Human Molecular Genetics, 2003, Vol. 12, No. 11 **1241–1252**
DOI: 10.1093/hmg/ddg148

Genetic and functional analyses of *FH* mutations in multiple cutaneous and uterine leiomyomatosis, hereditary leiomyomatosis and renal cancer, and fumarate hydratase deficiency

N.A. Alam[1,35,†], A.J. Rowan[1,†], N.C. Wortham[1,†], P.J. Pollard[1,†], M. Mitchell[2], J.P. Tyrer[3], E. Barclay, E. Calonje[4], S. Manek[5], S.J. Adams[6], P.W. Bowers[7], N.P. Burrows[8], R. Charles-Holmes[9], L.J. Cook[10], B.M. Daly[11], G.P. Ford[12], L.C. Fuller[13], S.E. Hadfield-Jones[14], N. Hardwick[15], A.S. Highet[16], M. Keefe[17], S.P. MacDonald-Hull[18], E.D.A. Potts[19], M. Crone[20], S. Wilkinson[21], F. Camacho-Martinez[22], S. Jablonska[23], R. Ratnavel[24], A. MacDonald[25], R.J. Mann[26], K. Grice[27], G. Guillet[28], M.S. Lewis-Jones[29], H. McGrath[30], D.C. Seukeran[31], P.J. Morrison[32], S. Fleming[33], S. Rahman[34], D. Kelsell[35], I. Leigh[35], S. Olpin[36] and I.P.M. Tomlinson[1,*]

[1]Molecular and Population Genetics Laboratory, [2]Computational Genome Analysis Laboratory and [3]Mathematics, Statistics and Epidemiology Department, Cancer Research UK, Lincoln's Inn Fields, London WC2A 3PX, UK, [4]Department of Dermatopathology, St John's Institute of Dermatology, St Thomas's Hospital, London SE1 7EH, UK, [5]Department of Histopathology, John Radcliffe Hospital, Oxford OX3 9PU, UK, [6]Torbay Hospital, Devon TQ2 7AA, UK, [7]Treliske Hospital, Truro, Cornwall TR1 3LJ, UK, [8]Addenbrookes Hospital, Cambridge CB2 2QQ, UK, [9]Warwickshire Hospital, Warwick CV3 5BW, UK, [10]St Mary's Hospital, Portsmouth PO3 6AD, UK, [11]Blackburn Royal Infirmary, Blackburn BB2 3LR, UK, [12]Dewsbury District Hospital, West Yorkshire WF13 4JU, UK, [13]88 Claylands Road, London SW8 1NJ, UK, [14]West Suffolk Hospital, Bury St Edmunds IP33 2QZ, UK, [15]Cannock Chase Hospital, Cannock WS11 2XY, UK, [16]7 Shilton Garth Close, Old Earswick, York YO3 9SQ, UK, [17]14 Forest Gardens, Lyndhurst, Hampshire SO43 7AF, UK, [18]Pontefract General Infirmary, West Yorkshire WF8 1PL, UK, [19]Halifax General Hospital, West Yorkshire HX3 0PW, UK, [20]North Hampshire Hospital, Basingstoke, Hampshire RG24 9NA, UK, [21]Leeds General Infirmary, Leeds LS1 3EX, UK, [22]Dermatologia H.U. Virgin Macarena Avda Dr Fedriani, No. 3 Sevilla 41071, Spain, [23]Department of Dermatology, Warsaw School of Medicine, Koszykowa 82a, Warsaw 02-008, Poland, [24]Stoke Mandeville Hospital, Buckinghamshire HP21 8AL, UK, [25]Independent Hospital, Tunbridge Wells TN3 0RD, UK, [26]Princess Margaret Hospital, Swindon SN1 4JU, UK, [27]BUPA Hospital, Harpenden AL5 4BP, UK, [28]Service Dermatologique CHR Brest Avenue, Foch-Brest 29200, France, [29]Wrexham Maelor Hospital, Wrexham LL13 7TD, UK, [30]Doncaster Royal Infirmary, South Yorkshire DN2 5LT, UK, [31]James Cook University Hospital, Middlesborough TS4 3BW, UK, [32]Department of Medical Genetics, Belfast City Hospital, Belfast BT9 7AB, UK, [33]Molecular and Cellular Pathology, University of Dundee, Ninewells Hospital, Dundee DD1 9SY, UK, [34]Biochemistry, Endocrinology and Metabolism Unit, Institute of Child Health, London, UK, [35]Centre for Cutaneous Research, St Bartholomew's and London School of Medicine and Dentistry, Queen Mary and Westfield College, Whitechapel, London E1 2AT, UK and [36]Neonatal Screening and Chemical Pathology, Sheffield Children's Hospital, Sheffield S10 2TH, UK

Received February 12, 2003; Revised and Accepted April 4, 2003

Germline mutations of the fumarate hydratase (*FH*, fumarase) gene are found in the recessive FH deficiency syndrome and in dominantly inherited susceptibility to multiple cutaneous and uterine leiomyomatosis

*To whom correspondence should be addressed.
†The authors wish it to be known that, in their opinion, the first four authors should be regarded as joint First Authors.

(MCUL). We have previously reported a number of germline *FH* mutations from MCUL patients. In this study, we report additional *FH* mutations in MCUL and FH deficiency patients. Mutations can readily be found in about 75% of MCUL cases and most cases of FH deficiency. Some of the more common *FH* mutations are probably derived from founding individuals. Protein-truncating *FH* mutations are functionally null alleles. Disease-associated missense *FH* changes map to highly conserved residues, mostly in or around the enzyme's active site or activation site; we predict that these mutations severely compromise enzyme function. The mutation spectra in FH deficiency and MCUL are similar, although in the latter mutations tend to occur earlier in the gene and, perhaps, are more likely to result in a truncated or absent protein. We have found that not all mutation-carrier parents of FH deficiency children have a strong predisposition to leiomyomata. We have confirmed that renal carcinoma is sometimes part of MCUL, as part of the variant hereditary leiomyomatosis and renal cancer (HLRCC) syndrome, and have shown that these cancers may have either type II papillary or collecting duct morphology. We have found no association between the type or site of *FH* mutation and any aspect of the MCUL phenotype. Biochemical assay for reduced FH functional activity in the germline of MCUL patients can indicate carriers of *FH* mutations with high sensitivity and specificity, and can detect reduced FH activity in some patients without detectable *FH* mutations. We conclude that MCUL is probably a genetically homogeneous tumour predisposition syndrome, primarily resulting from absent or severely reduced fumarase activity, with currently unknown functional consequences for the smooth muscle or kidney cell.

INTRODUCTION

Fumarate hydratase (FH, fumarase) catalyses the conversion of fumarate to malate as part of the tri-carboxylic acid cycle in the mitochondrial matrix. FH also exists in a cytosolic form which is thought to be involved in amino acid metabolism. FH is very highly conserved across species, although some lower organisms have additional fumarases which have relatively low homology to the human enzyme (1). FH is a homo-tetramer, with residues from three of the chains forming the active site of the enzyme (2).

Germline mutations in the fumarase (*FH*) gene are associated with two distinct conditions. In the homozygous or compound heterozygous state, bi-allelic *FH* mutations cause the fumarase deficiency syndrome. This inborn error of metabolism is characterized by gross developmental delay and death in the first decade (OMIM 606812). The germline mutations of 10 FH deficiency patients have been reported (3–5). In all of these cases, and in some patients who have not had mutation screening, affected individuals have been shown to have very low FH activity.

In the heterozygous mutant/wild-type state, *FH* acts as a tumour suppressor gene (6), with germline mutations causing a syndrome of multiple leiomyomata of the skin and uterus (multiple cutaneous and uterine leiomyomatosis, MCUL; OMIM 150800). MCUL has also been associated with an increased risk of type II papillary renal cancer and leiomyosarcoma (7,8) and this variant of the disease has been termed hereditary leiomyomatosis and renal cell cancer (HLRCC; OMIM 605839). There is limited evidence to show that the same *FH* mutations can occur in FH deficiency and MCUL patients, and that the parents of FH deficiency patients may be at risk of leiomyomata (6).

