Modeling tumor predisposing *FH* mutations in yeast; effects on fumarase activity, growth phenotype and gene expression profile

Antti Kokko[1,4], Sanna SK Ylisaukko-oja[1], Maija Kiuru[1], Maarit S Takatalo[2+], Paula Salmikangas[2++], Jarno Tuimala[3], Diego Arango[1], Auli Karhu[1], Lauri A Aaltonen[1] and Jussi Jäntti[4,5*]

[1]Department of Medical Genetics, Biomedicum Helsinki, Haartmaninkatu 8, P.O. Box 63, FIN-00014 University of Helsinki, Finland, [2]Folkhälsan Institute of Genetics, Department of Medical Genetics, University of Helsinki, Finland, [3]CSC - Scientific Computing Ltd. Box 405, FIN-02101 Espoo, Finland, [4]VTT Biotechnology, PO Box 1500, FIN-02044 VTT, Finland, [5]Institute of Biotechnology, University of Helsinki, P.O.Box 56, 00014 University of Helsinki, Finland

[+]Department of Biological and Environmental Sciences, Division of Biochemistry, Faculty of Biosciences, University of Helsinki, Finland, [++]Present address: National Agency for Medicines, Finland.

*To whom correspondence should be addressed at Institute of Biotechnology, University of Helsinki, P.O.Box 56, 00014 University of Helsinki, Finland. Fax: INT +358-9-19159570 E-mail: jussi.jantti@helsinki.fi, Tel: INT +358-9-1911 (direct: +358-9-19159722)

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The abbreviations used are: BIOB, E. coli BioB gene biotin synthetase; BIOC, E. coli bioC gene protein; BIODN, E coli bioD gene dethiobiotin synthetase; BSA, bovine serum albumin; CREX, Bacteriophage P1 cre recombinase protein; DTT, dithiothreitol; ECL, enhanced chemiluminesence; FGEA, functional gene enrichment analysis; HLRCC, the Hereditary Leiomyomatosis and Renal Cell Cancer; HRP, horseradish peroxidase; MAS, Micro Array Suite; NADP, nicotinamide dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SCD, synthetic complete dextrose; SDS, sodium dodecyl sulphate; TCA, tricarboxylic acid cycle; YPD, yeast peptone dextrose; YPDGE, yeast peptone dextrose glycerol ethanol;YPG, yeast peptone glycerol; YPGE, yeast peptone glycerol ethanol; YPL, yeast peptone lactate

This study describes the use of *S. cerevisiae* as a model organism to assess the effects of two missense mutations H153R and K187R in the fumarase gene causing tumor predisposition syndrome Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC). These mutations at fully conserved sites displayed some fumarase activity and normal growth phenotype demonstrating that cancer related *FH* mutations don't differ from their benign phenotype associated counterparts. These results give new insight to the effects of fumarase mutations without confounding input from the functional allele, and highlight the possible effect of yet unknown genetic modifiers – rather than simple genotype-phenotype associations – in the dramatically varying *FH* family phenotypes.

Abstract

Heterozygous mutations in the fumarase (*FH*) gene cause the tumor predisposition syndrome hereditary leiomyomatosis and renal cell cancer (HLRCC, MIM605839). While most families segregate a benign phenotype of multiple leiomyomas, others display a phenotype with early-onset renal cancer and leiomyosarcoma. Modifier genes may play a role in this, but an alternative explanation is simple genotype-phenotype association. *FH* mutations predisposing to cancer appear to be truncating or in fully conserved amino acids, suggesting that mutations severely affecting *FH* activity might predispose to malignancy. In the present study we analyzed two conserved fumarase mutations in yeast. H153R has been described in three cancer predisposition families; whereas all three reported K187R families have displayed the benign phenotype. Examining H153R and K187R should clarify whether cancer related *FH* mutations differ from their benign phenotype associated counterparts.

Yeast strains containing the two mutations, and knock-out and wild type references, were created and the growth phenotypes studied on selected carbon sources to assess mitochondrial function. Additionally Fum1 protein production and activity were measured, and the strains were subjected to transcriptional profiling.

