ORIGINAL ARTICLE

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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Background: Hereditary leiomyomatosis and renal cell cancer (HLRCC; OMIM 605839) is the predisposition to develop smooth muscle tumours of the skin and uterus and/or renal cancer and is associated with mutations in the fumarate hydratase gene (*FH*). Here we characterise the clinical and genetic features of 21 new families and present the first report of two African-American families with HLRCC.

Methods: Using direct sequencing analysis we identified *FH* germline mutations in 100% (21/21) of new families with HLRCC.

Results: We identified 14 germline *FH* mutations (10 missense, one insertion, two nonsense, and one splice site) located along the entire length of the coding region. Nine of these were novel, with six missense (L89S, R117G, R190C, A342D, S376P, Q396P), one nonsense (S102X), one insertion (111insA), and one splice site (138+1G>C) mutation. Four unrelated families had the R58X mutation and five unrelated families the R190H mutation. Of families with HLRCC, 62% (13/21) had renal cancer and 76% (16/21) cutaneous leiomyomas. Of women *FH* mutation carriers from 16 families, 100% (22/22) had uterine fibroids. Our study shows that expression of cutaneous manifestations in HLRCC ranges from absent to mild to severe cutaneous leiomyomas. *FH* mutations were associated with a spectrum of renal tumours. No genotype-phenotype correlations were identified.

Conclusions: In combination with our previous report, we identify 31 different germline *FH* mutations in 56 families with HLRCC (20 missense, eight frameshifts, two nonsense, and one splice site). Our *FH* mutation detection rate is 93% (52/56) in families suspected of HLRCC.

Those with hereditary leiomyomatosis and renal cell cancer (HLRCC; OMIM 605839) have 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 susceptibility to HLRCC.[2] Subsequently, we showed that mutations in the *FH* gene 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 catalyses 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 characterised by neurological impairment and encephalopathy.[4–6] 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 rate of occurrence of kidney cancer in parents is unknown.

Recently, we characterised the clinical and genetic features of the first 35 families we reported with HLRCC in North America.[3] Eighty nine per cent (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, seven were frameshifts (two insertions and five 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 13 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 tumours associated with HLRCC. Renal tumours were present in 15.6% of individuals who were screened for renal tumours. Renal cell carcinoma was associated with an aggressive disease course with nine of 13 patients dying 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

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additional 21 new HLRCC families and nine novel mutations in *FH*. We expand the phenotypes in HLRCC and investigate the correlation of specific mutations with phenotypic characteristics.

METHODS

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 tumours with pathological 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 tumours. 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 had CT scans of the chest, abdomen, and pelvis followed by renal ultrasound. The kidneys were scanned by CT before and after administration of approximately 120 cc of Ioxilan 300 (Cook Imaging, Bloomington, IN). High resolution (1 mm) sections were obtained through the chest at 10 mm intervals. Renal ultrasound was performed with 3–5 MHz grey scale and colour 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 who participated in the study were tested for germline mutations in *FH*. Patients in whom renal tumours were found were further evaluated by urologic oncology surgeons. Women in whom uterine leiomyomas were detected were evaluated by a gynaecologist.

Definitions

Histologically, cutaneous leiomyomas were a proliferation of interlacing bundles of smooth muscle fibres with a centrally located long blunt-edged nucleus. Renal tumours were diagnosed on the basis of histological examination of resected tumours, or on the basis of CT scans. Histologically, renal tumours had cytological 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 tumours. 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 dinucleotide micro-satellite marker located in intron 7 of *FH*. The fluorescently labelled PCR primers used to amplify microsatellite loci were purchased from Applied Biosystems (ABI; Foster City, CA). The PCR reaction was performed using ABI Prism True Allele PCR Premix. The alleles were separated on an ABI Genetic Analyzer 3100 and analysed 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 a confluent flask were washed with $1 \times$PBS, centrifuged at 600 g for 10 min, and resuspended in 1 ml of 250 mM sucrose, 25 mM HEPES, pH 7.4. The cells were then disrupted by gentle sonication for 5 s on ice. The cell lysate was centrifuged at 900 g at 4℃ for 8 min 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 described.[7] The increase in absorbance at 340 nm from NADPH formation was measured after adding fumarate (final concentration of 10 mM) into a reaction mixture of cell extract, 25 mM HEPES-KOH, pH 7.6, 0.4 mM NADP, 4 mM magnesium chloride, 5 mM potassium phosphate monobasic, and 0.2 U NADP-malic enzyme in total volume of 200 μl.

