparse (Robboy et al., 2000). In contrast, ULMSs generally have high levels of mitotic activity, hypercellularity, diffuse nuclear atypia, and coagulative tumor cell necrosis. Presently, the tumor necrosis alone, independent from the mitotic index or the degree of atypia, is considered to be sufficient for defining malignancy. If necrosis is absent, tumors combined with a significant (moderate or severe) atypia and ≥10 mitotic figures (MF)/10 high power fields (HPF) are considered ULMS (WHO Classification of Tumors 2003; Tumors of the Breast and Female Genital Organs) (Table 8). Typically ULMSs are also usually larger in size (>10 cm) than ULMs and have other disturbing cross-features (Hart, 2002).

Some histological variants of ULM, including mitotically active ULM, cellular ULM, and atypical ULM, can cause difficulties in diagnosis since they display features also typical of ULMS. Previously in ULMS diagnosis, emphasis has been on the mitotic counts so that any tumor with over 10 MF/10 HPF has been classified as malignant (Robboy et al., 2000). According to the recent citeria, when neither atypia nor coagulative necrosis is present, or when only mild atypia is present with no coagulative necrosis in a uterine tumor, the mitotic index can be as high as 10 MF/10 HPF for defining the tumor as an ULM. Even higher mitotic indexes can be acceptable, but >15 mitoses/10 HPF are suggested to be classified with definitions of a limited experience (WHO Classification of Tumors 2003; Tumors of the Breast and Female Genital Organs) or even with an uncertain malignant potential (Miettinen & Fetch, 2006). The cellular ULM accounts for less than 5% of ULMs and is characterized by a significantly greater cellularity compared to the surrounding myometrium (WHO Classification of Tumors 2003; Tumors of the Breast and Female Genital Organs). The atypia (nuclear enlargement, pleomorphism, hyperchromatism) is probably the most subjective feature to qualify. ULMs with atypical features have stricter limits for mitoses, the number of which still divides opinions. Tumors with <10 MF/10 HPF and significant atypia but without coagulative necrosis are, according to certain criteria, considered as atypical ULMs with a low (2-3%) risk of recurrence (Bell et al., 1994, WHO Classification of Tumors 2003; Tumors of the Breast and Female Genital Organs) and, according to those supporting the older criteria, tumors with the same characteristics and even with <5 MF/10 HPF should be considered as having uncertain malignant potential, and tumors with >5 MF/10 HPF as potentially malignant (Miettinen & Fetsch, 2006) (Table 8). Of note, this group of tumors, presently often classified as “atypical ULMs” (or bizarre, symplastic or pleomorphic ULMs), was previously termed as *in situ* ULMSs (Hart, 2002).

In addition to these features of smooth muscle tumors, sometimes myxoid or epitheloid changes can be seen in both ULMSs and ULMs. To determine the malignant potential, these types of tumors should be especially carefully evaluated. Curiously, benign ULMs rarely display also unusual growth patterns including intravascular, disseminating, and metastasizing growth (Robboy et al., 2000, WHO Classification of Tumors 2003; Tumors of the Breast and Female Genital Organs).

Table 8. Histological diagnostic criteria of uterine smooth muscle tumors according to the WHO Classification of Tumors, 2003 (Tumors of the Breast and Female Genital Organs)

Necrosis, atypia	*Mitoses/ 10 HPF*	*Diagnosis*	*Notes*
No coagulative necrosis, no or mild atypia	≤15	ULM	
No coagulative necrosis, no or mild atypia	>15	mitotically active ULM with limited experience	Some experts prefer designating tumors in this category with >15 MF/10 HPF as "a tumor of uncertain malignant potential" (Miettinen & Fetch, 2006)
No coagulative necrosis, diffuse and significant atypia*	<10	atypical ULM with low risk of recurrence	Some experts prefer designating tumors in this category with 2-9 MF/ 10 HPF at least "a tumor of uncertain malignant potential" (Miettinen & Fetch, 2006)
No coagulative necrosis, diffuse and significant atypia	≥10	ULMS	
Coagulative necrosis	any	ULMS	

* "leiomyoma with limited experience" can also be used for defining a tumor with significant but focal atypia

ULM is the most common gynecological tumor in women. In contrast, the incidence of ULMS is very low, and in a preoperative diagnosis of ULM, ULMS is found in about 0.5% of cases (Hart, 2002). Since the malignant uterine smooth muscle tumors are rare, the knowledge about the clinical behavior of these tumors is still somewhat limited and as delineated above, certain definitions are still divergent (Table 8). Anyhow, the direction during the years has been towards more stringent ULMS diagnosis criteria. In Finnish population altogether six ULMS cases have been reported in the context of *FH* germline mutation. Five of the ULMS patients are members of the HLRCC families (FAM1, FAM4, FAM6; study I) and one was found in the screening of early-onset ULMSs by Ylisaukko-oja and co-workers (2007a). Originally, in the case of the five ULMSs in the Finnish HLRCC families, atypia or increased mitotic activity had been according to the pathology reports (the number of mitotic figures rarely stated in the pathology reports) interpreted as indicative for malignancy and had been reported to the Finnish Cancer Registry as ULMSs. The ULMS diagnosis was assessed and confirmed from most of the tumor specimens at the time when the patients were diagnosed as *FH* mutation carriers (Launonen et al., 2001, Kiuru et al., 2002 & 2005, Ylisaukko-oja et al., 2006a).

