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UOK 262: Fumarate Hydratase (-/-) Hereditary Leiomyomatosis Renal Cell Carcinoma: *In Vitro* and *In Vivo* Model of an Aberrant Energy Metabolic Pathway in Human Cancer

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Abstract

Energy deregulation and abnormalities of tumor cell metabolism are critical issues in our understanding of cancer. Hereditary leiomyomatosis renal cell carcinoma (HLRCC) is an aggressive form of RCC characterized by germline mutation of the Krebs cycle enzyme, fumarate hydratase (FH), and is known to be highly metastatic and unusually lethal. There is significant utility in establishing preclinical cell and xenograft models for study of disorder of energy metabolism as well as development of new therapeutic approaches targeting of tricarboxylic acid (TCA) cycle deficient human cancers. Here we report the first immortal cell line derived from a patient having aggressive HLRCC-associated recurring kidney cancer, designated as UOK 262. We investigated gene expression, chromosome profiles, efflux bioenergetic analysis, mitochondrial ultrastructure, FH catabolic activity, invasiveness, and optimal glucose requirements for *in vitro* growth. UOK 262 cells have isochromosome 1q [i(1)(q10)] as recurring chromosome abnormality; demonstrate compromised oxidative phosphorylation and *in vitro* dependence on anaerobic glycolysis consistent with the clinical manifestation of HLRCC. Furthermore the cells display glucose-dependent growth, an elevated rate of lactate efflux, over-expression of the glucose transporter Glut 1 and lactate dehydrogenase (LDH) 5. Mutant FH protein was primarily present in edematous mitochondria, but its catalytic activity was nearly undetectable. UOK 262 xenografts retain the characteristics of HLRCC histopathology. Our findings indicate that the severe compromise of oxidative phosphorylation and rapid glycolytic flux in UOK 262 are an essential feature of this TCA cycle enzyme deficient form of kidney cancer. This tumor model is the embodiment of the “Warburg effect”. UOK 262 provides a unique *in vitro* and *in vivo* preclinical mode to study the bioenergetics of the Warburg effect in human cancer.

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Keywords

papillary kidney cancer; oxidative phosphorylation; glycolysis; *FH* gene; HLRCC: Hereditary Leiomyomatosis Renal Cell Carcinoma

1. Introduction

Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) is an inherited cancer syndrome in which affected individuals are at risk for the development of cutaneous and uterine leiomyomas as well as renal cell carcinoma (RCC). The cutaneous and uterine manifestations [1] and the renal manifestations [2] were described as autosomal dominant conditions in 1995. In 2001 the cutaneous, uterine and renal manifestations were described as a single entity by Launonen, et al. and the condition renamed hereditary leiomyomatosis and renal cell carcinoma (HLRCC).[3] Typically, HLRCC- associated renal lesions present as unilateral, solitary, high grade tumors that have a predisposition to metastasize early.[2,4-6]

The gene causing HLRCC encodes fumarate hydratase (FH), one of the essential metabolic enzymes of the tricarboxylic acid, or Krebs, cycle, and has been mapped using linkage analysis to the long arm of chromosome 1 at 1q42.3-q43.[4,7] Previous reports have indicated that these tumors display papillary type 2 histology.[7] Merino, et al. have described the unique features of HLRCC tumor cell morphology: not only a high Fuhrman grade (3 or 4) suggestive of papillary type 2 histological type, but also the presence of large eosinophilic nucleoli with a clear perinucleolar halo.[8] Use of these unique morphologic features has greatly improved the diagnostic accuracy for HLRCC kidney cancer.

Investigators have noted that a common feature of a number of types of cancer is preferential utilization and catabolism of glucose,[9-15] particularly in tumors with high malignant potential, that are poorly differentiated, and that proliferate rapidly. Despite the significant progress in identification of the genetic and molecular factors contributing to the malignant phenotype of HLRCC, gene/protein expression and cytogenetic studies of HLRCCs are limited, and there is no well-characterized tumor cell line model available. We have previously established and characterized renal tumor cell lines for studies of the *VHL* gene in clear cell kidney cancer[16], the *BHD* gene in the Birt-Hogg-Dubé syndrome[17], and the *TFE3* gene [18,19].

