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ORIGINAL ARTICLE

Molecular genetic analysis of FIH-1, FH, and SDHB candidate tumour suppressor genes in renal cell carcinoma

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Background: Overexpression of the hypoxia inducible factor 1 (HIF-1) and HIF-2 transcription factors and the consequent upregulation of hypoxia inducible mRNAs is a feature of many human cancers and may be unrelated to tissue hypoxia. Thus, the VHL (von Hippel-Lindau) tumour suppressor gene (TSG) regulates HIF-1 and HIF-2 expression in normoxia by targeting the α subunits for ubiquitination and proteolysis. Inactivation of the VHL TSG in VHL tumours and in sporadic clear cell renal cell carcinoma (RCC) results in overexpression of HIF-1 and HIF-2. However, RCC without VHL inactivation may demonstrate HIF upregulation, suggesting that VHL independent pathways for HIF activation also exist. In RCC, three candidate HIF activating genes exist—FIH-1 (factor inhibiting HIF), SDHB, and FH—which may be dependent or independent of VHL inactivation.

Aims: To investigate FIH-1, SDHB, and FH for somatic mutations in sporadic RCC.

Methods: Gene mutation was analysed in primary RCCs (clear cell RCCs, papillary RCCs, and oncocytomas) and RCC cell lines. SDHB mutation analysis was performed by denaturing high performance liquid chromatography followed by direct sequencing of aberrant PCR products. FH and FIH-1 mutation analysis were performed by single stranded conformational polymorphism and direct sequencing of PCR products.

Results: No mutations were identified in the three genes investigated.

Conclusions: There was no evidence to suggest that somatic mutations occur in the FH, FIH-1, or SDHB TSGs in sporadic RCCs.

Renal cell carcinoma (RCC) is the most common adult kidney tumour, accounting for approximately 2% of all adult malignancies. RCC is heterogeneous with most (~80%) sporadic RCCs classified as clear cell tumours, and papillary RCC (PRCC) being the most common non-clear cell form. PRCC may be further subdivided into type 1 and type 2 PRCC.[1] Although only about 2% of individuals with RCC have a family history, the elucidation of the molecular pathology of familial RCC syndromes has provided important insights into the pathogenesis of sporadic forms of RCC. Thus, germline mutations in the VHL tumour suppressor gene (TSG) cause von Hippel-Lindau (VHL) disease, which is characterised by a high risk of clear cell RCC, retinal and central nervous system haemangioblastomas, and phaeochromocytoma. Furthermore, somatic inactivation of the VHL TSG by mutations or promoter methylation is found in most sporadic clear cell RCCs.[2–4] Although the VHL protein (pVHL) has muliple functions, a major consequence of VHL inactivation in VHL tumours and sporadic clear cell RCC is overexpression of the hypoxia inducible transcription factors, HIF-1 and HIF-2. HIF-1 plays a key role in cellular responses to hypoxia, including the regulation of a wide repertoire of hypoxia inducible genes involved in energy metabolism (for example, GLUT1), angiogenesis (for example, VEGF), and apoptosis (for example, NIP3).[5 6] HIF-1 is a basic helix–loop–helix heterodimeric transcription factor consisting of HIF-1α and HIF-1β. Although HIF-1β is constitutively expressed, under normoxic conditions, the HIF-1α subunit is targeted for ubiquitination and proteolysis by the pVHL–E3 ligase complex.[7–10] A subset of clear cell RCCs do not show evidence of VHL inactivation,[2 4] and it has been suggested that upregulation of HIF-1 in clear cell RCC might involve both VHL dependent and VHL independent mechanisms.[11]

> "A major consequence of VHL inactivation in VHL tumours and sporadic clear cell renal cell carcinoma is overexpression of the hypoxia inducible transcription factors, HIF-1 and HIF-2"