Although the Finnish HLRCC families are clearly at a high risk of developing *FH* mutation-associated malignancies seen as a high incidence of RCC, the complete lack of ULMS cases in other populations has been somewhat surprising. Hence, we have now re-evaluated the histology of five out of the six Finnish ULMS cases in light of the latest World Health Organization (WHO) diagnosis guidelines (WHO Classification of Tumors 2003; Tumors of the Breast and Female Genital Organs). A tumor from the other ULMS patient of FAM1 (from year 1966) was not available for re-evaluation. The remaining four cases from the families were diagnosed between years 1975-1996. With the current criteria, the four tumors were now considered by a pathologist as atypical or proliferative ULMs (with a low risk of recurrence) (for details, see Appendix 1). Two of

the tumors were independently re-evaluated by two pathologists according to the current guidelines, and still the interpretations varied in the case of one of the tumors as being “an atypical ULM as worst” or “a borderline case”. True ULMSs are highly malignant and recurrence is mostly detected within two years. Overall five-year survival rates range from 15% to 25%, varying historically largely due to the use of different criteria for their diagnosis (WHO Classification of Tumors 2003; Tumors of the Breast and Female Genital Organs). No recurrence or metastases have been observed in the five members of the families originally considered as affected with ULMS. All of the patients, except one, were alive after several years (9, 25, 25 and, 28 years) from the point of ULMS diagnosis (based on Cancer Registry data of year 2003). The patient from whom no tumor block was available for re-evaluation died from the RCC. The sixth Finnish ULMS patient with a germline *FH* mutation found in the screening of early-onset ULMSs died of a metastatic disease 12 years after the diagnosis of the primary tumor (Ylisaukko-oja et al., 2006a, Ylisaukko-oja, 2007). The histopathological features of the tumor were also indicative for ULMS.

In conclusion, while the high relative risk obtained for ULMS is adequately determined based on the Cancer Registry data, the true malignant potential of the uterine tumors in HLRCC is likely to be much less dramatic. Of note, besides these four atypical ULMs, five additional patients with atypical ULMs were identified in studies I and II. Furthermore, two cases have been reported in North America in the context of HLRCC (Toro et al., 2003). Interestingly, because atypia (or mitotic activity) is not that common in ULMs in general (Docent J. Arola, personal communication), the unusual features in the tumors seem to be indeed characteristic of HLRCC, as strongly suggested by our Cancer Registry data analysis.

Since ULMSs are highly malignant, it is important to identify markers that would facilitate the differentiating diagnosis of benign and malignant tumors. For this purpose, the role of DNA ploidy, AgNOR counts, IHC for PCNA, MIB-1 (Ki-67), p53, estrogen receptor (ER), progesterone receptor (PR), and ERBB2 has been studied in uterine tumors (unrelated to HLRCC) (Layfield et al., 2000). The proposed indicators for ULMS, which have consistently been seen in two independent studies, include increased MIB-1 and p53 expression and loss of PR (Mittal & Demopoulos, 2001). Karyotypically ULMSs are much more complex than ULMs and commonly no unifying aberrations have been observed between these tumor types. However, recently, a set of cellular and atypical ULMs were shown, similarly to ULMS, to display loss of the short arm of chromosome 1. Hence, these variant types of ULMs were proposed to possibly represent a premalignant stage and have an increased malignant potential (Hodge & Morton, 2007).

6.3. Role of hypoxia and genetic instability in TCA cycle-deficient tumors (III)

Fast growing cancers are often deficient of oxygen and nutrients until the blood supply is adjusted to the altered situation. Transcriptional adaptation to hypoxia is mediated by

hypoxia-inducible factor HIF1, which drives e.g. the transcription of *VEGF*, thus supporting the neovascularisation and blood supply of a tumor. Typical of cancer cells is also genetic instability, which was recently suggested to result from hypoxia-caused defects in mismatch (MMR) and recombinational (HR) genes (Mihaylova et al., 2003, Koshiji et al., 2005, Shahrzad et al., 2005, Bindra et al., 2005a, Bindra & Glazer 2007a, see also 2.1.2.4.). The MMR proteins that were proposed to be down-regulated by hypoxia included, with some variability between studies, MLH1, PMS2, MSH2, and MSH6 (Mihaylova et al., 2003, Koshiji et al., 2005, Shahrzad et al., 2005). This evidence has led us to hypothesize whether pseudohypoxia resulting from a deficient TCA cycle (Selak et al., 2005, Isaacs et al., 2005, Pollard et al., 2005b) could cause genetic instability. Thus, HLRCC and HPGL tumor material was studied for stabilization of HIF1α and down-regulation of MMR as well as for microsatellite instability (MSI). Of the suggested MMR proteins MSH2 was chosen for our study object. The previous data on the direct association of HIF1α stabilization and MMR protein expression in clinical samples has been obtained from sporadic CRC tumor material (Koshiji et al., 2005).