We have previously reported a number of germline *FH* mutations from multiple leiomyomatosis patients (6). In this study, we provide extra data concerning the role of germline *FH* mutations in tumorigenesis. We report a variety of additional mutations in a sample of 46 MCUL probands. We have mapped these mutations to the *FH* gene and protein, and thereby gained clues as to their functional effects. We have compared the spectra of mutations in FH deficiency and MCUL, and determined whether or not these are ancestral changes. We have examined the mutations identified in the minority of MCUL patients who also develop renal carcinoma to see whether or not these mutations share any common features. We have assayed FH functional activity in the germline, including some MCUL patients in whom direct sequencing failed to identify any *FH* mutations. We have determined the *in vitro* stability of mutant FH proteins. Finally, we have screened for 'second hits' in skin and uterine leiomyomata from MCUL individuals with missense mutations and we have found evidence for a non-papillary renal carcinoma resulting from germline *FH* mutation.

RESULTS AND DISCUSSION

Mutations in MCUL patients

We identified 20 distinct germline *FH* mutations (Table 1) in 35 of 46 (76.1%) screened probands (21/35 previously reported) (6). In 11 probands (families 58, 83, 136, 167, 209, 270, 324, 722, 764.11, 764.4 and 765.3), no *FH* variants were identified by direct sequencing of genomic DNA or F-SSCP. The 20 identified variants (Table 1) consisted of 13 missense changes, two nonsense mutations, two frameshift mutations, one in-frame deletion and two different germline deletions of 1q43 encompassing both *FH* and several flanking genes [46,XY.ish del(1)(q43) (BA96F14+, BA467I20−, BA25B4−, RGS7−, FH−, KMO−, CHML−, OPN3−, EXO1−, BA561I11+) and 46,XX.ish del(1) (q43) (BA467I20+, BA25B4−, RGS7−, FH−, KMO−, CHML−, OPN3−, EXO1−, BA561I11+)]. It is of note that members of the families with these large deletions had no phenotype

Table 1. All reported germline *FH* mutations in MCUL patients

Mutation	Exon	Nucleotide change	Mutation type	Family numbers	Number of patients
Q4X	1	CAA→TAA	Nonsense	596	4
Codon 17 frameshift	1	GAACTAA→GAATAA	1 bp deletion	1[c]	1
R58X	2	CGA→TGA	Nonsense	14, 296, 333	7
N64T	2	AAC→ACC	Missense	129[c], 182[c], 250, 304, 409, 765.4	23
A74P	2	GCT→CCT	Missense	47	1
H137R	3	CAT→CGT	Missense	782	1
Q142R	3	CAG→CGG	Missense	765.2	2
2bpdel, codon 181	4	GAGTTT→GTTT	2 bp deletion	LGL11556[b], LGL12497[b]	2
I186T	4	ATC→ACC	Missense	239	1
delI186	4	ATCATCAAG→ATCAAG	3 bp deletion	36[c]	1
K187R	4	AAG→AGG	Missense	317.2, 764.13	6
R190H	4	CGT→CAT	Missense	317.3, 552	2
G239V	5	GGT→GTT	Missense	138	3
R300X	6	CGA→TGA	Nonsense	LGL12404[b]	1
E312K	6	GAA→AAA	Missense	765, 765.50	4
N318K	6	AAT→AAA	Missense	766[a]	1
S323N	6	AGT→AAT	Missense	67	1
G354R	7	GGA→AGA	Missense	8, 84.2, 88, 123, 164, 764.9	12
Codon 406 frameshift	8	ATGAATG→ATAATG	1 bp deletion	84	4
L464P	9	CTG→CCG	Missense	317	3
Whole gene deletion	N/A	N/A	Whole-gene deletion	297	4
Whole gene deletion	N/A	N/A	Whole-gene deletion	713	7
None detected	N/A	N/A		58, 83, 136, 167, 209, 270[c], 324, 722, 764.11, 764.4, 765.3[c], 391[c]	24

[a]This UK family had an individual with renal cell carcinoma.
[b]These are previously reported Finnish families with renal papillary cell carcinoma type II and uterine leiomyosarcoma (6).
[c]These are probands with no known family history of MCUL.

other than leiomyomata, despite having only one copy of five other genes in the region.

The most frequent germline mutation was N64T (Table 1), found in six probands and a total of 23 subjects. Next most frequent was G354R, found in six probands and a total of 12 subjects. A nonsense mutation, R58X, was found in three probands and a total of seven subjects. Another three missense mutations were found in two probands each: K187R (six subjects); E312K, (four subjects); and R190H (two subjects). All other mutations were found in one proband only.

Of seven probands with no known family history of skin or uterine leiomyomata, four (cases 1, 36, 129 and 182) were found to have germline mutations in *FH* (Table 1). Parental DNA was not available to determine in each case whether the proband had acquired a new mutation, a parent had been a non-penetrant carrier, or disease had gone unreported or been overlooked in other family members; the fact that proband 182 with mutation N64T had a haplotype consistent with a founding mutation (see below) suggests that the last is a likely cause.

Although none of our probands was knowingly related and a coarse haplotype analysis had found no evidence of founding mutations, we re-analysed patients with the five common *FH* mutations for the three microsatellites within the *FH* gene. In four cases (N64T, K187R, R190H, G354R), the *FH* mutation was present on a common haplotype spanning all three microsatellites. For the other mutation, R58X, the three probands shared an allele (frequency ~0.2) at only one microsatellite, $(CA)_{13}$, which is located in intron 2 immediately after the R58X mutation; the possibility of a founding mutation therefore remains. Some geographical links were identified: for mutation N64T, all probands were of UK caucasian origin and four currently reside in the same UK county; and for mutation R190H, both probands were of Spanish origin, although one lives in the UK and one in Spain.

FH mutations and renal cancer

Two distinct *FH* mutations (2bpdel, codon 181 and R300X) have previously been found in MCUL (HLRCC) families in which renal cancer and uterine leiomyosarcomata were present (6). None of our families had leiomyosarcoma, but one patient from family 766 had a renal cancer, which presented at 18 years of age with metastatic disease. No primary tumour was resected, but a lymph node metastasis was available and it is known that the morphology of renal cancers is generally preserved in metastases. Histological review showed a tumour composed entirely of closely packed tubular structures with a dilated lumen containing mucoid material (Fig. 1). The tubules were lined by a single layer of cuboidal epithelial cells with eosinophilic cytoplasm. There was at most moderate pleomorphism, Furhman grade 2, and infrequent mitotic figures. There was no evidence of clear cell change and, although some complex intra-tubular growth was seen, no papillary structures were found. The tumour cells stained for large molecular weight

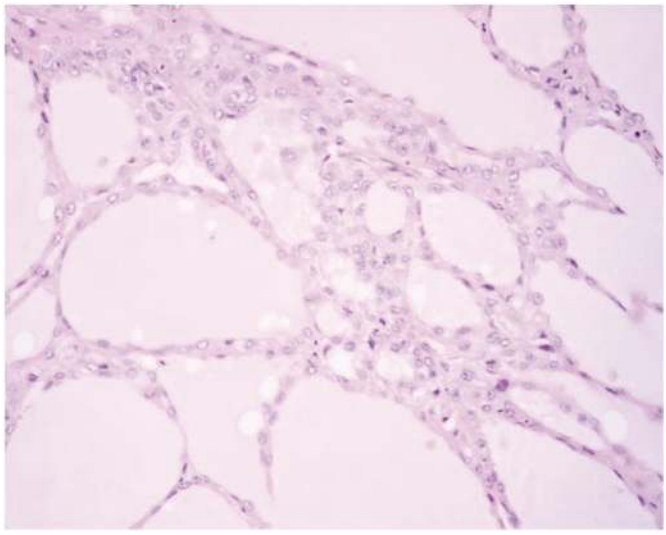

Figure 1. Renal cancer metastasis in MCUL family with N318K *FH* mutation. The tumour has a monomorphic architecture composed of tubules lined by eosinophilic cuboidal cells with moderate (grade 2) nuclear pleomorphism. No papillary growth pattern is seen and mitotic activity is low. Haematoxylin and eosin, original magnification ×120.

