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Overexpression of ubiquitous mitochondrial creatine kinase (uMtCK) accelerates tumor growth by inhibiting apoptosis of breast cancer cells and is associated with a poor prognosis in breast cancer patients

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ABSTRACT

Background: Ubiquitous mitochondrial creatine kinase (uMtCK), a mitochondrial isoenzyme of creatine kinase (CK), is a central controller of cellular energy homeostasis. Overexpression of uMtCK has been reported to be associated with a poor prognosis for several tumors. The aim of this study was to assess its association with breast cancer (BCa) and to further investigate its underlying mechanisms.
Method: We first detected uMtCK expression by immunohistochemistry in human BCa tissues and assessed the association with the prognosis of patients. We then evaluated uMtCK expression in crowded and normal condition cultures of several human BCa cell lines. After two stable clones of the MDA-MB-231 cell line with high expression of uMtCK were established, cell growth, apoptosis and mitochondrial apoptotic pathway protein expression were measured in these clones. Finally, tumorigenicity of the above cells was assessed using nude mice to explore the relationship between uMtCK expression and tumor progression.
Results: uMtCK expression was detected in 85.5% (47 of 55) of the invasive ductal carcinomas of breast tissue, not otherwise specified (IDC-NOS). Expression in BCa tissue was significantly associated with reduced progression-free survival (PFS; $P = 0.019$) and overall survival (OS; $P = 0.022$) of the patients. Up-regulation of uMtCK expression was identified in crowded BCa cells in culture, and the number of apoptotic cells was significantly decreased in uMtCK transfected MDA-MB-231 cell clones ($P < 0.01$). Stabilization of the mitochondrial membrane potential ($\Delta\Psi m$) and down regulation of cytochrome c (cyt c) and activated caspase 9, two components of mitochondrial apoptotic pathway proteins, were also identified in the same clones when cells were crowded in culture. *In vivo* studies revealed that the transfected tumor cells with uMtCK overexpression induced faster tumor growth in nude mice, along with accelerated animal body weight loss and a significantly lower tumor apoptotic index (AI) ($P < 0.001$).
Conclusion: The results indicated that uMtCK expression is associated with a poor prognosis in BCa and might serve as a tumor marker. *In vivo* and *In vitro* evidence suggests that uMtCK overexpression promotes tumor growth by inhibiting apoptosis of tumor cells through stabilizing $\Delta\Psi m$ and down regulating mitochondrial apoptotic pathway proteins. Exploration of therapeutic agents targeting the expression of uMtCK may have practical value for BCa patients.

Abbreviations: uMtCK, ubiquitous mitochondrial creatine kinase; CK, creatine kinase; BCa, breast cancer; PFS, progression-free survival; OS, overall survival; IDC-NOS, the invasive ductal carcinomas of breast tissue, not otherwise specified; $\Delta\Psi m$, mitochondrial transmembrane potential; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; cyt c, cytochrome c; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; AI, apoptotic cell index.

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1. Introduction

Creatine kinase (CK), a key enzyme in energy metabolism, reversibly catalyzes the transfer of a phosphate group from ATP to creatine and five different isoforms have been identified in mammals: three in the cytosol, namely CKB (brain type), CKM (muscle type) and CKMB (mixed type), and two in mitochondria, namely ubiquitous mitochondrial creatine kinase (uMtCK) and sarcomeric mitochondrial creatine kinase (sMtCK) [1–3]. uMtCK is a central controller of cellular energy homeostasis and is co-expressed with the cytosolic CK in many cells, particularly in tissues with high-energy demands, such as brain, placenta, kidney, testis, sperm, and endothelial cells [4,5].

Overexpression of CK has been demonstrated in a variety of solid tumors and tumor cell lines [6–10]. Elevated CK has been detected in the serum of patients with advanced cancer and is associated with a poor prognosis [7,9,11]. Overexpression of uMtCK has been reported in several cancers with poor prognoses, such as lung cancer, gastric cancer and prostate cancer [6,7,12]. Up-regulation has also been identified in cell lines, particularly in Hodgkin's disease cells [1]. Pang et al. discovered that uMtCK was unregulated in poorly differentiated prostate cancer tissue [13]. Cimino et al. studied gene expression profiling with microarray analysis in two sets of breast cancer (BCa) tissue from patients with distinct clinical outcomes and identified that uMtCK expression was significantly associated with both disease-free and overall survival of patients. They concluded that it could serve as an independent prognostic marker [14].