6.3.1. HIF1α

In the studied samples, 67% and 79% of the HLRCC tumors and *SDHB/C/D* mutation-associated PGLs, respectively, were found with a moderate or high level stabilization of HIF1α. None of the PHEOs (including one *SDHD* tumor) showed stabilization of the protein. An alternative pathway in the genesis of the remaining HIF1α negative PGLs/PHEOs could have been the HIF1-independent nerve growth factor (NGF) pathway. A defect in this route causes a failure in the developmental apoptosis of neuronal precursor cells, thereby retaining cell populations with a neoplastic capability (Lee et al., 2005, see also 2.4.).

Somewhat surprisingly, the majority (3/4, 75%) of the studied non-familial control ULMs displayed also significant levels of HIF1α. In previous studies, HIF1α has been detected with IHC strongly or moderately in HLRCC-associated RCCs and ULMs, respectively, and altered levels of HIF1α targets (e.g. *VEGF*, *TSP1*, *BNIP1*) and increased microvessel density have been seen in HLRCC-associated ULMs compared to sporadic ones (Pollard et al., 2005a & 2005b). Previous data on HIF1α in sporadic ULMs, however, could not be found. The number of control ULMs in our study was nonetheless quite small, and thus no far-reaching conclusions can be drawn. Similarly in our study, many non-familial PGLs (25/35, 71%) also showed stabilization of HIF1α. Some patients with apparently sporadic PGLs have been shown to harbor germline mutations in *SDHB* or *SDHD* (Neumann et al., 2002), but the non-familial PGLs on the tissue microarray (TMA) were screened for *SDHD* mutations with negative results. A small number of non-familial PGLs with up-regulated HIF1α could still be explained with an underlying germline *SDHB* mutation, which has been seen in 6.4% of apparently sporadic PHEOs/PGLs (Jimenez et al., 2006). Somatic mutations of *SDHB* have been suggested to also play a role in the tumorigenesis of truly sporadic PHEOs/PGLs, but only one case has been reported so far (van Nederveen et al., 2007). Of note, the carotid body, one of the most common origins of a PGL tumor, is an oxygen sensing organ. Hyperplasia of the carotid body and

a higher incidence of PGLs are more often seen in people living in high altitudes or suffering from certain medical conditions resulting in hypoxemia, such as chronic obstructive pulmonary disease (Saldana et al., 1973, Chedid & Jao, 1974, Pacheco-Ojeda et al., 1988). Moreover, expression profiles of some sporadic PGLs/PHEOs have been shown to display hypoxia markers similarly with *VHL* and *SDHB/D* tumors as well as expression of angiogenic proteins, such as VEGF (Jyung et al., 2000, Salmenkivi et al., 2003, Dahia et al., 2005). Hence, it is possible that the activation of HIF pathways due to real hypoxia is associated with PGLs in general, thereby explaining the HIF1α protein in the sporadic PGLs of our study. Of note, the response to hypoxic conditions and the activation of hypoxia pathways due to genetic alterations are not mutually exclusive but can in combination result in an enhanced hypoxia response (Semenza, 2003).

6.3.2. Mismatch repair and microsatellite instability

Despite the majority of the studied samples showing increased HIF1α levels, no MSI or reduction of MSH2 protein was detected in any of the TCA cycle-deficient or non-familial tumors. The presence of normal p53 has been suggested to be an essential factor in the HIF1α-driven down-regulation of MSH2 and subsequent MSI (Koshiji et al., 2005, To et al., 2005). Lack of p53, however, was not considered to be the cause of the negative result of our study, since the majority of the HLRCC-associated RCCs had been analyzed for *p53* mutations and p53 protein expression revealing no abnormality. Studies showing some association of increased immunoreactivity of p53 and/or *p53* mutations with tumorigenesis of (adrenal) PHEOs have been reported, mainly linking inactive p53 to the malignant outcome of a tumor (Yoshimoto et al., 1998, de Krijger et al., 1999, Gupta et al., 2000). In our study material, only three PGL tumors and none of PHEOs were malignant. Moreover, another study excluded the involvement of p53 mutations in the tumorigenesis of hereditary and sporadic PGLs. The results of the study showed an increased immunoreactivity of p53 in ~35% of the studied specimens, including both *SDHD* and sporadic tumors. The increased immunoreactivity of p53 is often seen in tumors with a *p53* mutation as the mutant p53 has a longer half-life. However, since no *p53* mutations were found in the tumors, the IHC result was suggested to be a consequence of hypoxic induction of a wild-type p53 protein rather than stabilization of a mutant p53 (van Nederveen et al., 2003). Thus, the lack of p53 as a cause of the absence of defective MMR in HIF1α-positive PLGs can be excluded. The requirement of p53 for the effect has anyhow become questionable, since the hypoxia induced repression of MMR has also been seen in cell lines deficient for p53 (Bindra & Glazer, 2007a, Bindra et al., 2007).

