LETTER TO JMG

Fumarate hydratase enzyme activity in lymphoblastoid cells and fibroblasts of individuals in families with hereditary leiomyomatosis and renal cell cancer

M Pithukpakorn, M-H Wei, O Toure, P J Steinbach, G M Glenn, B Zbar, W M Linehan, J R Toro

J Med Genet 2006;**43**:755–762. doi: 10.1136/jmg.2006.041087

Background: Hereditary leiomyomatosis and renal cell cancer (HLRCC) is the autosomal dominant heritable syndrome with predisposition to development of renal cell carcinoma and smooth muscle tumours of the skin and uterus.

Objective: To measure the fumarate hydratase (FH) enzyme activity in lymphoblastoid cell lines and fibroblast cell lines of individuals with HLRCC and other familial renal cancer syndromes.

Methods: FH enzyme activity was determined in the whole cell, cytosolic, and mitochondrial fractions in 50 lymphoblastoid and 16 fibroblast cell lines including cell lines from individuals with HLRCC with 16 different mutations.

Results: Lymphoblastoid cell lines (n=20) and fibroblast cell lines (n=11) from individuals with HLRCC had lower FH enzyme activity than cells from normal controls (p<0.05). The enzyme activity in lymphoblastoid cell lines from three individuals with mutations in R190 was not significantly different from individuals with other missense mutations. The cytosolic and mitochondrial FH activity of cell lines from individuals with HLRCC was reduced compared with those from control cell lines (p<0.05). There was no significant difference in enzyme activity between control cell lines (n=4) and cell lines from affected individuals with other hereditary renal cancer syndromes (n=22).

Conclusions: FH enzyme activity testing provides a useful diagnostic method for confirmation of clinical diagnosis and screening of at-risk family members.

Hereditary leiomyomatosis and renal cell cancer (HLRCC) (OMIM 605839) is an autosomal dominant heritable cancer syndrome with predisposition to the development of uterine leiomyomas (fibroids), cutaneous leiomyomas, and an aggressive form of renal cell carcinoma. The HLRCC locus was mapped to chromosome 1q42.3–q43.¹ Subsequently, germline mutations in the fumarate hydratase gene (*FH*) were reported to be responsible for the susceptibility to HLRCC.^{2–3} We previously showed that mutations in *FH* are associated with HLRCC in families in North America.^{3–4} In contrast to HLRCC, germline mutations in *FH* are associated with another distinct hereditary condition in which autosomal recessive inheritance leads to fumarate hydratase deficiency (FHD) (OMIM 606812) or fumaric aciduria. FHD is a metabolic disorder characterised by rapidly progressive neurological impairment and homozygous or compound heterozygous germline mutations of the *FH* gene.

FH codes for fumarate hydratase, the enzyme that catalyses the conversion of fumarate to malate in the tricarboxylic acid (TCA) or Krebs cycle. Similar to *E coli* fumarase C (Fum C), human fumarate hydratase is a member of the class II

fumarases. The class II fumarases belong to a family of proteins that includes aspartase, adenylosuccinate lyase, arginosuccinate lyase, and γ -crystallin.⁵ There are three highly conserved amino acid regions identified within the class II fumarase superfamily: region 1 spans H129 through T146, region 2 spans V182 through Q200, and region 3 spans G217 through E331 (amino acid numbering is based on the *E coli* fumarase C).⁶ The functional fumarate hydratase unit is a homotetramer in which each of the four active sites is made up of residues from three of the four subunits.⁷ In contrast, each of the four secondary binding sites, or B sites, is formed by atoms from a single monomer and mainly by residues H129 to N135.⁸ Each monomer consists of three domains. The individual domains are generated in a sequential fashion. The second domain consists of a five helix bundle. The core of the homotetramer is a 20 helix bundle generated from the second domain in each of the four monomers.

In this study, we report the whole cell cytosolic and mitochondrial FH enzyme activities in lymphoblastoid and fibroblast cell lines from affected individuals with HLRCC and their relatives. We also investigated the correlation of specific *FH* mutations with their FH enzyme activity levels. In addition, a homology model of the human FH tetramer shows that missense mutations associated with renal tumours are located in several different regions of the protein.

METHODS

Patients were evaluated at the National Cancer Institute (NCI) on the protocol approved by the NCI institutional review board. The study was conducted according to the Declaration of Helsinki principles. Family members who participated in the study gave their written informed consent. 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, Bethesda, Maryland. Each patient had a detailed examination of the skin including biopsies of lesions suspected to be leiomyomas. Clinical evaluation of patients for renal cell cancer and uterine leiomyomas was as previously described.³

Fumaric acid analysis

Ten millilitres of fresh urine from six consecutive individuals with clinical phenotypes of HLRCC were collected and stored frozen before testing at a certified laboratory. Urine organic acid content including fumaric acid was analysed by gas

Abbreviations: FH, fumarate hydratase; FHD, fumarate hydratase deficiency; HIF, hypoxia inducible factor; HLRCC, hereditary leiomyomatosis and renal cell cancer; HPH, HIF-prolyl hydroxylase; NCI, National Cancer Institute

Figure 1 Fumarate hydratase (FH) enzyme activity in lymphoblastoid cells from affected individuals with hereditary leiomyomatosis and renal cell cancer (HLRCC) and normal control (nmol/min/mg protein). The *FH* mutation is shown below each graph, with corresponding amino acid changes in parentheses. ND, no detectable mutation. The colour represents the cellular compartment: whole cell (purple), cytosolic (red), and mitochondrial (yellow). *Members of the same family.

chromatography–mass spectrometry. As no abnormality of urine organic acid was detected in any of the six subjects, we discontinued measurements of urine organic acid.