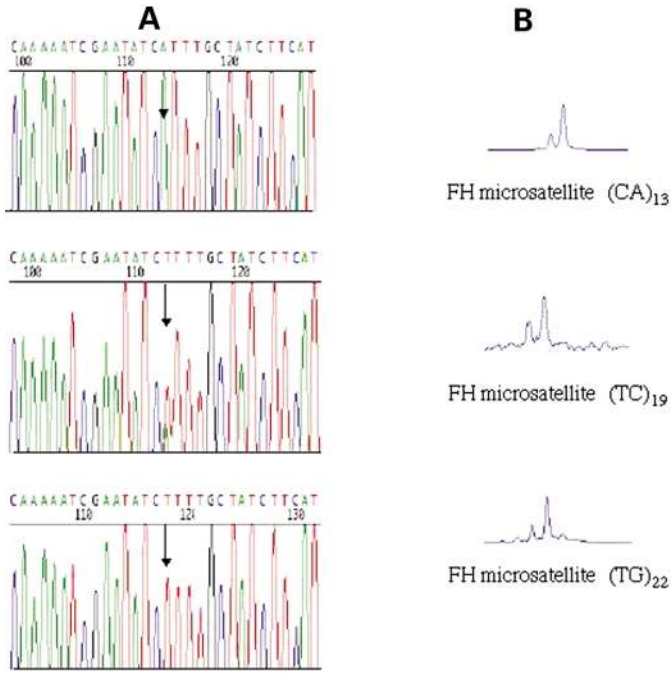

Figure 2. Loss of wild-type allele in renal carcinoma. (**A**) Wild-type control (top), constitutional DNA from family 766 proband showing A > T N318K (middle) and microdissected, metastatic renal tumour showing presence of T allele only (bottom). (**B**) Apparent homozygosity of renal tumour at three *FH* microsatellites.

cytokeratin using antibody 34β-E12, but not for cytokeratin 7, in common with all other renal cancers from MCUL/HLRCC cases. The family of the renal cancer patient had a missense N318K *FH* mutation. Neither this nor the Finnish families' mutations were identified in any other MCUL kindred. Although constitutional DNA was not available from the renal cancer patient, sequencing of DNA from the microdissected cancer showed only the mutant *FH* sequence, indicating loss of the wild-type allele, presumably as a 'second hit' (Fig. 2). Furthermore, genotyping of the 3 *FH* microsatellites showed the tumour to be homozygous for all three markers, which had been inherited from her mother who had leiomyomata (Fig. 2), again showing loss of the 'wild-type' allele.

The renal cancer reported here has features of a low-grade collecting duct tumour, a very rare entity which can undergo early metastasis (9). Our patient's cancer is therefore a different histological type from the type II papillary carcinomata reported previously in MCUL families by Launonen *et al.* (8) and others. However, Merino *et al.* (10) have recently reported a patient with MCUL who developed an oncocytic renal neoplasm, a type which may be related to collecting duct cancer.

Our histopathological and molecular data confirm that there is an elevated risk of rare types of renal carcinoma in MCUL families, and show that the HLRCC syndrome is indeed a variant of MCUL. Although the overall risk of renal cancer appears low in families ascertained on the basis of leiomyomata (1–2% in our series), it may be prudent to screen for renal cancers within families in which an individual has been affected by any type of renal neoplasm (8). The three *FH* mutations which have been associated with renal cancer do lie quite close together in the folded FH protein (see below), but are of different types and no genotype–phenotype relationship can currently be predicted for renal tumours.

Genotype–phenotype associations

We searched for dependence of phenotype on genotype. Phenotypic variables (age of presentation, number of skin lesions, presence of both cutaneous and uterine disease in females) were examined for associations with *FH* genotype [missense versus truncating changes, position of missense mutation in gene, common changes (N64T, G354R) versus others]. No significant associations were found (details not shown), consistent with the hypothesis that all *FH* mutations have similar functional effects (see below).

'Second hits' in leiomyomata

Previous studies had suggested that leiomyomata from patients with protein-truncating germline *FH* mutations almost always acquired a 'second hit' by loss of the germline wildtype allele (6–8,11). We extended this analysis to patients with missense germline *FH* mutations, since there is evidence from other genes, such as p53, in which dominant negative effects have been described [e.g. (12)], that missense mutations might not always require loss of the wild-type allele to have functional effects. Eighteen leiomyomata were studied: nine from three individuals carrying N64T; eight from three patients with G354R; and one from an individual with an S323N mutation. Seventeen of 18 tumours showed LOH at one or more markers (Table 2). In 15 tumours, the LOH pattern and haplotype analysis showed loss of the wild-type *FH* allele. In two tumours (T4 and T7), both skin leiomyomata from individuals with N64T mutations, the pattern of allele loss was suggestive of

Table 2. Loss of heterozygosity in MCUL leiomyomata

Family	Germline mutation	Patient	Tumour	Site	AL365184a	AC021359a	D1S180	D1S204	FH	D1S547	AL355992a	AL445221a
304	N64T	1	T1	u	NL	—	—	**LOH**		NI	—	NI
		1	T2	u	NL	NL	NL	**LOH**		NI	NL	NI
		1	T3	u	NL	—	NL	NL		NI	NL	NI
		1	T4	s	—	**LOH**	**LOH**	NL		NI	NL	NI
304	N64T	2	T5	s	**LOH**	**—**	**—**	**LOH**		NI	NL	NI
304	N64T	3	T6	s	NL	—	—	**LOH**		**NI**	**LOH**	**LOH**
		3	T7	s	—	**LOH**	—	NL		NI	**LOH**	**LOH**
		3	T8	s	—	**LOH**	**—**	**LOH**		NI	NL	**LOH**
		3	T9	s	**LOH**	**LOH**	**—**	**LOH**		**NI**	NL	**LOH**
84	G354R	4	T10	u	—	—	**LOH**	**LOH**		**LOH**	NI	NI
		4	T11	u	—	—	NL	**LOH**		NI	NI	NI
88	G354R	5	T12	u	**LOH**	**—**	**NI**	**NI**		**NI**	**LOH**	**LOH**
		5	T13	u	**LOH**	**—**	**NI**	**NI**		**NI**	**LOH**	NL
123	G354R	6	T14	u	—	—	—	**LOH**		NI	—	—
		6	T15	u	—	—	—	**LOH**		**NI**	**—**	**LOH**
		6	T16	u	—	—	—	**LOH**		NI	—	—
		6	T17	s	—	—	—	**LOH**		**NI**	**—**	**LOH**
67	S323N	7	T18	u	—	—	—	NI		**LOH**	—	—

LOH at each of eight microsatellite markers around *FH* (centromere–AL365184a–AC021359a–D1S180–D1S204–D1S547–AL355992a–AL445221a–telomere) is shown. Site of tumour = uterus (u) or skin (s). NL, no loss. NI, not informative. (—), Not done or failed. Possible extent of LOH is shown in bold.

homozygous deletion in the region of *FH*; perhaps in these tumours there was additional selection for loss of the small residual enzyme activity which may be conferred by the 64T allele. We did not find a 'second hit' in the one tumour in which we did not identify LOH.

FH deficiency-associated mutations

In addition to collecting data from previously published cases (3–5), we screened one FH deficiency patient and her parents for *FH* mutations (Table 3). We found that the child had inherited a novel missense mutation, P131R, and an A→G substitution at the −2 splice acceptor site of exon 2. The splice site variant led to aberrant splicing of *FH* mRNA, producing a novel shorter transcript and a reduced dosage of full-length transcript (data not shown). This finding was consistent with our subsequent detection of relatively high (92 nmol/mg/min) residual FH activity and a mild phenotype in this affected child. We had previously reported that the mother of a different, more severely affected FH deficiency patient (K190H/K190H) had developed multiple leiomyomata (6). However, despite being confirmed mutation carriers in their early 30s—by which age over 80% of MCUL patients have developed tumours (N.A. Alam *et al.*, unpublished data)—neither parent of the P131R/-2exon2 child had skin leiomyomata and the mother reported that no fibroids were present on a recent ultrasound screen in pregnancy. It is therefore possible that not all heterozygote *FH* mutations predispose to MCUL, or that some mutations have lower penetrance or cause disease of later onset.

