

Novel mutations in *FH* and expansion of the spectrum of phenotypes expressed in families with hereditary leiomyomatosis and renal cell cancer

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ABSTRACT

Introduction: Hereditary leiomyomatosis and renal cell cancer (HLRCC) (OMIM 605839) is the predisposition to the development of smooth muscle tumors of the skin and uterus and/or renal cancer. We previously showed that mutations in the fumarate hydratase gene (*FH*) are associated with HLRCC in North America

Methods: We performed a clinical evaluation to screen for cutaneous and uterine leiomyomas, and renal tumors in 21 new families with HLRCC. We used direct DNA sequencing analysis to screen for *FH* germline mutations in affected individuals and their family members.

Results: We identified *FH* germline mutation in 100% (21/21) of new families with HLRCC. In these 21 families, we identified 14 germline mutations located along the entire length of the coding region, including 10 missense, 1 insertion, 2 nonsense, and 1 splice-site mutations. Of these 14 different *FH* mutations, 9 were novel consisting: six missense (L89S, R117G, R190C, A342D, S376P, Q396P), one nonsense (S102X), one insertion (111insA) and one splice site mutation (138+1G>C). Four unrelated families had the R58X mutation and five unrelated families shared the R190H mutation. Sixty-two percent (13/21) of families with HLRCC had renal cancer. One hundred percent (22/22) of women *FH* mutation carriers from 16 families had uterine fibroids. Seventy-six percent (16/21) of our new families with HLRCC presented with cutaneous leiomyomas. Our study shows that in HLRCC there is variability of expression of cutaneous manifestations ranging from absent to mild to severe cutaneous leiomyomas. *FH* mutations were associated with a spectrum of renal tumors. In combination with our previous report, we identify 31 different germline *FH* mutations consisting of: 20 missense, 8 frameshifts, two nonsense and one splice site. To date we have seen 56 families with HLRCC. Our *FH* mutation detection rate is 93% (52/56) in families suspected of HLRCC.

Discussion: In this study we characterize the clinical and genetic features of 21 new families expanding the spectrum of phenotypes expressed in families with HLRCC. In addition, we present the first report of two African American families with HLRCC. No genotype-phenotype correlations were identified.

KEY WORDS: uterine leiomyomas (fibroids), cutaneous leiomyomas, mutations in the fumarate hydratase gene, genodermatosis, renal cancer

INTRODUCTION

Hereditary leiomyomatosis and renal cell cancer (HLRCC [OMIM 605839]) is an autosomal dominant predisposition to the development of uterine leiomyomas (fibroids), skin leiomyomas and renal cell cancer. The HLRCC locus was mapped to chromosome 1q42.3-q43 [1]. Germline mutations in the fumarate hydratase gene (*FH*) were reported to be responsible for the susceptibility to HLRCC [2]. Subsequently, we showed that mutations in the fumarate hydratase gene (*FH*) are associated with HLRCC in North America. [3]. The *FH* gene spans 22 kb and contains 10 exons. The first exon codes for a signal peptide. *FH* codes for fumarate hydratase, the enzyme that catalyzes the conversion of fumarate to malate in the Krebs cycle. It should be noted that mutations in *FH* also occur in fumarate hydratase deficiency (FHD [OMIM 136850]). Homozygous or compound heterozygous *FH* germline mutations cause autosomal recessive FHD, a metabolic disease characterized by neurologic impairment and encephalopathy [4, 5, 6]. Leiomyomas and renal cancer have not been reported in individuals affected with FHD. However, most individuals with FHD survive only a few months with very few surviving to early adulthood. Parents (heterozygous carriers) of affected FHD individuals have been reported to develop cutaneous leiomyomas similar to individuals affected with HLRCC [2]. The occurrence of kidney cancer in parents is unknown.

Recently, we characterized the clinical and genetic features of the first 35 families we reported with HLRCC in North America [3]. Eighty-nine percent (31/35) of our cohort of HLRCC families in North America had germline mutations in *FH*. We identified 20 different *FH* mutations, of which 18 were novel. Of these 20 mutations, 7 were frameshifts (2 insertions and 5 deletions) leading to premature truncation of the protein, and 13 were missense changes predicted to result in substitution of highly conserved amino acids [3]. In our previous study, we identified thirteen individuals from five HLRCC families with renal cancer [3]. Germline *FH* mutation analysis in five families with kidney cancer showed insertion/deletions in three families and missense mutations in two families. We found a spectrum of renal tumors associated with HLRCC. Renal tumors were present in 15.6% of individuals who were screened for renal tumors. Renal cell carcinoma was associated with an aggressive disease course with 9 of 13 dead of metastatic disease within 5 years of diagnosis.

Our previous study of HLRCC families at the National Cancer Institute (NCI) revealed that 55% (17/31) of *FH* mutations from our group were located before codon 250 in contrast to 92% of the mutations reported by the Leiomyoma Consortium [2]. Our current and previous studies support our original findings that *FH* mutations associated with HLRCC are distributed throughout the gene rather than clustering at the amino terminus of *FH* [3]. In this study we report an additional 21 new HLRCC families and 9 novel mutations in *FH*. We expand the phenotypes in HLRCC and investigate the correlation of specific mutations with phenotypic characteristics.