On nonfermentable lactate medium the fumarase knockout strains did not grow whereas the mutants showed no differences as compared to wild type yeast. While both mutant strains produced fumarase a considerable decrease in enzyme activity was seen in mutants with respect to wild type. Transcription of the majority of Krebs cycle enzymes was downregulated in response to mutations in fumarase.

In conclusion both mutants displayed some, albeit greatly reduced, fumarase activity. This activity was sufficient to support normal growth on nonfermentable carbon source, unlike the deletion

phenotype, demonstrating the significance of the residual activity. The findings support the hypothesis that modifier gene(s), rather than phenotype-genotype effects, display a major role in determining tumor phenotypes in families segregating *FH* mutations.

Introduction

The number of genetic diseases linked to mitochondrial function has been steadily growing and recent reports have associated defects in nuclear encoded mitochondrial proteins to cancer predisposition.[1-4] We and collaborators have previously reported that defects in one of the tricarboxylic acid cycle (Krebs cycle) genes predispose to dominantly inherited uterine leiomyomas (fibroids), skin leiomyomas and renal cell carcinoma. This autosomal dominant genetic disorder, named hereditary leiomyomatosis and renal cell cancer (HLRCC) (MIM 605839)[5-7] was linked to mutations in *fumarate hydratase* (*fumarase*, *FH*). Interestingly, homozygous mutations in *FH* have previously been reported to cause a severe neurological disorder, FH deficiency (MIM 606812).[8-10] Since the discovery of *FH* in tumor predisposition, the gene has been proposed to act as a tumour suppressor gene. However, the cellular and molecular mechanisms leading to tumor development remain unclear.

The striking diversity in phenotypes between families segregating *FH* mutations add to the puzzle. In particular, families from Finland have displayed a phenotype with high risk of early-onset renal cell carcinoma and uterine leiomyosarcoma.[11] Amazingly, no cases of uterine leiomyosarcoma have been reported outside this population, and also early-onset renal cell carcinoma appears to affect only a small number of *FH* mutation-positive families in other populations.[12-14] The data from all examined populations is compatible with the notion that some families are very prone to malignant tumors, whereas others are not.[5, 12-15] The most simple explanation for this is genotype-phenotype association. However, some data speak against this. In particular, R190H mutation has been described in 14 families, only one segregating renal cell carcinoma with five cases in the family.[11, 13] However, the mutations in cancer prone kindreds are either truncating or affecting fully conserved residues, suggesting that the severity of functional defect might relate to the occurrence

of malignant tumors.

As the mechanisms behind the HLRCC syndrome remain obscure and the most recent studies in human have examined the effect of tumorigenic fumarase mutations only in a heterozygous background[5, 12], yeast was selected as a model organism for functional studies of HLRCC in a homozygous situation, where the effect of a *FH* mutation could be evaluated more clearly. Yeast fumarase is highly homologous to the human form of the enzyme with close to 80 % of conserved amino acids of which 66 % are identical.[16] Experiments were designed to introduce fumarase amino acid mutations H153R and K187R in yeast and characterize these fumarase mutated yeast strains to provide information about the molecular level changes occurring in yeast. H153R is a founder mutation detected in three Finnish families with high risk of renal cancer and uterine leiomyosarcoma.[6, 11] K187R is a mutation strongly associated with benign phenotype. This missense mutation has been detected both in a FH deficient family as well as in UK families segregating leiomyomatosis and no cancer.[5, 8] Furthermore, K187R is one of the few *FH* mutations related to tumorigenesis which has been examined in human cells in homozygous form, and shown to display very low level of activity (3.7% of control activity).[17] It was attractive to examine whether the cancer related H153R had even lower or absent activity, and how low or absent activity would relate to the growth phenotype of yeast. As an extensively studied eukaryote with a known genome[18, 19], yeast is a useful and approachable tool to study molecular and cellular biology. Comparative analyses of predicted protein sequences suggest that many basic biological functions of eukaryotic cells such as DNA and RNA metabolism, protein folding, trafficking and degradation are carried out by a core set of orthologous proteins.[20]

In this study we show that in yeast strains with a fumarase mutation H153R or K187R the fumarase

enzyme activity decreases considerably, but performance of H153R is no worse than that of K187R. The residual activity is physiologically significant. Unlike strains with fumarase deletion (null for fumarase), H153R and K187R strains are able to grow normally on lactate (nonfermentable carbon source). Taken together, H153R in this model system does not display null activity, and little difference is observed between H153R and K187R. Thus these findings support the hypothesis that the dramatic differences in the respective human phenotypes, H153R families displaying much more cancers than K187R families, are likely to be due to unidentified genetic modifiers.