The expression of uMtCK at a protein level, as well as its role in tumor progression, has not yet been comprehensively studied in BCa. The aim of this study was to explore the associations of uMtCK expression with biological behavior and the prognosis of the invasive ductal carcinomas of breast tissue, not otherwise specified (IDC-NOS) by immunohistochemistry, cell culture and using an animal model, and to further investigate its underling mechanisms.

2. Materials and methods

2.1. Primary BCa specimens

Hematoxyline and eosin stained tissue sections of 55 patients with IDC-NOS that were diagnosed in March 2003 were retrieved from the archives of the Department of Breast Pathology and Research Laboratory, Tianjin Medical University Cancer Hospital (China), and were reviewed. All patients were female, and the mean age was 52.9 years (range, 35–76 years). None of the patients received preoperative radiation or chemotherapy, and all patients had complete follow-up data, with a mean follow-up of 56.8 months (10–101 months).

2.2. uMtCK immunohistochemistry

uMtCK immunohistochemistry was performed as previously described [13]. Cases were considered positive if cytoplasmic immunoreactivity was present in the cytoplasm of tumor cells. The staining was scored semi-quantitatively based on the staining intensity as following: −, no reactivity; 1+, weak reactivity; and 2+, strong reactivity.

2.3. Cell culture and cell crowding

Human BCa cell lines, MCF-7, T47D, BT-549, and MDA-MB-231, were purchased from the American Type Culture Collection (ATCC) and preserved in our lab. They were propagated and subcultured as recommended by ATCC.

Crowding experiments were performed as described in Murray's research [15]. Briefly, MDA-MB-231 cells were seeded and when the cells became 100% confluent, the medium was changed daily until the termination of the experiment at 72 h.

2.4. Establishment of uMtCK stable transfected BCa cells

The pcDNA3.1(−)/Myc-His B vector (Invitrogen Life Technologies) with a human uMtCK coding region in frame with a His-Myc tag was a gift from Professor Jian-Guang Zhou and was previously used in Pang's research [13]. The MDA-MB-231 cell line was transfected with the gene-specific expression vector by Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's protocol, and the empty vector was used as a control. Protein expression of uMtCK was evaluated by Western Blotting after the cells were cultured for 3 weeks in an appropriate medium containing 0.5 mg/ml of G418 (Sigma). Two stable clones overexpressing uMtCK were identified and were named MDA-MB-231-uMtCK clone 1 and MDA-MB-231-uMtCK clone 2, respectively. The control clone with empty vector stable transfection was named MDA-MB-231-null.

2.5. Cell growth curve analysis

The cell growth rate was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay. MDA-MB-231 cells were seeded 1000 per well in 96-well plates and incubated overnight for cell adherence. The culture media was then replaced by new media containing 0.5 mg/mL MTT, and the cells were further incubated for 4 h at 37 °C. Then 100 μL of dissolving reagent DMSO (Amresco, Inc.) was added to dissolve the formazan crystals. The absorbance was measured at a wavelength of 490 nm (A_{490}) on a microplate reader (Bio-Rad). Wells without seeded cells were used as a blank for the reading adjustment of each well. Each experiment was repeated independently seven times. The mean adjusted reading of the seven repeats in the first day after seeding (0 day) was defined as 1, and the reading changes in folds (A_{490} ratio) each day afterward were calculated.

2.6. Cell apoptosis assay

A cell crowding culture was performed as described above. Crowded cells were harvested and a cell apoptosis assay was performed using the method in Chen's research [16]. The lower right quadrant (LR) represents early-stage apoptotic cells that were positive for Annexin V-FITC and negative for propidium iodide (FITC+/PI−).

2.7. Measurement of mitochondrial transmembrane potential ($\Delta\Psi m$)

Crowded cells were harvested and Rhodamine-123 staining was used to detect the change in $\Delta\Psi$m via flow cytometry as described in Zhang's research [17]. The mean fluorescence intensity of Rhodamine-123 staining was used to compare the $\Delta\Psi$m of different cell clones. MDA-MB-231 cells without Rhodamine-123 staining were used as a negative control.

2.8. Western blot

Holoproteins in cell lysates were extracted, quantitated and Western Blotted as previously described [13] with exception of the following antibodies: anti-human uMtCK antibody (Abnova, Inc.); anti-β-actin antibody (Santa Cruz Biotechnology, Inc.); anti-6 × His tag antibody and anti-caspase-9 antibody (Cell Signaling

Technology, Inc.); and anti-cytochrome c (cyt c) antibody (Abcam, Inc.).