RESULTS

Mutation analysis

In this study we characterised the clinical and genetic features of 21 new families with HLRCC. Eighty three individuals from 21 families were screened for *FH* mutations. Using direct sequencing analysis, we identified *FH* germline mutations in 100% (21/21) of new families with HLRCC. Of the 42 individuals clinically affected, *FH* mutations were identified in 100%. Of those not clinically affected, 8.6% (4/46) had the family mutation. Direct sequence analysis of nine 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, one insertion, two nonsense, and one splice site mutation (figs 1–3). 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 (c.138+1G>C) mutation. Each missense mutation co-segregated with disease and was absent in more than 160 normal individuals. The 1 bp 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 compared to matched normal controls: L89S (mean 118 nmol/min/mg protein), S102X (mean 182 nmol/min/mg protein), and control (mean 513 nmol/min/mg protein).

Four unrelated families from diverse ethnic backgrounds and different geographical regions had the R58X mutation. We genotyped four families with the R58X mutation and five families with the R190H mutation with six microsatellite markers surrounding the HLRCC locus and one intragenic marker. Haplotype analysis of all four families with the R58X

Table 1 *FH* mutations in newly characterised families with HLRCC (n = 21)

Family	Exon	Mutation	Codon	Predicted result	Renal tumour	Skin leiomyoma	Uterine fibroid
4000	1	111insA*	K37	Frameshift	Yes	Yes	NW‡
1600		138+1G>C*		Unknown	Yes	No	Yes
920	2	172C>T	R58X	Nonsense	Yes	Yes	Yes
4800	2	172C>T	R58X	Nonsense	Yes	Yes	Yes
4200	2	172C>T	R58X	Nonsense	Yes	Yes	Yes
5800	2	172C>T	R58X	Nonsense	No	Yes	Yes
1400	2	191A>C	N64T	Missense	No	Yes	Yes
6000	3	266T>C*	L89S	Missense	Yes	No	Yes
5400	3	305C>G*	S102X	Nonsense	Yes	No	NW‡
3000	3	349A>G*	R117G	Missense	No	Yes	Yes
5200	4	568C>T*	R190C	Missense	Yes	No	NW‡
5000	4	569G>A	R190H	Missense	No	Yes	Yes
2000	4	569G>A	R190H	Missense	Yes	Yes	Yes
6200	4	569G>A	R190H	Missense	Yes	Unknown†	Yes
3600	4	569G>A	R190H	Missense	No	Yes	NW‡
4600	4	569G>A	R190H	Missense	Yes	Yes	NW‡
5600	6	891T>A	N297K	Missense	No	Yes	Yes
960	6	964A>G	S322G	Missense	No	Yes	Yes
2400	7	1025C>A*	A342D	Missense	No	Yes	Yes
4400	8	1126T>C*	S376P	Missense	Yes	Yes	Yes
3800	8	1187A>C*	Q396P	Missense	Yes	Yes	Yes
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. Nucelotide numbering is according to the cytosolic FH sequence (GenBank accession number NM_000143), with the A of ATG initiator codon as nucleotide position 1.
*Novel mutations; †status unknown; ‡no women evaluated.

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 the lack of available DNA from key members in four of the five families with the 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 a mutation hot spot or a founder mutation. There were no other tumours besides skin, uterine, or renal identified in the *FH* carrier family members in the 21 families with the exception of breast cancer in a woman from family 4600.

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 (fig 2). The son presented with gross haematuria at age 38. CT scan of the abdomen and pelvis revealed a 15 cm tumour in the left kidney. Upon microscopic examination after left radical nephrectomy, the renal cell carcinoma was reported as tubullo and papillary with cystic areas. Characteristic cytological features included amphophilic cytoplasm with large nuclei containing large eosinophilic-like inclusion nucleoli. Subsequently, the patient underwent a right partial nephrectomy and a right renal tumour demonstrated similar histology to the previously resected tumour 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 tumours. The histological 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) (fig 2). The patient's parents were screened for occult renal tumours 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 tumour in the right kidney. Subsequently, the father underwent right partial nephrectomy. Microscopic examination

showed renal cell carcinoma with solid and cystic lesions. No cutaneous lesions suspicious for leiomyomas were found on two independent dermatological examinations of the father and the son.

The proband of family 4000 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 tumour metastatic to retroperitoneal and mediastinal lymph adenopathy and multiple liver lesions. Microscopic examination showed renal cell carcinoma with focal papillary features and some clear cell areas (fig 1). Dermatological examination showed a single cutaneous leiomyoma. Sequencing analysis of the patient's genomic DNA revealed a 1 bp 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 tumours or cutaneous leiomyomas. However, there is a strong family history of kidney cancer in the proband's paternal family including his two aunts and a cousin (fig 1).