Materials and methods

Yeast strains

The yeast strains used in this study are listed in Table 1. All strains were derived from a wild type fumarase containing *Saccharomyces cerevisiae* strain (*FUM1*) congenic to W303-1A. Fumarase gene knockout strain (*fum1Δ*) was constructed from the wild type strain using a PCR-targeted gene disruption method utilising the *kanMX4* module.[21] This knockout strain was used as a template for the creation of different yeast strains. Integrative yeast expression vector containing different versions of the *FUM1* gene, these being the wild type fumarase ($FUM1^{U}$), mutant H153R ($fum1^{H153R}$), mutant K187R ($fum1^{K187R}$) or empty vector ($fum1^{vect}$) were integrated to the *ura3*-locus by linearization of the plasmids with StuI prior to transformation. The integration and copy number of the integrated genes were verified by southern blotting.

The strains were routinely grown on YPD containing 1% Bacto-yeast extract (Difco Laboratories), 2% Bacto-peptone (Difco Laboratories) and 2% dextrose (BDH Laboratory Supplies) at 30°C on petri dishes or in 10-50ml flasks on a shaker at 30°C, 250 rpm. Other YP -media used were YPDGE

containing 2% dextrose, 3% glycerol (Merck) and 2% ethanol (Primalco), YPGE containing 3% glycerol and 2% ethanol, YPG containing 3% glycerol and YPL containing 2% potassium lactate (BDH Laboratory Supplies. Yeast transformation was carried out essentially as described previously.[22] For plate growth test yeast cultures at OD_{600} 1.0 with dilutions 1:10, 1:100 and 1:1000 were plated on different carbon sources. Growth tests in YPD yeast cultures were done as 3 independent experiments with 3 tubes per strain. Averaged values of the 3 replicates per strain from a given experiment were used as the basis for the calculation of doubling times.

Mutagenesis

Yeast chromosomal DNA used in PCR reactions was extracted using standard protocols.[23] The mutagenesis PCR was done according to manufacturers instructions using QuickChange® site-directed mutagenesis kit (Stratagene). For mutagenesis a genomic *FUM1* fragment in pBluescript® SK was used as a template. The primers used (6715, 6716, 6717 and 6718) are listed in Table 2. All plasmids generated by PCR were verified by sequencing. *FUM1* and the different mutants were cloned as XhoI/XbaI fragments into yeast integrative (pRS406) plasmids.[24]

Fumarase activity assay and Western Blotting

Preparation of cell lysates for fumarase enzyme activity assays was done from yeast cell cultures collected at A_{600} 2.0, harvested by centrifugation (3min, 4000g, +22°C) and washed with 1ml of H_2O. Cells were resuspended into 400 µl of wash buffer (20mM Hepes-KOH pH 7.5 containing 2mM Dithiothreitol) and disrupted by vortexing (5×15sec., +4°C) with glass beads. The fumarase enzyme activities were measured from the amount of NADP consumed in the fumarate-to-malate reaction per minute per mg of total protein in the sample.[25]

Cell lysates for western blotting were collected and measured as described above, but breaking the cells in SDS buffer (2%) containing protease inhibitors (Complete Mini™, Roche). Lysates were

then heated (5min, 94°C) followed by centrifugation (20000g, 5min, +22°C). After SDS-PAGE and Western Blotting the fumarase protein bands were visualized with ECL detection reagents (Amersham Biosciences) using αFum1p serum (courtesy of Dr. Ophrey Pines, Hebrew University, Israel) and Goat Anti-Rabbit IgG (H+L) HRP conjugate (Bio-Rad Laboratories).