2.9. In vivo tumorigenicity study

Female nude athymic BALB/c mice, 6–8 weeks old (Vital River Laboratories, China) were employed. A total of 1×10^6 cells at the exponential growth phase were suspended in 100 μL serum-free RPMI 1640 containing Matrigel (1:1 vol/vol; BD Biosciences) and injected subcutaneously into the back of the mouse s.c. via a 27-gage needle. The tumor volume was measured and the mouse was weighed weekly. Tumor volumes were calculated by the formula $L \times W \times H \times 0.5236$. After 8 weeks, tumors were removed at necropsy and fixed in 4% neutral buffered formalin for 24 h, and were then embedded in paraffin. 4 μm sections were cut after tissues were embedded in paraffin. A terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay was then performed as previously described [18]. All nuclei with TUNEL positive signals were counted in three randomly selected fields for each case that contained at least 1000 tumor cells in each field. The counts were converted into the number per 100 tumor cells, and the mean of the three randomly selected fields were used to calculate the apoptotic index (AI).

2.10. Statistical analyses

The SPSS 15.0 software package was chosen for statistical analysis. Progression-free survival (PFS) and overall survival (OS) rates were estimated with the Kaplan–Meier product limit method. Statistical analyses were carried out using a *T* test for normally distributed data. The experimental points represent the mean ± standard deviation of 3–10 replicates. A Mann–Whitney *U* test was used to determine the significance of the differences between two groups of data with a skewed distribution, which is represented by the median (interquartile range); e.g. M (Q_R). A 2-sided $P < 0.05$ was considered statistically significant in all the analyses.

2.11. Ethics statement

All human tumor tissues were collected from Tianjin Medical University Cancer Hospital with written informed consent from patients prior to participation in the study. The protocols for collection and analysis of the samples were approved by the Institutional Review Board of the Tianjin Medical University Cancer Institute and Hospital in accordance with the current revision of the Helsinki Declaration. The Institutional Animal Care and Use Committee of the Tianjin Medical University Cancer Institute and Hospital approved use of the animal models in this study in accordance with the EU Directive 2010/63/EU for animal experiments.

3. Results

3.1. Tumor tissue uMtCK expression detected by immunohistochemistry and its association with patient prognosis

The expression of uMtCK protein was identified in 47 of 55 (85.5%) cases of IDC-NOS and 29 (52.7%) demonstrated weak cytoplasmic staining. The remaining 18 (32.7%) showed strong staining intensity (Fig. 1A). uMtCK expression was positively correlated with tumor stage ($P < 0.05$), and was expressed preferentially in the estrogen receptor negative tumors ($P < 0.05$). Kaplan–Meier univariate analysis revealed a reduced PFS ($\chi^2 = 7.980$, $P = 0.019$; Fig. 1B) and OS ($\chi^2 = 7.592$, $P = 0.022$; Fig. 1C) in patients with