The current study describes the properties of UOK 262, which was established from a metastatic HLRCC kidney tumor surgically removed from a patient with recurrent kidney cancer. Unlike other available renal carcinoma cell lines, UOK 262 cells provide an *in vitro* and *in vivo* model for studying the metabolic impact of fumarate hydratase deficiency in kidney cancer. This cell line provides a unique model with which to study the Warburg phenomenon in human cancer.

2. Materials and methods

2.1. Patient Characteristics

The patient was evaluated at the National Cancer Institute (NCI) on a Urologic Oncology Branch protocol approved by the NCI Institutional Review Board (IRB) and gave written informed consent for participation in this study. The presentation and clinical course of this patient were described previously.[6] In brief, the patient was an asymptomatic 48 year-old caucasian female who was initially found to have a large renal mass during evaluation, which was initiated because of a strong family history of renal cell carcinoma and because of cutaneous findings characteristic of HLRCC. The patient's past medical history was significant

for presence of multiple cutaneous leiomyomas on arms and trunk, as well as uterine fibroids requiring myomectomy and partial hysterectomy at an early age. She underwent an open right radical nephrectomy of a 10 cm renal tumor at an outside institution. The renal pathology revealed a renal cell carcinoma with tubulo-papillary and clear cell histology. The surgical margins were free of tumor.

Approximately 14 months after initial surgery, the patient was found to have a tumor recurrence in the retroperitoneum and retroperitoneal lymphadenopathy suspicious for metastatic disease. Metastatic work up revealed disease localized to the retroperitoneum. The patient underwent exploratory laparotomy and retroperitoneal lymph node dissection. Pathologic evaluation revealed the presence of the tumor in the right adrenal gland as well as in four retrocaval nodes. Given the identification of cutaneous leiomyomas, the early onset of uterine fibroids, prior renal carcinoma, and a strong family history of kidney cancer, the patient underwent germline genetic testing to confirm the diagnosis of HLRCC. She was found to have a germline mutation in the fumarate hydratase (*FH*) gene.[6] The patient was found subsequently to have disease progression in the mediastinum as well as new lesions on the calvarium. Despite an initial response to systemic chemotherapy, the disease progressed further and the patient died approximately twenty-one months after her original renal surgery.

2.2. Establishment of the UOK 262 immortalized cell line

The UOK 262 cell line was established from tumor tissue following tissue and cell culture protocols and techniques of the Urologic Oncology Branch as previously described with modifications.[17] The first passage was carried out two to three days after plating by a light treatment with 0.05% trypsin-EDTA, while monitoring the cells under an inverted tissue culture microscope. Subsequent passages (over 20) were carried out every 2-3 days by splitting 1 to 2 in the same manner.

2.3. Tumor xenografts in Nu/Nu nude mice

Animal usage was in accordance with the guidelines of the National Cancer Institute. Female athymic nude mice (Taconic, NY) were received at 4-5 weeks of age and kept in our animal facility for 2 weeks prior to injection with tumor cells. Approximately 5×10^6 UOK262 cells in 0.2ml (50:50 1× HBSS/Matrigel) were injected subcutaneously into athymic nude mice (in the right flank) to determine the tumorigenic potential as previously described [17]. Xenograft tumor specimens were re-implanted into additional mice, grown, harvested, and this process was repeated several times.

2.4. Glucose dependence of cell growth *in vitro*

Growth rates were compared under conditions of low (0.5g/L and 2.5g/L) and high (5g/L and 10g/L) glucose in DMEM (Invitrogen). Equal numbers of cells were plated into 6-well plates at ~30% initial confluence and were allowed to grow for 72 hours. Cells in particular wells were counted in triplicate at 24 hour intervals using a Cellometer™ Auto T4 image-based cell counter (Nexcelom Bioscience, MA).