Previously, expression of MMR proteins and genetic instability has been examined in sporadic tumors of the HLRCC and HPGL spectrum as well as PHEOs of a MEN2 background. In one study, benign MEN2A-associated PHEOs, and benign and malignant sporadic PHEOs were assessed for the MSI analysis of intronic sequences in some tumor suppressor genes (TSG; *p53*, *RB*, *WT1*, and *NF1*) as well as for sequence and IHC analyses of MLH1 and MSH2 (Blanes et al., 2006). The results showed a lower incidence of microsatellite lesions and down-regulation of MMR in locally invasive and benign

tumors suggesting MSI to be mainly involved in the tumorigenesis of malignant PHEOs. The association of hypoxia to the down-regulation of MMR was not witnessed in this study since the reduction of the proteins was seen in the peripheral and not in the central compartment of the tumors, in which hypoxia is more pronounced (Blanes et al., 2006). Moreover, in another study of benign familial (including one *SDHB* tumor) and sporadic PHEOs, the Bethesda MSI marker set showed no alterations in the studied microsatellites (Namour et al., 2006). Similar studies on PGLs have not been reported. The few studies on MSI in uterine tumors have shown instability in 30% ULMs (the number of microsatellite lesions was not available) and low instability in 18% of ULMSs (in one out of seven microsatellite markers) (French et al., 1998, Amant et al., 2001). In renal cancer, repression of mainly MSH3 and MLH1 has been occasionally observed (Leach et al., 2002, Deguchi et al., 2003). Some RCC cell lines seem to display MSI but renal tumors are usually microsatellite stable (Diakoumis et al., 1998, Kanomata et al., 1998, Cullinane et al., 2004). Thus, MSI and the down-regulation of MMR do not seem to be strongly associated with the studied tumor types in general. As the inherited MMR defect and predisposition to genetic instability leads to a restricted spectrum of tumors in HNPCC (Sieber et al., 2005), some kind of tissue or cell type-specific factor may play a role in the context of hypoxia-induced genetic instability as well.

The hypothesis of a HIF1α mediated effect of hypoxia on MSH2 function (Koshiji et al., 2005) has served as a model for our study on the influence of HIF1α in TCA cycle-deficient tumors. The study by Koshiji and co-workers suggested a mechanism in which HIF1α displaces the transcriptional activator c-MYC from *MSH2* promoter (Koshiji et al., 2005). However, observations suggesting repression of MMR (both MSH2 and MLH1) under hypoxia takes also place in the absence of HIF1α (in HIF1α deficient cells) have recently gained evidence (Mihaylova et al., 2003, Bindra & Glazer, 2007a). In the light of these results, the arrant HIF1α stabilization (pseudohypoxia) in the TCA cycle-deficient tumors might not have been capable of resulting in down-regulation of MMR. However, if postulating that the HIF1α observed in the non-familial PGLs could be due to real hypoxia, down-regulation of the MMR system could actually have been seen in them. Consequently, the presence of real hypoxia in the *SDHB/C/D* PGLs must also be considered. Although the recent evidence suggests co-repression of MSH2 and MLH1, the role of MLH1 has now been highlighted since the decrease of the MLH1 protein appears to be greater than that of MSH2 (Mihaylova et al., 2003, Bindra & Glazer, 2007a). Although a possible reduction in the MLH1 protein was not assessed in our material, absence of MSI in all the tumors was nevertheless evident.

The functional consequences of the hypoxia-driven MMR defect have been studied by MSI analysis or by analyzing the mutation status of a reporter gene in cell lines cultured in hypoxia (Mihaylova et al., 2003, Koshiji et al., 2005). Moreover, accumulation of K-ras mutations has been observed in cells cultured under hypoxic conditions (Shahrzad et al., 2005). Yet the *in vivo* evidence of the link between hypoxia, down-regulation of MMR, and subsequent consequences has remained light. Bindra and co-workers discussed in their recent review article the discrepancies between different studies related to the lack of *MLH1* reduction in one study by Koshiji and co-workers (2005) (Bindra et al., 2007). As

a possible explanation was suggested the different levels of hypoxia used; 1.0% and 0.01% O_2 in the study by Koshiji and co-workers (2005) and Mihaylova and co-workers (2003), respectively, thus proposing that the repression of MLH1 requires more severe hypoxia (Bindra et al., 2007). In normal tissue the oxygen tension varies being 50 mmHg on average which corresponds to 7% of O_2, whereas the mean tension of 10 mmHg corresponding to 1.5% of O_2 is seen in tumors. In a hypoxic tumor the tension can be zero (Greijer et al., 2005). Hence, it seems that *in vivo* a tumor should be almost anoxic at least to repress MLH1.

In conclusion, our results suggest that HIF1α is not sufficient to cause MSI and the down-regulation of MSH2 in TCA cycle-deficient tumors and non-syndromic PGLs. Therefore more data is required to determine the possible tissue specificity and other factors mediating the hypoxic effect, the differences in the significance of different MMR proteins, as well as the severity of hypoxia required for the repression of MMR, especially *in vivo*.