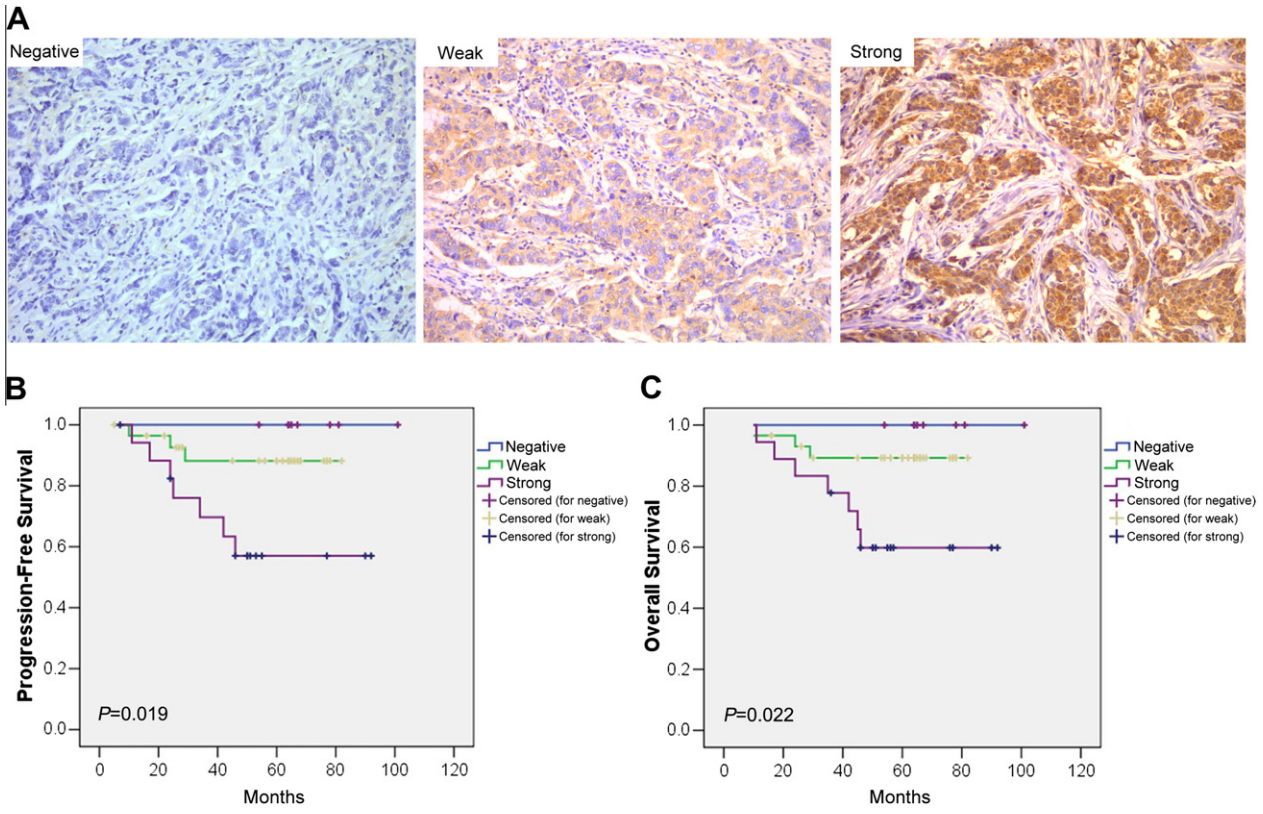

Fig. 1. uMtCK expression and its relationship with the prognosis of IDC-NOS. Examples of negative, weak and strong expression of uMtCK expressed in the cytoplasm of tumor cells (×200) are shown in (A). Kaplan–Meier curves of PFS (B) and OS (C) for uMtCK expression in 55 patients with IDC-NOS show the association of uMtCK expression with reduced PFS ($P = 0.019$) and OS ($P = 0.022$). *P* values were calculated using a log-rank test.

