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Breaking the ritual metabolic cycle in order to save acetyl CoA: A potential role for mitochondrial humanin in T2 bladder cancer aggressiveness

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ABSTRACT

Introduction: Cancer cells may exhibit outsourcing of their high energy need in order to avoid the intrinsic mitochondrial apoptosis. Reduced mitochondrial respiration and accumulation of mitochondrial genome mutations are among metabolic transformations in this regard. Mitochondrial humanin (MT-RNR2) is a small peptide with anti-apoptotic activities attributed to binding some pro-apoptotic proteins. *Aim of the work:* The current study aims at investigating the expression of mitochondrial humanin in bladder tumor cells and the possible casting of humanin anti-apoptotic action through orchestrating some of the mitochondrial metabolic enzymes. *Material and methods:* Here messenger RNA of humanin, succinate dehydrogenase, glutaminase, isocitrate dehydrogenase were compared in tissues from patients with T2 bladder carcinoma in comparison to tumor associated normal tissues from the same patients. Levels of lactate and mitochondrial pyruvate carrier (MPC1) mRNA were determined to scrutinize the prevalence of aerobic glycolysis. *Results:* The present study found that tumor cells had suppressed aerobic glycolysis, augmented mitochondrial respiration and interrupted tricarboxylic acid cycle, all of which were suggested to serve tumor aggressiveness. MT-RNR2 was found closely related to the alterations in mitochondrial activity. *Conclusion:* MT-RNR2 plays its anti-apoptotic role partly by avoiding deploying energy from complete oxidation of organic compounds to inorganic wastes. Thus MT-RNR2 can potentially serve as a new biomarker in the diagnosis of bladder carcinoma especially that it is present in blood circulation.
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Introduction

Bladder cancer is the ninth most common cancer throughout the world and is the most prevalent malignancy among Egyptian males (16%), producing >7900 deaths annually, which is strikingly higher than most other parts of the world [1]. Over 75% of bladder cancers in Egypt are transitional cell carcinoma (TCC) [2], which could be presented as superficial tumors confined to the mucosa (Ta disease) and lamina propria (T1) or presented as muscle invasive disease (≥T2) with no history of superficial disease. Low-grade Ta tumors have a high risk of recurrence (70%) but rarely progress to muscle-invasive tumors, conversely high-grade ≥T1 tumors show a high risk of progression [3]. High-grade bladder cancer progression follows complex sequential steps, not completely understood. Reprogramming of energy metabolism in cancer cells gives cells the ability to acquire nutrients autonomously and coordinately regulate metabolic pathways to support proliferation [4]. Years ago, Otto Warburg identified cancer cells reprogramming to involve decreased respiration and an enhanced lactate production in presence of oxygen, a phenomenon known as "aerobic glycolysis" or more recently "the Warburg effect" [5]. According to Warburg's original hypothesis, mitochondria ought to be irreversibly damaged. A decreased reliance on mitochondrial function would increase resistance to intrinsic apoptotic pathways [6]. Another functional consequence of mitochondrial uncoupling is decreased ROS production which would otherwise surge when cells switch to excess nutrient uptake [7]. Mitochondrial dysfunction theory in tumor cells was recently supported by finding of mutations in the nuclear DNA (nDNA)-encoded succinate dehydrogenase (SDH) A and SDHB subunits of oxidative phosphorylation

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(OXPHOS) complex II in gastrointestinal stromal tumor [8]. Moreover, mutations in OXPHOS genes encoded by the mtDNA were also found. Inactive cytochrome oxidase subunit II (COX II) concomitant to the normal silencing of p53 in cancer cells was among recorded mitochondrial genetic alterations [9]. Knowing that mutation in mitochondrial DNA is closely linked to disturbed metabolic environment required for tumor cells growth, led us to look closely to humanin (HN); a new recently discovered peptide within the mitochondrial genome [10]. Mitochondrial humanin (MT-RNR2) is a small, secreted 21 amino acid peptide with anti-apoptotic activities attributed to binding BAX, BID, and BIM, all pro-apoptotic proteins, perhaps by stabilizing their inactive conformations [11]. Humanin is a new member to be added to the communicating molecules from mitochondria to nucleus. HN could be encoded within one or more of the nuclear regions with 92–95% similarity to the original HN cDNA, dispersed in multiple copies throughout the human genome [12]. However, it was shown that, mitochondrial humanin (MT-RNR2) encoding for the prototype HNM was by far more activated than any of the nuclear *MT-RNR2*-like genes, and showed a moderate response to both STS and beta-carotene [13]. The expression of mitochondrial humanin in bladder tumor cells and the possible launching of the anti-apoptotic action of humanin through the metabolic switch by mastering the fate of pyruvate have not been studied before. Pyruvate, this three carbon compound could stay at the cytosol keeping its main carbon skeleton but in the reduced form called lactate and with the drop of energy to two ATP only (the Warburg effect). Pyruvate could instead be shuttled to the mitochondria by mitochondrial carrier protein (MPC1) where its carbon skeleton comes out as three molecules of carbon dioxide but in return the energy gain rise up to 38ATP. A completely different anabolic fate takes place if pyruvate could be processed appropriately in the mitochondria; by sticking one carbon at the methyl end or trimming one carbon from the carboxyl end to produce oxaloacetate and acetyl CoA respectively. These two compounds are the building blocks of glucose and fatty acids but only after moving back to cytosol in the transient forms malate and citrate respectively. So inspecting mitochondrial carrier protein closely will tell whether pyruvate is ready to manage all the backlog of tasks required to support the highly proliferating tumor cells or if it will suffice by adhering to the quiescent lactate form perhaps in order to avoid mitochondrial apoptotic and oxidative tide. The present study was also querying whether the Krebs' cycle is inhibited according to Warburg's or not. In this regard, the current study was conducted on a clinical samples rather than cell culture to reveal functional mitochondria and metabolic adaptation mechanisms which could have been masked because of the cell culture conditions in which a finite metabolic program is imposed on the cells as a function of the media composition and the optimal growth conditions [14]. The current study aims at finding out the expression of mitochondrial humanin in bladder tumor cells and the possible casting of humanin anti-apoptotic action through manipulating mitochondrial carrier protein and reorchestrating some of the Krebs' cycle related enzymes.