Lymphoblastoid and fibroblast cell lines

We made 50 lymphoblastoid and 16 fibroblast cell lines. There were 20 lymphoblastoid cell lines and 11 fibroblast cell lines from individuals from 20 families with identified *FH* germline mutations. We also established lymphoblastoid cell lines from a proband of a family with multiple cutaneous leiomyomas without detectable *FH* mutation by DNA sequencing, a patient with segmental cutaneous leiomyomas without detectable germline *FH* mutation, and an unaffected HLRCC family member. One fibroblast cell line was established from a patient with sporadic renal collecting duct carcinoma. Four lymphoblastoid cell lines and fibroblast cell lines matched by sex and race were purchased from Coriell Cell Repositories. In addition, we established 24 lymphoblastoid cell lines from nine individuals with Birt-Hogg-Dubé syndrome, 13 with von Hippel-Lindau disease, and two with sporadic kidney cancer.

Sequencing of the fumarate hydratase gene

DNA was extracted from peripheral blood leucocytes according to standard procedures. The genomic sequence analysis of *FH* gene was previously reported.^{3,4} The identified *FH* mutations

consisted of nine missense (N64T, L89S, R190C, R190H, H275Y, N297D, S376P, Q396P, Y422C), two nonsense (R58X, S102X), five frameshifts (780_781delGC, 782_788del, 1162delA, 1340delG, 1346_1347delTC), and one splice site (138+1G→C). Each mutation co-segregated with disease phenotype and was absent in more than 160 normal individuals.

Cell cultures

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.⁹ Skin biopsy tissues were obtained from probands with a confirmed clinical diagnosis of HLRCC and germline mutations in *FH*. Human skin fibroblasts were grown from skin tissues and cultured in Chang medium. Lymphoblastoid cells and fibroblast cells matched for race and sex were obtained from Coriell Cell Repositories and used as normal control.

Preparation of whole cell extract, cytosolic, and mitochondrial fractions

For each specimen of lymphoblastoid cell cultures, approximately 1×10^6 cells in confluent flask were washed with $1 \times$ phosphate buffered saline (PBS), centrifuged at $600 \times g$ for 10 minutes, and resuspended in 1 ml of 250 mM sucrose, 25 mM HEPES, pH 7.4. For each fibroblast cell culture specimen, one to two confluent T-25 flasks were treated with 0.05%

Figure 2 Pedigree of Family 1600, showing clinical phenotypes, FH mutation status, and fumarate hydratase (FH) enzyme activities. Colour diagram: skin = cutaneous leiomyoma; uterus = uterine leiomyoma; kidney = renal cancer.

trypsin in EDTA. The cell suspension was then washed with 1 × PBS, centrifuged at 600 ×g for 10 minutes, and resuspended in 1 ml of 250 mM sucrose, 25 mM HEPES, pH 7.4.

The cells then were disrupted by gentle sonication for five seconds on ice. The cell lysate was centrifuged at 900 ×g at 4°C for eight minutes to remove cell debris, after which 100 µl of supernatant were separated for whole cell extract. The remaining supernatant was transferred to a clean sterile tube and centrifuged at 14 000 ×g at 4°C for 10 minutes to produce a cytosolic fraction (supernatant) and a mitochondrial fraction (pellet). The mitochondrial fraction pellet was resuspended in 150 µl of 25 mM HEPES, pH 7.4. Both fractions were validated by citrate synthase and glucose-6-phosphate dehydrogenase enzyme activities, measured as previously described.^{10 11} All fresh extracts were kept on ice for enzyme assay.

Measurement of FH enzyme activity and cellular protein content

Protein quantification in the extract was undertaken by bicinchoninic acid calorimetric assay as previously described.¹² In vitro assay of fumarate hydratase enzyme activity was done by NADP-malic enzyme coupled assay as previously described.¹³

FH modelling

A homology model of human FH was constructed, based on the known crystal structures of a recombinant yeast fumarase (pdb code: 1yfm) and *E coli* Fum C with citrate bound (pdb code: 1fuq).⁸ The latter structure was used only to model FH residues L122 to P131. The SegMod algorithm¹⁴ in the program GeneMine was used to build the FH homotetramer, with each monomer being modelled from residues S6 to L464. The model was analysed using CHARMM.¹⁵ For each residue in the model, the nearest residue in the hybrid (1yfm/1fuq) template was determined based on the distance between α carbons.

RESULTS

FH enzyme activity in lymphoblastoid cell lines

Whole cell FH enzyme activity of lymphoblastoid cell lines from patients with HLRCC was significantly lower than whole cell FH enzyme activity of lymphoblastoid cell lines from normal controls ($p < 0.05$, Wilcoxon rank sum test) (fig 1). The mean FH enzyme activity of lymphoblastoid cells from patients with

HLRCC was 167 nmol/min/mg protein. We tested the enzyme activity in three members of Family 1600, consisting of the proband's father, brother, and cousin (fig 2). None of these individuals have kidney tumour, and only the brother had a cutaneous leiomyoma. They were all FH germline mutation carriers and their FH enzyme activities were very similar, with a mean level of 189 nmol/min/mg protein (fig 2).

