Analysis of fumarate hydratase mutations in a population-based series of early onset uterine leiomyosarcoma patients

Sanna K. Ylisaukko-oja1, Maija Kiuru1, Heli J. Lehtonen1, Rainer Lehtonen1, Eero Pukkala2, Johanna Arola3, Virpi Launonen1 and Lauri A. Aaltonen1*

1Department of Medical Genetics, University of Helsinki, Biomedicum Helsinki, Haartmaninkatu 8, Finland
2Finnish Cancer Registry, Institute for Statistical and Epidemiological Cancer Research, Liisankatu 21B, Helsinki, Finland
3Department of Pathology, Haartman Institute, University of Helsinki, Haartmaninkatu 3, Finland

Germline mutations in fumarate hydratase (FH) gene at 1q43 predispose to hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome. In HLRCC, the most common clinical features are benign tumours of the skin and uterus, and in a subset of the families, renal cell cancer (RCC) and uterine leiomyosarcoma (ULMS) occur frequently at young age. This study was conducted to evaluate the possible contribution of FH mutations in a population-based series of early onset (≤45 years) ULMSs. Eighty-one patients were identified through the national cancer registry, and samples from 67 cases (83%) were available for FH mutation screening and analysis of allelic imbalance (AI) at the FH locus. Seventeen percent of tumors showed AI. In the mutation analysis, a novel missense mutation K424R was found. The mutation was also found from the patient’s normal tissue. To study whether this variant has functional consequences, FH enzyme activity assay was performed in a cell model. The activity of the mutated protein was significantly reduced as compared to wild type (p = 0.009). This study shows that FH germline mutations can occur in seemingly non-syndromic cases of ULMS (1/67, 1.5%). It appears that on the population level hereditary FH defects do play a role in pathogenesis of sporadic early onset ULMSs, albeit rarely.

Key words: uterine leiomyosarcoma; fumarate hydratase; HLRCC; FH enzyme activity

Uterine leiomyosarcoma (ULMS) is a rare and highly malignant smooth-muscle tumor. It covers 1–3% of uterine malignancies and occurs mainly after menopause. ULMS has been presumed to be a malignant counterpart of benign uterine leiomyoma.1,2 The genetic background and pathogenesis of ULMS is poorly understood. ULMSs are typically associated with numerous inconsistent chromosomal aberrations.3–5 Some specific genetic changes have been associated with ULMS. Germline mutations have been observed in the TP53 gene in Li-Fraumeni syndrome, and somatic alterations have included mutations in TP53 and PTEN genes.6,7 In recent gene expression studies, a number of genes have been found to be differentially expressed in ULMS compared to benign leiomyoma.8 Recent cytogenetic studies have not demonstrated any chromosomal aberrations common to ULMS and leiomyoma.4

We have recently reported that heterozygous germline mutations in the fumarate hydratase (fumarase, FH) gene cause a novel tumor susceptibility syndrome, hereditary leiomyomatosis and renal cell cancer (HLRCC, OMIM 605839).9,10 Multiple leiomyomas of the skin and uterus is the most common feature of HLRCC, with nearly 100% prevalence.11 Renal-cell cancer (RCC) occurs only in a subset of HLRCC families.9,12–17 While ULMS has not been reported in the context of HLRCC by others, the risk for ULMS is markedly increased in Finnish HLRCC kindreds. Five cases, all at very young age (from 27 to 39 years), have occurred in our study; 15% of all women with FH germline mutations, providing a 71-fold risk (CI 8.6–260, large due to small numbers) as compared with the general population.15,12 Interestingly, 1 patient with ULMS, RCC and leiomyomas has been reported in 1973.13

The occurrence of ULMS in patients with FH mutation led us to hypothesize that FH mutations could also play a role in the pathogenesis of nonsyndromic early onset ULMS. This hypothesis was strengthened by findings in a small pilot series; 1 out of eight unselected ULMSs displayed a germline mutation.20 We collected a population-based material of all early onset (≤45 years) ULMS diagnosed in Finland between years 1981 and 2003 through a search from the Finnish Cancer Registry. Eighty-one patients fulfilled the criteria, and 67 tumors (83%) were available for FH mutation analysis. In addition, allelic imbalance (AI) at the FH locus (1q43) was studied.