Microarray preparation and hybridization

RNA extraction was done from 30ml YPD cultures harvested at A_{600} 3.0 by homogenising the samples with glass beads and breaking buffer (20mM Tris-HCl pH 7.5, 0.1M KCl, 2mM $MgCl_2$, 2mM DTT) in Mini-BeadBeater™ (BioSpec Products) for 6×30 seconds with 2 minutes on ice between the shakings. Total RNA was isolated from the lysate with RNEasy Midi Kit (Qiagen). The quality of RNA was analyzed using Agilent 2100 Bioanalyzer (Agilent Technologies). Array hybridizations to GeneChip YG-S98 arrays were done using 8 µg of total RNA as starting material according to the manufacturer's instructions (Affymetrix Inc.). Hybridization quality was controlled by inspecting that on each array the number of probes present on the chip, the 3' to 5' ratios of the hybridization controls (BIOB, BIOC, BIODN, CREX), average background intensity and noise were on similar levels. Probes with unsuccessful hybridization were left out of analyses by masking them out using the Microarray Suite (MAS) 5.0 software (Affymetrix Inc.).

Data normalization and filtering

The expression data were normalized by truncating small values to 0.01 and centering array intensities on the median. Gene intensities were scaled by dividing the expression measurements with the corresponding control sample value of *FUM1* wild type strain. The normalized expression data were filtered to remove expression values below detectable levels using the Affymetrix Detection Algorithm assigned flag calls. Subsequent analyses were restricted to genes with detectable expression in all 6 strains. Effect of biological variation resulting from strain processing

and transformation methods such as heat shock treatment and insertion of bacterial DNA containing vector was accounted for by filtering out probes with over 20% deviation in the expression values between the created wild type (WT) strain ($FUM1^U$) and the original WT strain (*FUM1*), resulting to a list containing 2113 probes. The threshold for differentially expressed probes was defined as probes from this list with at least 2-fold change in the expression value of mutant or deletion strain in comparison to the $FUM1^U$ strain. This resulted to a list of 270 probes differentially expressed genes in the yeast transcriptome. The enrichment of any functional groups or pathways in the list of differentially expressed genes compared to the initial list of 2113 comparable genes was analyzed using GoMiner software.[26] In addition the expression levels of genes involved in TCA cycle regulation were analyzed for differences between the wild type and fumarase modified yeast strains. The filtering rules used for transcriptome level analyses were discarded for analysis of this data set. All data manipulations and analyses except the functional group enrichment analysis were performed in GeneSpring 7.0 software (Silicon Genetics).

Results

Effect of fumarase mutation to yeast growth on fermentable and nonfermentable carbon sources

To assess the mitochondrial function of different fumarase mutants, integrant yeast strains were grown aerobically on different fermentable and nonfermentable growth media plates to study the effects of the mutations on the yeast growth (Figures 1A to 1F). On fermentative carbon sources SCD and YPD (Figures 1A and 1B) all strains had similar appearances and they showed no differences in growth rates or in the size of individual colonies. When grown on less fermentable carbon sources YPDGE and YPGE (Figures 1C and 1D) the colonies formed more slowly and different phenotypes in appearances were observed. On both YPDGE and YPGE plates, all clones containing the wild type or mutated fumarase appeared to grow equally well. However, both

deletion strains *fum1Δ* and *fum1*^{vect} showed inhibited growth compared to the mutant and wild type strains. This became more apparent on the YPGE medium when there was no glucose present. On nonfermentable culture media YPG and YPL (Figures 1E and 1F) yeast strains showed clear differences in growth. The strains containing wild type fumarase grew well on both media and the strains with fumarase mutations appeared to have similar phenotypes compared to the wild type**.** On YPG medium, the growth of the deletion strains *fum1Δ* and *fum1*^{vect} was nearly absent, and on YPL neither of these strains was able to grow at all.

Due to observed differences in growth abilities of the fumarase deletion strains on nonfermentable plates the yeast growth rates were measured also from YPD liquid cultures for the deletion strains *fum1Δ* and *fum1*^{vect} and wild type fumarase containing strains *FUM1* and *FUM1*^{U}. The measured mean doubling times for the *FUM1* and *FUM1*^{U} strains were 73.0 and 72.1 minutes and for the strains *fum1Δ* and *fum1*^{vect} 67.3 and 67.5 minutes respectively, indicating a trend that the fumarase knockout strains were dividing faster than the strains containing the wild type fumarase (supplementary data table 1). However, a t-test between the WT and KO strain doubling times showed no significant difference (p-value < 0.05) in the growth rates.