Renal tumours
In this study renal tumours occurred in 62% (13/21) of families with HLRCC (table 1). Twenty members from these 13 HLRCC families were affected with renal cell carcinoma. In 17 cases we confirmed the pathological diagnosis by histological 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 four deceased relatives who had died of kidney cancer, but no slides or pathology reports were available for review.

We found a histological spectrum of renal tumours associated with HLRCC. In family 4800, a father and his daughter were affected with collecting duct renal cell carcinomas described by 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, and tubullo-papillary elements. Renal tumour histology was also variable

within families and among families. In this report we identified three individuals who presented with bilateral and multifocal kidney tumours while the remaining 17 had solitary and unilateral kidney tumours.

Cutaneous leiomyomas

Seventy six per cent (16/21) of our new families with HLRCC presented with cutaneous leiomyomas (table 1). Clinically, cutaneous leiomyomas presented as firm skin-coloured to light brown-coloured papules and nodules. The number of lesions per individual ranged from none to more than 100. Forty eight per cent (10/21) of families had individuals affected with multiple cutaneous leiomyomas. There were three main patterns of presentation: 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 per cent 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 per cent (6/21) of the HLRCC families had mild cutaneous manifestations. Two families had an individual who exhibited only a single leiomyoma and kidney tumours. In addition, six individuals from four families presented with two to five cutaneous leiomyomas. Fifty per cent (3/6) of these individuals had cutaneous leiomyomas and renal tumours. 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 per cent (4/21) of the families did not have cutaneous manifestations. Sixty nine per cent (9/13) of *FH* gene mutation carriers from these four families without cutaneous manifestations had kidney tumours. The skin status of family 6200 is unknown since *FH* mutation carriers did not have a dermatological examination. In families without cutaneous leiomyomas, the clinical diagnosis of HLRCC was more challenging. Clues to the diagnosis of HLRCC were based on the kidney tumour cytological features, young age at presentation of kidney tumours, and aggressive clinical behaviour of tumours. Our study shows that expression of cutaneous manifestations in HLRCC is variable and ranges 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 to 15 tumours per patient and 1.0 to 8 cm in diameter. One hundred per cent (22/22) of women *FH* mutation carriers from 16 families had uterine fibroids (table 1). In the remaining five families only men participated in the study. Seventy three per cent (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 to 53 years of age. Sixty eight per cent (15/22) of women *FH* mutation carriers were diagnosed with uterine fibroids at 30 years of age or younger. Fourteen per cent (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 tumours but no skin leiomyomas.

Seventy three per cent (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 the other at 33 years of age. Subsequently, she underwent hysterectomy at age 39 for symptomatic relief of fibroids. Thirty six per cent (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 per cent of women in our cohort who had a hysterectomy or myomectomy had it at 30 years of age or younger.

Genotype-phenotype correlations

The two individuals with a single cutaneous leiomyoma and renal tumours had a nonsense mutation (R58X) or a frameshift mutation (c.111insA). 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, a broad spectrum of *FH* mutations were associated with a mild dermatological phenotype (1–10 leiomyomas) including: missense (R190H, S376P), nonsense (R58X), and frameshift (c.111insA) 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 an absence of cutaneous leiomyomas consisted of two missense (L89S and R190C), one nonsense (S102X), and one splice site (138+1G>C) mutation (table 1). Ten carriers of the above *FH* mutations ranging from 35 to 64 years of age had meticulous skin examinations on at least two different occasions 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 tumours consisted of nonsense mutations (R58X and S102X) in four families, missense mutations (R190H, R190C, S376P, L89S, and Q396P) in seven families, a frameshift mutation (c.111insA) in one family, and a splice site mutation (c.138+1G>C) in one family. In family 4800, a father and a daughter, who carried the R58X germline mutation, were affected with renal collecting duct carcinoma (CDC).

Families with the R190H and R58X mutations had a high frequency of kidney tumours. Seventy five per cent (3/4) of families with the R58X mutation and 60% (3/5) of families with the R190H mutation had renal tumours. Two families with R190H and a family with R58X were confirmed not to have kidney tumours following abdominal and pelvic CT screening. Family 5200 with a mutation at residue R190 also had kidney cancer. In this family both the father and the son presented with multifocal kidney tumours. Therefore, 67% (4/6) of families with mutations at residue R190 included individuals who developed kidney cancer compared with a 62% frequency of renal tumours among HLRCC families in this study. Therefore, given the relatively small numbers there is no difference between them. Families carrying the R190H and R58X mutations demonstrated variability of expression of renal tumours 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 characterise the clinical and genetic features of 21 new families with HLRCC. In addition, we present the first two African-American families reported to have HLRCC.

