

REVIEW

Warburg effect and renal cancer caused by errors in fumarate hydratase encoding gene

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Abstract: The several types of heterogeneous kidney cancers are interrelated by their primary sites of pathology. Despite its origin in the kidney, renal cell carcinoma (RCC) is associated with its varying genetic basis. Von Hippel-Lindau (VHL) syndrome is the earliest and, thus the most highly, characterized of genetic forms of kidney cancer, which is associated with alterations in the Von Hippel-Lindau (VHL) gene. As a result of his studies and investigations, Otto Warburg reached the conclusion that cancer's fundamental cause is altered mechanism. But this theory was disdained because of the discovery of tumor suppressor genes and oncogenes. Lately, the breakthrough finding about the tumor suppressing role of gene coding for enzymes involved in Krebs cycle has revived the interest in Warburg's hypothesis. This effect has led to the uncovering of the links between metabolic alterations, mitochondrial dysfunction and cancer. One such metastatic cancer characterized by the germ-line inactivating mutation of the gene coding for fumarate hydratase (FH), a Krebs cycle's enzyme, is hereditary leiomyomatosis and renal cell carcinoma (HLRCC). In this review paper, we have discussed the background of this carcinoma, the metabolic dysfunction causing it and its therapeutic solutions.

Keywords: Warburg effect, fumarate hydratase, oncometabolite, hereditary leiomyomatosis and renal cell cancer.

INTRODUCTION

In early 20th century, Otto Warburg discovered a particular metabolic phenotype in which cancer cells had high rates of glycolysis despite oxygen's presence, a condition he named as "aerobic glycolysis" (Warburg, 1956). He found these metabolic alterations to be associated with defects in mitochondrial respiration, particularly production of ATP (during the process of oxidative phosphorylation). This phenomenon is now proclaimed to be among one of the metabolic defects causing cancer formation and has been extensively studied for the development of novel therapeutic tools. However, this theory was disdained because of the identification and characterization of the mutated cancer predisposition genes including the tumor suppressor (anti-tumorigenic) genes and oncogenes (pro-tumorigenic). It was also thought that mitochondrial dysfunction is the key reason behind tumorigenesis (Frezza and Gottlieb, 2009). The last decade, however, vindicated Warburg's findings by the discovery of mutations in principal enzymes involved in metabolism which play a perfunctory part in tumor development (Warburg *et al.*, 1927; Kaelin, 2009). One such Krebs cycle's enzyme is fumarate hydratase (FH) which catalyzes the reversible conversion of fumarate to malate. It also plays a role as a tumor suppressor as the mutation in this enzyme is linked with the development of renal cysts, tumors and leiomyomata.

Due to the lack of conversion into malate caused by loss in fumarate hydratase's activity, fumarate gets accumulated inside the cell. This accumulated fumarate acts as 2-oxyglutarate-dependent oxygenases' competitive inhibitor, one of which is hypoxia-inducing factor (HIF) hydroxylases. This consequently activates the oncogenic HIF pathways (Yang *et al.*, 2012).

The evidence that metabolic enzymes play role in tumorigenesis

A number of evidences show that energy is produced by cancer cells via a heightened rate of glycolysis in cytoplasm. This is markedly contrasting to the relatively low glycolysis rate that is followed by pyruvate oxidation (Kim and Dang, 2006). Genes of well-characterized metabolic functions have been studied for cancer-associated mutations which include those encoding succinate dehydrogenase (SDH), isocitrate dehydrogenase (IDH1 and 2) and fumarate hydratase (FH; Semenza, 2011). The role of fumarate hydratase is of paramount importance in the Krebs cycle owing to its ability to prevent accumulation of fumarate. Germ-line mutations in fumarate hydratase are loss-of-function in nature which lead to renal carcinoma (collecting duct and type II papillary), inherited leiomyomas (benign tumors of smooth muscle), and hereditary leiomyomatosis and renal cell cancer (HLRCC). There is no activity of fumarate hydratase in HLRCC. Furthermore, the loss of allele's wild-type is the major characteristic in most of the tumors (Tomlinson *et al.*, 2002). Mutations in FH-encoding gene

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have also been reported to be involved in bladder, breast and testicular (Leydig cell) cancers (Sass *et al.*, 2001; Lehtonen *et al.*, 2006).