In addition, we found decreased FH enzyme activity (188 nmol/min/mg) in a patient from a Family 511 with multiple cutaneous leiomyomas who did not have a detectable FH mutation by DNA sequencing (proband No 16, fig 1). The father who is the proband of the family did not appear to represent a mosaicism as his son and daughter also had cutaneous leiomyomas and undetectable FH mutations by direct DNA sequencing in two independent laboratories. We also measured enzyme activity in a five year old boy who presented with segmental leiomyomas on his right thigh. No detectable FH mutation was identified in the patient's or his parent's peripheral blood leucocytes. His FH enzyme activity was 354 nmol/min/mg protein. This level was not different from normal control.

As an additional internal control we also measured the enzyme activity of sister of proband No 4 (fig 1). This individual did not have cutaneous leiomyoma, kidney cancer, or a detectable FH germline mutation. Her whole cell FH enzyme activity was 427 nmol/min/mg protein. This level was not significantly different from the whole cell FH enzyme activity of normal control (mean 394 nmol/min/mg protein). This provided a test to confirm her negative FH mutation carrier status obtained by direct DNA sequencing analysis.

We also compared the enzyme activity of patients with frameshift, nonsense, and missense mutation (fig 1). The enzyme activity in lymphoblastoid cell lines from four individuals with mutations in R190 (mean, 161 nmol/min/mg protein) was not significantly different from individuals with other missense mutations ($n = 4$) (mean, 158 nmol/min/mg protein). We found that frameshift mutations ($n = 5$) had similar enzyme activity (mean, 155 nmol/min/mg protein) to missense mutations ($n = 8$) (mean, 160 nmol/min/mg protein). The enzyme activity in lymphoblastoid cells from patients with nonsense mutations (R58X and S102X) (mean 170 nmol/min/mg protein) was also not significantly different from that of the patients ($n = 8$) with missense mutations (fig 1).

Figure 3 Fumarate hydratase (FH) enzyme activity in lymphoblastoid cell lines from affected individuals with Birt-Hogg-Dubé syndrome (BHD), von Hippel-Lindau disease (VHL), and normal control (nmol/min/mg protein). The colour represents the cellular compartment: whole cell (purple), cytosolic (red), and mitochondrial (yellow).

In addition, there was no significant difference in enzyme activity between control cell lines and cell lines from individuals with Birt-Hogg-Dubé syndrome ($n = 9$) or von Hippel-Lindau disease ($n = 13$) (fig 3). The mean FH enzyme activities of lymphoblastoid cells from patients with Birt-Hogg-Dubé syndrome and von Hippel-Lindau disease were 349 and 350 nmol/min/mg protein, respectively. In addition, the FH enzyme activity of lymphoblastoid cells from two patients with sporadic kidney cancer (mean 342 nmol/min/mg protein) was not significantly different from control cell lines.

We examined whether the FH enzyme activity was different in the mitochondria and the cytosol. We fractionated the cell extract into cytosolic and mitochondrial compartments in order to measure their respective FH enzyme activities. G-6-PD and citrate synthase enzyme activities were measured to confirm cytosolic and mitochondrial fractions, respectively. When compared with normal control, the cytosolic and mitochondrial compartments of lymphoblastoid cells from individuals with HLRCC showed a similar degree of significant reduction in FH enzyme activity (fig 1). The mean cytosolic FH activity of lymphoblastoid cells from individuals with HLRCC was 40% of mean cytosolic FH activity of normal control cells ($p < 0.05$, Wilcoxon rank sum test). Similarly, the mean mitochondrial FH activity of lymphoblastoid cells from individuals with HLRCC was 34% of mean mitochondrial FH activity from corresponding control cells ($p < 0.05$, Wilcoxon rank sum test).

Next, we made direct comparisons of cytosolic and mitochondrial FH enzyme activity in lymphoblastoid cells, first from individuals affected with HLRCC, and then in the controls. In the HLRCC affected individuals' lymphoblastoid cells, we found that the mean cytosolic FH enzyme activity was 3.8 times higher than the mean mitochondrial FH activity ($p < 0.05$, Wilcoxon

rank sum test). Similarly, in the control individuals' lymphoblastoid cells, we found that the mean cytosolic FH activity was 3.4 times higher than the mean mitochondrial FH activity ($p < 0.05$, Wilcoxon rank sum test) (fig 1).

FH enzyme activity in fibroblasts

All 11 fibroblast cell lines from individuals with HLRCC from nine families had significantly lower enzyme activity than normal control fibroblasts ($n = 4$) ($p < 0.05$ Wilcoxon rank sum test) (fig 4). The mean FH enzyme activity in fibroblast cell lines from individuals with HLRCC was 31.8% of mean activity in control fibroblast cell lines. We measured the FH enzyme activity of multiple members in two families with HLRCC. Family No 2005 consisted of a father and a daughter with cutaneous leiomyomas, kidney cancer, and an FH germline mutation. Their enzyme activities were very similar. The father's enzyme activity was 48 nmol/min/mg protein and the daughter's was 62 nmol/min/mg protein. In another family (Family No 1600) we tested the FH enzyme activity in the fibroblast cell lines of the proband's father and brother, both whom are FH germline mutation carriers (fig 4). Similarly, their fibroblast whole cell FH enzyme activities were very similar, at 81 nmol/min/mg protein (father) and 67 nmol/min/mg protein (brother).