Sequence analysis revealed a total of 14 different *FH* germline mutations. We identified nine novel mutations in *FH*. Sixty two per cent (13/21) of the new families with HLRCC had renal tumours. Some families with renal tumours shared the same mutation, that is, R58X (three families) and R190H (three families). *FH* mutations were associated with a spectrum of renal tumours.

In combination with our previous report, to date we have identified 31 different germline *FH* mutations consisting of 20 missense, eight frameshifts (three insertions and five deletions), two nonsense, and one splice site mutation (fig 4). Mutations were distributed throughout the gene except for exon 5. Exon 4 (seven different mutations) and exon 6 (eight different mutations) had the most mutations. Forty two per cent (13/31) of *FH* mutations were associated with kidney tumours and were also distributed throughout the gene. There was no association between the location or type of mutation and kidney cancer. Eighty seven per cent (27/31) of *FH* mutations were associated with skin leiomyomas (fig 4). Four *FH* mutations identified in families without skin leiomyomas were associated with kidney cancer. However, no specific type of mutation was associated with absence of skin lesions. In addition, 96% (27/28) of *FH* mutations were associated with uterine fibroids (fig 4). 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 tumours. 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 that the missense mutants have a dominant negative action. 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 per cent (17/52) of families had a mutation at the R190 residue. It remains to be determined whether R190 represents a founder mutation. Eight per cent (4/52) of HLRCC families had the R58X mutation. The R58X appears to be a hot spot since haplotype analysis excluded a founder effect. Furthermore, R58X has also been reported in three European families with HLRCC.[2 8] Alam and co-workers found that the three probands with the R58X mutation shared an allele (frequency ~0.2) at only one microsatellite, $(CA)_{13}$, which is located in intron 2 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, which were found in six families each.[8] Haplotype analysis of the six families with N64T was consistent with a founder mutation. It is of interest that, to our knowledge, the G354R mutation has been identified in families with HLRCC in North America.

Previously, using direct sequencing we were not able to 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 approximately 2.4 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 tumours in 32% (18/56) of our cohort of HLRCC families at NCI. To our knowledge, this constitutes the largest collection of reported HLRCC families with renal tumours; however, 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 tumours increased to 62% (13/21). In the current study, families were recruited based on cutaneous leiomyomas or a diagnosis of kidney tumours with cytological features characteristic of HLRCC. The increase in frequency of renal tumours in this study compared to our previous study may be due to differences in recruitment approaches. Therefore, there may be a selection bias for families with renal cancer. In addition, as our histological 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 bp 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 having CDC of the kidney. Some potential factors may explain the lower frequency of renal tumours previously reported in families with leiomyomatosis and/or *FH* germline mutations.[1 10] First, families were not extensively screened for renal tumours and occult kidney tumours may not have been detected. Optimal screening for renal tumours involves CT scans of the abdomen and pelvis. Alternatively, MRI of the abdomen and pelvis is sometimes used if needed. Papillary renal tumours maybe difficult to detect if patients are screened with renal ultrasound only as they are often isoechoic and can be missed.[11] Second, it is also possible that some *FH* mutations have low penetrance for renal tumours or specific *FH* mutations are not associated with renal tumours. Third, some pathologists may lack experience to recognise the histological features associated with HLRCC renal tumours. HLRCC associated renal tumours are rare and have been only recently described.[1 3] Therefore, many pathologists may not be familiar with their features.