Effect of FUM1 mutations on fumarase enzyme activity and protein production

In literature, both heterozygous and homozygous mutations in the human fumarase gene (*FH*) causing both modifying and truncating forms of protein with different levels of enzyme activities have been described.[5, 17] Previously, the K187R mutation has been one of the few mutations examined in homozygous state in human cells and shown to cause decreased activity.[17] We therefore wanted to analyze the effect of these mutations on fumarase activity in yeast.

The fumarase enzyme activities were measured *in vitro* on YPD medium from the original and integrant strains and are presented in Table 3. The results indicated that the fumarase activity levels differed considerably between the strains. The activity in the integrant strain $FUM1^{U}$, where wild type *FUM1* gene with its endogeneous promoter and terminator sequences had been integrated to the *URA3* locus, was considerably higher in comparison to all the other strains in the experiment. The wild type strain *FUM1* had only 44% of the activity compared to the integrant $FUM1^{U}$ strain. In the mutant strains $fum1^{H153R}$ and $fum1^{K187R}$ the activity had dropped to 1/11 fraction of the activity measured in the integrant strain $FUM1^{U}$. The deletion strains *fum1Δ* and $fum1^{vect}$ showed no detectable activity. Despite considerable decreases in enzyme activities the Western Blotting results showed that the wild type fumarase containing strains *FUM1* and $FUM1^{U}$ as well as the fumarase mutant strains $fum1^{H153R}$ and $fum1^{K187}$ all produced fumarase protein while in the fumarase deletion strains *fum1Δ* and $fum1^{vect}$ no production of fumarase was observed (data not shown).

Differentially expressed genes in FUM1 modified strains in yeast transcriptome

In order to get insight on the fumarase mutations at the yeast transcriptome level and to identify possible candidate genes or functional groups for the studies of HLRCC syndrome microarray experiments were performed. Of the 2113 probes with comparable expression data (supplementary data table 2) 270 probes consisting of 173 genes and 97 poorly characterized sequences were identified to be differentially expressed in the mutant or knockout strains by 2-fold change in comparison to the wild type fumarase containing strain $FUM1^{U}$. The expression levels were compared between the 4 integrant strains with identical genetic backgrounds, as this was thought to best resemble the differences resulting from modifications of the fumarase gene.

To test whether any functional group was significantly enriched in the differentially expressed genes a functional gene enrichment analysis (FGEA) was done for strains $fum1^{H153R}$, $fum1^{K187R}$ and

fum1^{vect}, where the list of comparable genes (2113 probes) was compared to lists of (2-fold) differentially expressed genes in each of the mutant and knockout strains. The FGEA used Gene Ontology (GO) annotations[27] to calculate whether any functional group was significantly ($p<0.05$) increased during the analysis. Due to the GO annotation structure a single gene could be present in multiple categories.

The FGEA found 116 functional groups that were enriched in the differentially expressed genes. Although only 1 group (GO term 16705, oxidoreductase activity acting on paired donors) was common to all strains with 2 shared genes (*JLP1* and *COX1*), multiple genes inside the groups were overlapping with significantly enriched groups in the other strains. The analysis indicated that the mutant strain *fum1*^{K187R} and the knockout *fum1*^{vect} resembled each other more than the other mutant strain *fum1*^{H153R}, which had fewer groups in common with the other strains. Groups common to both mutants were nucleotide-sugar metabolism (GO term 9225) and cyclin-dependent protein kinase regulator activity (GO term 16538), containing 2 genes each (*PMI40* and *GNA1*, *PCL2* and *CLB2*, respectively). Groups present in the knockout strain *fum1*^{vect} only contained genes related to functions such as myogenesis (*MLP1*) and early endosome (*TOM1*). The results of the functional gene enrichment analysis are presented in supplementary data tables 3, 4, 5, 6 and 7.