2.5. Measurements of extracellular acidification and oxygen consumption rate

The XF24 Extracellular Flux Analyzer (Seahorse Bioscience) was used to detect rapid, real time changes in cellular respiration and glycolysis rate. UOK 262 and control cells (786-O and HK-2, ATCC) were cultured in custom XF24 microplates. Analysis of extracellular acidification rate (ECAR) reflects lactate excretion and serves as an indirect measure of glycolysis rate, while O₂ consumption (OCR) reflects cellular respiration and is directly determined [20]. All measurements were performed following manufacturer's instructions and protocols, and the observed rates are reported in pMol/min for OCR and mpH/min for ECAR.

2.6. Cell invasion assay

In vitro invasiveness of UOK262 cells was evaluated using the RT-CIM™ system (ACEA Biosciences), and compared to VHL-deficient 786-O clear cell renal carcinoma cells and HRCE normal renal epithelial cells (LONZA, Cat# CC-2654). The upper and lower culture compartments of the device are separated by a polyester membrane of 8 µm pore size coated with fibronectin (10 µg/ml). The membrane itself contains microelectronic sensor arrays that register an impedance signal as cells pass through from upper to lower chamber, which serves as a reservoir for media. Prior to assay, cells were cultured overnight without serum. The wells were filled with serum-free DMEM in the top chamber and with DMEM containing 10% FBS in the bottom chamber. Both chambers were incubated for 30 min at 37°C. Then 4×10^4 cells of each cell line were added per well to the top chamber (serum free medium). All wells were in triplicate and invasion was monitored in real time for five days in a humidified incubator at 37°C and 5 % CO₂. Using software supplied by the manufacturer, we derived a dimensionless parameter, the Cell Index, to quantify the invasiveness of each cell line, and this value was plotted against incubation time.

2.7. Spectral Karyotyping (SKY), gene specific FISH analysis and DNA sequencing

The metaphase chromosomes were prepared from passage 16 of UOK 262 cells. Three bacterial artificial chromosome-based human genomic DNAs (name from 5'end: RP11-409K12(70Kb); RP11-509A24 (4Kb); RP11-527D7(157Kb) were used as gene specific probes for FISH analysis. The detailed methods for SKY and FISH analyses were previously described [21, 22]. The karyotype for UOK262 (see SKY analysis) has been entered into the NCBI SKY/CGH interactive online database (<http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi>). Public access takes effect upon publication of the present article. DNA sequencing was performed as previously described [17].

2.8. Cellular and ultrastructural imaging of mitochondria

Both UOK 262 cells and control human renal cortical epithelial (HRCE) cells were harvested in triplicate at passage 34. Cell pellet fixation and thin sectioning for ultrastructural studies was carried out as described previously [17] with minor modifications. The cell pellet was fixed in 2.5% PBS-buffered glutaraldehyde, post-fixed in 1.0% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated in a series of ethanol, and infiltrated with Epon-Aradite (Ted Pella, CA) for two days. Samples were polymerized at 60°C for 2 days. Ultrathin sections (approximately 100 nm) were cut using a Leica EM UC6 Microtome (Bannockburn, IL) and collected on film-supported slot grids. Sections were slightly counter-stained with uranyl acetate and lead citrate, and examined with a Philips CM120 transmission electron microscope (equipped with a Gatan GIF100 camera) operating at a beam energy of 80Kv. Images were acquired by using a Gatan high resolution CCD camera.

2.9. Antibodies, immunohistochemistry and immunoblotting

UOK262 cells (passage 16) were seeded in Lab-TEK II chamber slides (Nalge Nunc International), fixed in 3.7% formaldehyde in PBS, and permeabilized in 0.2% Triton- $\times$ 100. Cells were then washed three times in PBS and incubated for 1 hour at room temperature with either rabbit anti-human GLUT-1 (1:500 dilution; DAKO) or mouse anti human FH (1:200 dilution; #ab58232, Abcam). Antibodies used for mitochondria analysis include rabbit anti-human SOD2 (#ab13533, Abcam). Following three washes in PBS, cells were incubated with either goat-anti rabbit or goat-anti mouse IgG-Alexa fluor 488/Alexa fluoro594 (1:500 dilution, Invitrogen) for 30 min at room temperature, washed in PBS, and briefly counterstained with DAPI. After mounting, cells were observed under a Leica DMRxA fluorescent microscope and images were obtained with IPLab v.3.7 software. Antibodies used for Western blotting include β -actin (Sigma, # A2522) and FH (Abcam, #ab58232).