In this study, we identified the first family with 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 characterised
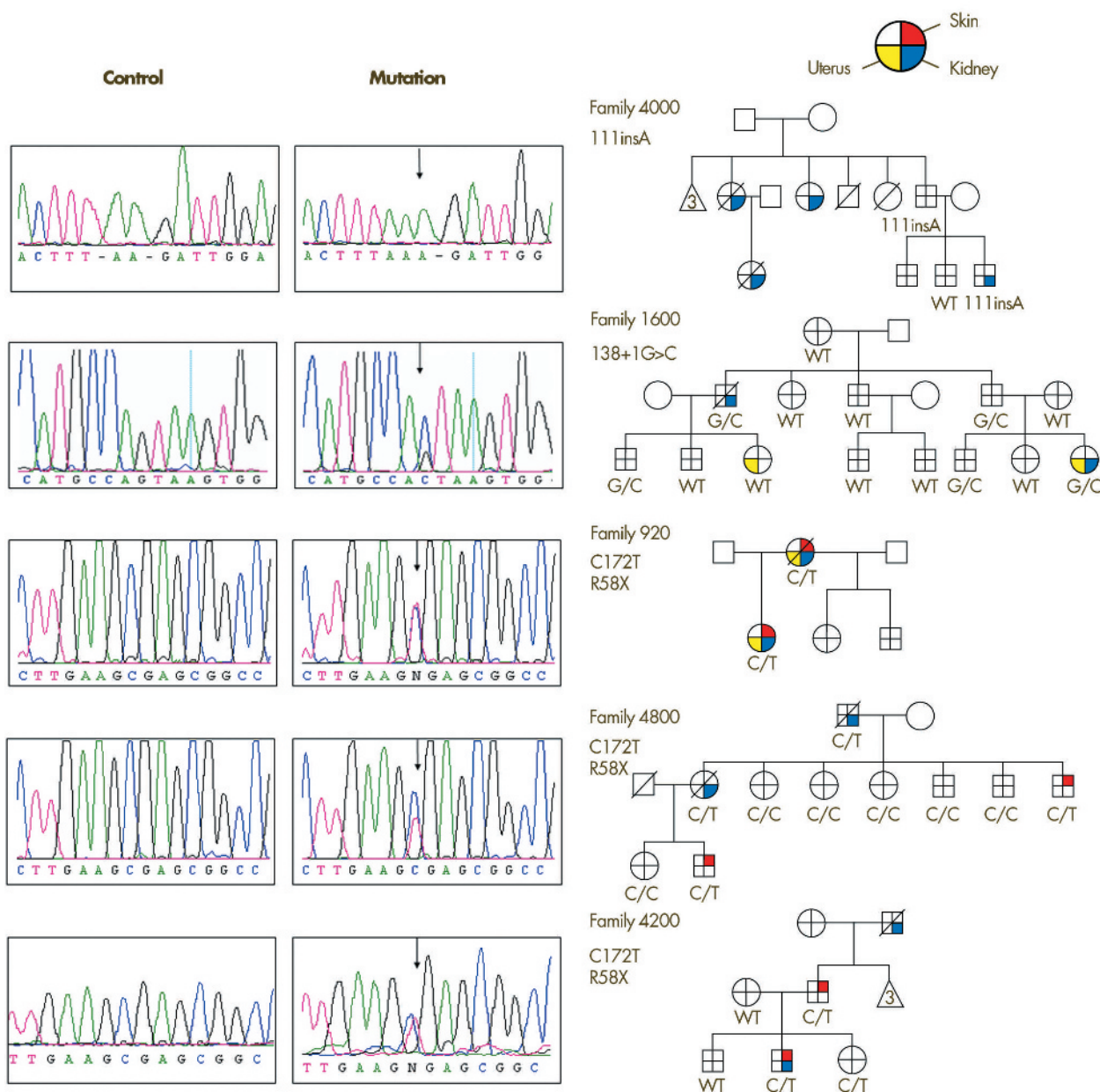
Figure 1 *FH* mutations in families 4000, 1600, 920, 4800, and 4200 newly characterised with HLRCC. Sequencing chromatograms of genomic DNA from control subjects and patients are shown on the left. The arrows indicate the position of the identified nucleotide changes. The corresponding pedigrees are shown on the right. WT, wildtype.

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 tumours was that the cytological features present in renal tumours were very consistent even though the architectural morphology of the tumours varied. Renal tumours associated with HLRCC were characterised by the presence of cells that had an abundant amphophilic cytoplasm and large nuclei with large inclusion-like eosinophilic nucleoli. These cytological features were originally attributed to type II papillary tumours in the original description[1]; however, they can be present in other histological types of renal tumours associated with HLRCC. HLRCC is associated with a histological spectrum of renal tumours. Most renal tumours in this study share some features with type II papillary renal cell

carcinoma. However, renal tumours associated with HLRCC are difficult to classify under the existing renal tumour classification schemes since they have distinct clinical and histological features. Therefore, renal tumours associated with HLRCC may in the future constitute a new renal pathological entity.