6.4. Characteristics of the alternative transcript of FH (FHv) (IV)

The function of each protein takes place in a certain subcellular compartment or compartments. This distribution is orchestrated by targeting signals (such as mitochondrial, nuclear, endoplasmic reticulum), specific to each compartment, as part of the protein peptides. The removal of the targeting signals is a mechanism to alter protein localization or even function. The differentially distributed protein isoforms can be encoded by different genes but can also be produced from a single gene by an alternative transcription or translation initiation, splicing, or post-translational modification (Danpure, 1995). The implication of these phenomena is the allowance of a greater diversity of gene products without enlargement of the genome. The alternative forms can be utilized in the regulation of protein function for example in the different stages of development, different cells and tissues, and in the response to internal and external signals.

6.4.1. Transcript and protein characteristics of FHv

FH operates in the mitochondrial TCA cycle and is targeted to the mitochondria by an N-terminal targeting signal, encoded by exon 1. The variant transcript of FH (*FHv*), part of which was originally described in the Aceview database, was found to contain exons 2-10 identical to *FH* and an alternative first exon (1b). The exon 1b of 245 bp in length mapped to the sequence of *FH* intron 1-2. The transcript was postulated to be produced by an alternative promoter, but no sequence suggestive of a core promoter in the 1b and near flanking sequences, however, could be found with *in silico* tools. Nonetheless, the existence of a further localizing promoter cannot be excluded. The alternative promoter usage results in transcripts with differing N-terminal parts, either varying only in the 5' UTR or also at the protein level (Landry et al., 2003, Hughes, 2006). In the case of FH, the

protein produced from the alternative transcript is different as it lacks exon 1 and is thus devoid of the mitochondrial targeting signal.

The eucaryotic mRNAs, in most cases, are translated from the first ATG (AUG) at the 5' end of the mRNA. This is based on the scanning model which postulates that the 40S ribosomal subunit runs along the 5' end of the mRNA until it reaches the first ATG (Kozak, 1995). The translation of FHv was studied with an overexpression construct which revealed in an immunoblotting three peptides of a closely same size. Exon 1b does not contain any ATGs that function as translation initiation codons but two CTGs (CUGs) which also can serve as initiation sites (Packham et al., 1997, Meiron et al., 2001). The subsequent first three ATGs are located in the following exons: two in exon 2, and one in exon 3. Mutating the CTGs and ATGs one by one, the three peptides were found to be produced from the ATGs in exons 2 and 3, and exon 1b thus to be 5' UTR. The predicted sizes of the peptides were 46.4, 45.2, and 41.7 kDa, the middle one seen to be produced most inadequately (in comparison, processed FH without the signal peptide is 50.2 kDa). Whether the initiation from multiple ATGs is an artifact or rational phenomenon is not known, but may be due to leaky ribosomal scanning (LRS). For efficient translation, an optimal sequence flanking an ATG is 5'-CCA/GCCATGG-3' of which a purine at the position -3 and G at the position +4 are the most important ones (Kozak, 1987). It has been shown that if the sequence surrounding the first ATG is compromised, LRS can occur leading to translation from both the imperfect first codon and additional downstream ATG(s) (Smith et al., 2005). In *FHv*, the second ATG in exon 2 ("ATG2") lacks an optimal flanking sequence whereas the first ATG in the exon 2 ("ATG1") and the first ATG in exon 3 ("ATG3") display a relatively favorable sequence context (nucleotide A at the positions -3 and +4, representing the third common flanking sequences of a functional initiator) (Kozak, 1987) (Figure 5). In addition to the poor ATG context, short and GC rich 5' UTR, and downstream secondary structure have been suggested to lead to LRS. Close adherence of the ATGs also has a strong influence but even 100 nucleotides spacing is possible (Kozak, 1991 & 1995). However, whether lack of G in the position +4 of the "ATG1" and quite close adherence of the ATGs could cause LRS and the translation also from the downstream initiators remains to be determined.

Interestingly, when the subcellular distribution of FHv was studied, the GFP-tagged FHv protein(s) were found to target the nucleus and cytosol. As expected, FH, both endogenously and as GFP-tagged was observed mainly in the mitochondria and to a lesser extent in the cytosol. No nuclear targeting signal in *FHv* was found with *in silico* modeling, but alternatively the nuclear localization can sometimes be determined by a post-translational glycosylation of the peptide (Rondanino et al., 2003, Guinez et al., 2004). The localization to the nucleus seemed to be specific to FHv since the protein produced from the cDNA construct of *FH* exons 2-10 (artificial ATG as an initiator) ($FH_{no\text{-}signal}$) remained in the cytosol. Moreover, the nuclear distribution of FH has also been seen in a previous study on endogenous fumarase of rat (Bowes et al., 2007). The cytosol is the "default" location of any protein and therefore it is not sure whether the putative FHv targeting signal to the nucleus is somehow inadequate to transfer all the protein or whether the FHv could also have a function in the cytosol. No difference in

the distribution was observed when translation from the three ATGs was inhibited in turn. In general, the extra-mitochondrial distribution of primarily mitochondrial proteins is not uncommon (Mueller et al., 2004).

The overexpression of FHv in cells showed no increase in FH enzyme activity. The FHv peptide lacks at least 32 N-terminal amino acids that are present in the processed (mature) FH. These amino acids may be essential for formation of the FH tetramer or catalytic capability of the protein, or both. The function of FHv, however, may be completely different from that of FH, and the enzymatic activity is thus unnecessary.

