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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.

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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).

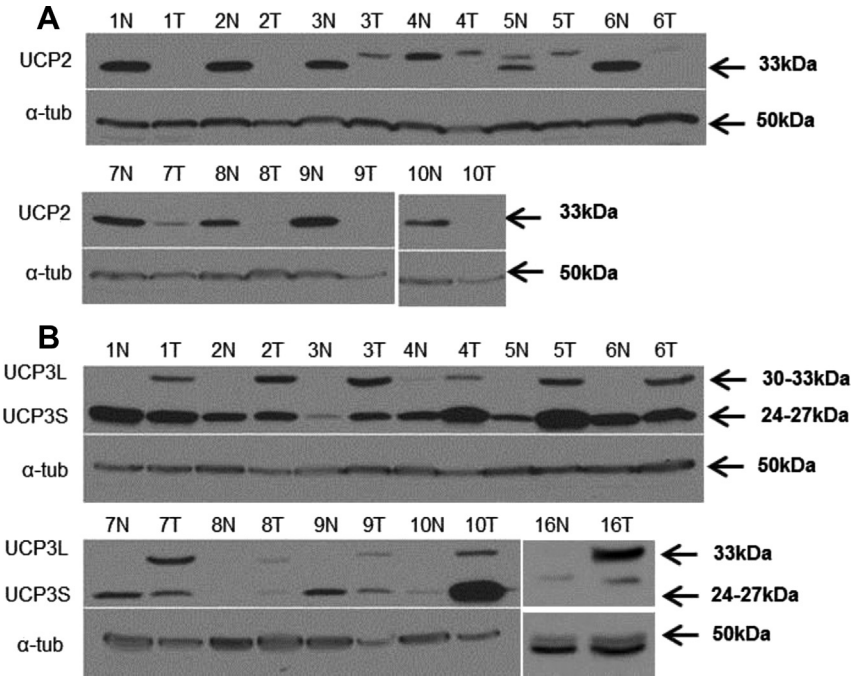

Fig. 1. Protein expressions of UCP2 and UCP3 in lysates of ccRCC tumor and patient-matched normal tissues. Tissue lysates prepared from ccRCC tumors and their corresponding normal counterparts were probed for uncoupling proteins, UCP2 and UCP3. Samples #10 and #16 were performed on separate blots. (A) Protein expression of UCP2 was absent in almost all tumor samples. (B) Two bands were detected for UCP3 due to the presence of two spliced variants, UCP3L and UCP3S. UCP3S lacked the sequence critical for uncoupling activity and therefore was not discussed. UCP3L was up-regulated in all ccRCC tumor samples except for #8 which was not derived from ccRCC (Supplementary Table 1). In sample #16, a nonspecific band below α-tub was observed but unidentified.