Germline mutations in the MET protooncogene cause hereditary type 1 PRCC,[12] and inherited mutations in the fumarate hydratase (FH) TSG cause familial leiomyomatosis and, in some kindred, susceptibility to type 2 PRCC. The FH gene encodes a mitochondrial Krebs cycle enzyme and the precise mechanism of tumour susceptibility in familial leiomyomatosis is unknown, although activation of hypoxia response pathways or altered citrate production have been suggested as possible explanations.[13] Inactivation of another Krebs cycle component, succinate dehydrogenase (SDH), has also been associated with tumorigenesis. Thus, germline mutations in three of the four subunits of SDH (mitochondrial complex II; SDHB, SDHC, and SDHD) cause familial head and neck paragangliomas, and germline mutations in SDHB and SDHD may also cause phaeochromocytoma

Abbreviations: DHPLC, denaturing high performance liquid chromatography; FH, fumarate hydratase; HIF, hypoxia inducible factor; LOH, loss of heterozygosity; PCR, polymerase chain reaction; PRCC, papillary renal cell carcinoma; pVHL, von Hippel-Lindau protein; RCC, renal cell carcinoma; SDH, succinate dehydrogenase; SNP, single nucleotide polymorphism; SSCP, single stranded conformational polymorphism; TBE, Tris/borate/EDTA; TSG, tumour suppressor gene; VHL, von Hippel-Lindau

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susceptibility.[14–16] SDHB inactivation in head and neck paraganglioma and phaeochromocytoma has been reported to result in activation of hypoxic response pathways.[17]

Recently, Mahon *et al* reported that a novel protein, FIH-1 (factor inhibiting HIF), interacted with HIF-1α and pVHL and repressed HIF-1α transcriptional activity.[18] We hypothesised that mutations in FH, FIH-1, or SDHB might cause or enhance HIF dysregulation and so be selected for in RCC.

METHODS

Tumour samples

DNA was extracted from 51 primary RCCs (25 clear cell RCCs and 26 PRCCs), four oncocytomas, and nine RCC cell lines (UMRC 2, UMRC 3, SKRC 18, SKRC 39, SKRC 45, SKRC 47, SKRC 54, KTCL 26, and 786-0).

Mutation analysis

SDHB mutation analysis was performed by denaturing high performance liquid chromatography (DHPLC) followed by direct sequencing of aberrant polymerase chain reaction (PCR) products. In brief, SDHB exons were amplified using primers and conditions described previously.[15] The GenBank accession numbers for SDHB exons 1 to 8 are: U17296, U17880, U17881, U17882, U17883, U17884, U17885, and U17886.

The eight coding exons of SDHB were analysed on the WAVE fragment analysis system (Transgenomics, Crewe, Cheshire, UK), using the Wavemaker software to calculate the melting profiles and the required elution gradient. Each fragment was analysed at the predicted melting temperature (and additional temperatures if necessary) to determine the optimal analysis conditions. Samples showing an aberrant profile were directly sequenced with the Big-Dye Terminator Kit (PE Applied Biosystems, Foster City, California, USA) and analysed on an ABI377 sequencer.

FH mutation analysis was performed by single stranded conformational polymorphism (SSCP). Thus, the nine exons of FH were amplified from the clear cell RCC DNA as whole exons, whereas the PRCC DNA was amplified in half exons (details available on request) using standard conditions: 30 ml reactions consisting of 50 ng DNA, 20pM of each primer, 5mM dNTPs, 2.5mM $MgCl_2$, 0.8 µl REDTAQ (Sigma, Poole Dorset, UK). Samples were initially denatured at 95℃ for 10 minutes, followed by 35 cycles of 45 seconds at 95℃, 45 seconds at 55℃, and one minute at 72℃, with a final extension phase of five minutes at 72℃. SSCP was carried out as described previously.[19] Briefly, standard 30 µl PCR reactions were performed for all samples and water controls, and 10 µl of each reaction added to an equal volume of formamide loading buffer (98% formamide, 10mM EDTA, 0.1% bromophenol blue). Samples were denatured by heating at 95℃ for five minutes, followed by cooling on ice before loading on to an 8% polyacrylamide gel containing 5% glycerol and 0.5× Tris/borate/EDTA (TBE) buffer. Gels were run overnight at 4℃ in 0.5× TBE buffer. DNA bands were visualised by silver staining as described previously.[20] Gels were dried under vacuum on to Whatman 3MM paper. PCR fragments were sequenced using the same primers as used to create the fragments using the Big Dye termination cycle sequencing kit (Applied Biosystems) and run on an ABI prism 377 DNA sequencer.