The cytosolic and mitochondrial compartments of individuals with HLRCC showed a similar degree of decreased FH enzyme activity. The mean cytosolic FH activity of fibroblasts from patients with HLRCC was 33.5% of the mean activity of normal control fibroblasts. The mean mitochondrial FH activity of fibroblasts from individuals with HLRCC was 22.8% of mean mitochondrial FH activity of normal control fibroblasts. In contrast, the FH activity of fibroblast from a patient with renal collecting duct carcinoma without

Figure 4 Fumarate hydratase (FH) enzyme activity in fibroblasts from affected individuals with hereditary leiomyomatosis and renal cell cancer (HLRCC) and normal control (nmol/min/mg protein). The FH mutation is shown below each graph, with corresponding amino acid changes in parentheses. The colour represents the cellular compartment: whole cell (purple), cytosolic (red), and mitochondrial (yellow). */**Members of the same family.

detectable cutaneous leiomyomas or FH mutation was not different from FH activity of normal control.

Next, we made direct comparisons of cytosolic and mitochondrial FH enzyme activity in fibroblast cells, first in HLRCC affected individuals and then in controls. It should be noted that the results differed from a similar comparison made in the lymphoblastoid cells. In the fibroblast cells, we found that cytosolic enzyme activity was not significantly different from the mitochondrial activity in both cell lines from HLRCC patients and normal control.

It is noted that fibroblast from a patient with the S376P mutation had higher enzyme activity than the other four missense mutations (fig 4). The enzyme activity in fibroblasts from a patient with nonsense mutation R58X (55 nmol/min/mg protein) was not different from that in fibroblasts from the patients with missense mutations (mean 69 nmol/min/mg protein).

Sequence alignments and FH modelling

The sequences of human fumarase, *E. coli* fumarase C, and yeast fumarase are very similar (fig 5). To date, including our previously described mutations,^{3,4} we have identified 20 missense mutations in FH. Missense mutations in FH occurred at evolutionarily conserved residues. Eighty five per cent of missense mutations (17 of 20) occurred at residues identical in yeast FH and 80% (16/20) of missense mutations occurred at residues identical in *E. coli* fumarase C.

A homology model of human fumarate hydratase (FH) (fig 6) was constructed. The nearest residue in the template was identical to the modelled residue for 1224 of the 1836 FH residues (66.7%). The α carbons of these 1224 conserved residues are within a root mean square distance of 0.55 Å from the corresponding atoms in the hybrid template.

We identified seven missense FH mutations^{3,4} in our NCI families that correspond to residues present within the three highly conserved regions characteristic of the fumarase superfamily. S144L and N145S were within the first highly conserved region (fig 5). Furthermore, S144L and N145S mutations occurred in residues that form the active site A. The S144L mutation led to a change from a non-charged hydrophilic to a non-charged hydrophobic amino acid. The N145S mutation resulted in an exchange of neutral hydrophilic residues.

We also identified four FH mutations (K187R, R190H, R190L, and R190C) within the second homology region (fig 5). The K187R mutation substituted one positively charged residue for another. R190H replaced a basic hydrophilic amino acid, arginine, with another hydrophilic amino acid, histidine, which may or may not be positively charged. The homology model (fig 6) shows that near R190 are several other charged residues, including lysine (K404, K187), aspartic acid (D195, D298), glutamic acid (E408, E312, E204), and arginine (R404). In addition, R190 forms a salt bridge with E312 in the modelled wild type (WT) protein.

