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Metabolic signatures of renal cell carcinoma

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ABSTRACT

Clear cell renal cell carcinoma (ccRCC) is characterized by the constitutive up-regulation of the hypoxia inducible factor-1. One of its target enzymes, pyruvate dehydrogenase (PDH) kinase 1 (PDHK1) showed increased protein expression in tumor as compared to patient-matched normal tissues. PDHK1 phosphorylated and inhibited PDH whose enzymatic activity was severely diminished, depriving the TCA cycle of acetylCoA. We and others have shown a decrease in the protein expressions of all respiratory complexes alluding to a compromise in oxidative phosphorylation (OXPHOS). On the contrary, we found that key parameters of OXPHOS, namely ATP biosynthesis and membrane potential were consistently measurable in mitochondria isolated from ccRCC tumor tissues. Interestingly, an endogenous mitochondrial membrane potential (MMP) was evident when ADP was added to mitochondria isolated from ccRCC but not in normal tissues. In addition, the MMP elicited in the presence of ADP by respiratory substrates namely malate/glutamate, succinate, α-ketoglutarate and isocitrate was invariably higher in ccRCC. Two additional hallmarks of ccRCC include a loss of uncoupling protein (UCP)-2 and an increase in UCP-3. Based on our data, we proposed that inhibition of UCP3 by ADP could contribute to the endogenous MMP observed in ccRCC and other cancer cells.

1. Introduction

Renal cell carcinoma (RCC) comprises a number of different types of cancer of which the conventional clear cell renal cell carcinoma (ccRCC) is the most aggressive, representing >80% of all RCC [1]. At least 8 oncogenes are implicated in kidney cancer [2]. Of these, the best known is von Hippel-Lindau (VHL) [3,4], the loss of which leads to stabilization of the hypoxia inducible factor 1 (HIF-1) due to a lack of its degradation by the ubiquitin proteasomal pathway. HIF-1 has been shown to elicit the Warburg effect of aerobic glycolysis [5] in many cancers including ccRCC [6]. Increased protein expression of the mitochondrial isoform, hexokinase II (HKII), the first committed enzyme in glycolysis has been reported [7]. Our study also showed that HIF-1 induces pyruvate dehydrogenase kinase 1 (PDHK1) to phosphorylate and inactivate pyruvate dehydrogenase (PDH) complex [8,9]. This would deprive the TCA cycle of acetyl-CoA with compromise in the generation of reducing equivalents and oxidative phosphorylation (OXPHOS). This study aimed to compare OXPHOS parameters such as ATP biosynthesis and membrane potential in mitochondria isolated from ccRCC and patient-matched normal tissue samples. As uncoupling proteins can de-link oxidation of respiratory substrates and phosphorylation of ADP, the possible contribution by UCP2 and UCP3 (but not UCP1 which is present mainly in adipose tissues) was examined. In contrast to our observation, the UCP2 isoform has been reported to be increased in many human cancers [10,11]. Likewise, UCP3 is increased in gastrointestinal adenocarcinoma and cancer cachexia [12,13]. When activated, all UCPs can mediate proton leak [14–16] which would reduce the mitochondrial membrane potential (MMP). On the contrary, tumor cells examined showed high MMP [17–19]. These observations led us to consider possible inhibition of UCP3 present in ccRCC by ADP which has been shown to affect this isoform more strongly than ATP [14].

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2. Materials and methods

2.1. Human kidney samples

Sixteen ccRCC tumor and patient-matched adjacent normal tissues were collected over a period of 2.5 years from the Department of Urology, National University of Singapore with approval from NHG (National Healthcare Group) Domain Specific Review Board (DSRB B/11/158) and prior consent from patients. Of these, data accrued from samples #1-10 and #16 are reported in this study. The particulars of patients and the classification of the tumors by histopathologists are shown in Supplementary Table 1. Isolation of intact mitochondria and mitochondrial extracts was carried out within 1–2 h after surgery by standard isolation procedure of differential centrifugation as described previously [20].