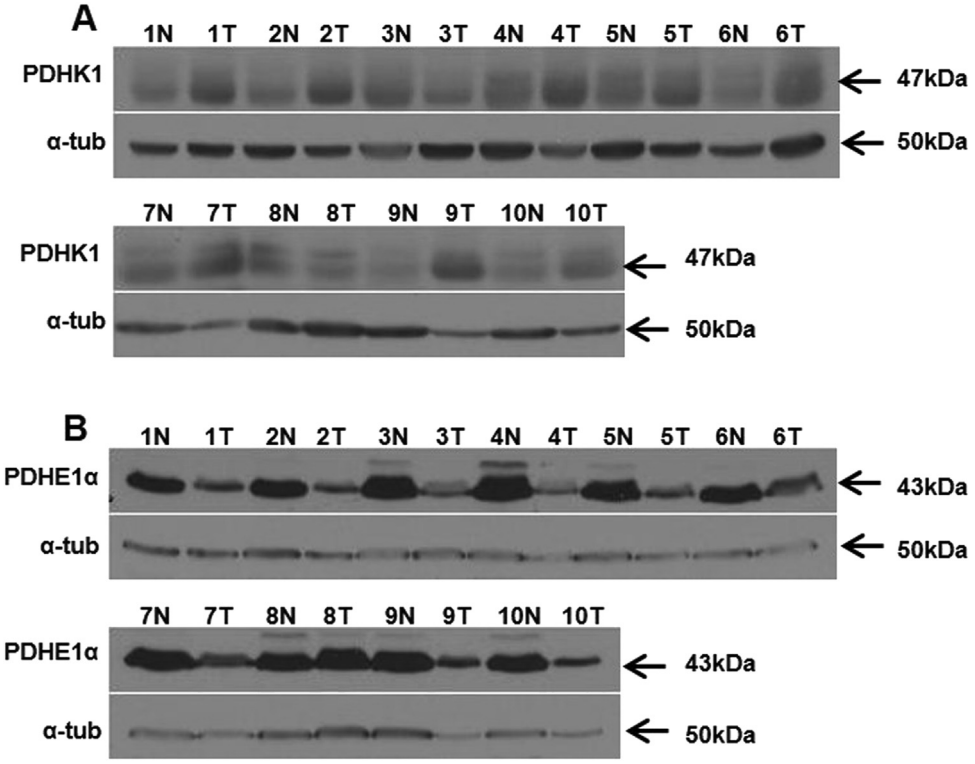

Fig. 2. Protein expressions of pyruvate dehydrogenase kinase (PDHK1) and Pyruvate dehydrogenase (PDH). Higher expressions of PDHK1 were observed in about 70% of ccRCC tumor (T) samples (A). PDH is regulated by phosphorylation of three serine residues on the E1α subunit [35,36] and an antibody against this E1α subunit was employed. The expression of PDHE1α subunit was lower in all tumor (except #8) samples (B). An inverse relationship between the protein levels of PDHK1 and PDHE1α was observed.