Human	-----MASQNSFRIEYDTFGELKVPNDKYYGAQTVRSTMNFKIG	39
Yeast	MLRFTNCSCKTFVKSSYKLNIRRMSSFRTEFDAFGEIHVPADKYGAQTQSFQNFKIG	60
E coli	-----MNTVRSEKDSMGADIVPADKLWGAQTQSFLEHFRIS	36
Human	GVTERMPFPVIAKFGILKRAAAEVNDYDGLDPKIANAIMKAADEVAEGKINDHFFLVVW	98
Yeast	GARERMPFLPLVHAFGVLKKSAAIVNESLGLDPKISKAIQQADEVASGLDDHFFLVVF	120
E coli	--TEKMPTSLIHALATKRAAAKVNEGLLLSEKASAIRQADEVLAGQHDDEFFLAIW	94
Human	QTGSGTQTMNVNEVISNRAIEMLGELGSKIPIVHPNDHVNKSQSSNDTFPTAMHIAAI	158
Yeast	QTGSGTQSNMNEVISNRAIEMLGELGSKIPIVHPNDHVNKSQSSNDTFPTAMHIAAI	179
E coli	QTGSGTQSNMNEVISNRAIEMLGELGSKIPIVHPNDHVNKSQSSNDTFPTAMHIAAI	154
Human	EVHEVLLPGLQKLDALDAKSKEFAQIIKIGRTHLQDAVPLTLGOEFGSYVQVKIAMTR	218
Yeast	QIQNELIPELTNLNALEAKSKEFDHIVKIGRTHLQDAVPLTLGOEFGSYVQVNGIQR	239
E coli	ALRQLIPQLTLTQTLEKSRADFIVKIGRTHLQDAVPLTLGOEFGSYVQVNGIQR	214
Human	IKAAMPRIYELAGGTAVGTGLNTRIGFAEKVAAKVAALTGLPFVTPAPNKEALAAHDAL	278
Yeast	VAHSLKTLFLAQGGTAVGTGLNTRIGFAEKVAAKVAALTGLPFVTPAPNKEALAAHDAI	299
E coli	IEYSLPHVAELALGGTAVGTGLNTRIGFAEKVAAKVAALTGLPFVTPAPNKEALATCDAL	274
Human	VELSGAMNTTACSLMKIANDIRFLGSGPRSGLGELILPENEPGSSIMPGKVNPTQCEAMT	338
Yeast	VECSGALNTLACSLFKIAQDIRYLGSGPRCGYHEMLPENEPGSSIMPGKVNPTQCEALT	359
E coli	VOAHGALKGLAASLMKIANDVRWLASGPRCGIGEISIPENEPGSSIMPGKVNPTQCEALT	334
Human	MVAQVMGNHVAVTVGGSNGHFELNVFKPMIMKIVLHSAARLLGDASVSFTENCVVGIQAN	398
Yeast	QVCQVMGNNAITFAGSQGFELNVFKPMIMKIVLHSAARLLGDASVSFTENCVVGIQAN	419
E coli	MLCCQVMGNDVAINMGASGNFELNVFRPMVIHNFQSVRLADGMESFNKHCavgIEPN	394
Human	TERINKLMNESMLVLTALNPHIGYDKAAKIATAHNGSTLKETAIELGYLTAEQFDEWV	458
Yeast	EPRHELLTKSLMLVLTALNPKIGYDAASKVAKNAHKGITLKEALELGVLTAEQFDEWV	479
E coli	RERINQLLNESMLVLTALNTHIGYDKAAEIAKKAHKEGLTLKAAALALGYLSEAEFDSWV	454
Human	KPKDMLGPK-----	467
Yeast	VPEHMLGPK-----	488
E coli	RPEQMGVSMKAGRHHHHH	472

Figure 5 Fumarate hydratase (FH) protein alignment showing sequences of amino acid comparisons between human, yeast, and *E. coli*. Red boxes indicate amino acid residues within the three highly conserved regions in the fumarase superfamily. Characters in yellow indicate the amino acid positions where missense or nonsense mutations were identified. Black triangles represent the amino acid position where frameshift mutations occurred.

The interaction between residues R190 and E312 will be weaker in the three mutants (R190H, R190L, and R190C).

Additionally, we identified two germline FH mutations (S322G and S323N) located in the signature sequence motif (GSSxMPxKxNPxxxE) in the third homology region (fig 5). The signature sequence motif has provided a means of establishing the evolutionary link between a broad superfamily of proteins. Therefore the simple exchange of amino acids, even

within the same class, predicts deleterious effects on the identity of the protein. The S322G and S323N mutations lead to exchanges of non-charged hydrophilic amino acids from serine to glycine and asparagine, respectively.

We also evaluated the amino acid location of FH mutations associated with kidney cancer. To date, and including our previous publication,^{3,4} we have identified six missense mutations in FH associated with kidney cancer. We also mapped the location of all these six FH missense mutations in the homology model constructed of human FH (fig 6). The five FH residues involved in the six mutations associated with renal tumours are located in regions of the tetramer that share considerable sequence identity with the yeast fumarase used as modelling template. For each of these five residues, every neighbouring residue with any atom within five Ångströms of the given residue, including the given residue itself, was identified and its conservation relative to the template was noted. The level of local sequence identity was: 89%(8/9) for L89, 88%(7/8) for R190, 91%(10/11) for H275, 64%(7/11) for S376, and 71%(5/7) for Q396. Thus the local identity for S376 and Q396 is comparable to the overall level of sequence identity (67%). However, the local identity for L89, R190, and H275 is higher than average. Missense mutations associated with kidney cancer were distributed throughout the protein without an obvious clustering pattern. Of interest, Q396 is on the surface of the protein and L89 is partially buried on the surface of the protein.

Figure 6 Three dimensional model of the fumarate hydratase (FH) homotetramer, showing positions of amino acid mutations in the protein structure (L89, R190, H275, S376, and Q396) in relation to the active site.

DISCUSSION

In this study, we identified the FH enzyme activity for the first time in fibroblast from patients with HLRCC. We also reported for the first time the FH enzyme activity associated with 14 FH mutations in patients with HLRCC. We found that lymphoblastoid cells and fibroblasts from individuals

with HLRCC had significantly lower FH enzyme activity than cells from normal controls.

Furthermore, the FH enzyme activities in fibroblasts from some patients with HLRCC were almost as low as the levels reported in some patients with FHD.^{16,17} Despite the low enzyme activity, the patients with HLRCC had no neurological impairment or increased urine fumaric acid excretion, as typically seen in patients with FHD. The reason for this distinction is unclear. A possible explanation is that FH expression in various tissues could be different. Therefore neural cells, kidney epithelial cells, and fibroblast cells could have different levels of expression, reflecting different metabolic needs in each tissue. A possible explanation for the lack of excretion of urine fumaric acid is that the fully functional FH allele in patients with HLRCC may be enough to prevent the accumulation of fumaric acid. The decreased enzyme activity in the family with undetectable FH mutation suggested that this family did carry an occult germline alteration that was not identified by genomic sequence analysis. In addition, Southern analysis of patient's genomic DNA did not suggest that they have a large deletion in the region of FH.