Location and functional consequences of FH mutations

We mapped the location of mutations in the *FH* gene and protein in order to gain clues as to their functional effects. In both MCUL and FH deficiency, mutations occurred throughout the gene (Tables 1 and 2, Fig. 3). There was overlap between mutations in the two conditions, although there were weak tendencies for FH deficiency-associated mutations to occur later in the gene and for more truncating/whole-gene deletion mutations to occur in the MCUL patients. We have previously hypothesized (6) that under-representation of protein-truncating and deletion mutations in FH deficiency might result from these being null alleles, which would be lethal at an ante-natal stage in a homozygous state or as a compound heterozygote with another null allele; missense alleles, by contrast, would retain some—albeit very limited—enzyme activity (even though they might act as dominant negatives, see below). Our data are consistent with this hypothesis, although FH deficiency is too rare a disease to provide unambiguous evidence.

In order to determine whether or not missense *FH* mutations occurred at particular sites of functional importance, an alignment of FH was constructed across seven species (Fig. 4). Overall, the FH protein was found to be highly conserved (47.3% identical, 18.2% conserved, 9.0% similar). There were evidently 'blocks' of higher levels of conservation which corresponded to residues which formed the enzyme's active site or activation site, or were involved in protein loops with important roles in folding and tertiary structure. Missense mutations occurred mostly at evolutionarily conserved residues. In MCUL families, 62% were at 'identical' sites, 15% at 'conserved' residues and 23% at 'similar' weakly conserved residues. In FH deficiency patients, 86% of missense mutations occurred at 'identical' residues, the remainder being at 'conserved' sites. Two missense mutations (K187R, R190H) were common to both MCUL families and FH deficiency patients. There was no significant difference between the tendency for mutations to occur at fully or strongly conserved residues in MCUL and FH deficiency.

The high level of homology observed in the sequence alignment allowed us to model the human FH sequence onto the only solved crystal structure of FH, that of *E. coli* fumarase C,

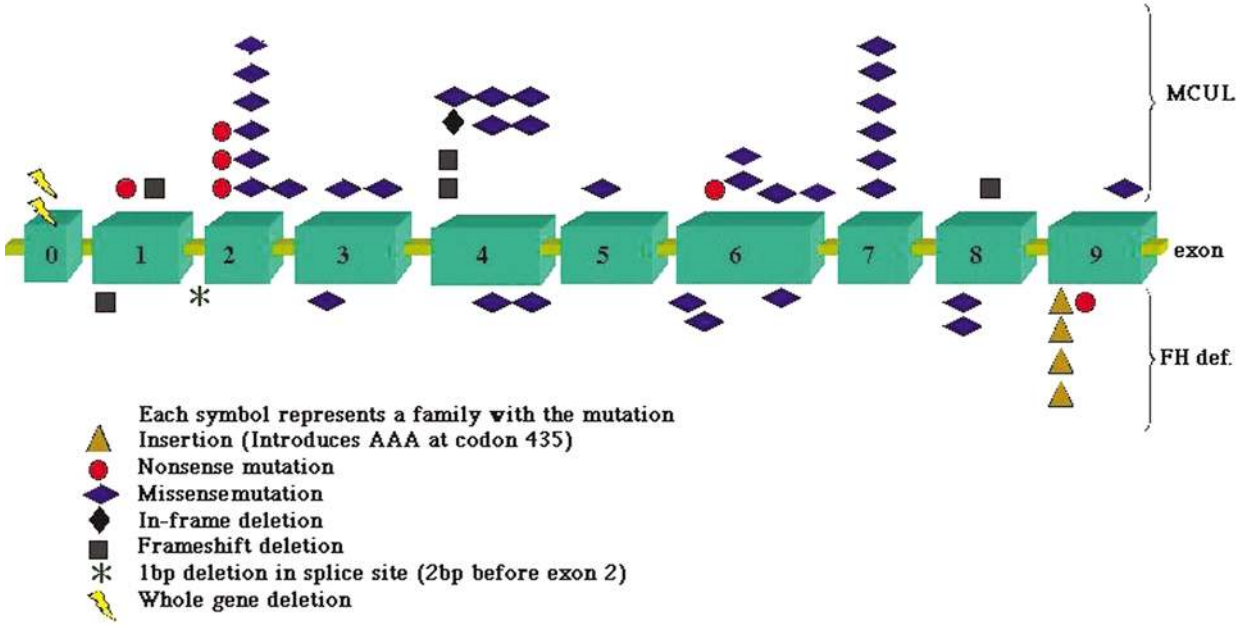

Figure 3. Location of MCUL and FH deficiency mutations within the *FH* gene.

Table 3. All reported germline *FH* mutations in FH deficiency patients

Reference	Mutation	Exon(s)	Mutation type	Activity (%)
Coughlin *et al.* (5)	A265T/A265T	6/6	Missense/missense	20
Coughlin *et al.* (5)	D383V/D383V	8/8	Missense/missense	<15
Coughlin *et al.* (5)	D383V/D383V	8/8	Missense/missense	13
This study	P131R/2-splice exon2	3/2	Missense/splicesite	13
Coughlin *et al.* (5)	435insK/?	9/?	Insertion/?	<12
Coughlin *et al.* (5)	435insK/?	9/?	Insertion/?	10
Coughlin *et al.* (5)	−269C/435insK	6/9	Missense/insertion	10
Gellera *et al.* (3)	R190H/435insK	4/9	Missense/insertion	<5
Coughlin *et al.* (5)	K187R/K187R	4/4	Missense/missense	3.7
Coughlin *et al.* (5)	66del74/W458X	1/9	Deletion/nonsense	<2
Bougeron *et al.* (4)	E319Q/E319Q	6/6	Missense/missense	<1

which has been reported to be a homotetramer containing two substrate binding sites (1,2). The first of these, identified as the catalytic site, utilizes residues from three of the four chains within the protein (Fig. 5A), and requires tetramerization to arrange these residues appropriately. We found the residues forming the catalytic site to be invariant across species (Fig. 4). A second substrate binding site, near to the active site, has also been identified. This second site utilizes residues from only one chain and has 1–2 orders of magnitude less affinity than the active site. In both prokaryotes and mammals this site has been identified as an activation site, which binds substrate at higher concentrations, resulting in an increase in the activity of the enzyme. The residues forming this site are also highly conserved across species (Fig. 4).

MCUL missense mutations were found at either end of the monomer (Fig. 5B), in the same domains as residues implicated in the active site, and, with the exception of the G354R mutation, in looped regions of the protein. The only active or activation site residue found to be mutated was histidine 137, which is a part of the activation domain. The other missense mutations could have one of two effects that would lead to a loss of enzyme activity. Firstly, mutations could affect the active site of the protein without affecting the structure; an example of this was the G239V mutation, which could alter the structure of an active site loop. Second, mutations could exhibit effects on the overall structure of the protein, resulting in misfolding or oligomerization, and thus formation of the active site, and so function. A potential member of this group was the S323N mutation. In the wild-type protein, this residue forms two strong hydrogen bonds with the amine group of lysine 430 on a different chain, an interaction apparently important for oligomerization (1,2). Interestingly, in at least two cases, MCUL mutations affected specific interactions between residues in the protein. Two such