2.2. Western blot analyses and antibodies

Snap-frozen tissues were thawed followed by the addition of lysis buffer (3 ml/g) containing 20 mM Tris–HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1X inhibitor cocktails for protease and phosphatase (Sigma). The tissue was homogenized and the supernatant after centrifugation at 16,000 × *g* for 20 min was used in Western blot analyses. The following antibodies were used: MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (MS604, MitoSciences, Oregon, USA), pyruvate dehydrogenase kinase 1 (PDHK1), pyruvate dehydrogenase E1 alpha (PDHE1α) (Invitrogen, CA, USA), α-tubulin (Sigma–Aldrich, St. Louis, MO, USA.), goat polyclonal, anti-actin (Santa Cruz, CA, USA), uncoupling protein 2 (UCP2) (Alpha Diagnostic, TX, USA) and UCP3 (CHEMICON International Inc., CA, USA).

2.3. Enzymatic activities of active and total pyruvate dehydrogenase (PDH)

Both active (dephosphorylated) and total (phosphorylated and dephosphorylated) PDH enzymatic activities were determined in freeze-thawed mitochondrial extracts as described [21].

2.4. Measurement of OXPHOS

2.4.1. Mitochondrial membrane potential (MMP)

The MMP was measured in freshly isolated mitochondria using JC-1, a specific mitochondrial probe [22] as described earlier [23].

2.4.2. Biosynthesis of ATP

The luciferin-luciferase assay was used and the luminescence produced was read in a luminometer (Victor 3, Perkin–Elmer) as described previously [20]. The concentrations of various respiratory substrates employed are shown in the respective figures.

2.5. Statistical analysis

Data were presented as means ± SD, and were analyzed by the Student's *t* test where a *p* value of <0.05 was considered to be significant for $n = 3$.

3. Results

3.1. Western blot analyses of tumor and patient-matched normal tissues

3.1.1. Uncoupling protein (UCP) 2 and 3

UCP2 was almost absent in all tumor as compared to patient-matched normal tissues (Fig. 1A). UCP3 exists as long and short isoforms designated as UCP3L and UCP3S, respectively. The short form does not contain the last 37 amino acids found in the long form of UCP3, UCP2 and UCP1 [24]. There was no consistent pattern of UCP3S between the tumor and normal tissues. However, the protein expression of UCP3L was present in all ccRCC samples but not in the normal counterparts (Fig. 1B). The exception was sample #8 which was later found not to be ccRCC (Supplemenatry Table 1). The loading control was α-tubulin.

3.2. Pyruvate dehydrogenase (PDH) complex

3.2.1. Pyruvate dehydrogenase kinase 1 (PDHK1)

This kinase phosphorylates and inactivates PDH and it is a target of HIF-1. Its protein expression was higher in 70% of ccRCC tumor (T) compared to the corresponding patient-matched normal tissues (Fig. 2A).

3.2.2. Pyruvate dehydrogenase E1α (PDHE1α)

The protein expression of PDH was probed using an antibody to one of its subunits, PDHE1α. PDH is regulated by phosphorylation of three serine residues on the E1α subunit. The PDHE1α expression levels were lower in tumor relative to the corresponding normal tissues (Fig. 2B) except again for sample #8 (Supplementary Table 1). It was noted that there was an inverse relationship between the expression of PDHE1α and its corresponding PDHK1 counterpart shown in Fig. 2A.

3.2.3. Total and active enzymatic activities

Both mitochondrial extracts from normal tissues showed significant total and active PDH enzymatic activities; the latter represents the dephosphorylated form. In contrast, both enzymatic activities were totally absent in the tumor extracts (Supplementary Fig. 1).

3.3. Protein expression levels of respiratory complexes

Respiratory complexes (RC) I, II, III, IV and V (corresponding to NADH dehydrogenase, succinate dehydrogenase, ubiquinol dehydrogenase, cytochrome c oxidase and ATP synthase or FoF_1-ATPase) were down-regulated in all ccRCC tumor tissues (Supplementary Fig. 2).

3.4. Oxidative phosphorylation

3.4.1. Biosynthesis of ATP

The rate of ATP biosynthesis from ADP by the oxidation of conventional respiratory substrates such as malate/glutamate (RCI substrates), succinate (RCII substrate) or TMPD/ascorbate (artificial RCIV substrates) was comparable in two pairs of tumor and normal tissues examined (Supplementary Fig. 3a and 3b). Interestingly, ATP generation from the oxidation of glutamate, α-ketoglutarate and isocitrate (products of glutamine metabolism) was significantly higher in mitochondria isolated from one ccRCC tumor tissue (#16) as compared to the matched adjacent normal tissue (Supplementary Fig. 3c). This was also observed in another pair of patient-matched tissue samples (data not shown).