Material and methods

Patient material

A population-based material of the early onset ULMS cases was derived through a search from the Finnish Cancer Registry. The study was approved by the Helsinki University Central Hospital’s ethics review committee, and samples were derived after obtaining the authorization from the National Authority for Medicolegal Affairs.

Altogether 392 ULMS cases were identified between years 1981 and 2003. Most of the cases were diagnosed at the age of 50 or above; 1.3% at the age of 0–29 years, 32% at the age of 30–49 years, 42% at the age of 50–69 years and 24% at the age of ≥70 years. In the present study, an age ≤45 was chosen as the definition of early onset ULMS. Altogether 81 individuals fulfilled this criterion, and paraffin-embedded tumor tissues for mutation analysis were available from 67 cases. The average age at diagnosis was 39.8 years (varying between 16 and 45 years, median 41). Most of the tumors originated from uterine corpus (70%) and represented grade 1 (35%) or 2 (36%). Eight patients (8/81, 10%) had another primary tumor: 2 breast cancers, 3 basal cell cancers, 1 endometrial stromal sarcoma, 1 lung hemangioepicytoma and 1 Hodgkin’s lymphoma. Eighteen patients (22%) had died of cancer, as detailed in Table I. Information on cancer history from the first-degree relatives was collected through church parish registries, the Population Register Centre and the Finnish Cancer Registry. Forty-two ULMS patients (42/81, 52%) had at least 1 first-degree relative diagnosed with cancer. These included 1 uterine cancer with unknown histology diagnosed at the age of 50.

Mutation analysis

The mutation analysis was performed from patients’ tumor tissues, and the presence of putative mutations was examined in the respective normal tissue. DNA extraction from paraffin blocks was performed according to standard procedures. The coding exons were amplified by PCR and analyzed by genomic sequenc-
ing. PCR conditions and the oligonucleotide primers used have been published earlier by Kiuru et al. (2002). The PCR products were purified using ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, Ohio), and the sequencing reactions were performed using the Big Dye Terminator v.3.1 kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Electrophoresis was performed on an ABI3730 Automatic DNA sequencer (Applied Biosystems). The analysis of FH exon 9 from 280 healthy controls was performed by denaturing high performance liquid chromatography (DHPLC) as described earlier by Lehtonen et al. (2003). For splice site predictions, GeneSplicer Web Interface (http://www.tigr.org/tdb/GeneSplicer/gene_spl.html), Splice Site Prediction at the Berkley Drosophila Genome Project web page (http://www.fruitfly.org/cgi-bin/seq_tools/splice.pl) and NetGene2 server (http://www.cbs.dtu.dk/services/NetGene2/) were used. To analyze the influence of the amino acid substitution to the protein structure, we used Swiss-Model protein modeling server at http://www.expasy.ch/swissmod/.

### Allelic imbalance analysis

The analysis of AI at 1q43 was performed using 3 flanking microsatellite markers of the FH gene (details from the authors on request). Fluorescent-labeled PCR products were detected by ABI3730 sequencer and analyzed by GeneMapper 3.0 (Applied Biosystems).