One hundred per cent 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, 68% (15/22) of women *FH*
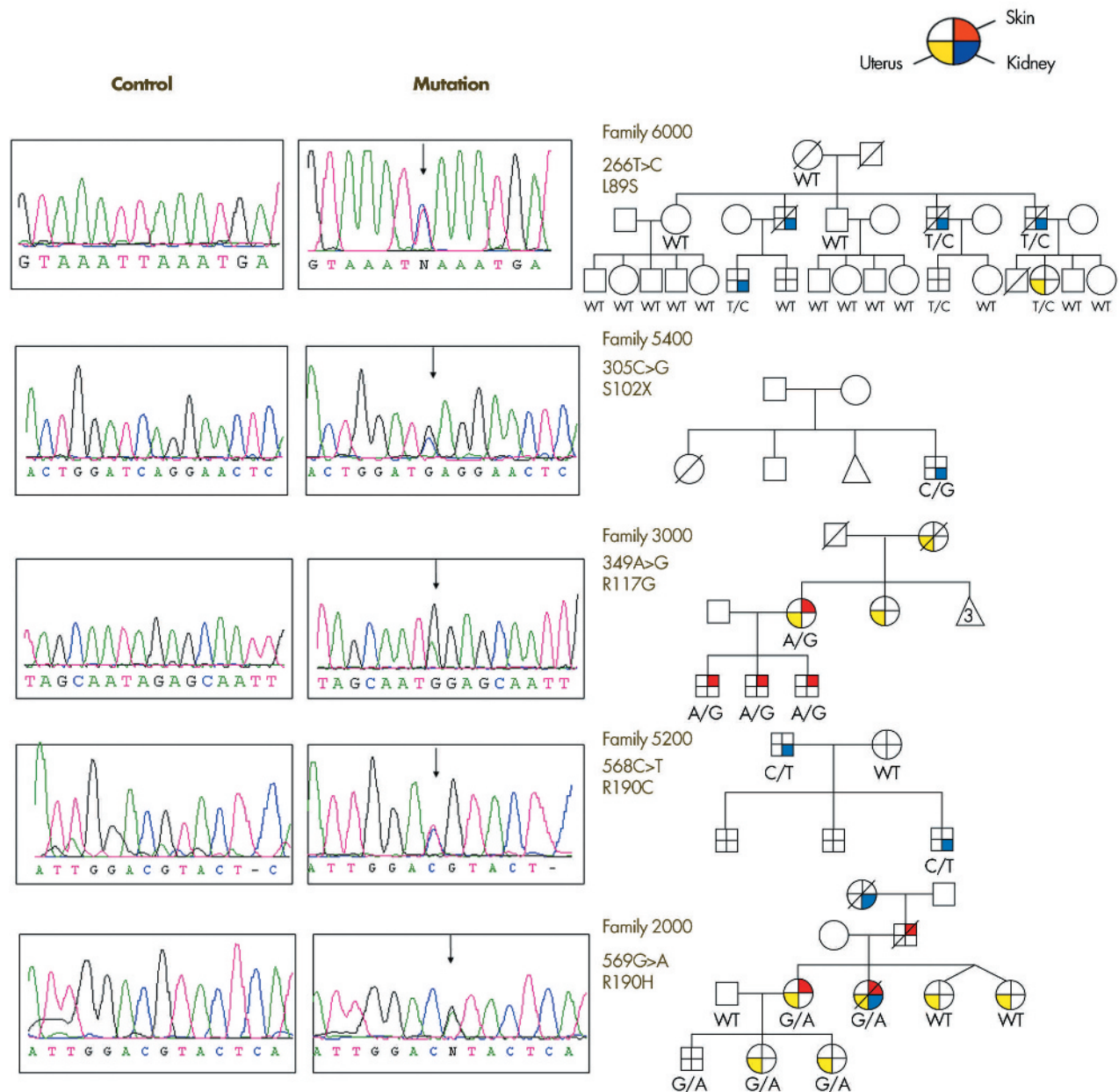
Figure 2 *FH* mutations in families 6000, 5400, 3000, 5200, and 2000 newly characterised with HLRCC. See fig 1 caption for explanations.

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 per cent of our cohort of women had a gynaecological 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 in women 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 it 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.

Sixty five per cent (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. In fact, we identified two *FH* mutations (S144L and N145S) corresponding to residues that form the active site and two germline *FH* mutations (S322G and S323N) that are located in the signature sequence motif.