Fumarate hydratase (FH) and its inactivation

Fumarate hydratase, also known as fumarase, is a homotetrameric enzyme (Wei *et al.*, 2006) which is highly conserved, and is located and functions in both the cytosol and the mitochondria of the cell. It functions in the mitochondria as a part of the Krebs cycle by catalyzing the hydration of fumarate and subsequent conversion to malate (fig. 1). This step is essential in producing cellular energy and generates several important molecular precursors. It is thought to contribute in cytosolic pathways which produce fumarate, including the purine nucleotide cycle and the urea cycle. Both forms of fumarate hydratase are encoded by the same gene and the difference in localization is due to the cleavage of the produced polypeptide into smaller fragments (Brosnan and Brosnan, 2004).

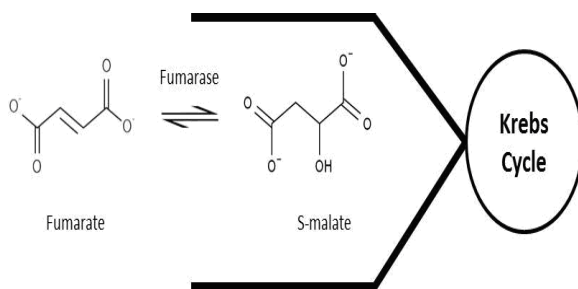


Fig. 1: The step catalyzed by fumarate hydratase (fumarase) in the mitochondrial Krebs cycle

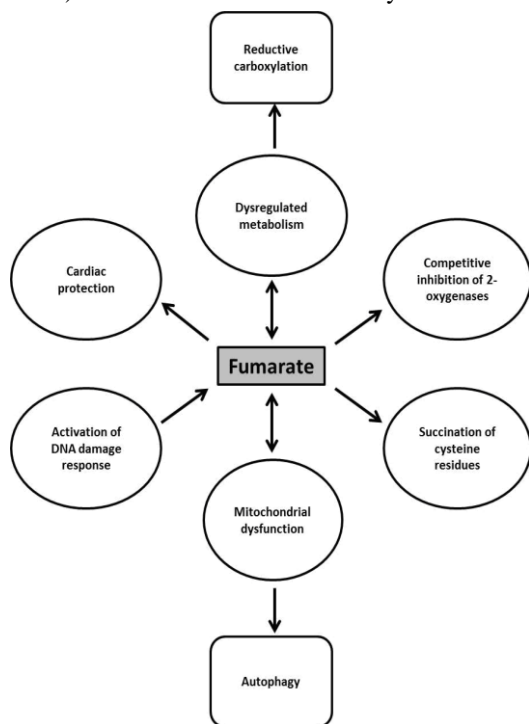


Fig. 2: Consequences of increased levels of cellular fumarate.

Presently it is not clear whether Krebs cycle's dysfunction contributes to tumorigenesis or it is the fumarate accumulation which contributes. However, FH deficiency has been found to be linked with mitochondrial dysfunction by generating a knockout mouse model lacking fumarate hydratase 1 (*Fh1*) (Pollard *et al.*, 2007; Adam *et al.*, 2011). It has been posited that dysfunction and disruption of the Krebs cycle results in changes in mitochondrial membranes' potential by causing an increase in the permeability of the organelle's outer membrane and subsequent autophagy. Cells and tissues deficient of fumarate hydratase accumulate very high levels of fumarate which result in multiple consequences one of which is oncogenic pathways' activation (Isaacs *et al.*, 2005). The subsequent mechanisms of fumarate accumulation have been described in fig. 2 in detail.

It is essential to determine the relative amounts of fumarate under various physiological conditions in distinct cellular compartments. In *Fh1* deficient mouse embryonic cell lines (MEFs), the level of fumarate was measured by using ¹H magnetic resonance spectroscopy metabolite analysis and was found to be approximately 8-10 fmol/cell (O'Flaherty *et al.*, 2010; Adam *et al.*, 2011). However, immunofluorescence studies on the MEFs have shown that an evident and striking change occurs in mitochondrial morphology (they become much enlarged) because of *Fh1* loss. The aforementioned discussion on the activation of oncogenic pathways includes the one driven by 2OG-dependent oxygenases. This has been discussed in detail in the next spread.