### Enzyme activity assay

To determine whether the amino acid change K424R (FHK424R) has functional consequences, FH enzyme activity assay in a cell model was performed. The wild type FH (FHWT) was used as a positive control and nontransfected cells as a negative control. The cDNA of the wild type FH was cloned into a pCI-neo Mammalian Expression Vector (Promega Corporation, Madison). The mutation K424R was created into a FHWT/pCI-neo construct by site-directed mutagenesis (QuickChange® Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA) using primers 5'-CCTCATA-TAGGGTATGACAGGGCAGAAAGATTGC-3' and 5'-GCAC- TCTTTGCTGCCCTGTACATACCATATTTAGG-3' to generate the 1432A>G substitution. The constructs were verified by sequencing. The wild type and mutated constructs (FHWT/pCI-neo and FHK424R/pCI-neo) were transfected into HEK293 cells (ATCC, Manassas, VA) by using a FuGENE 6 Transfection Reagent, according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN). To confirm the concordance of the transfection efficiencies of the different constructs, FH immunocytochemistry was performed to cells on coverslips, and the number of cells with FH overexpression was calculated.

Twenty-four hours after transfection, cells were centrifuged into pellet and resuspended into 1 ml of 20 mM HEPES-KOH buffer (pH 7.5) containing 2 mM dithiothreitol. Suspensions were sonicated on ice (2 times, 3 sec), and cell debris was removed by centrifugation (12,000 rpm, 3 min). FH enzyme activities were measured from the collected supernatants as described earlier by Hatch (1978) with minor modifications: reactions were performed in a total volume of 260 µl and chicken liver malic enzyme (Sigma, Missouri, US) was used. In short, the FH enzyme activity

### TABLE I – CLINICAL DATA OF PATIENT MATERIAL

| Clinical variable | N   |
|-------------------|-----|
| Age               |     |
| 41–45             | 45  |
| 36–40             | 26  |
| 31–35             | 5   |
| <30               | 5   |
| Died of cancer    |     |
| Age               |     |
| 41–45             | 12 (27) |
| 36–40             | 3 (12)  |
| 31–35             | 0 (0)   |
| <30               | 3 (60)  |
| Tumor site        |     |
| Corpus            | 57 (70) |
| Fundus            | 2 (2) |
| NUD               | 22 (27) |
| Tumor grade       |     |
| 1                 | 28 (35) |
| 2                 | 29 (36) |
| 3                 | 8 (10)  |
| Unknown           | 16 (20) |

Values in parentheses indicate percentages.

### FIGURE 1

- (a) FH mutation K424R detected in a 41-year old patient with ULMS. (b) No LOH was observed in the patient’s tumor tissue (T) when compared to normal tissue (N).

### TABLE II – FH VARIANTS FOUND IN MUTATION ANALYSIS

| Variant | Exon | Amino acid change |
|---------|------|-------------------|
| 927G>A  | 6    | No                |
| 1299C>T | 8    | No                |
| 1432A>G | 9    | K424R             |
| IVS2 -22A/T | Intrinsic | rs2275162   |
| IVS4 -44G/A | Intrinsic |                |
| IVS5 +24C/T | Intrinsic |                |
| IVS6 -14T/A | Intrinsic |                |
| IVS6 -15A/T | Intrinsic |                |
assay is based on the consumption of NADP in the fumarate-to-malate reaction per minute per milligram of total protein in the sample. Total protein levels of the cell lysates were measured using BCA Protein Assay Kit (Pierce, Rockford, IL).

Results

A population-based set of Finnish early onset ULMSs was analyzed to clarify the role of FH mutations in nonsyndromic ULMSs. Altogether, 81 patients fulfilled the adjusted criteria, and paraffin-embedded tissues were available from 67 tumors (83%). In the mutation analysis, a total number of 653 fragments out of 670 (97.5%) were analyzed successfully. A novel missense change K424R (1432A>G) was detected (1/67, 1.5%) (Fig. 1a and Table II).

In addition, 2 silent polymorphisms were observed in the coding region, 927G>A in exon 6 and 1299C>T in exon 8. A total of 5 intronic base pair substitutions were also detected but none of these were predicted to affect splicing in silico (Table II).

The patient with K424R mutation was diagnosed with grade 2 ULMS of the uterine corpus at the age of 41 and is alive after 12 years, although the disease had metastasized 10 years after diagnosis. The patient had no other tumors and no cancer was reported in her first-degree relatives. The missense change K424R was also found in patient’s normal tissue indicating a germline change. No loss of wild type allele was detected in the tumor tissue (Fig. 1b).