Subject and methods

Specimen collection

A heterogeneous group of 40 patients with bladder tumors, all of which had matched tumor-associated normal (TAN) samples were assessed but only 30 patients were eventually enrolled in this investigation. Diagnosis was done using cystoscopy and confirmed by pathology. Tissue samples were all gathered post-cystectomy at Demerdash Hospital Cairo, Egypt between May 2015 & August 2015. Cystectomy was undertaken as the proper treatment in order to control tumor spread as tumors were found to invade the muscularis layer.

Ethics and consent permission

This study obtained human research ethics approval from the Ethics Committee of the Faculty of Medicine, Ain Shams University. All participants provided their informed consent. Each patient provided 2 sets of samples. One set from the tumor and the other from the bladder wall distant from the tumor and grossly appeared healthy. The second set acted as the control. Each set was made of 3 samples, one tissue chip was fixed in 10% formalin for histopathological studying, the second was preserved in glutaraldehyde 2% for electron microscope scanning and transmission electron microscope, and the third was subjected to immediate snap freezing in liquid nitrogen and archival at -80C until further use.

The histopathological review aimed at selecting suitable samples appropriate to address specific questions where only high grade T2 tumors were chosen. Cancer stages were assigned according to the International Union Against Cancer tumor-node-metastasis staging system. Tumor grades were assigned using the World Health Organization criteria. The clinicopathologic characteristics of patients are shown in Table 1. Thirty bladder tumor specimens and thirty TAN tissues from the same subjects were found appropriate and so elected for gene expression profiling experiment using real-time quantitative (RQ)-PCR. Of note, all patients submitted in this study were men where during the time of study only male patients were encountered.

Specimen description

Laboratory analysis

Lactate analysis and RNA extraction

Tissue samples (50–100 mg) were homogenised using a hand-held homogenizer (Polytron PT1600E) in 1–2 mL of QIAzol reagent

Table 1
Clinic-pathological data for patients used for gene expression analysis.

	N (30)
Age	
Mean ± SD	60 ± 12
Range	45–78
Gender	
Men	30
Histological type	Transitional cell epithelium (urothelial)
Tumor growth pattern	
Non papillary solid tumor	30
Tumor growth stage	
T2a	14
T2b	16
Grade	
High	30
Cytological features	
Dysplasia	30
Moderate	12
Sever	18
Tumor size	
<3	2
≥3	28
Number of tumors	
Single	21
Multiple	9
Lymph node involvement	
No	30

(Qiagen). In brief, tumor and TAN samples were homogenised separately but on the same day. Tissue homogenate was divided into two parts. One part was used for lactate determination by using a lactate dehydrogenase kit (Sigma Chemical, St. Louis, MO)., for which deproteinization was done first to remove lactate dehydrogenase by loading the supernatant into a 10kda spin column (the eluate was used for subsequent assays). The level of lactate was expressed as micromoles per gram wet weight (μmole/g). RNA was extracted using RNeasy Plus Mini kits (Qiagen) according to the manufacturer's instructions. RNA was eluted in 60 μL volumes. RNA yield and purity was assessed using NanoDrop 2000 (Thermo Scientific, USA) and stored at −80 °C.