FIH-1 mutation analysis was performed by SSCP. PCR was performed in a final volume of 30 µl, containing 50 ng of human genomic DNA, 0.5µM of each primer (Invitrogen, Carlsbad, California, USA), 0.2µM dNTP mix, 1.5mM $MgCl_2$, and 0.4 U Taq DNA polymerase (Invitrogen). The PCR conditions were: 94℃ for five minutes, 35 cycles of 94℃ for one minute, annealing temperature (table 1) for one minute, and 72℃ for one minute, followed by a final extension of 72℃ for 10 minutes. Intronic primer pairs were designed to

Table 1 FIH-1 Primer sequences

Exon	Primer sequence	Annealing temperature
1	F: 5′-AATAGGCGGAGCTTCCGGTTCCGGT-3′ R: 5′-TACCTTCCTCCTCTCCCTTTAG-3′	59℃
2	F: 5′-CCTTGGCATTACTTCATTTG-3′ R: 5′-TTCTGCCACACCTTCAAACA-3′	54℃
3	F: 5′-ACTACATCTGTTCTCTGTGGGA-3′ R: 5′-TCAGGTTCATATGACTCCAAGCCAT-3′	57.5℃
4	F: 5′-TTTGGAACTTTTAGCTGGGAGGGCA-3′ R: 5′-ATTCCAGCCTAGGCATTACTAGAAT-3′	59℃
5	F: 5′-ATATTGTTCTCTCTCAGGGTTTAA-3′ R: 5′-TCAGAAGCGACACAGACAAGGCTTT-3′	58.5℃
6	F: 5′-AGACTGAACACCAGTACCTCTA-3′ R: 5′-GAGCCAGTTCCATCTGGAAA-3′	56℃
7	F: 5′-GCATTTCCTGAGCTACTGCTTCCTT-3′ R: 5′-TCTATCGAGAGAGAGGACACCAGA-3′	60℃
8a	F: 5′-ACTGGGTTGACCTTCAGGAAGATA-3′ R: 5′-TGTGGACGGGATAGCAGTCACCTG-3′	62℃
8b	F: 5′-CAG GTGACTGCTATCCCGTCCACA-3′ R: 5′-TTGTCTATGTATGTACGCACACGTA-3′	63℃
8c	F: 5′-AGTCTGTCAACTTCGGAATGTGTGC-3′ R: 5′-TCTTTCACCCCATTAGAGTCCCGGT-3′	60℃
8d	F: 5′-ACCGGGACTCTAATGGGGTGAAAGA-3′ R: 5′-TTGCACCCCTAGTGTGGATACGCAC-3′	58℃
8e	F: 5′-TCCTAGCTGTGGCTTCTCTAGGT-3′ R: 5′-GCTTAGGATAGGTCAGGCAGTACTT-3′	58℃
8f	F: 5′-CCGGGAAGTACTGCCTGACCTATCC-3′ R: 5′-AAGCTGAAGGTACGAGGTGGTCCA-3′	62℃
8g	F: 5′-ATAGGCTTGCGTCTTAAAGCCAGCT-3′ R: 5′-ACTGAATAGATGTGAATGATGGCCC-3′	60℃
8h	F: 5′-GGCAGCTGTGATTGCACAACTTGAA-3′ R: 5′-CCTCTCAGTTCATCAGTGCACTATA-3′	58℃
8i	F: 5′-AAGCTCAGTTATAGTGCACTGATGA-3′ R: 5′-AAGTGTGACTGTGGCCAAGTT-3′	60℃

F, forward; R, reverse.