In general genes that were over 2-fold over- or underexpressed in the list of comparable genes included probes related to cell respiratory functions such as *PET130* (Protein required for respiratory growth), *JLP1* (similar to alpha-ketoglutarate dioxygenase), *ACS1* (acetyl CoA synthetase) and *HST3* (Homolog of *SIR2*) and cell cycle regulation such as *PCL2* (G1 cyclin), *CDC21* (thymidylate synthase), and *CLB2* (B-type cyclin). Interesting genes involved in RAS-GTPase signalling such as *RPI1* (ras inhibitor), *PXL1* (LIM domain-containing protein that may

modulate signaling by the GTPases Cdc42p and Rho1p) and *RNA1* (GTPase activating protein (GAP) for Gsp1p) were also present. Differentially expressed genes with connections to mitochondrion and protein trafficking, such as *GGC1* (Mitochondrial GTP/GDP transporter) were also present. In addition to transcriptome level the effect of fumarase mutation was analyzed from the expression levels of cell cycle regulating genes. In total 108 probes related to cell cycle regulation were found that were comparable between the two wild type strains *FUM1* and $FUM1^{U}$. Of these, only 11 genes were found to have over 2 fold change in at least one of the mutant strains $fum1^{H153R}$, $fum1^{K187R}$ or $fum1^{vect}$ in comparison to the wild type strain $FUM1^{U}$. When the expression levels were looked on a pathway level no significant differences were distinguished. Complete list of differentially expressed genes and the cell cycle regulating genes is presented in supplementary data tables 8 and 9.

Microarray gene expression levels on TCA cycle probes

The expression level analysis of the 15 TCA cycle probe expression levels consisted of *CIT1, ACO1, IDH1, IDH2, KGD1, KGD2, LPD1, LSC1, LSC2, SDH1, SDH2, SDH3, SDH4, FUM1* and *MDH1* probe measurements. Results of this analysis are presented in Figure 2 and supplementary data table 10. In general, most of the 15 TCA genes appeared to be expressed at a decreased level in the knockout strain $fum1^{vect}$ and in the mutant strains $fum1^{H153R}$ and $fum1^{K187R}$ with the two mutant strains showing greater decrease in TCA genes expression levels when compared to the wild type fumarase containing strain $FUM1^{U}$. The only exception to this was *IDH1*, where observations from both of the mutant strains $fum1^{H153R}$ and $fum1^{K187R}$ as well as the knockout strain $fum1^{vect}$ showed increase in expression levels relative to the wild type fumarase containing strain $FUM1^{U}$. The increase was 40% in the knockout strain, and less in the mutant strains.

The mutant strains also showed 40% decrease in expression levels in all of the 4 SDH subunit

encoding genes, *KGD1* and *KGD2* as well as *LPD1*. There was no detectable fumarase expression in the knockout strain $fum1^{vect}$, while both of the mutant strains showed 30% decrease on fumarase expression levels compared to the strain $FUM1^{U}$ containing the wild type fumarase. Analyses of the strains revealed that the created WT strain $FUM1^{U}$ had obtained three copies of the vector insert containing the fumarase gene and the mutant strains $fum1^{H153R}$ and $fum1^{K187R}$ two copies each. When the fumarase expression values were divided by the number of genes inserted to the genome, no significant differences were seen in the mutant strains $fum1^{H153R}$ and $fum1^{K187R}$ in comparison to the wild type strain $FUM1^{U}$.

Discussion

Fumarase defects have been found to predispose to an autosomal dominant tumor predisposition syndrome HLRCC. The exact molecular mechanisms linking defective FH to malignant tumors are not known. The question on relation between FH defects and cancer is further complicated by the strikingly uneven occurrence of renal cell carcinoma and uterine leiomyosarcoma in families segregating *FH* mutations.[11] This could be explained by phenotype-genotype correlations, e.g. only severe mutations predisposing to cancer. While the mutation data indeed provides some basis for such a hypothesis[11], it is also clear that the same mutation can occur both in cancer predisposed and apparently low-risk families. We addressed this issue by examining high penetrance cancer predisposition associated missense mutation H153R and benign phenotype associated missense mutation K187R, in yeast background. Wild type and deletion mutant fumarase strains served as references.