2.10. Gene expression analysis by quantitative real time (qRT) PCR

Gene expression was analyzed by real time RT-PCR for *FH* and four other genes: *GLUT-1*, *LDH 5*, Succinate dehydrogenase subunit C (*SDH C*), peroxisome proliferative activated receptor gamma, and coactivator 1 alpha (*PGC-1α*). In brief, total RNA was extracted from 1.0×10^6 UOK262 cells using Trizol[®] (Invitrogen). Twenty nanogram of total RNA was reverse-transcribed for cDNA synthesis using random hexamers in a final volume of 20 ul, and 1 ul of the resulting cDNA was used for PCR amplification in an ABI 7000 real-time PCR system (Applied Biosystems) as recommended by the manufacturer. Primers and fluorogenic probes were designed by Applied Biosystems in the form of Taqman[®] Gene Expression Assays [assay# Hs00197884_ml (*GLUT-1*), Hs00855332_g1 (*LDH5*), Hs00818427_ml (*SDH C*) and Hs00173304_ml (*PGC-1α*)]. mRNA expression for these genes in UOK 262 was normalized to a housekeeping gene (Cyclophilin A) and CT values were further normalized to those obtained from a normal HRCE cell line. Samples were run in duplicate and CT values obtained were compared by the DeltaCT method. Results are expressed as fold-change to that of HRCE values from two independent experiments.

2.11. Measurement of FH enzyme activity

Whole cell protein quantification in the extract of UOK 262 and control cells was undertaken by the bicinchoninic acid (BCA) colorimetric assay as previously described [23]. *In vitro* FH enzyme activity was measured by NADP-malic enzyme coupled assay as previously described [24]. In brief, the increase in absorbance at 340nm from NADPH formation was measured after adding fumarate (Sigma) at final concentration of 10 mM into a total volume of 1 ml reaction mixture of cell extract at 30°C for 10 min. All spectrophotometric measurements were conducted using a Beckman DU-530 spectrophotometer.

3. Results

3.1. Glucose uptake by PET imaging in vivo and glucose dependence of cultured tumor cells *in vitro*

As described above, upon completion of the metastatic work up, the disease from the patient appeared to be localized to the retroperitoneum and was best viewed by abdominal and pelvic computed tomography (CT) as well as by positron emission tomography (PET). Anatomic imaging of the tumor mass is detected by CT (Fig.1A). Figure 1B shows PET imaging with fluorodeoxyglucose (FDG) to identify a regional lymph node metastasis based on enhanced glucose uptake.

In order to determine if UOK 262 cells displayed increased glucose dependence *in vitro*, we monitored their growth in media containing concentrations of glucose varying from 0.5g/L to 10g/L (Fig. 1C). In contrast to the clear cell RCC cell line 786-O, UOK 262 cells required a glucose concentration of at least 2.5 g/L for survival and of at least 5 g/L for optimal growth.

3.2. Invasiveness of UOK 262 cells

We observed that UOK 262 cells in culture exhibited an atypical branching morphology, and histopathologic features notably similar to HLRCC, e.g., large nuclei with prominent nucleoli and atypical perinucleolar clearing (white arrows, Fig. 1D). We next compared the *in vitro* invasive potential of UOK 262 with that of HRCE and 786-O cells. Figure 1E displays the real-time quantitative assessment of invasion for each cell line, and shows that UOK 262 is more invasive than 786-O, while untransformed HRCE cells are not invasive.