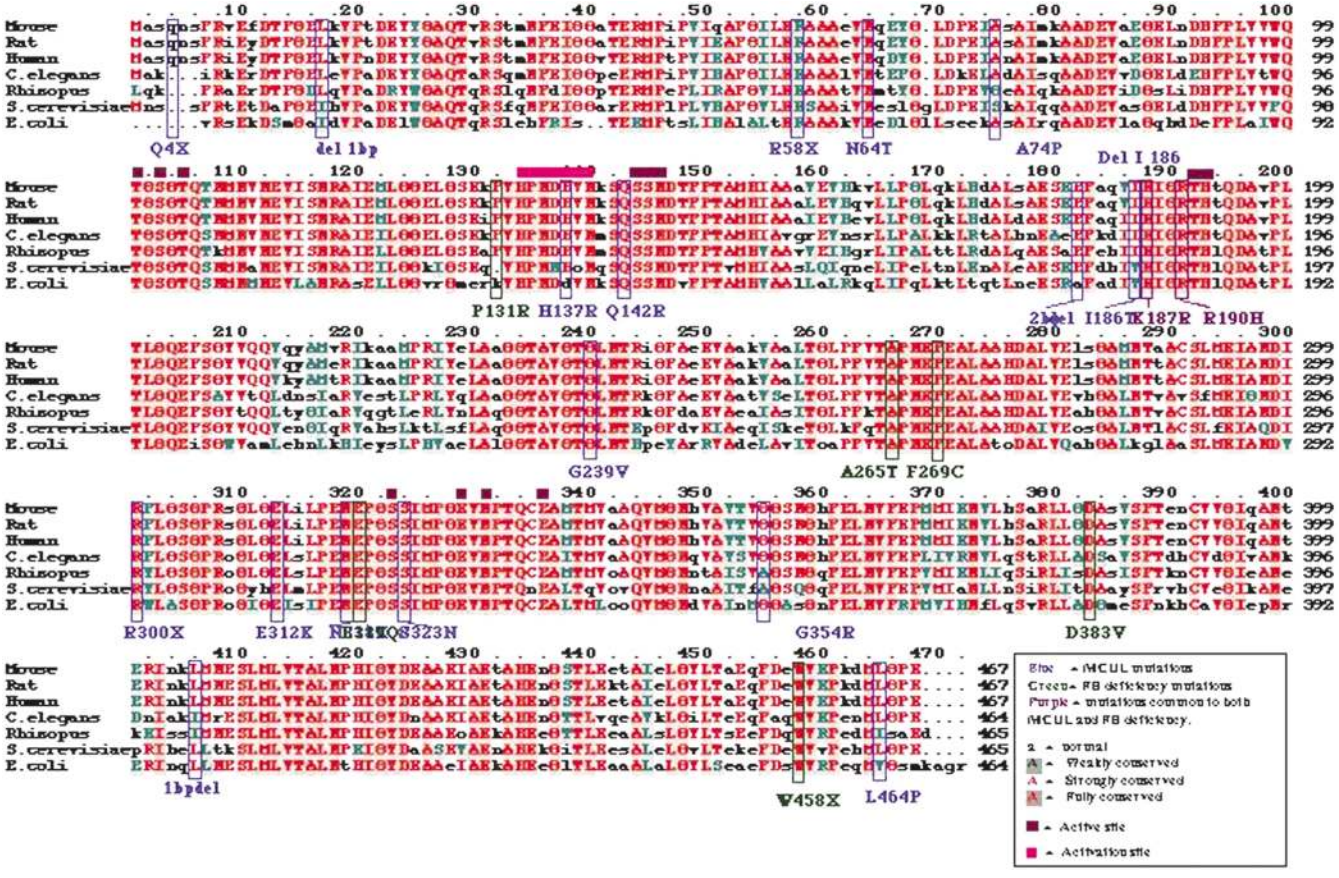

Figure 4. Multiple sequence alignment of *FH*. Mutations are indicated by the coloured boxes. Amino acids with similar properties are classified into strong and weak groups by the software used: strong groups—STA, NEQK, NHQK, NDEQ, QHRK, MILV, MIL-, HY, -YW; and weak groups—CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, -VLIM, H-Y. Mutations found in MCUL and FH deficiency are shown.

interactions were the charge–charge interaction between lysine 187, arginine 190 and glutamate 312—an interaction that appears to greatly stabilize one of the active site loops—and the hydrogen bonding interaction between glutamine 142 and asparagine 64. In both cases, the residues involved in each interaction were mutated in different patients; additionally, in the former case, other probands had mutations at isoleucine 186. We also examined the location of the three mutations associated with renal carcinoma. Although all were located in the same domain of the protein, the mutations were of different types (two truncating, one missense) and did not congregate at any precise location.

Mutations in FH deficiency patients demonstrated a similar pattern to MCUL mutations, with a majority at either end of the monomer (Fig. 5C). The locations of these mutations within the monomer were very similar to those seen in MCUL patients. For example, the E319Q mutation occurs on the same loop as the S323N and E312K MCUL mutations, and could have an effect on the conformation of the active site. Other observed mutations may have an effect on the conformation of the active site; for example, both alanine 265 and phenylalanine 269 form hydrophobic interactions that appear to help stabilize looped regions of the protein in the region of the active site. Another observed mutation, P131R, lies in the region of the activation site. Another mutation, D383V, mapped to the external face of

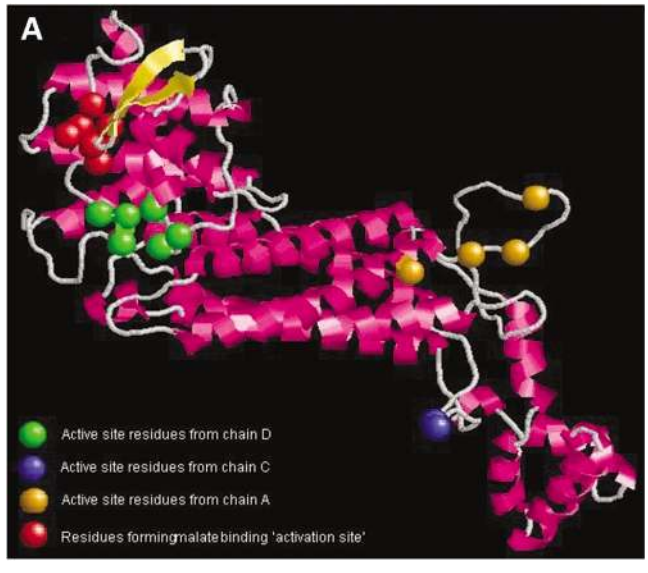

Figure 5. Mapping of MCUL and FH deficiency mutations onto the crystal structure of *E. coli* fumC. (**A**) Active and activation site residues. (**B**) MCUL mutations in relation to active site residues (front and rear views). (**C**) FH deficiency mutations in relation to active site residues; mutations not shown on this figure are 66del74 and a splice site mutation in intron 2.