PATIENTS AND METHODS

Patients

Patients were evaluated at the NCI on an Urologic Oncology Branch protocol approved by the NCI–Institutional Review Board. Family members who participated in this study gave written informed consent. Families were recruited based on one family member with cutaneous leiomyomas or one family member with the diagnosis of kidney tumors with pathologic features characteristic of HLRCC. All families with HLRCC were invited to participate in the study regardless of the number of affected individuals in the family. Patients and family members were evaluated for clinical features of HLRCC at the Clinical Center of the National Institutes of Health, and/or on field trips. Patients were interviewed for a history of cutaneous leiomyomas, uterine fibroids, hysterectomies and renal tumors. Each patient had a detailed examination of the skin including biopsies of lesions suspected to be leiomyomas. To detect occult malignancies, all family members who came to the NCI were examined by CT scans of the chest, abdomen and pelvis followed by renal ultrasound. The kidneys were scanned by CT before and after administration of approximately 120cc of Ioxilan 300 (Cook Imaging Corp. Bloomington, IN). High resolution (1mm) sections were obtained through the chest at 10 mm intervals. Renal ultrasound was performed with 3-5 Mhz gray scale and color doppler transducers with one of two units (Acuson Sequoia, Mountain View, CA, or ATL HDI 8000 Bellevue WA). Women who still had a uterus were examined by an MRI of the pelvis and transvaginal ultrasound. All family members that participated in the study were tested for germline mutations in *FH*. Patients in whom renal tumors were found were further evaluated by Urologic Oncology surgeons. Women in whom uterine leiomyomas were detected were evaluated by a gynecologist.

Definitions

Histologically, cutaneous leiomyomas were a proliferation of interlacing bundles of smooth muscle fibers with a centrally located long blunt-edged nucleus. Renal tumors were diagnosed on the basis of histological examination of resected tumors, or on the basis of CT scans. Histologically, renal tumors had cytologic features of amphophilic cytoplasm with large nuclei containing large eosinophilic like-inclusion nucleoli. Solid renal lesions greater than 1 cm in diameter with greater than 20 Hounsfield units enhancement were considered renal tumors. Uterine fibroids were documented by history, review of medical records, physical examination, MRI, CT, and/or ultrasonography. Hysterectomy was documented by history, absence of uterus on CT of the pelvis, trans-vaginal ultrasound and review of medical records.

Sequencing of the fumarate hydratase gene

DNA was extracted from peripheral blood leukocytes according to standard procedures. The genomic sequence containing *FH* was determined by BLAT alignment of the mitochondrial *FH* precursor cDNA (Acc. No NM_000143) with the assembled genomic sequence (NCBI Build 34). Methods for identification of

exon/intron boundaries and high-throughput DNA sequencing were as previously described [3].

Haplotype analysis

DNA was extracted from cells of peripheral blood or buccal swabs according to standard procedures. Seven genetic markers flanking the HLRCC locus were selected for haplotype analysis. The order of seven polymorphic markers crossing 33 Mb on the genome assembly (NCBI build 34) is: cen-D1S2833-S1S2709-D1S2875-AY299638-D1S2836-D1S2215-D1S2682. AY299638 is the di-nucleotide micro-satellite marker located in intron seven of FH. The fluorescently labeled PCR primers used to amplify micro-satellite loci were purchased from Applied Biosystems (ABI). The PCR reaction was performed using ABI Prism True Allele PCR Premix. The alleles were separated on an ABI genetic analyzer 3100 and analyzed by the ABI GeneMapper software 3.0.

Lymphoblastoid cell lines

Lymphoblastoid cells were made from EBV transformation of lymphocytes and cultured in RPMI 1640, 10% fetal calf serum, 1% non-essential amino acid, 1% sodium pyruvate and 2% L-glutamine. For each specimen of lymphoblastoid cell cultures, approximately 1×10^6 cells in confluent flask were washed with 1x PBS, centrifuged at 600g for 10 minutes and resuspended in 1 ml of 250mM sucrose, 25mM HEPES, pH 7.4. The cells then were disrupted by gentle sonication for 5 seconds on ice. The cell lysate was centrifuged at 900g at 4c for 8 minutes to remove cell debris.

Measurement of fumarate hydratase enzyme activity

In vitro assay of fumarate hydratase enzyme activity was measured by NADP-Malic enzyme coupled assay as previously been described [7]. The increase in absorbance at 340nm from NADPH formation was measured after adding fumarate (final concentration of 10mM) into reaction mixture of cell extract, 25mM HEPES-KOH, pH 7.6, 0.4 mM NADP, 4 mM Magnesium Chloride, 5 mM Potassium Phosphate monobasic and 0.2 unit of NADP-Malic enzyme in total volume of 200 microliter.