6.4.2. mRNA expression of FHv

The translation of FHv was studied with overexpression constructs since an FHv-specific antibody was not available for immunoblotting and immunofluorescence analyses. Some endogenous bands in addition to FH (~50 kDa) were detected in the FH immunoblotting of cell lines but evidence that some of those would have represented the endogenous FHv could not be obtained. mRNA expression, however, was analyzed from human tissue cDNA panels and different (mainly cancer) cell lines. *FHv* was ubiquitously expressed in the tissues and cell lines studied. The expression of *FHv* was evident both in the fetal and adult tissues but was somewhat lower (as was of *FH*) in fetal tissues. This suggests *FHv* is not specifically involved in developmental processes as sometimes is the case in the usage of alternative transcripts (Ayoubi & van de Ven, 1996). However, the expression of *FHv* was hundreds of times lower compared to *FH*. Alternative promoters or splicing can also be utilized when a tissue specific expression or response to a certain external stimulus is required (Ayoubi & van de Ven, 1996). Since cellular stresses have been shown to induce, for example, alternative splicing of several genes (e.g. *VEGF* and *COX1*), the induction of *FHv* was tested in various stress conditions. Neither glucose nor serum deprivation, nor hydrogen peroxide had any effect on *FHv* expression; nor did short-term hypoxia. When hypoxic (1% O_2) exposure was prolonged to 72-96 hours, an increase in *FHv* expression was noted in several cell lines (9 out of 15). The most evident response (a four-fold increase) was observed in a uterine leiomyosarcoma cell line (HTB115) suggestive of some cell or tumor type specificity in the expression of *FHv*. Subsequently the stress treatment range for this cell line was extended to heat exposure (42°C) which resulted in a gradual nine-fold increase in *FHv* in two hours compared to the untreated control cells. No major changes were observed in the expression of *FH* in either condition, although down-regulation of this factor central to aerobic metabolism could have been conceivable.

An elevated temperature, causing acute or chronic stress in cells, can induce the so-called heat shock response, which includes activation of heat shock factors (HSFs). HSFs drive the transcription and synthesis of proteins of the heat shock family (HSPs), as well as several other proteins (Pirkkala et al., 2001, Sonna et al., 2002, Baird et al., 2006). HSPs are involved in the folding of proteins, regulation of the cellular redox state, and in protein turnover, events which are related to the survival of the cell or induction of apoptosis if the stress becomes too severe (Sonna et al., 2002). In addition to heat, hypoxia is also

known to result in the heat shock response, as in the case of induction of HSP70 and downstream pathways in chronic hypoxia (Fei et al., 2007). Moreover, hypoxia-induced HIF1 has been shown to regulate HSF and activate the heat shock pathway (Baird et al., 2006). On the other hand, heat induction of HIF1α has been suggested to be mediated by HSP90 (Katschinski et al., 2002). Heat and hypoxia pathways are thus connected. Our results suggest that *FHv* could be regulated by stress-induced factors and be related to, for example, stress-induced apoptosis. Long-term hypoxia especially was detrimental to the cells and some differences in the appearance (rounding, detaching from surface) between the studied cell lines was seen, though no clear correlation between the condition of the cells and the increase in *FHv* could be observed. Alternatively, FHv could play a part in the adaptation of a cell to a disadvantageous environment or have a role in anaerobic metabolism, for example. However, no evidence of heat shock (HSE) or hypoxia response elements (HRE) for HSF/HIF binding in *FHv* could be found with *in silico* tools. Interestingly, the rarer HSF1 function as a negative regulator of the RAS pathway does not necessitate actual binding to the DNA sequence (Chen et al., 1997). Moreover, at least in the case of hypoxia response, a variety of transcription factors other than HIF (e.g. NFκB and p53) are activated. The activation of different pathways in hypoxia is probably cell type-specific and dependent on the degree and length of the hypoxia (Cummins & Taylor, 2005).

In conclusion, the differential subcellular distribution and the lack of FH enzyme activity strongly suggest that FHv has a role distinct from the TCA cycle. The hypotheses of the function of FHv in the response to hypoxia and elevated temperature are tempting, although regulatory elements required for at least HIF or HSF binding were not found in the *FHv* sequence. Whether stabilization of HIF1α in FH-deficient tumors could enhance transcription of *FHv* is an intriguing idea as well. Somewhat disturbingly, mRNA expression of *FHv* was clearly lower compared to *FH*, and the endogenous FHv protein could not be verified with our analyses. Notable is that FH is one the most essential proteins needed in a cell and therefore transcribed and translated efficiently, thus proving a challenging comparison point. The *FHv* transcript was originally derived from ovarian tissue, which was not included in the cDNA tissue panel analyzed for *FHv* expression. Whether expression of FHv would be significantly higher in ovarian tissue than in the studied tissues remains to be determined. There are examples of tissue-specific alternative promoter usage, e.g. the 100-fold lower expression of α-amylase in the liver compared to the parotid gland (in this case, the alternative promoter produces transcripts differing only by the 5' UTR) (Shibler et al., 1983). Low expression of certain regulatory transcripts may indeed be expedient as it may be critical that their transcription and translation is kept at a low level (Wera et al., 1995, Kozak, 1996). Moreover, the existence of a similar, alternative exon 1 in the tumor suppressor *SDHB* supports the relevance of the variant *FH* transcript. Anyhow, hypotheses on function of FHv still need to be verified.