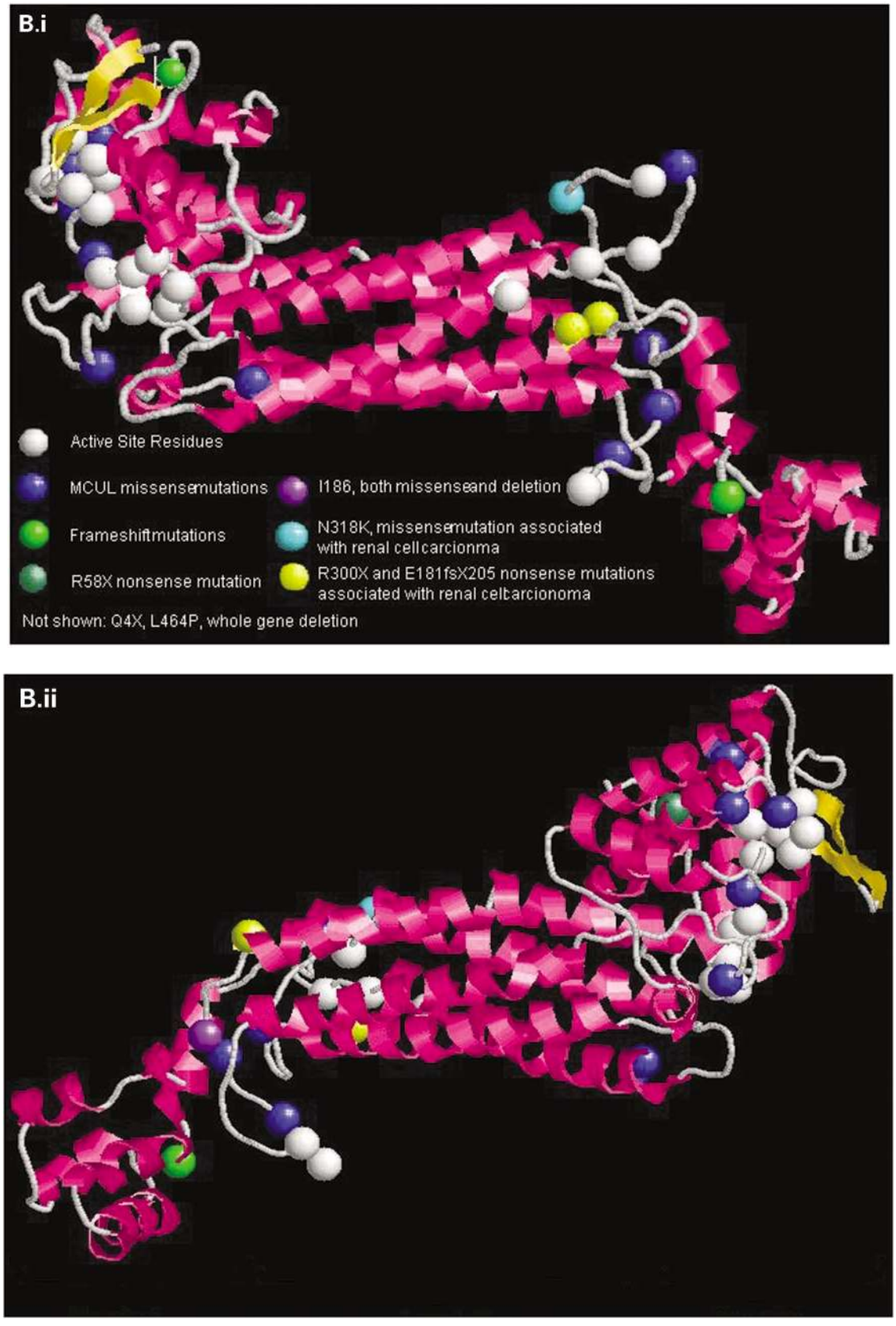

Figure 5. *Continued.*

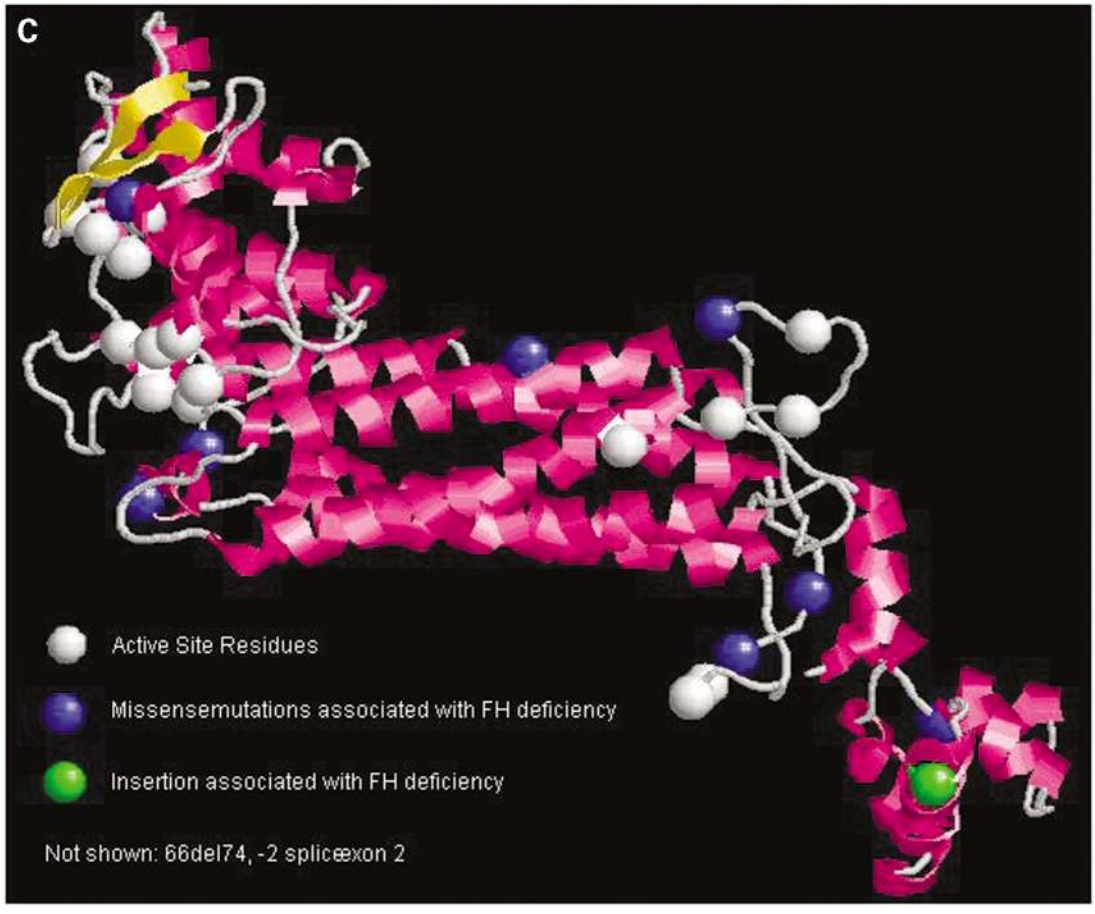

Figure 5. *Continued.*

an outward facing α-helix and the effects of this change are not clear. Finally, as in MCUL, one mutation (435insK) mapped to domain 3 of the protein, with potential effects on oligomerization. What could not readily be understood from this comparison was why some parents of FH deficient patients do not exhibit MCUL. The distribution of the mutations between the two conditions was very similar, as were the possible effects of the mutations on the protein.

Overall, consideration of the probable functional consequences of the missense *FH* mutations strongly suggests that they lead to greatly reduced fumarase activity. This contention is supported both by our previous work, which showed undetectable FH activity in tumours from patients with missense changes and in-frame deletions, and by analyses of FH deficiency patients (4,5), which show low or very low activity associated with most missense mutants. Our failure to find any clear genotype–phenotype association in MCUL is consistent with a model in pathogenic mutations have very similar functional effects.

mRNA and protein stability

Our previous results (6) had suggested that missense, but not truncating, *FH* mutants might act as dominant negatives in the germline of MCUL patients—presumably through alteration of the FH tetramer's conformation—although we had found no evidence of any resulting clinical effects. The fact that we did not find evidence of dominant negative action in patients with truncating *FH* mutations suggested that the mutant mRNA or protein might be unstable in these cases. We screened four patients with protein-trucating mutations (Q4X, codon 17 frameshift, R58X and codon 406 frameshift) and detected mutant mRNA from these patients' lymphoblastoid lines. However, western blotting did not show any truncated proteins (data not shown). Even allowing for the possibility that the very early mutations may not have produced a detectable protein product, the data from the other two patients indicate that truncated FH proteins appear to be unstable, as predicted. Thus, although we cannot entirely exclude protein instability in the tetrameric state, it is likely that truncating *FH* mutations are functionally null alleles.