RESULTS

Mutation analysis

In this study we characterized the clinical and genetic features of a total of 21 new families with HLRCC. Eighty-four individuals from 21 families were screened for *FH* mutations. Using direct sequencing analysis we identified *FH* germline mutation in 100% (21/21) of new families with HLRCC. Of the 48 individuals clinically affected, *FH* mutations were identified in 100%. Of those not clinically affected, five (three from family 1600; one from family 4000 and one from family 6000) had the family mutation. Direct sequence analysis of 9 coding exons and splice site junctions of *FH* revealed 14 germline mutations in 21 new families with HLRCC (**Table 1**). In these 21 families, we identified 14 germline mutations located along the entire length of the coding region, including 10 missense, 1 insertion, 2 nonsense, and 1 splice-site mutations. The nine novel *FH* mutations identified consisted of six missense (L89S, R117G, R190C, A342D, S376P, Q396P), one nonsense (S102X), one insertion (c.111insA) and one splice site mutation (c.138+1G>C). Each missense mutation co-segregated with disease and was absent in more than 160 normal individuals. The one base-pair insertion in exon 1 (c. 111insA) was confirmed by subcloning. Point mutations were detected in all 21 families. We measured the FH enzyme activity in lymphoblastoid cell lines from two probands with novel mutations. Their FH activity was significantly decreased from match normal controls: L89S (mean 118 nmole/min/mg protein), S102X (mean 182 nmole/min/mg protein) and control (mean 513 nmole/min/mg protein).

Four unrelated families from diverse ethnic backgrounds and different geographic regions had the R58X mutation. We genotyped four families with the R58X mutation and five families with R190H mutation with six microsatellite markers surrounding the HLRCC locus and one intragenic marker. Haplotype analysis of all four families with the R58X mutation showed that the families did not share a common haplotype. This suggests that R58X is not a founder mutation. Therefore, R58X may represent a hot spot mutation. Due to lack of available DNAs of key members in four of the five families with R190H mutation for genotyping, we could not generate a definitive haplotype for all affected members with the R190H mutation. It remains to be determined if the R190H mutation represents either a mutation hot spot or a founder mutation. There were not other tumors beside skin, uterine or renal identified in the *FH* carrier family members in the 21 families with the exception breast cancer in a woman from family 4600. Family 5400 is of Iraqi ancestry.

Table 1. FH Mutations in Newly Characterized Families with HLRCC (N=21)

Family	Exon	Mutation	Codon	Predicted Result	Renal Tumor	Skin Leiomyoma	Uterine Fibroid
4000	1	111insA*	K37	Framshift	●	●	○
1600		138+1G>C*		Unknown	●	○	●
920	2	172C>T	R58X	Nonsense	●	●	●
4800	2	172C>T	R58X	Nonsense	●	●	●
4200	2	172C>T	R58X	Nonsense	●	●	●
5800	2	172C>T	R58X	Nonsense	○	●	●
1400	2	191A>C	N64T	Missense	○	●	●
6000	3	266T>C*	L89S	Missense	●	○	●
5400	3	305C>G*	S102X	Nonsense	●	○	○
3000	3	349A>G*	R117G	Missense	○	●	●
5200	4	568C>T*	R190C	Missense	●	○	○
5000	4	569G>A	R190H	Missense	○	●	●
2000	4	569G>A	R190H	Missense	●	●	●
6200	4	569G>A	R190H	Missense	●	○	●
3600	4	569G>A	R190H	Missense	○	●	○
4600	4	569G>A	R190H	Missense	●	●	○
5600	6	891T>A	N297K	Missense	○	●	●
960	6	964A>G	S322G	Missense	○	●	●
2400	7	1025C>A*	A342D	Missense	○	●	●
4400	8	1126T>C*	S376P	Missense	●	●	●
3800	8	1187A>C*	Q396P	Missense	●	●	●
Total families affected:					62% (13/21)	76%(16/21)	100%(16/16)

Mutations are named according to the recommendations for the Nomenclature system for Human Gene Mutations. Nucleotide numbering is according to the cytosolic FH sequence (GenBank accession number NM_000143), with the A of ATG initiator codon as nucleotide position 1. The asterisk indicate the novel mutations. The filled ovals indicate affected members with renal tumor, skin leiomyoma and/or uterine fibroid. Gray filled ovals indicate status unknown. Gray filled ovals in the column of uterine fibroid reflect that no women were evaluated. The clear ovals indicate lack of renal tumor, skin leiomyoma and/or uterine fibroid.