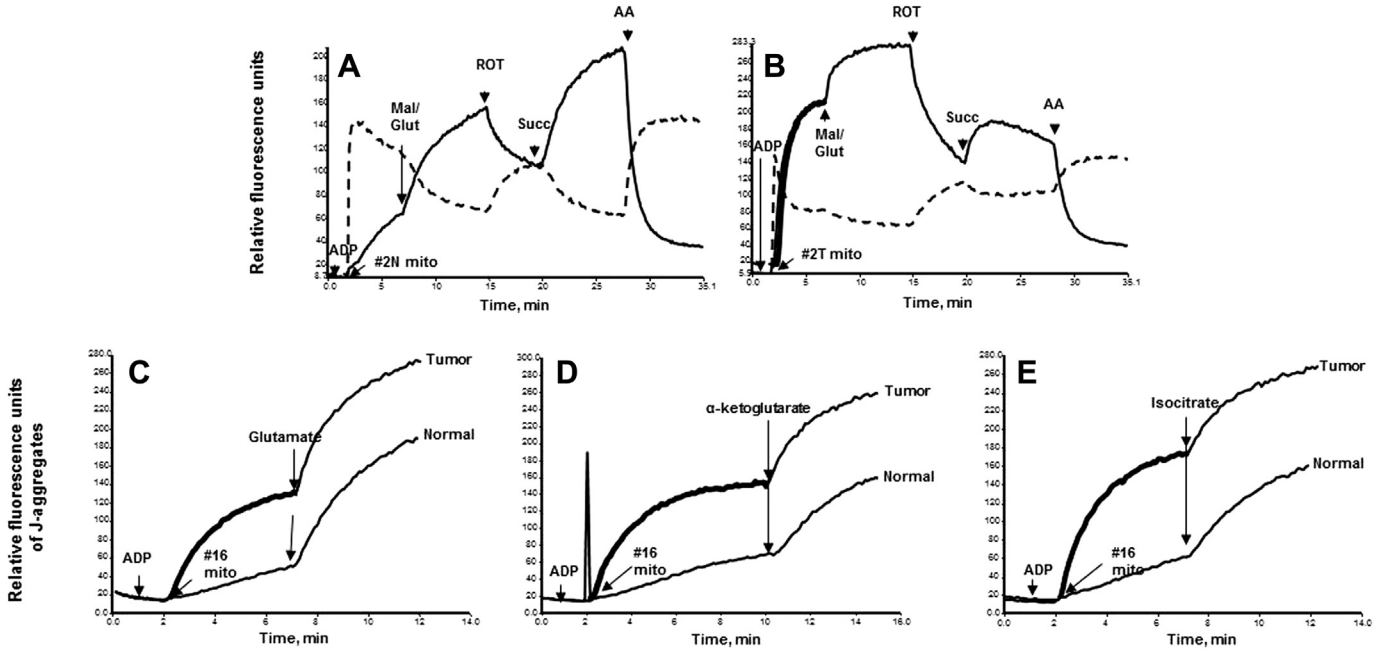

Fig. 3. Membrane potential in intact mitochondria isolated from ccRCC tumor and their corresponding normal tissues. The basal MMP, evident in the presence of 125 μM ADP but before the addition of respiratory substrates was higher in mitochondria isolated from ccRCC tumor tissues as shown by the highlighted lines as compared to the matched adjacent normal tissues. X- and y-axes represent, respectively, the intensity of fluorescence and time (min). Solid lines denote the red fluorescence of the JC-1 aggregates and dotted lines the green fluorescence of the monomers in A and B. The two fluorescence intensities showed a reciprocal relationship. The MMP was induced by adding 5 mM each of mal/glut which was inhibited by 5 μM rotenone (ROT) followed by re-energization by 10 mM succinate (Succ) with subsequent inhibition by 4 μg/ml antimycin A (AA), an inhibitor of respiratory complex III, which finally collapsed the MMP (A and B). Respiratory substrates, 5 mM glutamate, 2.5 mM α-ketoglutarate and 5 mM isocitrate (C, D and E) were added to induce the MMP. These are representative traces using sample #16. Only the intensity of the red fluorescence of the J-aggregates was shown in these three figures.

3.4.2. Mitochondrial membrane potential (MMP) with added ATP and different respiratory substrates

Mitochondria isolated from ccRCC tumor exhibited consistently higher MMP compared to the normal matched tissues on adding 125 μM ADP but before the introduction of mal/glu as highlighted by the steeper gradient in Fig. 3B compared to Fig. 3A). We designated this as "basal" MMP present in state 2 respiration, i.e. in the presence of ADP. The MMP was further increased by mal/glu and decreased by rotenone, which inhibited NADH dehydrogenase. The MMP elicited by the subsequent addition of succinate appeared to be compromised in sample #2T (tumor) compared to the normal (#2N) tissue (Fig. 3A and B). Similarly other respiratory substrates: glutamate, α-ketoglutarate and isocitrate were also able to generate a higher MMP in the presence of ADP in tumor compared to normal tissues (Fig. 3C, D and E). Taken together, the introduction of ADP alone induced consistently a higher MMP in mitochondria prepared from all ccRCC tumor samples, identified by the highlighted lines in Fig. 3.

3.4.3. Inhibition of MMP induced by ADP

From the above sets of data, it was apparent that endogenous respiratory substrate/s in the mitochondrial preparations could contribute to the MMP induced by ADP. Addition of rotenone almost completely abolished this phenomenon although part of the inhibition was attributed to the ethanol used to dissolve rotenone (Fig. 4A).

3.5. Endogenous MMP without added ADP and respiratory substrate

Mitochondria isolated from ccRCC but not those from patient matched normal tissues showed an endogenous MMP when both ADP and respiratory substrate were omitted in the incubating medium (i.e. state 1 respiration) as illustrated by a representative of three similar traces from different ccRCC tissues (Fig. 4B). This inherent MMP was maintained for a few min followed by a gradual decline until the addition of 125 μM ADP.

4. Discussion

4.1. Metabolic signatures of ccRCC

Despite the intra-tumor heterogeneity in ccRCC [27], it was surprising to find relatively distinct metabolic signatures of HKII, LDH5 (reported previously and confirmed in our study: data not shown), PDHK1, PDH and respiratory complexes I to V in tissues of patients with ccRCC, diagnosed as grade 1 to 4 tumor types and with or without malignancies (Suppl. Table 1). Our data supported the conclusion that these probably represent early events which could persist during the progression of some renal carcinomas [26]. The profiles of loss or decreased UCP2 and up-regulated UCP3 represent additional hallmarks of ccRCC.