The analysis of AI at the FH locus on chromosome 1q43 was examined by using 3 microsatellite markers. Altogether 52 of 62 available tumor-normal DNA pairs were informative for 1 or more markers, and 9 tumors (9/52, 17%) showed AI at the FH locus (Fig. 4).

Discussion

Altogether five ULMSs have so far been diagnosed in the Finnish HLRCC families. Even though ULMS occurs typically after menopause, all HLRCC patients with ULMS have been diagnosed at young age (<40 years). The occurrence of ULMS in Finnish HLRCC patients with FH mutation led us to hypothesize that FH germline mutations could also play a role in the pathogenesis
of nonsyndromic early onset ULMS. The Finnish Cancer Registry enabled identification of this unique population-based set of 81 nonsyndromic early onset ULMS cases of which 67 (83%) were available for the DNA analyses.

In the analysis of 67 tumors, a novel missense change, K424R was detected (1/67, 1.5%). The change was found in both tumor and normal tissues. The alteration was not detected in healthy controls and it occurred at a conserved residue (Fig. 2). To study the influence of the change to the protein function, FH enzyme activity was measured. The activity of the mutated protein was significantly reduced (p = 0.009) being ~43% of the activity the protein in the model system used. This result indicates that K424R is a pathogenic mutation. However, no LOH or additional somatic mutations were detected in the tumor DNA. The FH activity in lymphoblastoid cell lines from HLRCC patients with heterozygous FH mutation is reduced as compared with controls.10,13,17 Of note, lymphoblastoid cell lines with missense mutation tend to have a lower enzyme activity than deletion/truncating mutants, probably due to a dominant negative action of the missense mutations.1,3

Because ULMSs are known to display chromosomal instability and FH is presumed to be a tumor suppressor gene, the AI status of ULMS at locus at 1q43 was studied. Seventeen percent of tumors showed AI at 1q43. Barker et al. (2002) reported AI at 1q in half of the analyzed ULMSs, but in some of the cases AI was not confined to the vicinity of FH. In addition, in previous CGH analyses of ULMSs, chromosomal gains at 1q have been reported.2,8 Thus, it is possible that another yet unidentified gene at 1q is involved in tumorigenesis of ULMS.

Taken together, a novel germline FH mutation K424R was detected from a population-based series of 67 early onset nonsyndromic ULMS. In a previous work, we detected 1 germline mutation in 18 unselected ULMS cases.20 Taken these 2 studies together the frequency of FH germline mutations in nonsyndromic ULMS in Finland is 2/85 or 2.4%, though it should be noted that the 2 series had different inclusion criteria.

Overall, numerous sporadic tumor types have earlier been analyzed in several independent studies and FH mutations have rarely been found.20,23–26 Thus far, only 3 somatic FH mutations have been identified, in 1 soft-tissue sarcoma and 2 uterine leiomyomatas.12,24 Barker et al. (2002, 2005) analyzed altogether 35 ULMSs but no FH mutations were found.23,26 Here too, no somatic mutations were identified in ULMSs. However, based on this and previous works by us FH is clearly involved in genesis of a subset of early onset ULMSs, especially in the context of the HLRCC syndrome.7,20

Five cases of ULMS have occurred in the Finnish HLRCC families. Thus altogether 6 ULMS cases with FH germline mutation have been detected in Finland, all at young age,15,18 but no cases have been reported in other populations despite of multiple published studies on HLRCC phenotype.10,11,13,14,16,17,27,28 Additional genetic modifiers are likely to play a role in explaining this rather dramatic difference in phenotype. Identification of these modifying factors would likely to be relevant in view of understanding the genesis of uterine leiomyosarcoma in the context of FH defects, and their possible role in nonsyndromic ULMS would be an important focus for future studies towards understanding the mechanisms of malignant degeneration in smooth muscle tumors.

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