African-American families with HLRCC

Among the 21 new families studied we identified two African-American families with HLRCC. Previous reports of *FH* mutations in HLRCC have not included African-American families [3]. Family 5200 consisted of a father and a son with renal cell carcinoma (**Figure 1**). The son presented with gross hematuria at age 38. CT scan of the abdomen and pelvis revealed a 15 cm tumor in the left kidney. Upon microscopic examination after left radical nephrectomy, the renal cell carcinoma was reported as tubulopapillary with cystic areas. Characteristic cytologic features included amphophilic cytoplasm with large nuclei containing large eosinophilic inclusion nucleoli. Two months later, he underwent a right partial nephrectomy and the right renal tumor demonstrated similar histology to the previously resected tumor on the left kidney. A few months later, he developed metastatic renal cell carcinoma to other areas on his right kidney and omentum. This presentation was atypical as most cases of HLRCC have unilateral and solitary renal tumors. The histologic features and the early age of onset associated with aggressive clinical disease suggested the diagnosis of HLRCC. Direct sequencing of genomic DNA revealed an *FH* missense mutation (R190C) (**Figure 1**). His parents were screened for occult renal tumors and an *FH* germline mutation. The germline *FH* mutation identified in the son was also confirmed in the father. CT scan of the abdomen and pelvis of the father, a 68-year-old man, revealed a 2 cm tumor in the right kidney. Subsequently, the father underwent right partial nephrectomy. Microscopic examination showed renal cell carcinoma with solid and cystic areas. No cutaneous lesions suspicious for leiomyomas were found on two independent dermatologic examinations of the father and the son.

The proband of Family 4000 (**Figure 1**) is a 26 year-old African American man who presented with metastatic kidney cancer and a 6 cm left supraclavicular node. He had a 8 cm left kidney tumor metastatic to retroperitoneal and mediastinal lymph adenopathy and multiple liver lesions. Microscopic examination showed renal cell carcinoma with focal papillary features. Dermatologic examination showed a single cutaneous leiomyoma. Sequencing analysis of the patient's genomic DNA revealed a one base pair insertion in exon 1(c.111insA). This mutation was confirmed by subcloning. The patient's father, who is an *FH* mutation carrier, did not have renal tumors or cutaneous leiomyomas. However, there is a strong family history of kidney cancer in the proband's paternal family including his aunt and a cousin (**Figure 1**).

Renal tumors

In this study renal tumors occurred in 62% (13/21) of families with HLRCC (**Table 1**). There were 20 members from these 13 HLRCC families affected with renal cell carcinoma. In 17 cases we confirmed the pathological diagnosis by histologic review of the specimens. In three cases the diagnosis of renal carcinoma was based on local pathology reports because pathological specimens were not available for review. Renal carcinoma occurred in nine family members clinically affected with cutaneous leiomyomas. In addition, we

identified six deceased relatives who died of kidney cancer but no slides or pathology reports were available for review. Family 6000 (**Figure 1**) had four individuals with kidney cancer. Only one individual is a kidney cancer survivor. It is of interest that 69 % (9/13) families with HLRCC who presented with kidney cancer had a family history of kidney cancer.

We found a histologic spectrum of renal tumors associated with HLRCC. In Family 4800 a father and his daughter were affected with collecting duct renal cell carcinomas described by outside pathology reports. No slides or tissue blocks were available for review. One individual in Family 3800 had cystic and tubulo-papillary architecture with clear cell areas. Renal cell carcinomas associated with HLRCC had mixed features with cystic, papillary, tubulopapillary elements. There was also variability of renal tumor histology within families and among families. In this report we identified three individuals who presented with bilateral and multifocal kidney tumors while the rest had solitary and unilateral kidney tumors. A twin from Family 2000 who was wild type for *FH* had an angiomyolipoma on her right kidney.

Cutaneous leiomyomas

Seventy-six percent (16/21) of our new families with HLRCC presented with cutaneous leiomyomas (**Table 1**). Clinically cutaneous leiomyomas presented as firm skin-colored to light brown-colored papules and nodules. The number of lesions per individual ranged from none to more than 100. Forty-eight percent (10/21) of families had individuals affected with multiple cutaneous leiomyomas. There were three main patterns of presentations: grouped, disseminated, and disseminated and segmental. Lesions were present on the trunk and extremities and two individuals developed multiple leiomyomas on their head and neck. Ninety percent of individuals with cutaneous leiomyomas complained of sensitivity to light touch associated with the cutaneous lesions. Only one individual from one family had a history of a wide surgical excision of a cutaneous leiomyosarcoma on her leg.

Twenty-nine percent (6/21) of the HLRCC families had mild cutaneous manifestations. Two families had an individual who exhibited only a single leiomyoma and kidney tumors. In addition, six individuals from four families presented with two to five cutaneous leiomyomas. Fifty percent (3/6) of these individuals had cutaneous leiomyomas and renal tumors. These individuals had aggressive kidney cancer but mild skin manifestations. Each of these patients was unaware that they had cutaneous leiomyomas until they were seen at NCI.

Nineteen percent (4/21) of the families did not have cutaneous manifestations. Sixty-nine percent (9/13) of *FH* gene mutation carriers from these four families without cutaneous manifestations had kidney tumors. The skin status of Family 6200 is unknown since *FH* mutation carriers did not have a dermatologic examination. In families without cutaneous leiomyomas the clinical diagnosis of HLRCC was more challenging. The clues to the diagnosis of HLRCC were

based on the kidney tumor cytologic features, young age at presentation of kidney tumors and aggressive clinical behavior of tumors. Our study shows that in HLRCC there is variability of expression of cutaneous manifestations ranging from absent to mild to severe involvement with cutaneous leiomyomas.