The fumarase enzyme activity as well mRNA and protein levels were measured in the strains. It was of interest that the performance of H153R was no worse than that of K187R. No fumarase was

detected in the knockout strain as expected. To examine whether the residual activity detected was physiologically meaningful the created yeast strains were tested for growth phenotype using combinations of fermentative and non-fermentative carbon sources containing dextrose, glycerol, ethanol or lactate. The fumarase knockout strains could not use their mitochondrial respiratory chain on non-fermentative carbon source while the fumarase mutant and wild type strains showed normal growth.

Microarray studies of fumarase mutated yeast strains consisted of global analysis of genes most differentially expressed in the fumarase mutants and the knockout and a functional gene enrichment analysis. In the latter experiment Gene Ontology (GO) annotations were used to assess whether any functional group had enriched during the analysis. While the FGEA showed different functional groups between the three compared strains ($fum1^{H153R}$, $fum1^{K187R}$ and $fum1^{vect}$), many of the genes inside the functional groups and between the strains were the same. This indicated that in general, the mutants showed similar expression profiles to the fumarase knockout strain.

The expression level of most TCA cycle genes was decreased, *IDH1* being the only exception in the mutant strains, and *CIT1*, *IDH1* and *LSC2* in the knockout strain. In general, the expression level decrease was more severe in the mutants, which may result from point mutations allowing the expression of the proteins although being partially defective. Thus fumarase could still display interactions with other cellular components possibly even unrelated to respiration, such as cytosolic forms of the TCA cycle enzymes or other yet unknown interaction partners. Interestingly, the TCA cycle results on *IDH1* were similar to those seen in human *FH* deficient myomas (our unpublished data), indicating that the mutations in the yeast fumarase may have similar effects to those observed in patients with HLRCC. The expression levels of the TCA cycle enzymes were compared to other

published results[28], where a fumarase knockout yeast was studied using 2-color microarrays and galactose growth medium. Although variation to the previously published results existed, in general the results were comparable and the observed differences were thought to result from experimental variation on assays done on different growth media and microarray platforms and strains with different genetic backgrounds.

Genes responsive to fumarase mutations or deletion were composed of multiple categories with functions integral to cell regulation, respiration, GTPase activity and including genes related to mitochondrial trafficking. The array results reflected observations seen in activity assays and growth tests. When expression levels of cell cycle regulating genes were analyzed in different fumarase mutants no significant differences were observed between different mutant strains. This correlates well with the finding that no growth rate differences were observed between the mutants.

The key observation of this study was the physiologically relevant residual activity of renal cancer and uterine leiomyosarcoma phenotype associated H153R fumarase mutation and a similar result with K187R mutation associated with the benign form of HLRCC, multiple cutaneous and uterine leiomyomatosis. This finding supports the hypothesis that HLRCC is not a classical one gene cancer predisposition syndrome with genotype-phenotype variations in severity, but that modifier genes appear to play a key role in genesis of malignant tumors in the context of *FH* germline mutations. Identification of those genetic modifiers should shed much light in tricarboxylic acid cycle related carcinogenesis.

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Tables

Table 1. Yeast strains used and created in the study.

strain code	genotype
FUM1	*MATa ade2 can1 his3 leu2 trp1 ura3*
fum1Δ	*MATa ade2 can1 his3 leu2 trp1 ura3 fum1::kanMX4*
$FUM1^{U}$	*MATa ade2 can1 his3 leu2 trp1 ura3::(pRS406-URA3, FUM1) fum1::kanMX4*
$fum1^{H153R}$	*MATa ade2 can1 his3 leu2 trp1 ura3::(pRS406-URA3,* $fum1^{H153R}$*) fum1::kanMX4*
$fum1^{K187R}$	*MATa ade2 can1 his3 leu2 trp1 ura3::(pRS406-URA3,* $fum1^{K187R}$*) fum1::kanMX4*
$fum1^{vect}$	*MATa ade2 can1 his3 leu2 trp1 ura3::(pRS406-URA3) fum1::kanMX4*

Table 2. Oligonucleotide primers used in the study.