4.2. Is mitochondrial function compromised in ccRCC?

The gene frequently linked to ccRCC is von Hippel-Landau (VHL) which has been reported to translocate to mitochondria [28] suggesting that it could affect mitochondrial function. The occurrence of OXPHOS in cancer cells has been controversial ever since Warburg hypothesized that aerobic glycolysis was due to "irreversible damage" to mitochondrial respiration [29]. We and others had observed decreased protein expressions of the respiratory complexes in ccRCC [25,26]. This could be the result of a global defect in mitochondrial biogenesis which was consistent with our observation of decreased expressions of the nuclear transcription factors: peroxisome proliferator-activated receptor γ co-activator 1α (PGC-

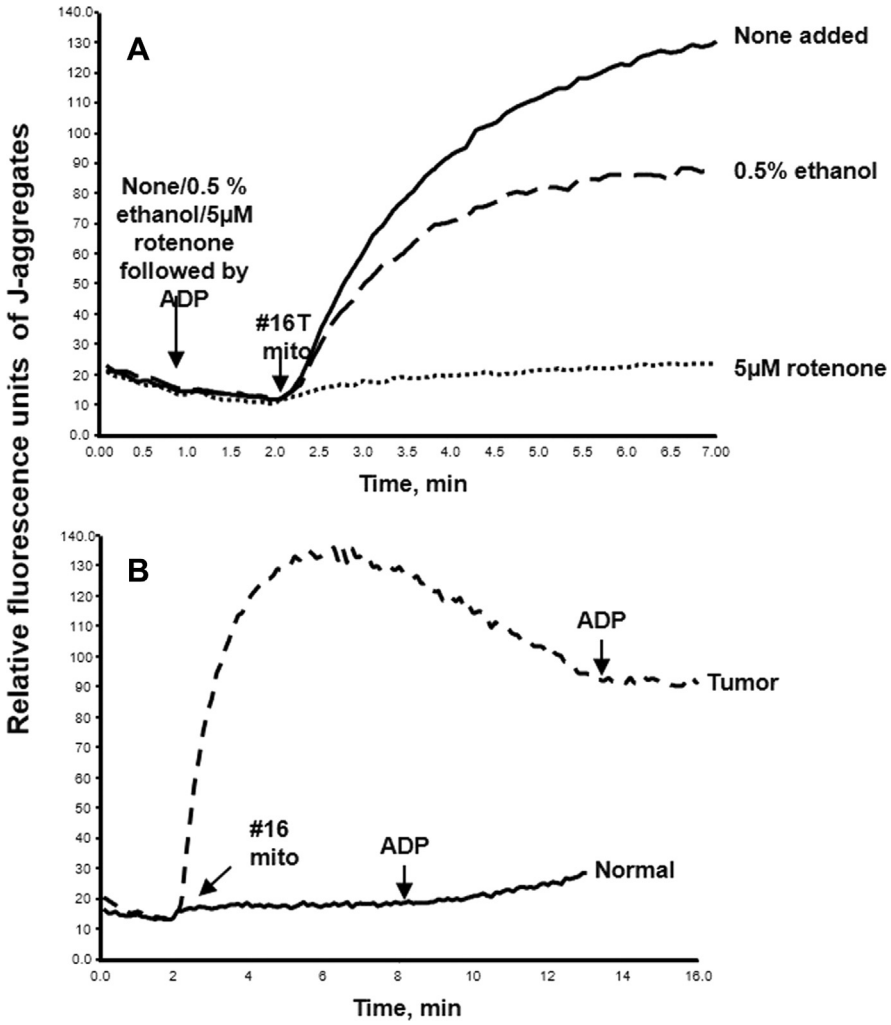

Fig. 4. (A) **Inhibition of MMP induced by ADP in mitochondria isolated from ccRCC tumor**. 5 μM rotenone, an inhibitor of respiratory complex I, effectively inhibited the basal MMP. Part of the inhibition was due to ethanol used to dissolve rotenone. Only the intensity of the red fluorescence of the J-aggregates is shown. (B) **Endogenous MMP in mitochondria isolated from ccRCC tissue**. Mitochondria isolated from ccRCC tumor sample #16, but not from the patient-matched normal tissue, exhibited an MMP without the addition of ADP and respiratory substrate i.e. under state 1 respiration as shown by the intensity of the red fluorescence of the J-aggregates. This endogenous MMP was sustained for a few min followed by a decline which was prevented by the addition of 125 μM ADP.

1α), mitochondrial transcription factor A (mtTFA) and nuclear respiratory factor 1 (NRF-1) (data not shown). Decreased biogenesis of OXPHOS complexes has been attributed to a deficiency of VHL [30] resulting in the HIF-1 mediated reduction in mitochondrial mass and oxygen consumption [31]. All these observations together with our identification of up-regulated UCP3 (known to induce uncoupling in mitochondria) alluded to a deficit of OXPHOS in ccRCC. However our study showed otherwise.

4.3. Mitochondrial function in ccRCC

4.3.1. Evidence of OXPHOS with added ADP ± respiratory substrates

We showed that mitochondria freshly isolated from ccRCC tumor tissues have the ability to perform essential mitochondrial activities of OXPHOS, even though standard isolation procedures are deemed to disrupt mitochondrial structure and function [32]. Indeed, we have demonstrated ATP biosynthesis via coupling of oxidation of numerous respiratory substrates to the phosphorylation of ADP and to the induction of the MMP. OXPHOS is considered the single most useful measure of mitochondrial function in isolated mitochondria [33]. However, in our study, these measurements were determined by adding ADP and respiratory substrates which