Reverse transcription

RNA was reverse transcribed to first strand cDNA using QuantiTect Reverse Transcription Kit (Qiagen) (cat. No. 205310) and RT Primer Mix (1 μg,). Negative control samples were included in each set of reactions. The purified RNA sample is incubated in gDNA Wipeout Buffer at 42 °C for 2 min to effectively remove contaminating genomic DNA. Template RNA and reverse-transcription master mix were left to react and incubated at 42 °C for 15 min then the reaction was inactivated by incubation at 95 °C for 3 min. The cDNA synthesis was repeated using Oligo-dt only containing Omniscript RT Kit (Qiagen) (cat. No. 205111). Samples were stored at −20 °C.

Real-time quantitative PCR

Amplification efficiency. The amplification efficiency of each assay is an important consideration in the determination of relative quantities of gene expression by RQ-PCR. PCR efficiency impacts greatly on the accuracy of the calculated expression result and is influenced by PCR reaction components. For 100% efficiency a doubling of the amount of DNA will occur at each cycle, while for 80% and 70% the amount of DNA will increase from 1 to 1.8 and 1.7, respectively. Resultantly, small differences in efficiency can greatly affect the calculation parameters involved in the determination of gene expression values. Amplification efficiencies for each gene assay in this study were calculated applying the formula $E = (10^{-1}/\text{slope}^{-1}) \times 100$, using the slope of the plot of Cq versus log input of cDNA (10-fold dilution series). A threshold of 10% above and below 100% efficiency was applied.

Data analysis

Endogenous control

Relative quantification is the most widely adopted approach whereby quantification of gene expression is normalised relative to an endogenously expressed control (EC) gene. Central to the reliable determination of gene expression is the choice of control gene. Beta actin (ACTB) has been used in previous studies of bladder cancer [15]. ACTB was used to normalize expression values in the present study.

RT-qPCR of mRNA

The expression of each gene was analyzed by RQ-PCR using QuantiTect SYBR Green PCR Kit (Cat. No. 204141) and Applied Biosystems StepOnePlus instrument. For MT-RNR2, the cDNA produced by both QuantiTect and Omniscript RT Kit were run on RQ-PCR. Amplification efficiencies for each gene assay in this study were calculated using the slope of the plot of Cqversus log input of cDNA (10-fold dilution series) by applying the formula

$$E = 10 - 1/\text{slope}$$

To convert E into a percentage: % Efficiency = $(E - 1) \times 100\%$.

Negative controls for each gene target under assay containing all PCR components without template DNA were used to ensure that the reagent mix was free of contamination. On each plate, an interassay control was included to account for any variations between runs. A reaction volume of 25 μl was used. For each well 3 μL of cDNA from each sample was added to 22 μL of PCR reaction mix which consisted of 12.5 μL QuantiTect SYBR Green PCR Master Mix, nuclease free water and 1 μL gene expression assay primer-probe mix (Applied Biosystems). PCR initial activation step of 95 °C for 15 min is required to activate HotStarTaq DNA Polymerase. Standard fast thermal cycling parameters of 40 cycles of 95 °C for 15 s and 60 °C for 60 s were applied in accordance with the manufacturer's recommendations.

Relative quantification

Cycle threshold (Cq) is defined as the PCR cycle number at which the fluorescence generated from amplification of the target gene within a sample increases to a threshold value of 10 times the standard deviation of the base line emission and is inversely proportionate to the starting amount of the target cDNA.

Gene study

The mitochondrially encoded gene humanin (MT-RNR2) has its ORF located within a functional mtDNA gene producing rRNA molecules. Since HN is encoded from a gene within a gene it is challenging to measure the mRNA coding for the HN peptide and not the 16S rRNA gene transcript. Therefore, cDNA synthesis was done twice using oligo dt containing primers and primer mixture. HNM primer used in RQ-PCR was selected specifically to 16 s rRNA located between 1671 and 3229 within mitochondrial genome. Moreover, the relative abundance of the mitochondrial rRNA containing the putative peptide-coding sequence could have been accompanied by the existence of highly homologous but significantly less abounding nuclear *MT-RNR2*-like mRNA species. Although most nuclear MT-RNR2-like sequences (NUMTs) are considered pseudogenes, bioinformatics-based evidence suggests that at least some of the nuclear MT-RNR2-like sequences might be functional genes (13). Interestingly, these HN-like genes are dispersed in multiple copies throughout the human genome, but they were not identified in bladder (13). Therefore, the current study screened the expression of HN1, HN2, HN6, HN10 in some random samples.

In addition to mitochondrial pyruvate carrier (MPC1) and humanin (MT-RNR2), the current study enrolled number of mitochondrial enzymes (Table 2). Succinate dehydrogenase or Complex II is a four-subunit enzyme that straddles the inner mitochondrial membrane, such that it can easily participate in both the electron-transport chain (membrane-associated) and Krebs cycle (mitochon drial-matrix-associated). This complex is anchored to the membrane by two membrane-spanning proteins —SDHC and SDHD. The peripheral portion of this complex is made up of a flavoprotein (SDHA) and an iron–sulphur subunit (SDHB), and projects into the mitochondrial matrix. The presence and proper functioning of both SDHA and SDHB are required for catalytic activity. The active site of the enzyme is located within SDHA and contains covalently bound FAD. SDHA subunit was selected to be studied. Primers were selected to produce a product length of 303 (Table 3).