3.3. UOK 262 retains HLRCC histopathologic features

Histopathologic analysis of subcutaneous xenograft tumors (Fig. 2A) derived from UOK 262 revealed that UOK 262 retained the characteristic features of retroperitoneal nodal HLRCC tumor (Fig. 2B) that it was derived from; both are characterized by large eosinophilic nuclei/nucleoli and atypical perinucleolar clearing [8]. These characteristics were noted in the patient's primary kidney tumor as well as in a retroperitoneal lymph node metastasis (image not shown).

3.4. Cytogenetics and identification of isochromosome i(1)(q10) in UOK 262 cells

Three different DNA probes that encompass the entire *FH* gene genomic region were used to determine gene copy number by FISH. SKY was also performed to detect alterations at the chromosome level. The results are shown in Figures 2C (FISH) and 2D (SKY). FISH analysis shows that all three *FH* genomic DNA fragments from the bacterial artificial chromosome-based human genomic DNA (from 5' end of the *FH* genomic region: RP11-409K12 (~70Kb); RP11-509A24 (~4Kb); and RP11-527D7 (~157Kb) are present in both the normal chromosome 1 and in an isochromosome 1 [i(1)(q10)], at band 1q42.3 (in UOK 262 at passage 34). Isochromosome i(1)(q10) in UOK 262 consists of two long arms of chromosome 1 fused together at the centromere region. We have repeated the FISH analysis for cells at both early and late passage (p6 and p63, respectively) and confirmed that UOK 262 cells contain isochromosome 1q i(1)(q10) as a clonal abnormality regardless of passage number.

Ten metaphase spreads were karyotyped by SKY and each spread revealed multiple clonal, as well as unique abnormalities, and also multiple numerical and structural aberrations (indicative of genomic instability). The clonal aberrations are shown in Fig. 3D. Composite karyotype: 47, X, -X, +1, i(1)(q10), +5, der(21)t(15;21)(q15;p11.2), +22. The karyotype for UOK262 has been entered into the NCBI SKY/CGH interactive online database (<http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi>) [21;22]. Public access takes effect upon publication of the present article.

3.5. UOK 262 cells harbor an FH germline mutation at exon 8

The patient possessed an *FH* germline mutation at nucleotide 1187 in exon 8, resulting in a Gln396Pro amino acid change, which was determined as previously described. [13] In order to determine whether UOK 262 cells contain the identical *FH* germline mutation, we sequenced genomic DNA from early and late passages of the cell line. The DNA sequencing chromatograms are shown in Figure 3A. As expected, we verified the same nucleotide change as the patient's germline missense mutation, which is an A to C change, in early and late passage UOK 262. We detected only the germline mutation sequence in the tumor cells, confirming a loss of heterozygosity of the wild type allele at this nucleotide position. An amplified, single mutant allele is retained in UOK 262 (Fig 2C shows 4 copies of chromosome 1q in passage 34 cells).

3.6. Mitochondrial morphology, subcellular localization of mutant FH protein, and FH enzymatic activity in UOK 262

Ultrastructural examination of UOK 262 cells showed a larger number of mitochondria in each cell than was seen in normal renal cell epithelium (HRCE). Evaluation of UOK 262 mitochondria revealed edema, and various degrees of disintegration and disruption of the internal membrane cristae (Fig. 3B).

Recent reports [25;26] have shown that, in most human cells and tissues, FH protein is mainly or exclusively localized to the mitochondria. As we have shown that UOK 262 cells contain only a mutated version of the *FH* gene, we asked whether the predicted full length mutant protein is present and localized in the mitochondria. Immunofluorescence was used to detect

both FH and superoxide dismutase 2 (SOD2) (the mitochondrial form of the SOD protein family). The *in situ* distribution of both FH and SOD2 in UOK 262 is shown in Figure 4A. The upper panel depicts FH (labeled with Alexa Fluor® 488), and the middle panel depicts SOD2 expression (labeled with Alexa Fluor® 594), which is specifically localized in the mitochondrial matrix. The lower panel shows a merged image of both FH and SOD2 staining patterns. The mutant FH protein in UOK 262 is primarily co-localized with SOD2, confirming its presence in the mitochondrial matrix.