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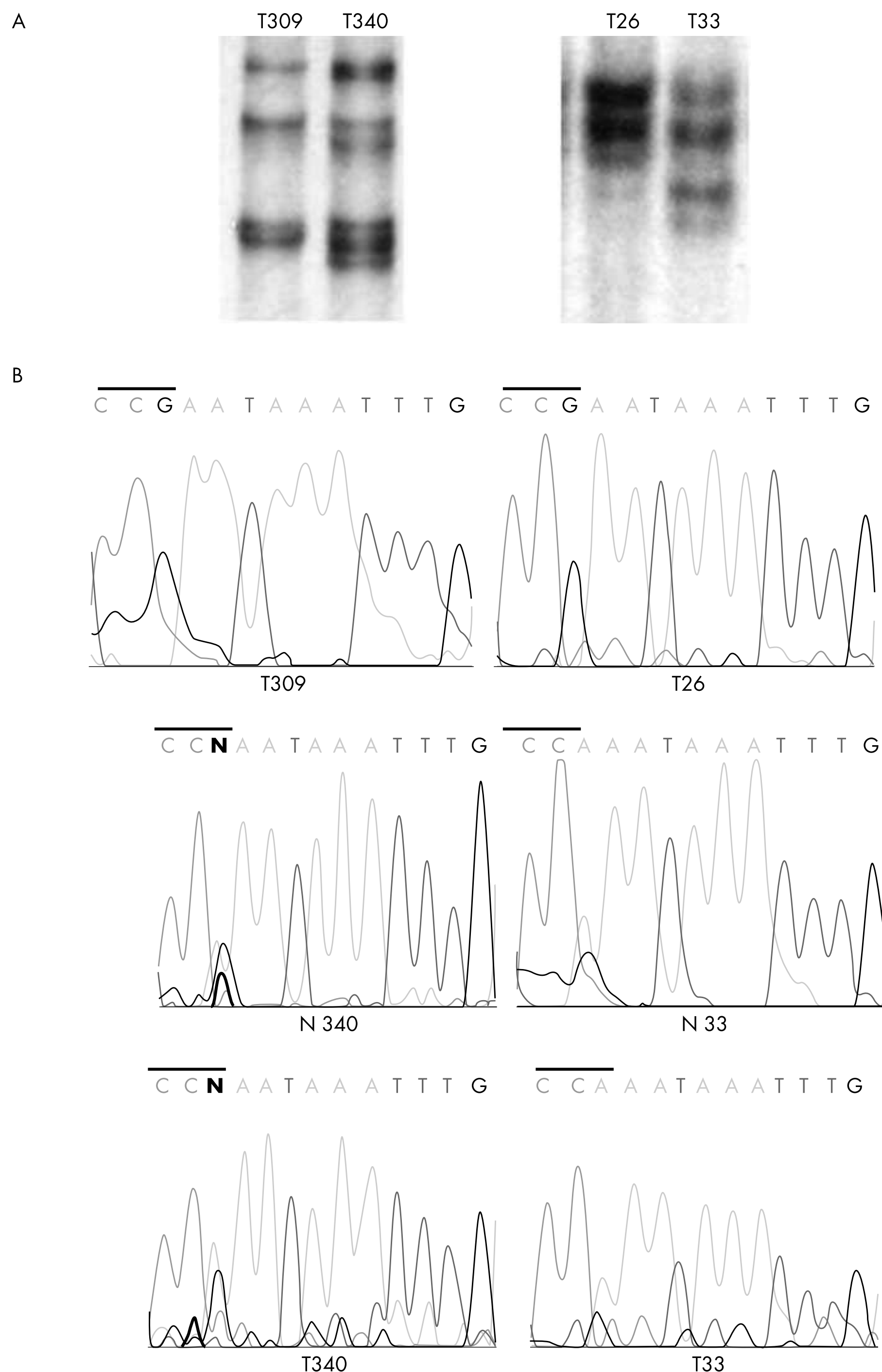