FH most likely acts as a tumour suppressor in familial leiomyoma since LOH studies have shown loss of the wild type allele in cutaneous, uterine, and renal tumours, and FH
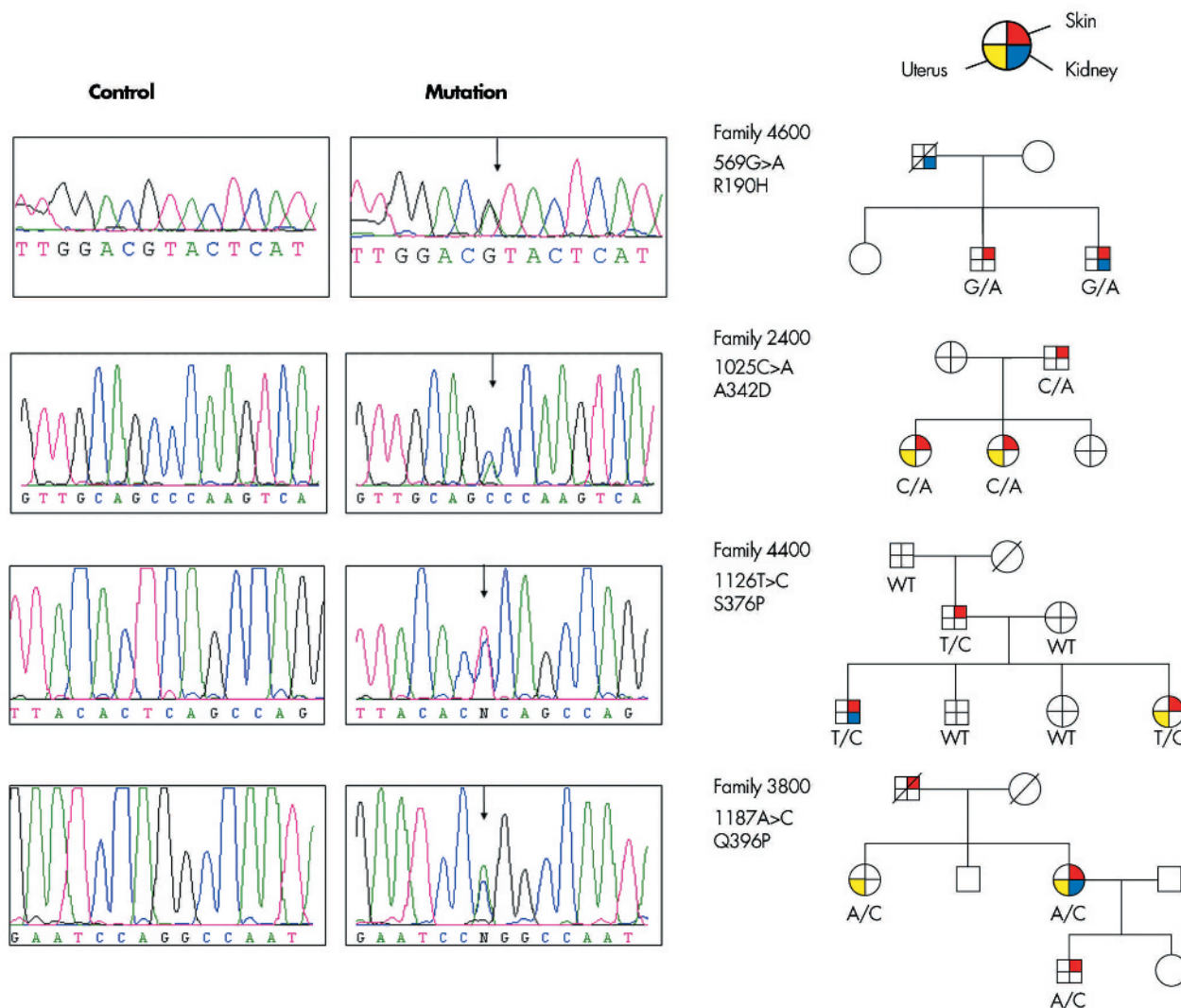
Figure 3 *FH* mutations in families 4600, 2400, 4400, and 3800 newly characterised with HLRCC. See fig 1 caption for explanations.