We further evaluated FH gene expression by real time PCR and Western blot analysis, and we compared FH expression in UOK 262 cells to its expression in both 786-O and HK-2 renal cells. Although FH expression appears somewhat decreased in UOK 262 compared to the other cell lines (Fig. 4B, top and middle panels), Western blotting confirms that the expressed FH protein is full-length, as predicted. Importantly, the enzymatic activity of FH protein in UOK 262 cells is not detectable, and is at least 60-fold lower than in either 786-O or HK-2 cells (Fig. 4B, bottom panel).

3.7. UOK 262 cells over-express genes and proteins involved in glycolysis

We initially examined whether glycolytic activity may be elevated in UOK 262 cells by immunocytochemistry. UOK 262 cells were strongly positive for GLUT-1 and LDH5 expression (Fig. 5A and 5B). We validated these data by determining mRNA expression using real time PCR. We examined *GLUT-1* and *LDH5* expression, as well as expression of other mitochondrial enzymes/proteins including the Krebs cycle enzyme *SDH C* and *PGC-1 α* , the master regulator of mitochondrial gene expression. When compared to normal renal epithelium, we detected a 2-fold upregulation of *PGC-1 α* , > 3-fold upregulation of *SDH C*, and, consistent with our immunocytochemical data, an approximately 15-fold upregulation of *LDH5* and a >100-fold upregulation of *GLUT-1* (Fig. 5C).

3.8. Significant attenuation of mitochondrial respiration in UOK 262 cells concomitant with increased glycolytic flux

We next explored the relative contributions of mitochondrial respiration, as measured by oxygen consumption rates (OCR), and glycolysis, as measured by extracellular acidification rate (ECAR), to energy production in UOK 262 cells. For comparison, we obtained similar data for 786-O and HK-2 cells. The basal OCR for UOK 262 cells was 21.98 ± 0.7 pmol/ 5×10^4 cells/min; for HK-2 cells it was 89.46 ± 0.81 pmol/ 5×10^4 cells/min; and for 786-O cells it was 195.98 ± 1.89 pmol/ 5×10^4 cells/min (Fig. 5D). Based on instrument sensitivity, an OCR ≤ 20 pmol/min is indistinguishable from signal noise due to low levels of non-mitochondrial oxygen utilization (mitochondrial respiration accounts for ~ 90% of cellular oxygen consumption, while cellular protein oxidation accounts for the remaining 10%). [27] These data thus confirm that mitochondrial respiration is near zero in UOK 262 cells. By measuring ECAR in parallel with oxygen consumption, we found that glycolytic flux is markedly elevated in UOK 262 compared to the other cell lines (Fig 5E), consistent with upregulation of *LDH5*, and with our recent study showing that these cells are obligate fermenters and must rely fully on glycolysis for energy production. [28]

4. Discussion

Nearly a century ago Otto Warburg made the remarkable observation that cancer cells prefer to utilize glycolysis to produce ATP, even in the presence of normal levels of oxygen (the Warburg effect), and he proposed that abnormalities in energy metabolism were a fundamental aspect of cancer. [15,29-31] Hereditary leiomyomatosis renal cell carcinoma (HLRCC) is a novel form of inherited kidney cancer in which affected individuals are at risk for the development of cutaneous and uterine leiomyomas and kidney cancer. [1-3] HLRCC is

characterized by germline, inactivating mutation of the Krebs cycle enzyme, fumarate hydratase [4], and is unique among kidney cancers in its remarkable propensity to grow quickly and metastasize early. [6] Detection of fumarate hydratase mutation in a high percentage of HLRCC families [4,13,32-33] has enabled early identification of disease in at risk individuals, allowing for initiation of therapy when tumors are still small and potentially curable. However, in spite of aggressive monitoring, patients remain at risk for the early development of advanced disease. The dependence of HLRCC tumors on glycolysis, coupled with their impaired mitochondrial respiration, mark this hereditary renal cancer as a unique example of Warburg's hypothesis. [15] The data in this report suggest that strategies to interfere with glycolytic flux may represent a targeted approach to therapy for HLRCC.