The subcellular measurement of FH enzyme activity in the mitochondria and cytoplasm of cells from patients with HLRCC has not been reported previously. In this study we showed that cells from HLRCC patients with different types of FH mutation had a decrease in FH enzyme activity in both cytosolic and mitochondrial compartments. This finding suggested that mutations of the FH gene in HLRCC may not affect the subcellular distribution of the enzyme.

The FH gene encodes for two isoforms of fumarate hydratase enzyme. The function of the cytosolic FH isoform is thought to be involved in amino acid metabolism. The mitochondrial FH isoform catalyses the conversion of fumarate to malate as part of the TCA cycle in the mitochondrial matrix. Studies measuring FH enzyme activities in different cellular compartments have been conducted in various organisms.¹⁸ A study in *Saccharomyces* indicated that FH translational products are initially targeted to mitochondria by a characteristic signal sequence.¹⁹ The FH proteins are partially imported and processed at the mitochondrial outer membrane, after which approximately 70–80% of FH proteins are released back in cytosol while the remaining portion is fully imported into the mitochondrial matrix. It has been shown that *Saccharomyces* transfected with vectors containing FH mutations target FH proteins to mitochondria but it fail to distribute them properly to the cytosol.²⁰ In neurones, cytosolic FH is minimal while the mitochondrial FH represents the majority of the whole cell FH enzyme activity.¹⁸ In lymphoblastoid cells, we found that cytosolic FH enzyme was more active than the mitochondrial counterpart. In contrast, both cytosolic and mitochondrial FH enzymes were equally active in fibroblasts. The reason for this difference in tissues is unclear. It is possible that differences in FH enzyme activity in cytosol and mitochondria reflect various metabolic needs in each cell type. Moreover, EBV transformation of lymphocytes and rapid growth rate of lymphoblastoid cells could affect the mitochondrial targeting or processing of FH, or both.

Similar to our findings, Alam and colleagues showed a decrease in FH enzyme activity in lymphoblastoid cell lines from affected individuals with HLRCC compared with the cells from controls.²¹ In contrast to Alam and colleagues,²¹ who only measured whole cell FH enzyme activity in lymphoblastoid cell lines, in our study we measured FH enzyme activity in both fibroblasts and lymphoblastoid cells. Our study expands the previous reports as we also fractionated and measured the FH enzyme activity in the cytosolic and mitochondrial compartments of cells from individuals with HLRCC. We did not find significant

differences in FH enzyme activity between missense and frameshift mutations. In contrast, Alam and co-workers showed that lymphoblastoid cells from affected individuals with truncating FH mutations had significantly higher enzyme activity than cells from HLRCC individuals with missense mutations. One possible explanation for this discrepancy is that our sample size may have been too small to demonstrate the significance. In addition, the difference in results may reflect the specific FH mutations studied by each group of investigators, though the same three mutations (N64T, R190H, and R58X) were involved in both studies. Our investigation included cell lines with 14 FH mutations that were not tested in the study of Alam *et al.*²¹ Future studies involving large numbers of patients will provide enough statistical power to investigate whether there is a correlation between FH enzyme activity and types of FH mutation. In our previous study, no clear genotype–phenotype correlations could be identified but our data suggested that families with R190H and R58X mutations tend to contain a high frequency of individuals with kidney tumours.⁴ This early finding also needs to be investigated in a larger group of families.

The amino acid position of some FH mutations suggests functional significance. Mutations at S144 and N145 may have profound effects on the FH enzyme activity as these residues are highly conserved in evolution and they form the active site. We also investigated the region of R190 in our model because R190 is the residue where the most FH mutations occur (R190H, R190C, and R190L) and R190H is the most frequent mutation identified.³ As R190 is adjacent to residues that form the active site, R190 mutants may also affect the conformation of the active site. Similarly, the S322G and S323N mutations may affect the enzymatic activity as the nearby residues K328 and N330 are part of the active site. In the WT protein, S323 forms two strong hydrogen bonds with the amine groups of K430 on a different monomer. This interaction has been found to be important for oligomerisation.^{7,8} Similarly, the report by Alam and co-workers found that the majority of FH mutations in HLRCC occurred in a region around the active site of the FH protein.²¹

FH most probably acts as a tumour suppressor gene in HLRCC as loss of heterozygosity has been shown in cutaneous and uterine leiomyomas² as well as in renal tumours,²² and FH enzyme activity is low or absent in these tumours. The enzymatic conversion of fumarate to malate is preceded by the conversion of succinate to fumarate by the succinate dehydrogenase (SDH) complex. The SDH complex of enzymes is located at the inner mitochondrial membrane. Germline mutations in SDH genes lead to the genetic predisposition to develop paragangliomas and pheochromocytomas.^{23,24} Furthermore, renal cancer has been reported in families with germline mutations in SDH genes.²⁵ The hypoxia inducible factor (HIF) proteins and products of their target genes are shown to be overexpressed in HLRCC tumours.^{22,26} However, the mechanisms of how mutations in FH and SDH and TCA cycle dysfunction lead to tumour formation remain to be elucidated. A recent study showed that succinate, which accumulates as a result of SDH inhibition, leads to the stabilisation of HIF by inhibiting HIF-prolyl hydroxylase enzyme in the cytosol.²⁷ Similarly, we recently demonstrated that increased intracellular fumarate directly impairs HIF-prolyl hydroxylase (HPH) function by competitively inhibiting α -ketoglutarate, a key co-factor of HPH.²² As a result, increased stability of intracellular HIF promotes the upregulation of several target genes, including VEGF and Glut-1. These investigations suggest that the von Hippel-Lindau–HIF pathway may be involved in the mechanism of tumorigenesis in both SDH and FH mutations.