Glutaminase catalyze the hydrolysis of glutamine to glutamate and ammonia. Primers were selected to produce product length 142.

Isocitrate dehydrogenases catalyze the oxidative decarboxylation of isocitrate to 2-oxoglutarate. These enzymes belong to two

Table 2
Function, subunits and isoforms of some mitochondrial metabolic enzymes.

enzyme name	Enzyme function	Subunits	Selected subunit	Isoforms	Selected isoform
Succinate Dehydrogenase	Oxidation of succinate to fumarate with reduction of ubiquinone to ubiquinol	4 subunits: 2 structural: SDHC and SDHD. 2 catalytic: SDHA and SDHB	SDHA		
Glutaminase	Hydrolysis of glutamine to glutamate and ammonia	–	–	–	–
Isocitrate dehydrogenase	Oxidative decarboxylation of isocitrate to 2-oxoglutarate	4 subunits; two alpha subunits, one beta subunit, and one gamma subunit	Alpha subunit designated by IDHA	IDH1 and IDH2: NADP + dependent IDH3:NAD + dependent	IDH3A

distinct subclasses, one of which utilizes NAD(+) as the electron acceptor and the other NADP(+). NAD(+)-dependent isocitrate dehydrogenase isoform (IDH3) is localize to the mitochondrial matrix together with one of NADP(+)-dependent isocitrate dehydrogenase isoforms. NAD(+)-dependent isocitrate dehydrogenases (IDH3) catalyze the allosterically regulated rate-limiting step of the tricarboxylic acid cycle. Each isozyme is a heterotetramer that is composed of two alpha subunits, one beta subunit, and one gamma subunit. Primers were selected to produce a product length of 71 for the alpha subunit of one isozyme of NAD(+)-dependent isocitrate dehydrogenase (IDH3A).

Statistical analysis

The average threshold cycle (Cq) and the comparative ΔΔCq method were calculated for the expression of the gene and normalized to the mean Cq-value of ACTB. Fold change (RQ) was used to compare gene expression between samples and was calculated as $2^{-\Delta\Delta Cq}$. For the TAN sample, $2^{-\Delta\Delta Cq}$ equal zero and 2^0 equal one, so that the fold change in gene expression relative to TAN equal one, by definition. For tumor samples, a higher negative ΔΔCq value, represents higher expression. Statistical analysis was carried out with IBM SPSS Statistics 17.0 (SPSS Inc.). Data was tested for normal distribution graphically using histograms and also using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Parametric tests were used where appropriate. Correlation analysis used Spearman's Rho and Pearson's correlations coefficient for nonparametric and parametric data respectively. . Differentially expressed genes were identified with the paired *T* test. P values < 0.05 were considered statistically significant.

Results

All patients submitted in this study were men where during the time of study only male patients were encountered. As per histology, all the cases had transitional cell carcinoma. Tumor growth pattern showed that all the cases had non papillary solid tumor. With regard to tumor grade, there were no cases that had papillary urothelial neoplasm of low malignant potential (PUNLMP) or low grade tumor where the present study involved only the patients who needed cystectomy. As such, all cases had high grade tumor with characteristic dysplasia. All the selected thirty cases were at the progressive T2 stage. The thirty cases with T2 stage were further sub classified where fourteen subjects were staged at T2a and sixteen subjects were staged at T2b. T2a stage was defined as cancer invading less than one-half of the depth of muscular propria (pT2a) while in T2b stage, cancer invades greater than one-half of the muscle wall [16]. Most of the cases were unifocal cases while only nine cases were multifocal. In this study, lactate tissue level was measured. The level of lactate in tumor tissues was found not significantly different from that in the adjacent tumor associated normal tissues. Lactate level in tumor tissues was 2.54 μmole/g which is generally considered as low level [17].

Prior undergoing QPCR, amplification efficiency was determined for both the target and the reference genes using the slope of the plot of Cq versus log input of cDNA (10-fold dilution series). The slopes for the target and the reference genes were found identical and having the value −3.5. By applying the formula

$$E = 10^{-1/\text{slope}}$$

$$E = 1.93$$

$$\%\ \text{Efficiency} = (E - 1) \times 100\%$$

$$\%\ \text{Efficiency} = 93\%$$

Therefore, rather than calculating fold change in gene expression as $2^{-\Delta\Delta Cq}$, it was calculated as $1.93^{-\Delta\Delta Cq}$

Upon measuring the quantity of beta actin (ACTB) as a reference gene in bladder tissue samples of the enrolled patients using qRT-PCR, the mean ± standard deviation, maximum value, minimum value, and the range of Cq value of ACTB were 23.17 ± 0.82, 21.69, 25.13, and 3.44, respectively, in the bladder cancer group, and 22.7 ± 1.2, 18.59, 26.49, and 7.9, respectively, in the control group. As such, the Cq values of the housekeeping gene ACTB that was used for normalization of real-time qRT-PCR were not significantly different between the bladder cancer and control groups.