7. CONCLUSIONS AND FUTURE PROSPECTS

To clarify the clinical characteristics in *FH* germline mutation carriers, Finnish HLRCC/FHD families were broadly examined for the associated tumors and analyzed for cancer risk. The Cancer Registry risk analysis provided for the first time calculated risk values for renal cell cancer (RCC) and uterine leiomyosarcoma (ULMS). The analysis estimated the risk (standardized incidence ratio) in association of HLRCC/FHD to be 6.5- and 71-fold for RCC and ULMS, respectively, compared to that of the general population. In young individuals the risk was even more pronounced. However, if the most recent - more stringent - classification for ULMS is applied, it now seems likely that the patients with an *FH* mutation are prone to develop atypical or proliferative ULMs (with a low risk of recurrence), and formation of ULMS as currently defined is uncommon. Naturally, the risk estimate for RCC only reflects that of other populations. As a novel finding, the loss of *FH* was shown to possibly be involved in the genesis of breast and bladder cancer, though increased incidence of these tumors could not be proven with the Cancer Registry risk analysis. Moreover, kidney cysts were frequently detected in young *FH* mutation carriers. In the context of FH deficiency were for the first time observed malignant manifestations. The Spanish RCC case showed that the HLRCC-associated renal tumors can also include conventional (clear cell) type RCC. This data points to the possibility of *FH* mutation carriers among young patients with conventional cancer when other genetic factors (e.g. *VHL* mutation) do not seem to play a role. In these cases, a simple diagnosis method could be FH immunohistochemistry.

To clarify the molecular pathways in association of FH and SDH defects, the hypothesis of the role of hypoxia/HIF1α-induced repression of mismatch repair (MMR) was analyzed in HLRCC and HPGL tumor material. The analysis confirmed previous observations of HIF1α stabilization in these tumors. Increased HIF1α seen also in some sporadic paragangliomas (PGL) probably results from real hypoxia present in PGLs in general. However, any of the tumors displayed no repression of mismatch repair protein MSH2 or increased MSI. Thus, our results showed that at least HIF1α alone is not sufficient for causing MSI in these tumors and more data is required to determine the other required components as well as the implication of the pathway *in vivo*. The alternative transcript of *FH* (*FHv*) was found to contain instead of exon 1, an alternative exon 1b, which seemed to remain untranslated. Differential subcellular distribution, lack of FH enzyme activity, low mRNA expression compared to *FH*, and induction by cellular stress suggest that FHv has a role distinct from FH, for example in apoptosis or survival. However, the endogenous protein expression as well as the physiological significance of the variant requires further elucidation.

In summary, these studies suggested the association of novel tumor types and subtypes with loss of *FH*. The dynamic accumulation of the new data from clinical follow-up and genetic studies provides essential updates to the characteristics, classification, and prognosis of tumors. Awareness of these changes in the collection and analysis of tumor material for research purposes is also an important issue that has emerged from this

study. The evidence of inhibition of PHDs, factors of the degradation machinery of HIF1α/HIF2α, and up-regulation of the hypoxia pathway target genes by succinate and fumarate is strong. The actual implication of the stabilization of HIF1α on TCA cycle associated tumorigenesis is, however, still under investigation. The results presented here suggest that the putative tumorigenic effect of HIF1α in TCA cycle-deficient tumors does not involve increased genetic instability. Physiological significance of the alternative transcript of *FH* (*FHv*) remains obscure, but as being responsive to cellular stresses it would be intriguing to consider it operating in some tumorigenesis-associated pathway.

In the future, the research on TCA cycle-associated tumorigenesis will undoubtedly continue to elucidate the functional consequences of the loss of FH and SDH as well as search for possible therapeutics. Introduction of α-ketoglutarate derivatives has successfully been shown to restore normal PHD activity in FH- and SDH-deficient cells, suggesting a therapy possibility for the cancers associated with TCA cycle dysfunction (MacKenzie et al, 2007). Alternatively, since the energy production in TCA cycle-deficient cells seems to be dependent on the anaerobic pathways, inhibitors of glycolysis enzymes have been proposed as putative therapeutics agents. Weak inhibitors would minimally harm aerobic tissues but the tumor tissue dependent on glycolysis would be more severely affected (Catherino et al., 2007). A valuable model for testing the future drugs will be the recently developed kidney-specific *Fh1* knock-out mouse (Pollard et al., 2007). In general, advanced RCC has a limited response to drug treatments. The novel targeted therapies for RCC include molecules sorafenib and sunitinib which are broad-spectrum inhibitors of RTKs activated by HIF targets such as VEGF, PDGF, and TGFα (Grandinetti & Goldspiel, 2007). Thus, these novel therapeutic agents may also provide an alternative for the growth suppression of HLRCC renal tumors with an overactive HIF pathway.