Uterine fibroids

Within these 21 new families, women with HLRCC complained of early onset of fibroid-associated symptoms including irregular menses, menorrhagia and pain. Uterine fibroids were numerous and large ranging from 1-15 tumors per patient and 1 to 8 centimeters in diameter. One hundred percent (22/22) of women *FH* mutation carriers from 16 families had uterine fibroids (**Table 1**). In the remaining five families no women participated in the study. Seventy-three percent (16/22) of these women with fibroids had cutaneous leiomyomas. The skin status of two women was unknown. The age of diagnosis of uterine fibroids ranged from 19-53 years of age. Sixty-eight percent (15/22) of women *FH* mutation carriers were diagnosed with uterine fibroids at 30 years of age or younger. Fourteen percent (3/22) of women *FH* mutation carriers had uterine fibroids as their only manifestation of disease. In addition, one woman in Family 1600 had uterine fibroids and renal tumors but no skin leiomyomas.

Seventy-three percent (16/22) of *FH* mutation carriers underwent a myomectomy or hysterectomy to treat fibroids. Of these 16 women, 12 underwent a hysterectomy only, two had a myomectomy only and one had a myomectomy at age 25 and a hysterectomy at age 35. Another woman had two myomectomies; one at 26 and another at 33 years of age. Subsequently, she underwent hysterectomy at age 39 for symptomatic relief of fibroids. Thirty-six percent (8/22) and 68% (15/22) of women *FH* mutation carriers had a hysterectomy or myomectomy for symptomatic relief of fibroids at 30 years of age or younger and at 40 years of age or younger, respectively. Fifty percent of women in our cohort who had a hysterectomy or myomectomy had surgery at 30 years of age or younger.

Genotype-phenotype correlations

The two individuals with a single cutaneous leiomyoma and renal tumors had nonsense mutation (R58X) or a frameshift mutation (K37->STOP). Germline mutations in the four families who presented with individuals with two to five cutaneous leiomyomas consisted of two missense mutations (R190H and S376P) and a nonsense mutation (R58X). Therefore, there was a broad spectrum of *FH* mutations that were associated with a mild dermatologic phenotype (1-10 leiomyomas) including: missense (R190H, S376P), nonsense (R58X) and frameshift (K37->STOP) mutations. Two of these mutations (R190H, S376P) led to changes in highly conserved amino acids and two (R58X and K37->STOP) are predicted to produce a truncated protein.

Germline mutations associated with absence of cutaneous leiomyomas consisted of two missense (L89S and R190C), one nonsense (S102X), and one splice site

(138+1G>C) (**Table 1**). Ten carriers of the above *FH* mutations ranging from (35-64) years of age had meticulous skin examinations at least two different times within 1-3 years, but no signs of cutaneous leiomyomas or subcutaneous leiomyomas (screened by palpation) were identified. Therefore, there was no apparent association between the type of mutation and mild or absent cutaneous leiomyoma phenotypes.

The germline mutation spectrum of the 13 families with renal tumors consisted of four families with nonsense mutations (R58X and S102X), seven families with missense mutations (R190H, R190C, S376P, L89S, and Q396P), one family with a frameshift mutation (c.111insA) and a family with a splice site mutation (c.138+1G>C). Family 4800 had a father and a daughter, who carried the R58X germline mutation, affected with renal collecting duct carcinoma.

Families with the R190H and R58X mutations had a high frequency of kidney tumors. Seventy-five percent (3/4) of families with the R58X mutation had renal tumors and 60% (3/5) of families with the R190H had renal tumors. Two families with R190H and a family with R58X were confirmed to lack kidney tumors following abdominal and pelvic CT screening. Family 5200 had the R190C mutation and both the father and the son presented with kidney cancer. Therefore, 67% (4/6) of families with mutations at residue R190 included individuals who developed kidney cancer compared with a 62% frequency of renal tumors among HLRCC families in this study. Therefore, given the relatively small numbers there is not a difference. Families carrying the R190H and R58X mutations demonstrated variability of expression of renal tumor within families who share the same germline mutation. In addition, there was no association between the mutations and the type of renal cancer.

DISCUSSION

In this study we characterize the clinical and genetic features of 21 new families with HLRCC. In addition, we present the first two African-American families reported with HLRCC. Sequence analysis revealed a total of 14 different *FH* germline mutations. We identified 9 novel mutations in *FH*. Sixty-two percent (13/21) of the new families with HLRCC had renal tumors. Some families with renal tumors shared the same mutation; i.e R58X (3 families) and R190H (3 families). *FH* mutations were associated with a spectrum of renal tumors.