Fumarate as 2-oxoglutarate-dependent oxygenases' competitive inhibitor

A small molecular component of normal metabolism the accumulation of which results in metabolic dysfunction/dysregulation and, consequently, allows a progression towards the development of cancer is referred to as an oncometabolite. Several complex steps might be included during this course, including activation, disruption, or inhibition of the pathways. Studies on the oncometabolites provide an opportunity of a detailed study and devising therapeutic routes for a variety of cancers. Here fumarate has been discussed as an oncometabolite in FH-deficient cells (Yang *et al.*, 2012).

Genetic and histological characterization of FH mutations have suggested a casual association between dysfunctions of the Krebs cycle and activation of hypoxia inducible transcription factors (HIFs), which are chief regulators in giving response to low levels of oxygen. FH-linked tumorigenesis is thought to be caused by the increased levels of several oncogenic pathways by HIF (Gottlieb and Tomlinson, 2005). HIF activation promotes tumorigenic process by inducing metabolic alterations observed in these tumors. In fact, HIF has been reported to plan and control the genetic and metabolic reprogramming required for the sustenance of tumor cell

growth, vascularization and proliferation (Ivan *et al.*, 2001; Ashrafian *et al.*, 2010). Selak *et al.* first proposed the link between Krebs cycle dysfunction and activation of HIF. It has also been proven that fumarate causes 2-oxoglutarate-dependent oxygenases' competitive inhibition, in particular the HIF PHDs (prolyl hydroxylases). Fumarate therefore mimics hypoxia (in a phenomenon called pseudohypoxia), causes the stabilization of the HIF complex and activates its oncogenic target genes (Isaacs *et al.*, 2005; Hewiston *et al.*, 2007).

The aforementioned phenomenon can be explained by studying HIF's dysregulation caused by the VHL (von Hippel-Lindau) pathway. VHL is a tumor suppressor gene which is involved in renal cell carcinoma's development. Its protein product, hence called pVHL, is a part of the signaling complex which regulates oxygen-dependent gene expression of the cell. Intact VHL complex has ubiquitin ligase activity which aims at specific proteins for their degradation (Pause *et al.*, 1997). Hypoxia inducible factor 1 α and 2 α (HIF-1 α and HIF-2 α , respectively) are two examples of such targeted proteins which are also oxygen homeostasis' key regulators (Kaelin, 2005). Genes which HIFs target include erythropoietin (EPO), transforming growth factor- α (TGF- α), platelet-derived growth factor (PDGF), glucose transporter 1 (Glut 1), and vascular endothelial growth factor (VEGF). In order for the VHL complex to recognize HIF, the latter's conserved proline residues must be hydroxylated. This hydroxylation is conducted by HIF prolyl hydroxylases (also known as HIF PHDs or HPHs). Ascorbate and iron ions are a pre-requisite as co-factors for the functioning of PHDs, and molecular oxygen along with 2-oxoglutarate (2-OG) are needed as co-substrates. During hypoxia, there is a limited amount of molecular oxygen because of which HIF remains unhydroxylated. In this state VHL complex does not cause the ubiquitination of HIF which is then consequently available for the activation of the aforementioned genes' transcription (Zhang *et al.*, 2006).

When VHL is present in its mutated form, HIF escapes degradation and, therefore, creates a pseudo hypoxic condition. Nonetheless, pVHL's tumor suppressor function is extenuated in xenograft mouse models; reintroduction of pVHL belittles the formation of tumors. On the contrary, in the presence of HIF-1 α mutant, the tumor-suppressing ability of pVHL remains intact (Maranchie *et al.*, 2002). Recent evidences have shown the ability of fumarate and succinate to cause competitive inhibition of the activity of other members of the super family of enzymes 2-oxoglutarate-dependent oxygenase, inclusive of TET proteins and histone demethylase enzymes (HDMs), both of which are vital in gene expression's non-genetic regulation (Frizzell *et al.*, 2011; Xiao *et al.*, 2012).