Figure 1 FH analysis. (A) Polymorphism identified by single stranded conformational polymorphism analysis. (B) Sequencing of T340 and T33 demonstrated a c.798 G → A (P266P) substitution in both tumour and matched tissue DNA samples.

amplify exons 1–7 of FIH-1, and nine overlapping primer pairs were used for the large exon 8 (table 1). Where variant band patterns were observed, representative samples were sequenced on the ABI Prism 377 DNA analyser using the BigDye Terminator V3 according to the manufacturer's protocol.

Loss of heterozygosity analysis

PCR amplification of the dinucleotide repeat microsatellite sequence markers D1S547 (73 kb from FH; heterozygosity, 0.77), D10S1266 (18 kb from FIH; heterozygosity, 0.75), and D1S2697 (134 kb from SDHB; heterozygosity, 0.70) was performed on 20 RCCs and corresponding normal tissue. The PCR conditions were 95℃ for five minutes, 30 cycles of 95℃ for 30 seconds, 55℃ (D1S547, 56℃) for 30 seconds, and 72℃ for 30 seconds, followed by a final extension step of five minutes at 72℃.

Cell culture

The RCC derived cell lines; SKRC 18, SKRC 39, SKRC 45, SKRC 47, SKRC 54, KTCL 26, UMRC 2, UMRC 3 and 786-0 were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum. Demethylation was carried out by the addition of 2μM 5-aza-2-deoxycytidine to the

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growth medium for four days. Cells were grown until approximately 70% confluent, pelleted by centrifugation at 370 ×*g*, and the DNA was extracted using a Nucleon (Amersham, Little Chalfont, Buckinghamshire, UK) extraction kit.

RESULTS

FH mutation and expression analysis in RCC

Fifty five RCC DNA samples (nine from cell lines and 46 from primary tumours (26 PRCCs and 20 clear cell RCCs)) were examined for evidence of FH mutations. No abnormalities were detected in the nine RCC cell lines. Four of 46 primary tumours (three clear cell RCCs and one PRCC) had a silent single nucleotide polymorphism (SNP; c.798 G → A; P266P) (fig 1A, B). Loss of heterozygosity (LOH) analysis at D1S547 (73 kb from FH) revealed no evidence of allele loss in 15 informative clear cell RCCs.

SDHB mutation and LOH analysis in sporadic RCC

Twenty nine sporadic primary RCCs (25 clear cell RCCs and four oncocytomas) were analysed for somatic SDHB mutations by DHPLC. No mutations were identified, but two clear cell RCCs contained SNPs (IVS2 +33 A → G and IVS2 +35 G → A) within intron 2 and one tumour demonstrated a silent SNP (c.18 A → C ; A6A) in exon 1 (fig 2). LOH analysis at D1S2697, a microsatellite marker 134 kb from SDHB, demonstrated allele loss in one of 18 informative clear cell RCCs.

FIH-1 mutation and LOH analysis in clear cell RCC

Mutation analysis of the entire FIH-1 coding sequence and intron–exon boundaries was performed in 15 clear cell RCCs without VHL gene mutations. A missense substitution P121A was detected in seven of 15 RCCs. In all cases, the P121A substitution was also present in the germline. None of the tumours harbouring the P121A variant demonstrated LOH. The P121A variant was also detected in nine of 20 normal control samples (fig 3). LOH analysis at D10S1266 (which maps 18 kb from FIH-1) showed no evidence of allele loss in eight informative clear cell RCCs.