FH enzyme activity testing could provide a useful diagnostic method for confirming the clinical diagnosis or screening at-risk family members. There are only a few certified laboratories that can carry out FH testing. For fibroblast culture, media have to be available and rapid access to a laboratory is required to establish the culture. Thus the best indication to test FH enzyme in patients with a suspected diagnosis of HLRCC may be when no FH mutation is identified by direct DNA sequencing. The major disadvantage of using lymphoblastoid cell lines for assay is the time needed for lymphocyte transformation and the establishment of cell culture. The advantage of using fibroblasts to measure FH enzyme activity is that fibroblast culture usually takes a shorter time (10 to 14 days) than lymphoblastoid cell line transformation and culture (four to eight weeks). Therefore fibroblasts are a good alternative to lymphoblastoid cells in FH enzyme assay.

In conclusion, FH enzyme activity can be useful in the diagnosis of HLRCC in cases with atypical presentation and undetectable FH mutations. Furthermore, FH enzyme activity testing is of value in laboratory investigations to elucidate the mechanism of HLRCC.

ACKNOWLEDGEMENTS

We wish to thank the families for their cooperation and participation in our study of HLRCC. This publication was funded with federal funds from the intramural programs (DCEG and CCR) of the National Cancer Institute. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government.

Authors' affiliations

M Pithukpakorn, Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA

M Pithukpakorn, M-H Wei, O Toure, G M Glenn, J R Toro, Genetic Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Rockville, Maryland, USA

M-H Wei, Program of Division of Cancer Epidemiology and Genetics, SAIC-Frederick Inc, Frederick, Maryland, USA

P J Steinbach, Center for Molecular Modeling, Center for Information Technology, National Institutes of Health

B Zbar, W M Linehan, Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health

Conflicts of interest: none declared.

Correspondence to: Jorge R Toro, Genetic Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, 6120 Executive Boulevard, Executive Plaza South, Room 7012, Rockville, MD 20892-7231, USA; toroj@mail.nih.gov