Table 3
list of genes selected for RQ-RT-PCR validation.

Gene name	Gene symbol	Gene ID	Location	BP	Forward primer	Reverse primer	Amplicon size
Mitochondrial pyruvate carrier	MPC1	51660	6q27	18.095	AGGTGAAAACGCAACTCCTTGA	AAGAATGCGTTCTGCGTAGC	113
Succinate dehydrogenase	SDHA	6389	5p15	46.594	GGTGCCTGAGAACGAATGGA	TGACAGGTGGTGTTCACAGAG	303
Glutaminase	GLS	2744	2q32-q34	84.732	TTGGAAAAGAGCCGAGTGGA	CCCCCATTTCCTGAGGATCAC	142
Isocitrate dehydrogenase	IDH3A	3419	15q25.1-q25.2	21.217	TTGCCTTCATGAGAAACCGTCT	GGGCCAATACCATCTCCTGG	71
Humanin	MT-RNR2	4550	mtDNA (1671–3229)	1.559	ACCGGAGTAATCCAGGTCGG	AGGCGCTTTGTGAAGTAGGC	91
Actin beta	ACTB	60	7p22	3.454	GAGGCCAAGTGTGACTTTGTG	AGGCCAGGAACTCCCCAATA	225

Bladder tumor tissue at the mRNA level demonstrated a higher MPC1 expression (2.13 ± 1.66) compared to adjacent normal tissues (0.48 ± 1.71) (P < 0.001). Regarding fold change (Fig. 1); MPC1 mRNA level in the bladder tumor in was about 4.4-fold higher than in tumor-adjacent normal tissues (Table 4).

The expression of isocitrate dehydrogenase A was significantly lower in bladder tumor (2.01 ± 2.33) compared to TAN tissues (7.48 ± 2.03) (P < 0.001). Isocitrate dehydrogenase expression in bladder tumor was 3.7-fold lower than in the normal samples.

Expression levels of SDHA increased from tumor-associated normal (0.57 ± 2.02) to tumors (10.32 ± 2.44) (P < 0.001) by 18 folds.

Glutaminase showed increase in expression from tumor associated normal (0.32 ± 2.03) to tumor tissues (1.46 ± 2.33) (P = 0.048).. Additionally, up-regulation of glutaminase (GLS) was found to be about 4.6-fold higher compared to that of the normal samples.

MTRNR2 mRNA analysis both with primer mix and oligo dt primers produced equal results. Random screening for the expression of some of the nuclear MT-RNR2-like isomers like HN1, HN2, HN6 and HN10, has not detected their expression.

Comparing tumor (3.98 ± 3.37) to normal (0.19 ± 2.62) (P < 0.001), humanin (MT-RNR2) showed significant 20.6-fold increase.

The relationship between MT-RNR2 and various metabolic enzymes was further investigated using Pearson correlation (Table 5). Preliminary analysis was performed to ensure no violation of the assumption of normality, linearity and homogenecity. Strong positive correlation between MT-RNR2 and SDHA in tumor was observed (Fig. 2), while strong negative correlation between MT-RNR2 and IDH was found (Fig. 3). With regard to the correlation among the studied mitochondrial enzymes and protein, there was a strong positive correlation between SDHA and MPC1 (See Fig. 4) (See Fig. 5).

Table 4
Cq, ΔΔCq and RQ for the studied genes.

Gene symbol	Cq	ΔΔCq	RQ
MPC1	22.01	−2.27	4.46
SDHA	19.62	−4.39	17.97
GLS	22.68	−2.32	4.63
IDH3A	22.14	2	0.27
MT-RNR2	21.08	−0.461	20.59

Table 5
Significant correlation among bladder tumor group.