Germline fumarase activity

We assayed germline fumarate hydratase activity in 38 MCUL probands [some reported previously, in (6)] for whom lymphoblastoid cell lines were available, and 31 controls (Fig. 6). Twenty-three of the probands had missense changes, one had an in-frame deletion, five had truncating mutations, two had whole-gene deletions and seven had no identified *FH*

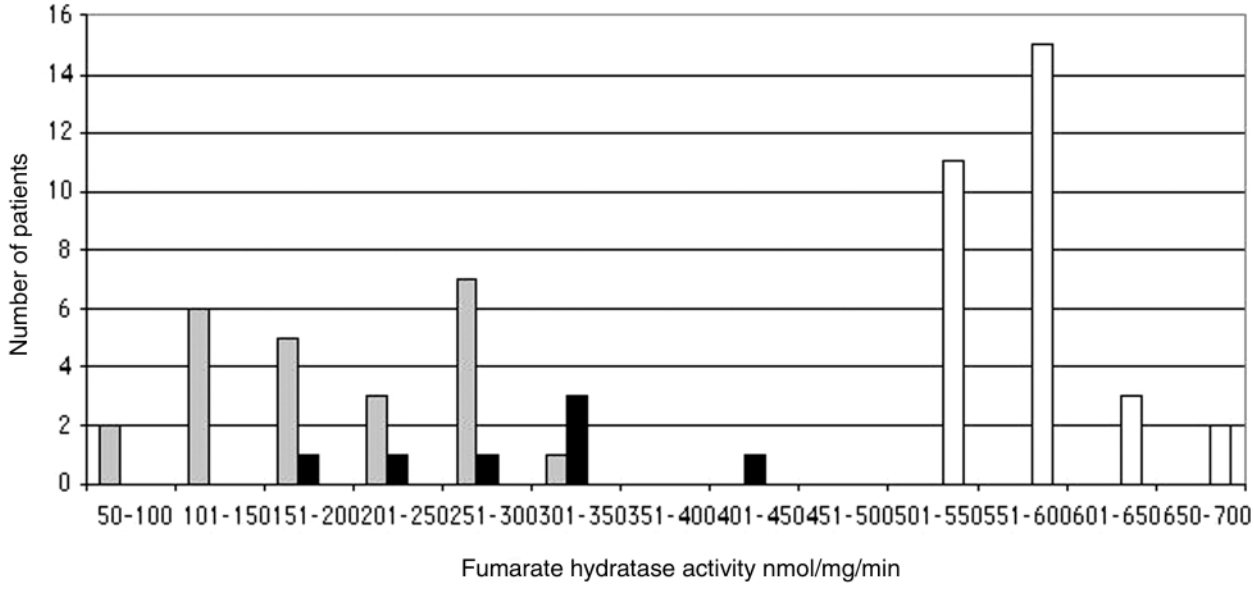

Figure 6. Fumarase activity in lymphoblastoid cell lines from MCUL patients with missense/in-frame deletion (grey) and truncating/null (black) germline mutations compared with spouse controls (white).

mutation. We showed that *FH* mutant patients had significantly lower levels of activity (mean 226, median 232, range 96–438) than controls (mean 565, median 555, range 509–681) ($P < 0.00001$, Mann–Whitney test), but higher activity than two FH deficiency patients, who had 3% (17 nmol/mg/min) and 16% (92 nmol/mg/min) FH activity compared with the control mean. When we compared patients with missense changes to those with truncating mutations or large deletions, we found that the former had significantly lower activity (mean 208, median 200, range 96–341 versus mean 296, median 306, range 163.5–438, $P = 0.0226$, Mann–Whitney test). The FH activity of MCUL patients with known *FH* mutations thus ranged from 17 to 78% of the control mean. Use of an enzyme activity threshold of 475 nmol/mg/min provided nominal 100% sensitivity and specificity for indicating an *FH* mutation.

Of the seven probands without detected *FH* changes, five had diminished enzyme activity (cases 209, 136, 764.11, 722 and 764.4 had activities of 145, 257, 293, 303 and 385 nmol/mg/min, respectively), suggesting that these individuals did carry occult germline changes in *FH* (although mutations at another locus leading to decreased FH function cannot entirely be excluded). The other two patients without detected germline *FH* mutation had normal fumarate hydratase activity (cases 765.3 and 270, with activities of 593 and 612 nmol/mg/min, respectively). These were both female probands with only a single cluster of skin leiomyomata, no fibroids and no known family history of disease. These individuals may therefore have been true 'sporadic' cases or *FH* mosaics.

It is notable that some MCUL patients (Fig. 5) have very low FH activity (minimum detected value, 96 nmol/mg/min) in the germline, despite having one normal copy of the gene (as shown by direct sequencing of gene and promoter, data not shown). The most plausible explanation is dominant negative action of the missense mutant, preventing fully functional tetramers from forming in all but 1/16 of molecules. The FH activity of some MCUL patients is nearly as low as that of some FH deficiency patients. Despite this, we confirmed that none of our MCUL patients suffered any of the adverse developmental consequences which typify FH deficiency. The explanation for this apparent discrepancy is unclear, but it is possible that physiological up-regulation of FH levels and/or activity can occur more readily in the MCUL patients who produce protein from one fully-functional *FH* allele.

Conclusions

We have identified underlying germline *FH* mutations in the great majority of patients with MCUL/HLRCC. Most patients without identified *FH* mutations have diminished FH activity, suggesting that MCUL is a genetically homogeneous condition. Most of the common *FH* mutations may be derived from founders. The FH functional assay appears to be as specific as, and more sensitive than, direct sequencing for identifying FH dysfunction, but the requirement for cultured cells means that it may best be reserved as a backup investigation if no mutation is identified by DNA analysis. We have identified only a small number of non-familial cases with neither germline FH mutations nor diminished FH function, suggesting that true 'sporadic' cases of skin leiomyomata are rare.

MCUL-associated mutations probably either result in truncated/absent protein or are missense changes, most of which target residues close to the enzyme's active site and lead to deficient enzyme activity. Almost all leiomyomata and renal cancers from MCUL families show loss of the wild-type *FH* allele and hence low or absent FH activity results in these tumours. Some parents of patients with bi-allelic *FH* mutations do develop leiomyomata, but this may not be the case for all FH deficiency-associated mutations. The consequences of deranged FH activity in the smooth muscle cell and the tumorigenic pathways remain opaque, but our data suggest that

gross derangement of the Krebs cycle is the proximate cause and the combined genetic data provide a sound basis for future functional studies.

MATERIALS AND METHODS

Study subjects

We recruited probands principally through a circular letter to UK dermatologists. Probands were ascertained on the basis of a personal history of multiple skin leiomyomata, since these tumours are a more specific indicator of multiple leiomyomatosis than uterine fibroids. Additional family members were identified through the proband. All subjects were examined for the presence of skin leiomyomata by a geneticist or dermatologist. Histological confirmation of skin leiomyomata was obtained wherever possible. Patients were considered to have uterine leiomyomata if these had been previously diagnosed by pelvic ultrasound, hysteroscopy or examination after myomectomy or hysterectomy. Forty-six probands and a further 60 affected relatives were recruited. We identified one patient, the daughter of the proband from family 766, who had died of metastatic renal cancer at age 18. We also identified two unrelated FH deficiency patients and examined them and their parents for skin leiomyomata. Additional data on FH deficiency mutations were obtained from published reports. The study was performed with full informed consent and Ethical Review Board approval.

Samples

DNA was extracted from blood samples from study subjects using standard methods. In addition, we also cultured lymphoblastoid cell lines from 38 probands. We obtained fixed, paraffin-embedded, archival specimens of seven skin leiomyomata and 11 uterine fibroids from seven affected individuals. We also obtained archival material of a lymph node metastasis from the renal cancer from the daughter of the proband from family 766.

Mutation screening

Mutation screening was performed on probands by direct sequencing of genomic DNA in forward and reverse orientations, using reported oligonucleotides, the Applied Biosystems Big Dye terminator reaction kit and the 377 semi-automated DNA sequencer (6).

Genotyping for founder mutations

We identified three intronic polymorphic microsatellites within the *FH* gene: a $(CA)_{13}$ repeat between exons 2 and 3; a $(TG)_{22}$ repeat between exons 3 and 4 and a $(TC)_{19}$ repeat between exons 7 and 8. Oligonucleotides were designed to amplify each of these markers: FH$(CA)_{13}$ (ATTGGCATGCACAAAGCC TA, TGGCATCCCCAGTCTCTATC); FH$(TG)_{22}$ (CCATTC ATCCTACTTCTTTCGT, GGGTGACAGAGTGAAGCACA); and FH$(TC)_{19}$ (TGTTTCCTTGGTTATAGTGCCTTA, CC TGGCTGAGTGTAACACATTT) using an annealing temperature of 55°C and otherwise standard PCR conditions. Individuals and, where possible, families with identical *FH* mutations were genotyped at these microsatellites in order to determine whether or not they shared common haplotypes.