Since HLRCC is a quite newly identified and rare syndrome, any international consensus on the patient surveillance has not yet been established. Only some provisional recommendations have been presented based on the present knowledge about the clinical outcome of HLRCC (Pithupakorn & Toro, 2006; http://www.geneclinics.org). The surveillance guidelines applied to Finnish HLRCC patients at present include the follow-up of uterine tumor formation and progression by gynecological examination and ultrasound annually to every two years (Dr K. Aittomäki, personal communication). Since RCCs in HLRCC seem to have a very aggressive nature, the frequent, at least biannual, screening of the kidneys is specifically emphasized. As being sensitive and safe, a contrast enhanced MRI is recommended as the primary method (Kujala et al., 2007; http://www.uroweb.org/publications/eau-abstracts-online/?AID=14154). However, to reach a comprehensive consensus for HLRCC patient surveillance practices, a more long-term experience is required.

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APPENDIX 1

FH mutation carriers of the Finnish HLRCC families and the FHD family. Number of all family members identified in each family is in parenthesis after the family ID.

Family/ Patient	*Sex**	*Skin lesions*	*Uterine tumors (age at diagnosis)*	*RCC (age at diagnosis)*	*Renal cysts*	*Other tumors (age at diagnosis)*
FAM1 (137)						
M1	F		Leiomyomas (44)			
M2	F		Leiomyomas (34)			Breast cancer (53)
M4	F	Yes	Leiomyomas (39) (atypical)		Yes	Adrenal gland adenoma (63)
M5	F	Yes	Leiomyosarcoma (39) / atypical/proliferative ULM**		Yes	Breast cancer (61) Myeloma (62) Non-Hodgkin's lymphoma (63)
M7	F		Leiomyomas (29)	Yes (42)	Yes	
M9	M (-57)					
M13	F	Yes		Yes (33)		
M16	F	Yes	Leiomyomas (33)			
M17	F		Leiomyomas (39)	Yes (39)		
M18	F		Leiomyomas (37)			Cancer of unknown origin (42)
M19	F		Leiomyosarcoma (35)**	Yes (35)		
M24	F		Leiomyoma (25)			
M25	M	Yes				
M26	F		Leiomyomas (44)			
M27	F		Leiomyomas (48)			Adrenal gland adenoma (61)
M31	F (-67)					
M33	F	Yes				
FAM2 (97)						
B1	F			Yes (33)	Yes	
B2	M			Yes (26)		
B3	F		Leiomyomas (32)			Chronic lymphatic leukemia (48) Adrenal gland adenoma (40)
B5	M					Prostate cancer (63) Adrenal gland adenoma (65)
B6	F (-85)					
B7	F		Leiomyomas (40)		Yes	
B10	F (-77)					Liver hemangioma (23)
B11	M (-84)					
FAM3 (59)						
C1	M	Yes				
C3	M	Yes				
C4	F (-74)				Yes	
C5	F	Yes	Leiomyomas (34) (atypia)	Yes (35)	Yes	
C6	M	Yes				

C8	F	Yes	Leiomyomas (27) (atypia)			
C9	F	Yes	Leiomyomas (20)			
C10	F	Yes				
FAM4 (208)						
D1	F	Yes	Leiomyomas Leiomyosarcoma (30) / atypical/proliferative ULM**			Breast cancer (55)
D3	M				Yes	Hodgkin's lymphoma (25)
D5	M					Salivary gland adenoma (54)
D6	F		Leiomyomas			Fibrocystic lesions of the breast
D7	M			Yes (68)		
D10	F		Leiomyomas Leiomyosarcoma (27) / atypical ULM**			
D0***	F		Leiomyoma (36) (atypia)			
FAM5 (37)						
E1	F	Yes (30)	Leiomyoma (45)	Yes (50)	Yes	
E2	F	Yes (39)	Leiomyomas (37)	Yes (36)		
E3	M			Yes (71)	Yes	
E4	M (-51)				Yes	
E7	M				Yes	Esophagus cancer (53)
E11	F			Yes (90)		Basal cell cancer (83)
E12***	F					Liver/bile duct cancer (82)
FAM6 (42)						
F1	F		Leiomyosarcoma (32) / atypical/proliferative ULM**			
FAM 7 (6)						
H1	M	Yes			Yes	
FAM8**** (282)						
G1	M (-51)					
G2	F (-57)					
G3	M				Yes	Bladder cancer (71)
G5	M					Basal cell cancer (70)
G6	F					Breast cancer (50)
Total of mutation carriers identified / total of family members identified 54/868						

ULM; uterine leiomyoma. *In parenthesis the year of birth of unaffected (CLM/ULM/RCC) individuals. **Tumors originally diagnosed as uterine leiomyosarcomas agree the most recent diagnosis criteria of atypical ULMs. Some tumors displayed in addition increased proliferation (mitotic activity). Tumor from patient M19 was not re-evaluated. ****Obligate *FH* mutation carrier. ****FH deficiency family. Homozygous carriers are not listed. Tumor names in bold indicate biallelic inactivation of *FH* in the tumor tissue (see also Tables 3 and 5).