Parameter	r^2	P-value
MPC1 and SDHA	0.51	P = 0.0034
Humanin (MT-RNR2) and SDHA	0.74	P < 0.0001
Humanin (MT-RNR2) and IDH3A	−0.55	P = 0.0013

Discussion

Currently, bladder cancer represents a great burden due to the limited remedial response. T2 bladder carcinoma is an onerous high grade tumor that invades into the muscle wall and requires scrutiny. The invasive capacity of malignant tumors is closely associated with cell proliferation which is underpinned by the ability to acquire nutrients and coordinately regulate metabolic pathways endowed by mutations [18]. So Warburg's observations posed a conviction of how the relatively inefficient energy pathway could be exploited in recruiting more glucose molecules [18]. In contrast to Warburg's, tumor cells in the current study had low lactate level concomitant with upregulated MCP1 expression which was associated with succinate dehydrogenase overexpression. These findings suggest that Warburg's does not conform to this type of invasive bladder tumor and propose energy exhaustion in anaplerosis and the resulting hunger as the great turn on for the metabolites recruiting process. The present study found upregulated glutaminase (GLS) and succinate dehydrogenase (SDHA) enzymes in tumor cells relative to TAN. Glutamine is the most abundant amino acid in plasma, at concentrations of 0.6–0.9 mmol/l, [19]. Glutaminase (GLS) is a mitochondrial enzyme that catalyzes the deamination of glutamine to produce ammonium ions and glutamate. Glutamate, by transamination or deamination, yields α-ketoglutarate; a Krebs cycle intermediate. Therefore, glutamine has been considered as an anabolic intermediates and a bioenergetic substrate [20]. Nevertheless the up-regulated biosynthesis in tumors require an increasing amount of energy as well as anabolic intermediates which could only be met by cordial collaboration between glycolysis, pentose phosphate shunt, and most importantly Krebs cycle in a functional mitochondria. Consequently, mitochondrial dysfunction and inactive oxidative phosphorylation in Warburg's original hypothesis are controversial especially, as the oxidative processes carried out in mitochondria are highly dependent on a functional oxidative phosphorylation. So the findings in this study of upregulated GLS and SDHA add to the context of building up in functional mitochondria of tumor cells. Similar findings of mitochondrial activation have been found elsewhere. Breast cancer cells obtain their

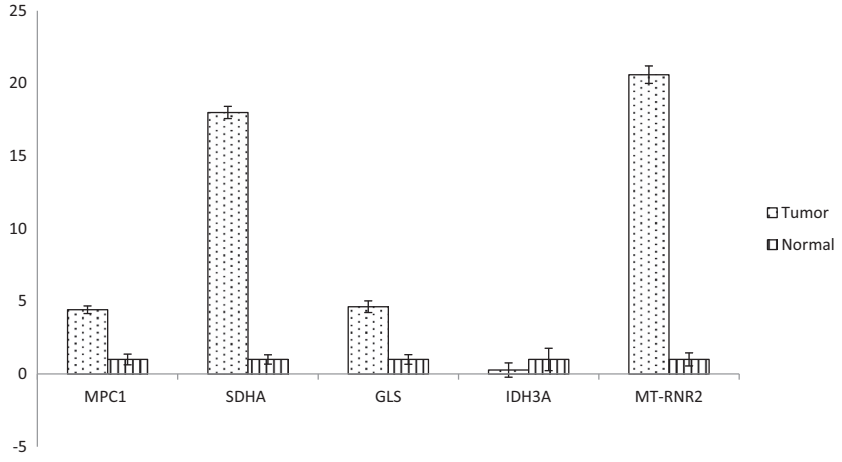

Fig. 1. Expression pattern of MPC1, SDHA, IDH3A, GLS and mt-RNR2 in bladder carcinoma and tumor associated normal.

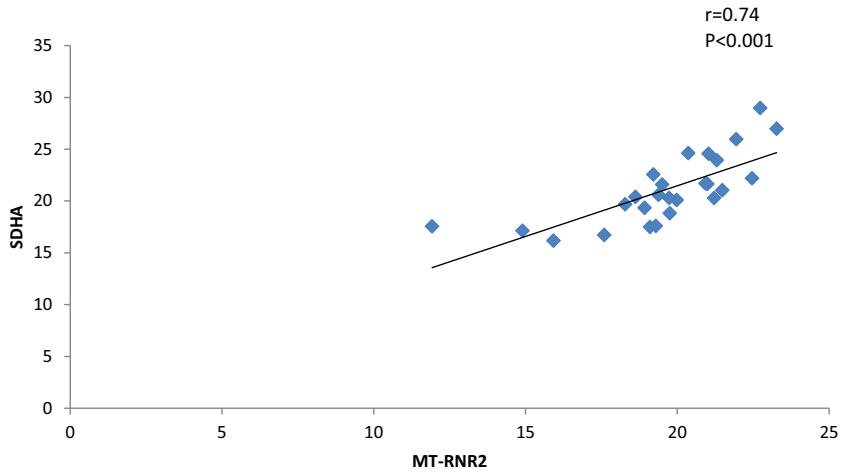

Fig. 2. Significant correlation between SDHA and MT-RNR2 among bladder tumor group.