HLRCC and the associated renal cancer

Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) is a syndrome characterized as an autosomal dominant inherited cancer which may cause the development of uterine and cutaneous leiomyomas in affected individuals (Launonen *et al.*, 2001). Cutaneous leiomyomas mostly occur in the *arrectores pilorum* muscles attached to the hair follicles. These are energy sensing organs as they contract and trap air when it is cold to provide insulation. There risen lesions are often painful and sensitive to cold or touch. The treatment is often symptomatic. The affected females are prone to the development of the early-onset multiple uterine fibroids (Wei *et al.*, 2006).

Patients of HLRCC are also at a risk for developing bilateral and/or multifocal, early-onset renal cysts and papillary renal tumors (it is reported in 60% of the cases) (Schmidt *et al.*, 1997). Papillary renal cancer can be characterized as type I or type II, the former one following an indolent pattern of growth and being associated with germ-line mutation in MET gene. Type II is a lethal disease associated with aggressively rapid growth and early metastasis (Schmidt *et al.*, 1997). This cancer normally develops in young individuals because of which a life-long abdominal imaging is required which has to start by the age of 10 years. However, other renal cancer syndromes, including von Hippel-Lindau (VHL), Birt-Hogg-Dubé syndrome and hereditary papillary renal cell carcinoma, may be treated with renal parenchyma-sparing surgical resection (Iliopoulos *et al.*, 1995; Grubb *et al.*, 2007).

Fumarate hydratase as the HLRCC gene and the Warburg effect

As discussed above, fumarate hydratase is an important Krebs cycle's enzyme the germ-line mutation of which characterizes HLRCC. In 90% cases of HLRCC, mutations in the gene encoding FH, including missense, frameshift and complete or partial deletions, are detected. FH, gene locus at chromosome 1q42.2, undergoes a 2-hit, loss-of-function leading to HLRCC tumors. The second hit is represented by the loss of the somatic allele found in uterine leiomyomas and HLRCC-associated renal tumors (Wei *et al.*, 2006).

As discovered by Otto Warburg, FH-deficient renal cancer goes through a metabolic shift to aerobic glycolysis. The cell line UOK262 is specified by faulted oxidative phosphorylation and increased rates of glycolysis, which is assessed by decreased rate of oxygen consumption and increased rate of extracellular acidification. All these glycolytic and tumorigenic features of the cell line can be reversed by restoring the FH activity. FH-deficient renal cancer cells are dependent uniformly on glycolysis and, therefore, glucose for the production of ATP required for rapid proliferation (Tong

et al., 2011). This is concluded on the basis that HLRCC-associated renal cancer shows continuously high fluorodeoxyglucose uptake as shown by positron emission tomography (PET) scan imaging (Lehtonen *et al.*, 2004).

FH-deficient renal cancer is also characterized by a metabolic shift to reductive carboxylation via glutamine-dependent pathway (Mullen *et al.*, 2012). ¹³C metabolite analysis was used to determine this. Even though this cancer witnesses a substantial decrease in the flow of the glucose-derived pyruvate in the mitochondria, it is still dependent on this metabolic flux for its critical biochemical reactions. Thus FH-deficient renal cancer cells require glutamine as the major carbon source for increased synthesis of fatty acids which is, in turn, required for rapid proliferation in this fastest-growing form of kidney cancer (Mullen *et al.*, 2011).

Disruption to metabolism caused by the loss of FH activity

The loss in FH's activity results in Krebs cycle dysfunction that further threatens the cellular activity in terms of macromolecular precursors' production, meeting the energy requirements, and ultimately its survival. Several mechanisms have been discovered and studied through which FH-deficient cells combat these problems. One such mechanism involves impaired respiration and increase in the rate of aerobic glycolysis which is presumed to be an adaptation in order to meet the energy requirements of the cell by of the Krebs cycle-independent production of ATP (Sudarshan *et al.*, 2009). Increased rate of glutaminolysis has also been studied by using stable isotope labelling of a murine renal cell deficient in *Fh1*. This vindicated the idea that the most important carbon source in the Krebs cycle is glutamine.