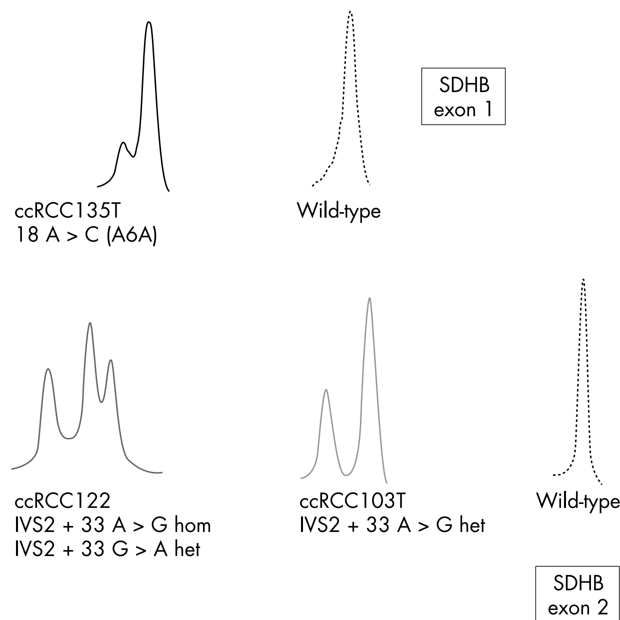

Figure 2 SDHB analysis. Denaturing high performance liquid chromatography analyses of SDHB exons 1 and 2 in samples with sequence variants.

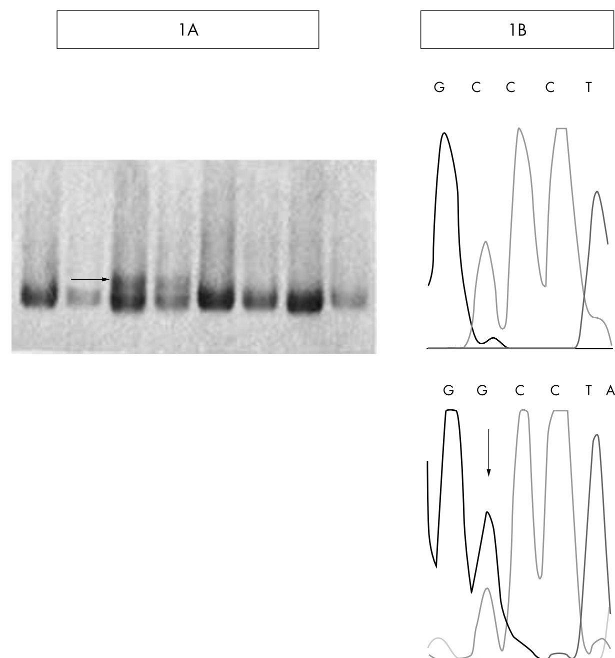

Figure 3 FIH-1 mutation analysis. Electropherograms showing normal sequence (top) and a heterozygous C121G substitution with the single stranded conformational polymorphism shift.

DISCUSSION

Upregulation of HIF-1 and HIF-2 expression in cancer occurs in response to tissue hypoxia and/or genetic alterations,[21] such as VHL inactivation in clear cell RCC.[7] In addition, cells transfected with the v-src oncogene overexpress HIF-1, and inactivation of downstream genes, including VEGF[22] and p53, is reported to enhance HIF-1α concentrations and augment HIF-1 dependent transcriptional activation of VEGF in response to hypoxia.[23] Thus, multiple genetic mechanisms exist for enhanced HIF-1 expression in human cancers.