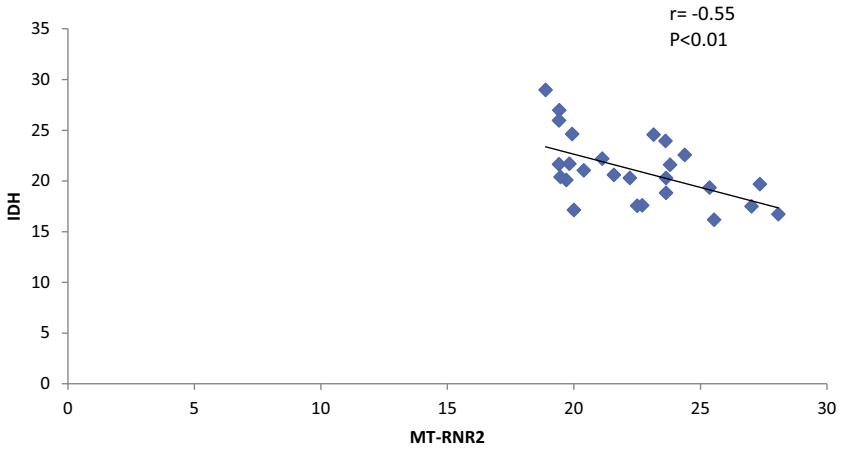

Fig. 3. Significant correlation between IDH and MT-RNR2 among bladder tumor group.

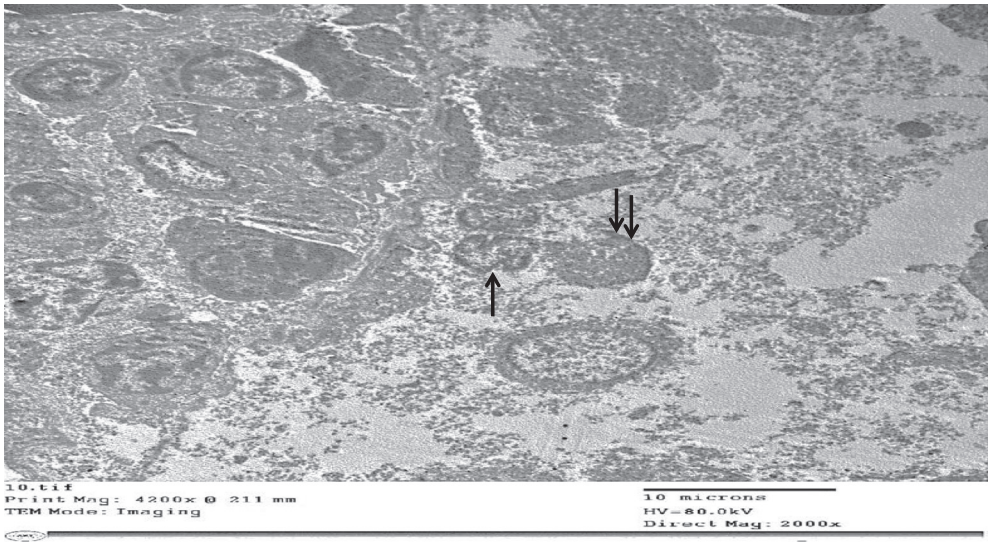

Fig. 4. Transmission electron micrograph of T2 transitional cell carcinoma which is characterized by invasion into muscularis propria. Infiltrating tumor cells permeate the thick muscularis propria wall. It shows a cluster of different shape malignant cells, some cells show karyorrhexis (arrow) and karyolysis(double arrow). The cells also show different shapes nuclei and granular cytoplasm.TEM X 2000.