enzyme activity is low or absent in tumours from individuals with leiomyomas.[2] However, the mechanisms by which FH defects promote tumourigenesis are unknown. Possible mechanisms include hypoxia, apoptosis, and oxidative stress. It is of interest that germline mutation in another mitochondrial 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 *SHD-B* have been reported.[19] In one family with a germline *SDH-B* mutation (c.847-50delTCTC), two members had renal cell carcinoma and paraganglioma, and in another family, a son with clear cell renal cell carcinoma and his mother with a cardiac paraganglioma both had a germline *SDH-B* R27X mutation. Furthermore, all three of these renal cell carcinomas showed LOH at *SDH-B*. Taken all together, the literature suggests that mitochondria dysfunction through various mechanisms may lead to the formation of kidney tumours. Hypoxia mediated pathways have been shown to be implicated in tumourigenesis, 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.[20–22] When normal oxygen levels are present, this complex binds to HIF1-alpha and HIF2-alpha 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 tumourigenesis. Inactivation of *VHL* leads to the development of highly vascular tumours.[24] This is the mechanism that has been shown in VHL and it is possible that HLRCC and VHL share a common pathway or share some part of the pathway that is key for tumourigenesis. Recently, we showed that accumulation of fumarate and succinate following pharmacological 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 evidenced by an increased 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 SDH leads to HIF accumulation via inhibition of HIF prolyl hydroxylase in 293
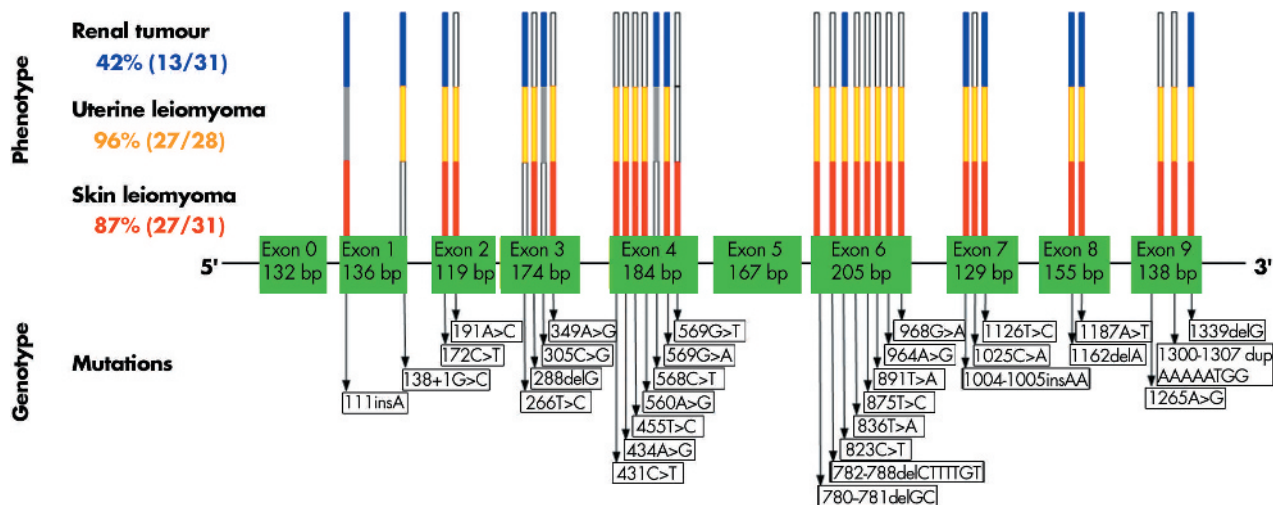
Figure 4 Distribution of *FH* mutations and the genotype-phenotype in HLRCC. The lower vertical arrows denote *FH* mutations. The upper vertical bars show the phenotype corresponding to the specific *FH* mutation. The colours in the vertical bars indicate the phenotype: blue, renal tumour; yellow, uterine fibroid; and red, skin leiomyoma. The empty bars indicate the absence of phenotype and the grey bars denote unknown phenotype status.

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 tumourigenesis in HLRCC.

It has been shown that mitochondria play a key major role in apoptosis. 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.[29] It is also possible that the mitochondrial 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.[30] In addition, glutamate can rescue carcinoma cell lines from apoptosis and can have a proliferative effect in different cell types.[31] Therefore, it is possible that tumourigenesis associated with HLRCC can lead to anti-apoptosis.

In conclusion, in this study we characterise the clinical and genetic features of 21 new families and expand the spectrum of phenotypes expressed in families with HLRCC. Patients with HLRCC can have a range of clinical presentations including multiple cutaneous leiomyomas, a single skin leiomyoma, no cutaneous lesions, multiple renal tumours, a single renal tumour, absence of renal tumours, uterine fibroids, and/or various combinations of these phenotypes. Furthermore, this variability of expression is present within and among families with HLRCC. Although individuals with HLRCC have diverse racial and ethnic backgrounds including African-American, patients with Eastern European heritage are over-represented. However, 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 a spectrum of aggressive renal tumours. Appropriate surveillance and genetic counselling is needed in the clinical evaluation of patients with HLRCC.

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ELECTRONIC-DATABASE INFORMATION

The following web pages were accessed: GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for mitochondrial *FH* precursor); National Center for Biotechnology Information, http://www.ncbi.nlm. nih.gov/; NCBI Blast Home Page, http:// www.ncbi.nlm.nih.gov/BLAST/ (for BLAST 2); 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); Wise2, http:// www.ebi.ac.uk/Wise2/.

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