Received 19 January 2006

Revised version received 9 March 2006

Accepted for publication 14 March 2006

Published Online First 5 April 2006

REFERENCES

- Launonen V, Vierimaa O, Kiuru M, Isola J, Roth S, Pukkala E, Sistonen P, Herva R, Aaltonen LA. Inherited susceptibility to uterine leiomyomas and renal cell cancer. *Proc Natl Acad Sci USA* 2001;**98**:3387–92.
- Tomlinson IP, Alam NA, Rowan AJ, Barclay E, Jaeger EE, Kelsell D, Leigh I, Gorman P, Lamlum H, Rahman S, Roylance RR, Olpin S, Bevan S, Barker K, Hearle N, Houlston RS, Kiuru M, Lehtonen R, Karhu A, Vilkki S, Laiho P, Eklund C, Vierimaa O, Aittomäki K, Hietala M, Sistonen P, Paetau A, Salovaara R, Herva R, Launonen V, Aaltonen LA; Multiple Leiomyoma Consortium. Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. *Nat Genet* 2002;**30**:406–10.
- Toro JR, Nickerson ML, Wei MH, Warren MB, Glenn GM, Turner ML, Stewart L, Duray P, Toure O, Sharma N, Choyke P, Stratton P, Merino M, Walther MM, Linehan WM, Schmidt LS, Zbar B. Mutations in the fumarate hydratase gene cause hereditary leiomyomatosis and renal cell cancer in families in North America. *Am J Hum Genet* 2003;**73**:95–106.
- Wei MH, Toure O, Glenn GM, Pithukpakorn M, Neckers L, Stolle C, Choyke P, Grubb R, Middleton L, Turner ML, Walther MM, Merino MJ, Zbar B, Linehan WM, Toro JR. Novel mutations in FH and expansion of the spectrum of phenotypes expressed in families with hereditary leiomyomatosis and renal cell cancer. *J Med Genet* 2006;**43**:18–27.
- Woods SA, Miles JS, Roberts RE, Guest JR. Structural and functional relationships between fumarate and aspartate. Nucleotide sequences of the fumarate (fumC) and aspartate (aspA) genes of *Escherichia coli* K12. *Biochem J* 1986;**237**:547–57.
- Simpson A, Bateman O, Driessen H, Lindley P, Moss D, Mylvaganam S, Narebor E, Slingsby C. The structure of avian eye lens delta-crystallin reveals a new fold for a superfamily of oligomeric enzymes. *Nat Struct Biol* 1994;**1**:724–34.
- Weaver TM, Levitt DG, Donnelly MI, Stevens PP, Babaszak LJ. The multisubunit active site of fumarate C from *Escherichia coli*. *Nat Struct Biol* 1995;**2**:654–62.
- Weaver T, Banaszak L. Crystallographic studies of the catalytic and a second site in fumarate C from *Escherichia coli*. *Biochemistry* 1996;**35**:13955–65.
- Frisan T, Levitsky V, Masucci M. Generation of lymphoblastoid cell lines (LCLs). *Methods Mol Biol* 2001;**174**:125–7.
- Srere PA, Brazil H, Gonen L. The citrate condensing enzyme of pigeon breast muscles and moth flight muscles. *Acta Chem Scand* 1963;**17**:S129–34.
- WHO Scientific Group. Standardization of procedures for the study of glucose-6-phosphate dehydrogenase. Report of a WHO Scientific Group. *WHO Tech Rep Ser* 1967;**366**:1–53.
- Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;**150**:76–85.
- Hatch MD. A simple spectrophotometric assay for fumarate hydratase in crude tissue extracts. *Anal Biochem* 1978;**85**:271–5.
- Levitt M. Accurate modeling of protein conformation by automatic segment matching. *J Mol Biol* 1992;**226**:507–33.
- Brooks BR, Brucoleri RE, Olafson BD, States D, Swaminathan S, Kerplur M. CHARMM: a program for macromolecular energy, minimization, and dynamics calculations. *J Comput Chem* 1983;**4**:187–217.
- Petrova-Benedict R, Robinson BH, Stacey TE, Mistry J, Chalmers RA. Deficient fumarate activity in an infant with fumaric acidemia and its distribution between the different forms of the enzyme seen on isoelectric focusing. *Am J Hum Genet* 1987;**40**:257–66.
- Coughlin EM, Christensen E, Kunz PL, Krishnamoorthy KS, Walker V, Dennis NR, Chalmers RA, Elpeleg ON, Whelan D, Pollitt RJ, Ramesh V, Mandell R, Shih VE. Molecular analysis and prenatal diagnosis of human fumarate deficiency. *Mol Genet Metab* 1998;**63**:254–62.
- Akiba T, Hiraga K, Tuboi S. Intracellular distribution of fumarate in various animals. *J Biochem (Tokyo)* 1984;**96**:189–95.
- Knox C, Sass E, Neupert W, Pines O. Import into mitochondria, folding and retrograde movement of fumarate in yeast. *J Biol Chem* 1998;**273**:25587–93.
- Sass E, Karniely S, Pines O. Folding of fumarate during mitochondrial import determines its dual targeting in yeast. *J Biol Chem* 2003;**278**:45109–16.
- Alam NA, Rowan AJ, Wortham NC, Pollard PJ, Mitchell M, Tyrer JP, Barclay E, Calonje E, Manek S, Adams SJ, Bowers PW, Burrows NP, Charles-Holmes R, Cook LJ, Daly BM, Ford GP, Fuller LC, Hadfield-Jones SE, Hardwick N, Highet AS, Keefe M, MacDonald-Hull SP, Potts ED, Crone M, Wilkinson S, Camacho-Martinez F, Jablonska S, Ratnaveil R, MacDonald A, Mann RJ, Grice K, Guillet G, Lewis-Jones MS, McGrath H, Seukeran DC, Morrison PJ, Fleming S, Rahman S, Kelsell D, Leigh I, Olpin S, Tomlinson IP. Genetic and functional analyses of FH mutations in multiple cutaneous and uterine leiomyomatosis, hereditary leiomyomatosis and renal cancer, and fumarate hydratase deficiency. *Hum Mol Genet* 2003;**12**:1241–52.
- Isaacs JS, Jung YJ, Mole DR, Lee S, Torres-Cabala C, Chung YL, Merino M, Trepel J, Zbar B, Toro J, Ratcliffe PJ, Linehan WM, Neckers L. HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: Novel role of fumarate in regulation of HIF stability. *Cancer Cell* 2005;**8**:143–53.
- Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, van der Mey A, Taschner PE, Rubinstein WS, Myers EN, Richard CW, Cornilisse CJ, Devilee P, Devlin B. Mutations in SDHB, a mitochondrial complex II gene, in hereditary paraganglioma. *Science* 2000;**287**:848–51.
- Baysal BE. On the association of succinate dehydrogenase mutations with hereditary paraganglioma. *Trends Endocrinol Metab* 2003;**14**:453–9.
- Vanharanta S, Buchta M, McWhinney SR, Virta SK, Peczkowska M, Morrison CD, Lehtonen R, Januszewicz A, Jarvinen H, Juhola M, Mecklin JP, Pukkala E, Herva R, Kiuru M, Nupponen NN, Aaltonen LA, Neumann HP, Eng C. Early-onset renal cell carcinoma as a novel extraparaganglial component of SDHB-associated heritable paraganglioma. *Am J Hum Genet* 2004;**74**:153–9.
- Pollard PJ, Briere JJ, Alam NA, Barwell J, Barclay E, Wortham NC, Hunt T, Mitchell M, Olpin S, Moat SJ, Hargreaves IP, Heales SJ, Chung YL, Griffiths JR, Dalgleish A, McGrath JA, Gleeson MJ, Hodgson SV, Poulson R, Rustin P, Tomlinson IP. Accumulation of Krebs cycle intermediates and over-expression of HIF1alpha in tumours which result from germline FH and SDH mutations. *Hum Mol Genet* 2005;**14**:2231–9.
- Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB, Gottlieb E. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-1alpha prolyl hydroxylase. *Cancer Cell* 2005;**7**:77–85.