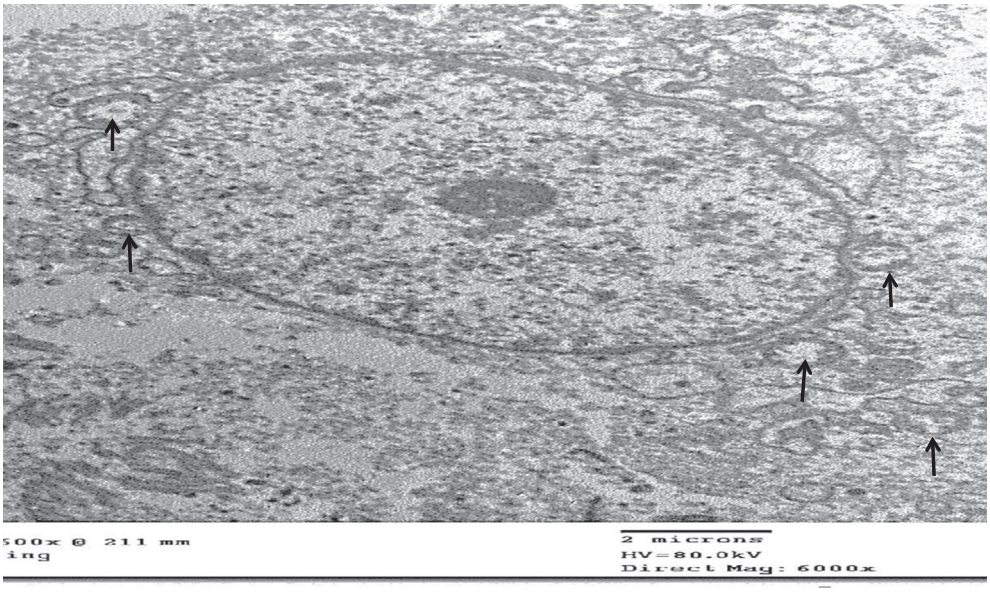

Fig. 5. High magnification of an irregular tumor cell with T2 transitional cell carcinoma shows intact mitochondria (arrow). TEM X 6000.

energy mainly from mitochondria [21], and this seems to be the case for other epithelial cancers as well, for which the activity of cytochrome oxidase (complex IV) is up-regulated causing a hyperactivation of mitochondrial metabolism [22]. A similar feature has been observed in liver and brain cancer, where a fully functional oxidative phosphorylation has been characterized [23]. Mitochondrial oxidative metabolism probably prevails in brain tumors in vivo, as it has been demonstrated that glioblastomas display a preference for glucose oxidation when implanted in mouse brain [24]. But still eluding Warburg's and retaining active mitochondria are integral part of metabolism in normal cells, leaving tumor cells' success in proliferation to remain an unanswered question.

In the present study, tumor cells exhibited isocitrate dehydrogenase down expression which seems to afford cancer cells the leverage of achieving uncontrolled growth. Provided that MPC1 was activated, it seems that Kerbs' cycle goes through the first step only to produce citrate; the precursor of the cytoplasmic acetyl coA which is channeled for fatty acids synthesis. The inactive citrate dehydrogenase will cause the cycle to derail. The found upregulated GLS and SDHA in tumor cells relative to TAN, hints that Krebs' cycle is then resumed after the instant halt but through a new molecule, α keto glutarate. Hence in aggressive tumor cells, rather than feeding the energy producing cycle with the two carbon compound acetyl coA which would surely vanish in the energy producing process as CO_2, the cycle will be rather fed with the five carbon compound α keto glutarate. This way, energy maintenance will maintain proliferation and prohibit apoptosis [25]. Interestingly, is it is a five carbon not a three carbon substrate to start with while the end of oxidative decarboxylation processes is when reaching a two carbon compound.

In accordance with this hypothesis, Vander Heiden, stated that for a cell to proliferate, the bulk of glucose cannot be committed to carbon catabolism for ATP production as there is a higher fold need for glucose to satisfy the carbon requirement of the acyl chain itself [18]. However, in contrast to the current study, the author reasoned that the carbon requirementwould be provided by the Warburg's effect, overlooking that most of the lactate produced is excreted from the cell as waste [19] and thus wasting carbons rather than availing them. In the current study, humanin (MT-RNR2) expression was found to be upregulated in association with activation of succinate dehydrogenase and inactivation of isocitrate dehydrogenase. MT-RNR2 gene is an anti-apoptotic factor which has protective characteristic in different cell types [26]. MT-RNR2 was found to be overexpressed in GC and was suggested to be one of the fundamental mechanisms in chemoresistance of GC cells [27]. The current results suggest that MT-RNR2 could modify the energy produced by mitochondria, a finding suggested before [28]. Furthermore, the current study suggests that MT-RNR2 could have an important role in acetyl CoA saving process through breaking the ritual cycle embraced by normal cells. So in bladder cancer cells, considering the high expression level of MT-RNR2 which is renowned for its anti-apoptotic role as well as its reverse association with IDH, this study assumes that MT-RNR2 plays its anti-apoptotic role partly by avoiding the deployment of energy from the complete oxidation of organic compounds to inorganic wastes. Thus MT-RNR2 can potentially serve as a new biomarker in the diagnosis of BC since it is present in blood circulation [29].

One limitation to the current study is being confined to high grade tumors of aggressive behavior as no low grade tumors were included. On the other hand it still has two points in its favor: first, no compromise to histopathology was exhibited as no tissues were charred during resection; second no undue resectioning to healthy tissue was performed in order to obtain control samples; a condition that may have added extra morbidity to the procedure and may have enquired more ethical elaboration. Future studies are required to find out whether Warburg's is the ruling metabolic pathway in low grade non-invasive bladder tumor and whether MT-RNR2 is the causative of Warburg's retreat upon progression to the high grade invasive stage. In conclusion, aggressiveT2 bladder carcinoma exhibited high expression of mitochondrial humanin (MT-RNR2) which was closely associated with prevalence of mitochondrial respiration where TCA cycle was interrupted except when being fed with glutamine.

Competing interest

The authors declare that they have no competing interests.

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