Glutamine-to-bilirubin pathway has been observed in FH-deficient cells; it involves the biosynthesis and degradation of heme. This results in partial production of NADH in mitochondria and, consequently, bilirubin is excreted from the FH-deficient cells. It was proven that FH-deficient cells cannot survive with the inhibition of the enzyme heme oxygenase. Hence its inhibition may result in specific death of tumor cells and sparing the non-FH-deficient cells in HLRCC-associated renal cancer (Frezza *et al.*, 2011). This is a stereotypical feature of transformed cells and may lead towards the malignancy of the cells deficient in FH.

A similar mechanism is glutamine-dependent reductive carboxylation which causes the Krebs cycle to partially reverse. Isoforms of IDH reductively carboxylate 2OG and generate isocitrate. This is then further metabolized to citrate, oxaloacetate and acetyl coenzyme A (AcCoA). The concentrations of these metabolites, initially decreased due to blockage of the Krebs cycle, are brought back to normal by oxaloacetate's reduction to malate

(Leonardi *et al.*, 2012; Lu *et al.*, 2012). Acetyl coenzyme A is essential for protein acetylation and fatty acid synthesis (Metallo *et al.*, 2012). However, these adaptations may contribute to oncogenic transformation owing to the changes in primary metabolism. Anaplerosis and the urea cycle may also be contributing in this phenomenon but they need to be studied extensively before deriving any conclusion.

Therapeutic strategies

As mentioned before, a shift to aerobic glycolysis takes place in FH-deficient renal cancer cells because of the latter being dependent on high level of adenosine triphosphate (ATP) production for rapid cell proliferation. Several different approaches have been tried for targeting glucose metabolism I such a Warburg cancer, one of the approaches being the targeting of crucial glycolytic enzymes such as lactate dehydrogenase A (LDHA), human pyruvate kinase M2 (PKM2) and mitochondrial hexokinase II (HKII) (Hamanaka *et al.*, 2012). Xie *et al.* studied the over expression of LDHA in HLRCC to rule out the option of FH-deficient cells being sensitive to LDHA blockage. In fact, LDHA blockade increased FH-deficient cells' apoptosis.

Another approach is to use englerin A, a sesquiterpene isolated from the stem and root bark of *Phyllanthus engleri*. It has been shown to have selective inhibition against renal cell carcinoma growth. It is the protein kinase C- θ to which englerin A binds, activates the former and limits the access to glucose thus inducing an insulin-resistant phenotype. At the same time, englerin A induces glucose dependence by inducing the activation of HSF1 transcription factor in protein kinase C- θ . Thus, by making the cells dependent on glucose and simultaneously starving the cells of glucose helps it to kill several types of kidney cancers, including FH-deficient renal cell carcinoma (Soubrier *et al.*, 2013). Another approach undergoing clinical trial is targeting FH-deficient kidney cancer Vasculature with anti-VEGF and Glut-1 reagents in patients with metastatic HLRCC-associated kidney cancer. The effect of bevacizumab and erlotinib are being particularly evaluated for this purpose (Yang *et al.*, 2012).

CONCLUSION

After the extensive studies to find genetic evidence of the role of metabolic enzymes in tumorigenesis, different approaches and techniques have led to a conclusion that germ-line mutations in the enzyme encoding FH are linked with HLRCC and other tumors. This has led to the deduction and vindication of contribution of accumulation of fumarate in the activation of oncogenic pathways because of its role as 2-oxoglutaate dependent oxygenases' competitive inhibitor. Subsequent studies led to the unraveling of the function of HIF and its escape

from pVHL during pseudo hypoxia. Thus fumarate hydratase is now characterized as not only a key enzyme of the Krebs cycle but as a tumor suppressor gene, the mutation in which may lead to the situation called Warburg effect. Several therapeutic strategies and approaches have been and are being devised in order to directly target the genes and, in some case, the inhibited metabolites. One may hope that these approaches may become the basis for the development of an effective therapy/treatment for patients suffering from Krebs cycle mutation cancer, and may provide an insight to other cancers which too involve a metabolic shift to aerobic glycolysis and faulted oxidative phosphorylation.

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