Renal cancer

This metastatic tumour (family 766) was reviewed by a specialist renal histopathologist (SF). After microdissection to remove a small quantity of normal tissue, we extracted DNA using a simple proteinase K digestion. We carried out direct *FH* sequencing and genotyping it for the three polymorphic microsatellites in *FH*.

'Second hits' in familial tumours

Haematoxylin and eosin-stained sections of 7 skin leiomyomata and 11 uterine fibroids from seven individuals were reviewed by a gynaecopathologist (SM) or a dermatopathologist (EC) as appropriate. After microdissection and DNA extraction as above, loss of heterozygosity (LOH) analysis was carried out using eight microsatellite markers (6), (further details available from the authors) previously reported as being derived from clones around *FH* (www.sanger.ac.uk/HGP/Chr1/). After analysis using the ABI377 sequencer and Genotyper software, corrected allelic ratios of less than 0.5 in tumours were considered to indicate loss of heterozygosity. The origin of the lost allele (mutant or wild-type) was determined by haplotype reconstruction.

Sequence alignment and structural studies

Clustal X PPC (1.64b) and Mac Boxshade (2.18) were used to align human FH with the equivalent mouse, rat, *C. elegans*, *Rhizopus*, *S. cerevisiae* and *E. coli* enzymes. Mutations observed in MCUL and FH deficiency patients were then modelled onto the crystal structure of *E. coli* fumarase C. The *E. coli* homologues of mutated residues in human FH were identified using the multiple sequence alignment. The high level of homology between the two protein sequences provided confidence that modelling onto the *E. coli* structure would produce an accurate model. Figures were drawn using Rasmol V2.7.1.2 using the protein databank file 1FUO.

RT–PCR analysis

cDNA was synthesized from RNA isolated from the lymphoblastoid cells of affected family members using the FastTrack mRNA purification kit (Invitrogen, Paisley, UK) followed by randomly primed cDNA synthesis using the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Amersham, UK). For RT–PCR analysis, the *FH* gene from the 5′-UTR to 3′-UTR was amplified in two regions using oligonucleotide pairs: FHCDNA1.F, 5′-TTTTACCCAAGCTCCCTCAG-3′, FHCDNA1.R, 5′-GAGCAGCCAGAGCTTCAAAT-3′; and FHCDNA2.F, 5′-GCCATGCCAAGAATCTATGAG-3′, FHCD NA2.R, 5′-CCGTTTTTAAGAAATGGGAGTC. RT–PCR was performed by adding 2 µl of cDNA to a 50 µl reaction containing 20 pmol of each primer and 200 µM dNTPs, 10 mM Tris–HCL(pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton-100 and 0.5 U of *Taq*. A denaturing cycle of 94°C for 4 min was

followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. PCR product size and quality were checked on a 1.2% agarose gel.

Western blotting

Fumarase was characterized for size and semi-quantitative protein levels using standard western techniques. Briefly, 1×10^6 patient lymphoblastoid cells were detergent-lysed, denatured and separated on a 10% acrylamide gel followed by charged transfer to PVDF membrane (Millipore, Watford, UK). Patient and control samples were probed with rabbit anti-fumarase (Nordic Immunological Laboratories, Tilburg, Netherlands) and detected using enhanced chemi-luminescence (ECL; Amersham Pharmacia Biotech, Amersham, UK). β-actin was used as a control.

FH functional activity

For 38 probands from whom lymphoblastoid cell lines were available, FH activity was assayed by measuring substrate-specific catalysis by extracted protein *in vitro* as described previously (13). Fumarate hydratase activity was also measured in 31 UK controls without leiomyomata or any malignancy.

ACKNOWLEDGEMENTS

We are grateful to the Equipment Park, Cancer Research UK London Institute and to the patients and families involved in the study.

REFERENCES

1. Weaver, T.M., Levitt, D.G., Donnelly, M.I., Stevens, P.P. and Banaszak, L.J. (1995) The multisubunit active site of fumarase C from *Escherichia coli*. *Nat. Struct. Biol.*, **2**, 654–662.
2. Weaver, T. and Banaszak, L. (1996) Crystallographic studies of the catalytic and a second site in fumarase C from *Escherichia coli*. *Biochemistry*, **35**, 13955–13965.
3. Gellera, C., Uziel, G., Rimoldi, M., Zeviani, M., Laverda, A., Carrara, F. and DiDonato, S. (1990) Fumarase deficiency is an autosomal recessive encephalopathy affecting both the mitochondrial and the cytosolic enzymes. *Neurology*, **40**, 495–499.
4. Bourgeron, T., Chretien, D., Poggi-Bach, J., Doonan, S., Rabier, D., Letouze, P., Munnich, A., Rotig, A., Landrieu, P. and Rustin, P. (1994) Mutation of the fumarase gene in two siblings with progressive encephalopathy and fumarase deficiency. *J. Clin. Invest.*, **93**, 2514–2518.
5. Coughlin, E.M., Christensen, E., Kunz, P.L., Krishnamoorthy, K.S., Walker, V., Dennis, N.R., Chalmers, R.A., Elpeleg, O.N., Whelan, D., Pollitt, R.J. *et al.* (1998) Molecular analysis and prenatal diagnosis of human fumarase deficiency. *Mol. Genet. Metab.*, **63**, 254–262.
6. Tomlinson, I.P.M., Alam, N.A., Rowan, A.J., Barclay, E., Jaeger, E.E., Kelsell, D., Leigh, I., Gorman, P., Lamlum, H., Rahman, S. *et al.* (2002) Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. *Nat. Genet.*, **30**, 406–410.
7. Kiuru, M., Launonen, V., Hietala, M., Aittomaki, K., Vierimaa, O., Salovaara, R., Arola, J., Pukkala, E., Sistonen, P., Herva, R. *et al.* (2001) Familial cutaneous leiomyomatosis is a two-hit condition associated with renal cell cancer of characteristic histopathology. *Am. J. Pathol.*, **159**, 825–829.
8. Launonen, V., Vierimaa, O., Kiuru, M., Isola, J., Roth, S., Pukkala, E., Sistonen, P., Herva, R. and Aaltonen, L.A. (2001) Inherited susceptibility to uterine leiomyomas and renal cell cancer. *Proc. Natl Acad. Sci. USA*, **98**, 3387–3392.
9. Fleming, S. and Lewi, H.J. (1986) Collecting duct carcinoma of the kidney. *Histopathology*, **10**, 1131–1141.
10. Merino, M., Torres-Cabala, C., Zbar, B., Chian-Garca, C. and Linehan, W. (2003) Hereditary leiomyomatosis and renal cell carcinoma syndrome (HLRCC): clinical, histopathological and molecular features of the first American families described. *US Can. Acad. Pathol.* (in press).
11. Alam, N.A., Bevan, S., Churchman, M., Barclay, E., Barker, K., Jaeger, E.E., Nelson, H.M., Healy, E., Pembroke, A.C., Friedmann, P.S. *et al.* (2001) Localization of a gene (MCUL1) for multiple cutaneous leiomyomata and uterine fibroids to chromosome 1q42.3–q43. *Am. J. Hum. Genet.*, **68**, 1264–1269.
12. Varley, J.M., Thorncroft, M., McGown, G., Appleby, J., Kelsey, A.M., Tricker, K.J., Evans, D.G. and Birch, J.M. (1997) A detailed study of loss of heterozygosity on chromosome 17 in tumours from Li–Fraumeni patients carrying a mutation to the TP53 gene. *Oncogene*, **14**, 865–871.
13. Hatch, M.D. (1978) A simple spectrophotometric assay for fumarate hydratase in crude tissue extracts. *Anal. Biochem.*, **85**, 271–275.