Xie, et al. developed a novel A549 surrogate FH deficient cell line which had increased lactate levels, enhanced HIF1 α levels and increased expression of GLUT1 and vascular endothelial growth factor (VEGF). Furthermore, Xie et al. showed that LDH-A inhibition in the A549 FH deficient cells increased apoptosis via ROS production and increased oxygen consumption. [34]

In the present report we describe and characterize a human cell line, UOK 262, that was derived from a patient with HLRCC-associated kidney cancer. We recently showed glucose-mediated generation of cellular reactive oxygen species (ROS) and ROS dependent HIF1 α in UOK 262. [28] In the current study, we show that this cell line, which harbors an inactivating germline *FH* mutation and displays loss of heterozygosity, can grow as a xenograft in nude mice and exhibits additional notable characteristics: (a) autonomous growth that requires elevated extracellular glucose levels; (b) impaired oxidative phosphorylation; (c) a highly invasive phenotype; (d) pronounced up-regulation of genes involved in glucose uptake and metabolism; (e) and retention of the morphologic characteristics of HLRCC kidney cancer. UOK 262 is thus a useful cell model for studying the underlying molecular derangements associated with impaired oxidative phosphorylation in cancer and for evaluating novel therapeutic approaches for this disease.

The majority of fatalities from HLRCC are caused by tumor invasion and metastasis. In the patient in this report, FDG PET scanning clearly imaged the metastatic, recurrent tumor. This is an example of "functional imaging"; i.e., a tumor with constitutive impairment of mitochondrial respiration that is dependent on glucose transport and glycolysis for energy production is clearly imaged by FDG-PET imaging. Over-expression of GLUT-1, visualized by RT-PCR and immunohistochemistry, as well as the notable dependence of UOK 262 on high glucose for survival/proliferation, indicates that the in-vitro model is phenotypically similar to HLRCC tumor tissue. [9,34] Whether the strong expression of glycolytic enzymes and enhanced glycolytic flux in UOK 262 is a direct consequence of loss of FH activity and resultant impairment of mitochondrial respiration is yet to be determined, but we and others have previously shown that molecular knockdown/knockout of FH in other cell lines leads to rapid up-regulation of GLUT-1 expression, glucose uptake and glycolysis. [9,34] Further, our recent study has shown that these events are reversible upon reintroduction of functional FH, suggesting that lack of FH activity is sufficient to establish the phenotypic behavior of UOK 262 cells. [28]

Isochromosome 1q frequently occurs in Wilms tumor and sarcomatoid tumors [35,36]. As we showed in our sequencing results in UOK 262 cells, only the germline mutant allele was retained, despite having four copies contained within the intact long arm of chromosome 1 (both homologues), as well as in the isochromosome [i(1)(q10)], that has two long arms of 1 fused at their centromeres. Isochromosome 1q may prove a marker for HLRCC. Zhang *et al.* [37] reported that chromosome-specific marker analysis from hereditary nonpolyposis colorectal cancer (HNPCC) indicated that loss of the wild-type allele predominantly occurs

through locus-restricted recombinational events, i.e., gene conversion, rather than mitotic recombination or deletion of the respective gene locus. Of interest, if the extra copy number of chromosome arm 1q [including i(1)(q10)] containing the *FH* mutant allele is being selected for, it may have a role in UOK 262 tumorigenesis and *in vitro* immortality. Alternatively, the absolute dependence of UOK 262 on glycolysis may itself determine the tumorigenicity and metastatic potential of these cells.

The morphology of mitochondria in UOK 262 cells suggests that mitochondrial function may be impaired and that the mitochondrial membrane/matrix may be disrupted. In UOK 262, mutant *FH* protein was targeted to mitochondria, and it was strongly associated with the mitochondrial matrix. Whether the mitochondrial localization of enzymatically inactive *FH* plays a role in the mitochondrial swelling and cristae disruption seen in UOK 262 is currently being investigated.