represent at best a "potential" of the isolated mitochondria for OXPHOS. Nevertheless, our data showed that residual electron transport system in ccRCC could support OXPHOS when respiratory substrate/s and ADP are made available. Furthermore, an MMP could be generated without any exogenous respiratory substrate. We concluded that respiratory complex I substrate/s, present in the isolated mitochondria, contributed to this observation based on the inhibitory action of rotenone. Unfortunately, it was not possible to measure retrospectively ADP and respiratory substrates in our samples. We concluded that the induced MMP could be the result of decreased uncoupling activity of UCP3 by ADP; this adenine nucleotide exhibited stronger inhibitory action than ATP on UCP3 [14].

4.3.2. Evidence of OXPHOS without added ADP and respiratory substrates

Of particular interest was our demonstration of an endogenous MMP without adding ADP and respiratory substrate to mitochondria isolated from ccRCC which was not the case of normal mitochondria. This represents the inherent state of mitochondria isolated from ccRCC tissues. Such a differential MMP had previously been reported in cancer cells in culture [17–19] and it formed the

basis of targeting mitochondria with cationic drugs for chemotherapy [34].

4.4. Limitations of our study

One obvious limitation of our study was the lack of replicates in our metabolic studies for comparison between tumor and normal tissues in many sets of data, resulting in only semi-quantitative analyses. This was because of the small quantity of mitochondria isolated from limited freshly resected human surgical samples. Even when replicates were performed, triplicates were the norm, because of our focus on measuring OXPHOS parameters which required the use of intact mitochondria. This was further constrained by the decrease in quality of mitochondria with time. Although our study showed that respiratory substrates and ADP contributed to the induction of MMP, it was not possible to measure retrospectively these intermediary metabolites in our mitochondrial preparations. In addition, assays with JC-1 lack normalization, unlike the conventional probes such as TMRM and TMRE for quantitative measurement of MMP. Nevertheless, MMP measurements with JC-1 were quite reproducible in our hands and they were suitable for fast semi-quantitative comparisons. Lastly, the protein expression levels of the respiratory complexes represent essentially data reported previously [25,26]. However, they were included to provide evidence that OXPHOS was measurable in the same ccRCC tissues with compromised electron transport system.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.130.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.130.

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