There is increasing evidence of the importance of germline mutations of genes involved in the Krebs cycle in tumour formation. Thus, germline mutations in FH and in three subunits of succinate dehydrogenase (SDHB, SDHC, and SDHD) are associated with tumour susceptibility. Although the precise mechanisms of tumour suppression by FH and SDHB have not been defined, dysregulation of hypoxia response pathways has been suggested as a possible mechanism. The VHL TSG has multiple functions and it is not clear to what extent HIF regulation and non-HIF related pVHL functions contribute to pVHL mediated tumour suppression. However, HIF-2 overexpression has been suggested to be oncogenic per se.[24] In addition, although HIF dysregulation occurs at an early stage of tumorigenesis in VHL disease, the HIF response appears to increase during tumorigenesis and may be augmented by other genetic events.[25]

> "The FIH-1 single nucleotide polymorphisms we have identified will be useful for further investigating the role of FIH-1 in modifying the hypoxia response and susceptibility to human disease"

Although inactivation of FH, SDHB, or FIH-1 would be a plausible explanation for non-VHL dependent HIF dysregulation in RCC, we found no evidence of mutations in these genes. Germline, dominantly inherited, mutations in FH cause susceptibility to cutaneous and uterine leiomyomatosis

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Take home messages

- Although inactivation of the FH, SDHB, or FIH-1 tumour suppressor genes would be a plausible explanation for non-VHL dependent hypoxia inducible factor dysregulation in sporadic renal cell carcinoma, we found no evidence of mutations in these genes
- However, we cannot exclude epigenetic inactivation of these genes

and RCC. In addition, homozygous (or compound heterozygous) FH mutations cause the autosomal recessive syndrome fumarate hydratase deficiency, which is characterised by neurological impairment and developmental delay. While our study was in progress two other groups also investigated whether FH mutations occur in sporadic counterparts of tumours associated with germline FH mutations. Thus, Kiuru *et al* undertook FH mutation analysis in 41 uterine leiomyomas, 10 cutaneous leiomyomas, 52 RCCs, 53 sarcomas, 29 prostate carcinomas, and 15 lobular breast carcinomas.[26] Only one somatic FH mutation (in a sarcoma) was detected (although a germline mutation was detected in a uterine leiomyosarcoma and a cutaneous leiomyoma). In addition, Barker *et al* analysed 26 leiomyosarcomas and 129 uterine leiomyomas (from 21 patients) for somatic FH mutations but detected none.[27] Although many genes responsible for a familial cancer syndrome also showed frequent mutations in sporadic tumours, there are well known exceptions—for example, BRCA1 and BRCA2 mutations are infrequent in sporadic breast and ovarian cancers.[28 29] However, Kiuru *et al* only investigated 12 non-clear cell RCCs, of which five were the sporadic counterparts of the type 2 PRCC seen in some kindreds with germline FH mutations.[26] We analysed a further 46 RCCs and preferentially included PRCC (26 of 46). Combining our data with those of Kiuru and colleagues,[26] somatic FH mutations have not been detected in a total of 60 clear cell RCCs or in 38 non-clear cell RCCs (including 16 type 2 PRCCs). Thus, there is little evidence that somatic FH mutations are frequent in sporadic RCC. There are now many examples of TSGs that are inactivated commonly by promoter methylation and are mutated rarely. We cannot exclude epigenetic inactivation of FH in RCC, although preliminary studies in nine RCC cell lines did not show evidence of transcriptional silencing (Morris *et al*, 2003, unpublished observations).

FIH-1 has been shown to be an asparaginyl hydroxylase.[30 31] Hydroxylation of an asparagine residue is the C-terminal transactivation domain of HIF-1α under normoxic conditions inhibits binding of the p300 coactivator, repressing transcriptional activation. Thus, a definitive analysis of FIH-1 in human cancer might involve selective sampling of tumours to ensure that tumour regions displaying HIF-1 upregulation were analysed in detail. We detected a germline missense substitution at codon 41, in the pVHL binding region of FIH-1.[32] However, we found no conclusive evidence that germline or somatic mutations in FIH-1 contribute to RCC development. Polymorphic variants in the components of the HIF pathway might influence the genetic response to hypoxia so that the FIH-1 SNPs we have identified will be useful for further investigating the role of FIH-1 in modifying the hypoxia response and susceptibility to human disease.

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