In combination with our previous report, to date we have identified 31 different germline *FH* mutations consisting of: 20 missense, 8 frameshifts (3 insertions and 5 deletions), two nonsense and one splice site (**Figure 2**). Mutations were distributed throughout the gene except exon 5. Exon 4 (7 different mutations) and exon 6 (8 different mutations) had the most mutations. Sixty-five percent (20/31) of the mutations resulted in the substitutions of single amino acid residues that were highly conserved throughout evolution. Missense mutations are important in that they may indicate residues in the fumarate hydratase protein that are functionally important. Forty-two percent (13/31) of *FH* mutations were associated with kidney tumors and were also distributed throughout the gene. There was no association between the location or type of mutation associated with kidney cancer. Eighty-seven percent (27/31) of *FH* mutations were associated with skin leiomyomas (**Figure 2**). Four *FH* mutations identified in families without skin leiomyomas were associated with kidney cancer. However, there was not a specific type of mutation associated with absence of skin lesions. In addition, 96% (27/28) of *FH* mutations were associated with uterine fibroids (**Figure 2**). Even though no clear genotype-phenotype correlations could be identified in this study, our data suggest that families with R190H and R58X mutations tend to have a high frequency of individuals with kidney tumors. This early finding needs to be investigated in a larger group of families.

Recently, we started measuring *FH* enzyme activity in cell lines of patients with HLRCC. In agreement with a previous report, we found significantly lower *FH* enzyme activity in lymphoblastoid cells from individuals with missense (L89S) and nonsense (S102X) mutations compared with normal controls. Previously, Tomlinson and co-workers also measured *FH* enzyme activity in lymphoblastoid cell lines from patients with cutaneous leiomyomas and controls [2]. All lymphoblastoid cell lines with *FH* mutations examined had decreased fumarase enzyme activity. However, lymphoblastoid cell lines with *FH* missense mutations had significantly lower enzyme activity than lymphoblastoid cell lines with *FH* truncating or large gene deletions. In addition, it is of interest that some patients had very reduced *FH* activity despite having one normal copy of the gene. A plausible explanation of these findings is dominant negative action of the missense mutants. This is conceivable as fumarase functions as a homotetramer. Thus, four wild type monomers are needed for the formation of

functional fumarase. A missense mutation in one allele would result in only one out of 16 fully functional wild type tetramers and would prevent fully functional tetramers from forming. Another possible explanation for the functional effects of missense mutations is that they may alter important protein-protein interactions.

To date we have evaluated 56 families with HLRCC. Using direct sequencing we detected germline mutations in *FH* in 93% (52/56) of our families. The *FH* mutation detection rate reported by the Leiomyoma Consortium was 60% in their cohort of European families with leiomyomatosis [2]. Thirty-three percent (17/52) of families had a mutation at the R190 residue. It remains to be determined whether R190 represents a founder mutation. Eight percent (4/52) of HLRCC families had R58X mutation. The R58X appears to be a hot spot since haplotype analysis excluded a founder effect. Furthermore, R58X had also been reported in three European families with HLRCC [2, 8]. Alam and co-workers found that the three probands with the R58X shared an allele (frequency ≈ 0.2) at only one microsatellite, (CA)₁₃, which is located in intron two immediately after the R58X mutation [8]. Therefore, the possibility of a founding mutation could not be excluded. The most frequent *FH* germline mutations reported in series of patients with HLRCC in Europe were N64T and G354R found in six families, respectively [8]. Haplotype analysis of the six families with N64T was consistent with a founder mutation. It is of interest that to our knowledge neither the N64T nor the G354R mutations have been identified in families with HLRCC in North America.

Previously, using direct sequencing we did not identify mutations in four families with HLRCC [3]. Southern analysis did not show large deletions of the entire *FH* gene in two of these four HLRCC families (data not shown). However, large deletions of roughly 2.4 Mb and 1.9 Mb including the entire *FH* gene have been reported in families with HLRCC [2]. These families were not phenotypically different from our 52 families with *FH* germline mutations.

To date we have identified renal tumors in 32% (18/56) of our cohort of HLRCC families at NCI. To our knowledge, this constitutes the largest collection of HLRCC families with renal tumors reported but many families were ascertained through renal cancer screening. In our previous study, families were recruited based on the affected status of multiple cutaneous leiomyomas [3]. In that cohort of families, the frequency of families with renal cancer was only 15%. In contrast in the current study, the frequency of families with renal tumors increased to 62% (13/21). In the current study, families were recruited based on cutaneous leiomyomas or the diagnosis of kidney tumors with cytologic features characteristic of HLRCC. The increase in frequency of renal tumors in this study from our previous study may be due to the difference in recruitment approaches. Therefore, there may be a selection bias for families with renal cancer. In addition, as our histologic diagnostic acumen improved, we identified more cases of kidney cancer likely to be HLRCC.