primer	primer name	sequence	purpose
6692	kanMX reverse	CCTCGACATCATCTGCCC	deletion verification
6693	*FUM1* -126 Down	TTTCTCCATTACATACAGTC	-"-
6694	*FUM1* +1548 up	TCACAAATTCTCTCCACTTC	-"-
6962	*FUM1* P^{start} (F)	GCATTCTCGAGGACGTGTCCTTAAACCCTTC	-"-
6963	*FUM1* T^{end} (R)	GCATTTCTAGAACCGATATTTGCTGCTAAACTTC	-"-
6715	*fum1*^{K187R}F	GGAATTTGACCATATTGTGAGGATCGGTAGAACACACTTGC	mutagenesis
6716	*fum1*^{K187R}R	GCAAGTGTGTTCTACCGATCCTCACAATATGGTCAAATTCC	-"-
6717	*fum1*^{H153R}R	GGTTGCAATGATTGTTTGGACGGACTTGTTTAGATCCAATC	-"-
6718	*fum1*^{H153R}F	GATTGGATCTAAACAAGTCCGTCCAAACAATCATTGCAACC	-"-
6719	*FUM1* -852 Down (R)	AAGAGCTAGAGTCACTCC	sequencing
6720	*FUM1* +1260 Up (R)	GACTCATTCACAATCGCG	-"-
6721	*FUM1* +1677 Up (R)	AACTCTTGACCTAGTGTC	-"-
6722	*FUM1* +2082 Up (R)	ACTTGAGTCAATGCCTCG	-"-
6723	*FUM1* +2600 Up (F)	AGAGGAGCATAGGCATAC	-"-
6724	*FUM1* +2216 Up (F)	TATTCATTTAGAGTGCAC	-"-
6725	*FUM1* +1800 Up (F)	TCGATGTCAAGATAGCCG	-"-
6726	*FUM1* +1402 Up (F)	AGTTATTTCCAATCGTGC	-"-
4320	T7	GTAATACGACTCACTATAGGGC	-"-
4321	T3	AATTAACCCTCACTAAAGGG	-"-

F, forward primer; R, reverse primer

Table 3. Measured fumarase enzyme activities on fermentable YPD (dextrose) media. The created mutant strains *fum1*^{H153R} and *fum1*^{K187R} showed considerable decrease in fumarase activity in comparison to the wild type strain while there was no detectable activity in the knockout strains *fum1*^{vect} or *fum1Δ.*

Strain	YPD (nmol/min/mg)	standard deviation (nmol/min/mg)
FUM1	17.7	0.4
fum1Δ	0.0	0.0
FUM1^{U}	40.1	0.7
fum1^{H153R}	3.6	0.0
fum1^{K187R}	3.6	0.6
fum1^{vect}	0.0	0.0

Figure Legends

Figure 1. Growth tests of fumarase mutant (2, 3), knockout (1, 4) and wild type (5, 6) strains on various carbon sources plated in dilution series. The carbon sources used were either fermentable (A, B), partially fermentable (C, D) or nonfermentable (E, F) and represent a hierarchically descending order (from A to F) in the yeast metabolic pathways, where fermentable carbon sources can be utilized either in mitochondrial tricarboxylic acid (TCA) cycle or in cytosolic energy production and nonfermentable carbon sources only in the TCA cycle. On fermentable carbon sources all strains grew equally well. When strains were grown on gradually less fermentable media knockouts (1 and 4) started to lose their ability to grow due to complete loss of fumarase production while mutant (2 and 3) and WT (5 and 6) strains continued growing.

Figure 2. Gene expression levels of 15 yeast TCA cycle enzymes in the fumarase mutant (*fum1*^{H153R}, *fum1*^{K187R}) and knockout (*fum1*^{vect}) strains. Majority of the genes had downregulated expression relative to the wild type fumarase containing strain *FUM1*^{U} in both the mutants and the knockout strains with greater decreases in the mutant strains. *IDH1* was observed to be upregulated in all mutant strains. Fumarase gene expression levels were similar to wild type strain *FUMI*^{U} in mutant strains *fum1*^{H153R} and *fum1*^{K187R} when gene copy number was accounted for, whereas no detectable expression was measured from the knockout strain *fum1*^{vect}.