In summary, the fumarate hydratase $\pm$ UOK 262 kidney cancer cell line represents a genetically defined example of the Warburg phenomenon in a human cancer. It provides both an opportunity to study the molecular pathways that are deregulated in cancer when mitochondrial respiration is compromised as well as a platform to test novel therapeutic strategies that target energy deregulation in human cancer.

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Fig.1.

(A) Abdominal CT scan reveals a recurrent, right retroperitoneal tumor mass. (B) FDG PET imaging of HLRCC patient reveals strong glucose uptake in the lymph node tumor metastasis. (C) The *in vitro* growth of UOK 262 in 0.5g/L, 2.5g/L, 5g/L, and 10g/L D-glucose. The cells' proliferation and survival were glucose dependent. (D) The morphology of UOK 262 cells in culture, showing active branching and the unique HLRCC pathological features: large nuclei with predominant nucleoli and atypical perinucleolar clearing (white arrows; 40× magnification). (E) Real-time assay of the invasive potential of UOK 262 cells.

Fig. 2.

(A) Hematoxylin-eosin (H&E) staining of tumor tissue from xenograft derived from UOK 262 cells (3×10^6); (B) H&E staining of tissue from metastatic HLRCC kidney cancer removed surgically from the patients retroperitoneum; (C) Gene-specific FISH and (D) SKY analysis of UOK 262 cells: *FH* gene genomic probe RP11-527D7 (157 Kb) is present in both normal copies of chromosome 1 and in an i(1)(q10), band 1q42.3. Karyotyping by SKY revealed clonal and also multiple numerical and structural aberrations. Composite karyotype: 47, X, -X, +1, i(1)(q10), +5, der(21)t(15;21)(q15;p11.2), +22

Fig. 3. (A) DNA chromatograms of fumarate hydratase (*FH*) gene sequence surrounding nucleotide 1187. DNA was prepared from Wild type control sequence (top panel), early passage 6 UOK 262 (middle panel), and late passages 49 (lower panel) UOK 262. (B) Transmission electron microscopy images of mitochondria from UOK262 cells (top panels) and from normal human renal cortical epithelial (HRCE) cells (bottom panels). Mitochondria of UOK 262 tumor cells are edematous and show disruption of internal membrane cristae. Magnification indicated as a scale bar on each image.

Fig. 4.

(A) Double immunofluorescence labeling for protein expression *in situ*. Mutant FH is primarily localized to mitochondria of UOK 262 cells. Alexa-Fluor®- 488 labeling with mouse monoclonal antibody to human FH (top panel); Alexa Fluor®- 594 labeling with rabbit antibody to human superoxide dismutase 2 (SOD2) which is a marker of the mitochondria matrix (middle panel); overlap of signals obtained with both FH and SOD2 antibodies showing co-localization FH and SOD2 (lower panel). (B) RT-PCR (top panel) and Western blot (middle panel) showing that both mutant *FH* mRNA and protein are present in UOK 262 cells. FH enzymatic activity in UOK 262 cells (bottom panel) is undetectable.

Fig. 5.

Immunohistochemistry, real time PCR analysis, and real time measurement of mitochondrial respiration rate and lactate efflux (glycolysis rate) in UOK 262 cells: (A) GLUT-1 protein is strongly expressed at branching tumor cell membrane; (B) LDH 5 is strongly expressed in a murine xenograft of UOK 262 cells; (C) Comparison of gene expression in UOK262 cells (green columns) with HRCE normal renal epithelial cells (black columns). (D) Basal cellular respiration rate (Oxygen consumption rate, OCR) and (E) Basal extracellular acidification rate (ECAR) of UOK 262 and control cells. Results from (D) and (E) were expressed as means $\pm$ SD; n=5. Both OCR (pMoles/min) and ECAR (mpH/min) were normalized against cell number and expressed as rate per 5×10^4 cells.