To our knowledge, only four HLRCC families with renal cancer have been reported by other groups [1,8, 9]. Germline mutations in *FH* have been reported in three Finnish kindreds with papillary type II renal cell carcinoma. Two families shared a 2-base pair deletion in codon 181, and the other had the R300X mutation [2]. In addition, an *FH* missense mutation (N318K) was reported in an HLRCC British patient described as collecting duct carcinoma of the kidney. There are a few potential factors that may explain the lower frequency of renal tumors previously reported in families with leiomyomatosis and/or *FH* germline mutations [1, 10]. First, families were not extensively screened for renal tumors and occult kidney tumors may not have been detected. Optimal screening for renal tumors involves CT scans of the abdomen and pelvis. Alternatively, MRI of the abdomen and pelvis is sometimes used, if needed. Papillary renal tumors maybe difficult to detect, if patients are screened with renal ultrasound only. Papillary tumors are often isoechoic and can be missed on renal ultrasound [11]. Second, it is also possible that some *FH* mutations have low penetrance for renal tumors or specific *FH* mutations are not associated with renal tumors. Third, some pathologist may have lack of experience recognizing the histologic features associated with HLRCC renal tumors. HLRCC-associated renal tumors are rare and were only recently described [1, 3]. Therefore, many pathologists may not be familiar with their features.

In this study, we identified the first family of CDC of the kidney with *FH* germline mutations consisting of a daughter and father, both affected with CDC of the kidney and *FH* mutations carriers. Previously, two cases described as CDC of the kidney among patients with HLRCC were reported [3, 8]. Interestingly, this is consistent with reports in the literature of cytogenetic abnormalities found in CDC of the kidney. Monosomy of chromosome 1 and LOH of 1q is reported to occur in 60-80% of cases of CDC [12, 13]. Furthermore, our patients with HLRCC share some clinical features typical of the clinical presentation of CDC of the kidney characterized by an aggressive course with evidence of metastatic disease at the time of presentation and poor prognosis [14]. The most significant finding in our collection of cases with renal tumors was that the cytologic features present in renal tumors were very consistent even though the architectural morphology of the tumors varied. Renal tumors associated with HLRCC were characterized by the presence of cells that had an abundant amphophilic cytoplasm and large nuclei with large inclusion-like eosinophilic nucleoli. These cytologic features were originally attributed to type II papillary tumors in the original description [1]; however, they can be present in other histologic types of renal tumors associated with HLRCC. HLRCC is associated with a histologic spectrum of renal tumors. Most renal tumors in this study share some features with type II papillary RCC. However, renal tumors associated with HLRCC are difficult to classify under the existing renal tumor classification schemes since they have distinct clinical and histologic features. Therefore, renal tumors associated with HLRCC may constitute in the future a new renal pathological entity.

One hundred percent of our new cohort of 22 women who were identified as *FH* mutation carriers had uterine fibroids. This is similar to our previous report, in which 100% of women with cutaneous leiomyomas had uterine fibroids [3]. Therefore, cutaneous leiomyomas are a good marker of affection status for uterine fibroids. In the general population, the reported prevalence rates of uterine fibroids ranged from 22 to 77% with the highest prevalence in women aged 40-44 years [15, 16]. In this study, sixty-eight percent (15/22) of women *FH* mutation carriers were diagnosed with uterine fibroids at age 30 or younger. Thus, women with HLRCC had a higher prevalence of uterine fibroids and younger age at diagnosis of uterine fibroids than women in the general population.

In agreement with our previous report, uterine fibroids associated with HLRCC are associated with increased morbidity and increased secondary infertility. Seventy-three percent of women in our cohort of women had a gynecologic procedure including hysterectomy or myomectomy for symptomatic uterine fibroids. Hysterectomy surveillance in the United States from 1994 to 1999 showed that hysterectomy occurred most frequently for those aged 40-44 years. Furthermore, in the United States 52% of women who have a hysterectomy have the surgery at 44 years of age or younger [17]. In contrast, 50% of our cohort of women with HLRCC who had a hysterectomy or myomectomy had the surgery at 30 years of age or younger. In conclusion, HLRCC is associated with early onset of uterine fibroids and early hysterectomy when compared with women in the general population in the United States. The young age of onset of symptomatic uterine fibroids significantly impacts the childbearing years of women with HLRCC.

FH most likely acts as a tumor suppressor in familial leiomyoma since LOH studies have shown loss of the wild type allele in cutaneous, uterine and renal tumors, and the enzyme activity of *FH* is low or absent in tumors from individuals with leiomyomas [2]. However, the mechanisms by which *FH* defects promote tumorigenesis are unknown. Possible mechanisms include hypoxia, apoptosis, and oxidative stress. It is of interest that germline mutation in another mitochondria enzyme in the Krebs cycle, succinate dehydrogenase (*SDH*), leads to the predisposition to develop paragangliomas and pheochromocytoma [18]. Furthermore, three cases with kidney cancer and germline mutations in *SDH-B* have been reported [19]. One family with germline *SDH-B* mutation (c.847-50delTCTC) had two members with RCC and paraganglioma; and another family comprised of a son with clear-cell RCC and his mother with a cardiac paraganglioma both had a germline *SDH-B* R27X mutation. Furthermore, all three of these RCCs showed LOH at *SDH-B*. Taken all together the literature suggests that mitochondria dysfunction through various mechanisms may lead to the formation of kidney tumors. Hypoxia mediated pathways have been shown to be implicated in tumorigenesis, especially in kidney cancer. In von-Hippel-Lindau (VHL) syndrome the over accumulation of hypoxia inducible factor (HIF) leads to increased transcription of anti-apoptotic and proliferative genes such as

vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and epidermal growth factor receptor (EGFR). The VHL protein (pVHL) forms a complex with Elongin B and C, and Cullin 2 to form the VHL complex VCB [20, 21, 22]. When normal oxygen levels are present this complex binds to HIF1- α and HIF2- α for ubiquitin-mediated degradation [23]. When VHL is mutated the complex cannot bind HIF, and HIF accumulates along with the associated increase in multiple factors promoting tumorigenesis. Inactivation of *VHL* leads to the development of highly vascular tumors [24]. Although this mechanism has been shown in VHL, it is possible that HLRCC and VHL share a common pathway or share some part of the pathway that is key for tumorigenesis. Recently, we showed that accumulation of fumarate and succinate following pharmacologic inhibition of FH and SDH leads directly to inhibition of HIF prolyl hydroxylase by competing with 2-oxoglutarate, a required co-factor of the enzyme [25]. These treatments resulted in accumulation of transcriptionally active HIF, as evidence by an increase level of both Glut-1 and VEGF transcripts. Treating cells with siRNA specific for FH has a similar effect. Similarly, Selak et al. [26] reported that succinate accumulation secondary to loss of succinate dehydrogenase leads to HIF accumulation via inhibition of HIF prolyl hydroxylase in 293 cells transfected with siRNA to various SDH subunits. ROS generation does not appear to be involved in either fumarate or succinate-dependent HIF induction [25, 26]. Recently, it has been shown that VEGF and PDGF are over expressed in leiomyomas [27, 28]. Taken together, these observations suggest that HIF may play a role in tumorigenesis in HLRCC.

It has been shown that mitochondria play a key major role in apoptosis. Pollard and co-workers recently suggested that mitochondria dysfunction associated with mutation in *FH* can lead to a decreased tendency for cell to undergo apoptosis [29]. Bax and Bcl-2 form pores in the mitochondrial membranes through which cytochrome C can escape to the cytoplasm to form the apoptosome with Apaf-1 and caspase 9 [30]. It is also possible that the mitochondria dysfunction associated with mutation in *FH* can alter the integrity of the mitochondrial membranes, can prevent apoptosis, and/or can lead to accumulation of anti-apoptotic metabolites. Glutamine has been shown to have anti-apoptotic effects in Jurkat cells via up regulation of glutathione and Bcl-2 [31]. In addition, glutamate can rescue carcinoma cell lines from apoptosis and have a proliferative effect in different cell types [32]. Therefore, it is possible that tumorigenesis associated with HLRCC can lead to anti-apoptosis.

In conclusion, in this study we characterize the clinical and genetic features of 21 new families and expanded the spectrum of phenotypes expressed in families with HLRCC. Patients with HLRCC can present with a range of clinical presentations including multiple cutaneous leiomyomas, a single skin leiomyoma, no cutaneous lesions, multiple renal tumors, a single renal tumor, absence of renal tumors, uterine fibroids and/or various combinations of these phenotypes. Furthermore, this variability of expression is present within and among families with HLRCC. Individuals with HLRCC have diverse racial and ethnic

backgrounds including African-American; however, patients with Eastern European heritage are over-represented. HLRCC can occur in patients without a typical ethnic background. In this study, we did not find apparent genotype-phenotype correlations in HLRCC. HLRCC is associated with clinically significant uterine fibroids and spectrum of renal tumors that are aggressive. Appropriate surveillance and genetic counseling is needed in the clinical evaluation of patients with HLRCC.

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Electronic-Database Information

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for mitochondrial *FH* precursor National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/> [First citation in article](#))

NCBI Blast Home Page, <http://www.ncbi.nlm.nih.gov/BLAST/> (for BLAST 2) [First citation in article](#)

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for multiple cutaneous leiomyomatosis, HLRCC, and FHD)

UCSC Genome Bioinformatics, <http://genome.ucsc.edu/> (for Genome Browser and BLAT) [First citation in article](#)

Wise2, <http://www.ebi.ac.uk/Wise2/> [First citation in article](#)

FIGURE LEGENDS

Figure 1 *FH* mutations in newly characterized families with HLRCC. Sequencing chromatograms of genomic DNA from control subjects and patients are shown at left. The arrows in the chromatograms indicate the position of the identified nucleotide changes. The arrow in the pedigree for Family 2000 indicates the individual with angiomyolipoma of the kidney. The corresponding pedigrees are shown in the right.

Figure 2 Distribution of *FH* mutations and the genotype-phenotype in HLRCC. The lower vertical arrows denote *FH* mutations. The upper vertical bars show the phenotype corresponding to the specific *FH* mutation. The colors in the vertical bars indicate the phenotype; presence of renal tumor (blue), uterine fibroid (yellow) and skin leiomyoma (red). The empty bars indicate the absence of phenotype and the gray bars denote unknown phenotype status.

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Novel mutations in FH and expansion of the spectrum of phenotypes expressed in families with hereditary leiomyomatosis and renal cell cancer

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