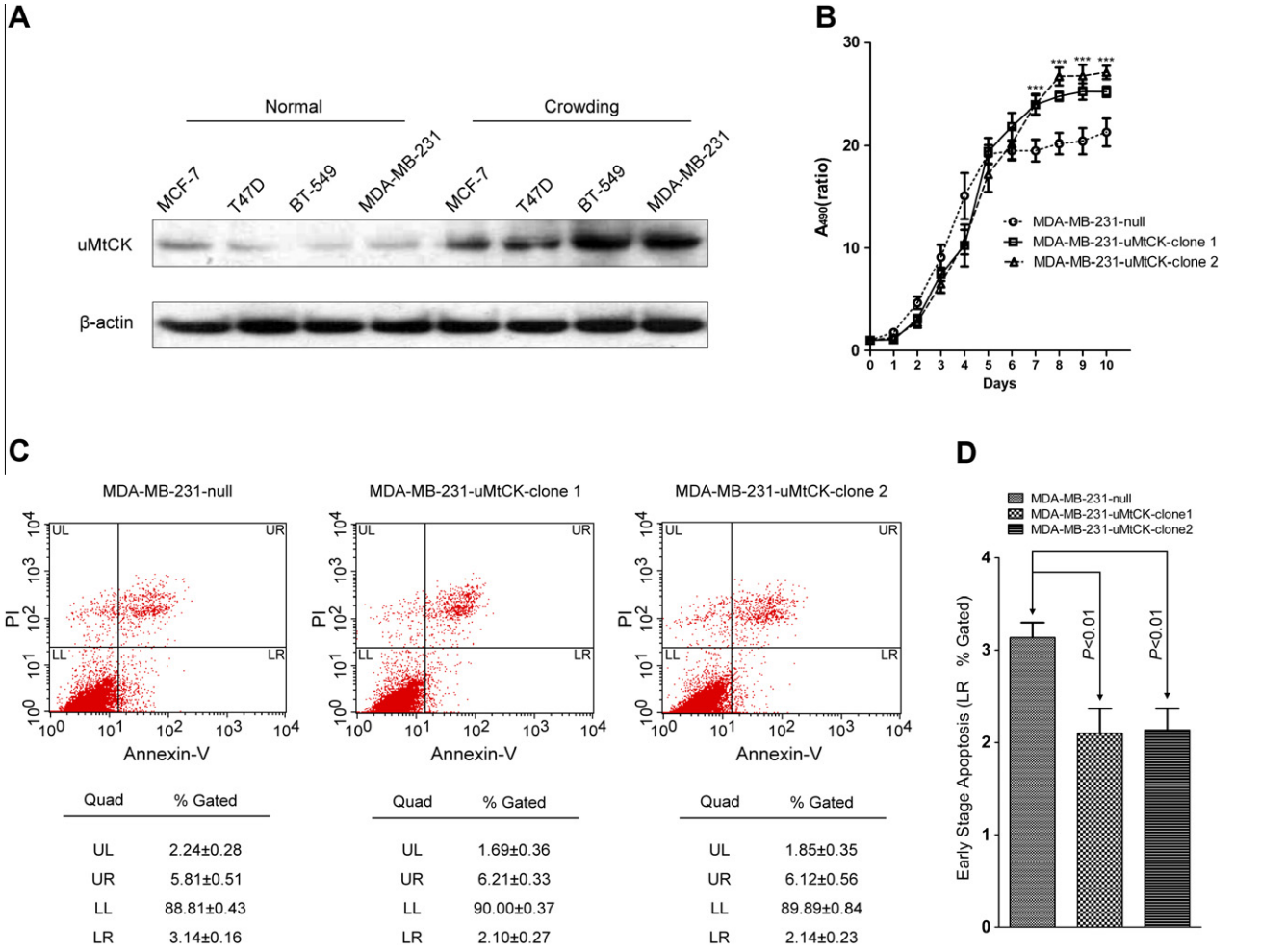

Fig. 2. uMtCK expression and phenotypes of BCa cells. (A) Western Blot analysis suggested that uMtCK is upregulated in crowded MCF-7, T47D, BT-549 and MDA-MB-231 BCa cells. (B) MTT assay suggested that exogenous overexpression of uMtCK rescues MDA-MB-231 cells from growth inhibition caused by cell crowding (***$P < 0.001$). (C) Flow cytometric analysis of PI and Annexin-V quantifies apoptosis in uMtCK overexpressed crowded MDA-MB-231 cells. (D) The numbers of early apoptotic cells were significantly decreased compared to the control group.

up-regulation of uMtCK in their tumors. Additional clinicopathological features, the tumors and their expression of uMtCK are shown in Table S1.

3.2. uMtCK expression of crowded cells and its association with cell growth and apoptosis

Thirty micrograms of holoprotein was extracted from the crowded and normally cultured cells (60% coverage) from each of the BCa MCF-7, T47D, BT-549 and MDA-MB-231 cell lines for immunblotting with anti-uMtCK specific antibody. β-actin served as an internal control. It was found that uMtCK was significantly upregulated in the crowded cells (Fig. 2A).

The MTT assay for the proliferation of the MDA-MB-231 cell line demonstrated that after 100% confluence (7–10 days), the fold changes (A_{490} ratio) in cell growth by adjusted readings of both MDA-MB-231-uMtCK clone 1 and MDA-MB-231-uMtCK clone 2 were significantly higher than those of the MDA-MB-231-null control ($P < 0.001$; Fig. 2B).

Flow cytometry analysis showed that exogenous overexpression of uMtCK inhibited MDA-MB-231 cell apoptosis when cells were crowded (Fig. 2C). The number of early apoptotic cells was significantly decreased compared with that of the control group ($P < 0.01$; Fig. 2D).

3.3. uMtCK overexpression and the initiation of mitochondrial apoptotic pathway in crowded BCa cells

Apoptosis is associated with a loss of $\Delta\Psi m$ and Rhodamine-123 is a commonly used indicator of $\Delta\Psi m$ via flow cytometry [19]. When cells were crowded, the Rhodamine-123 staining peak of both MDA-MB-231-uMtCK clones significantly shifted to the right, in comparison with that of the MDA-MB-231-null cells (Fig. 3A). Meanwhile, the mean fluorescent intensity of both of the uMtCK transfected cell clones was 15% higher than that of the control clone ($P < 0.001$; Fig. 3B). These results indicated that overexpression of uMtCK stabilized $\Delta\Psi m$ when cells were crowded.

To determine whether alteration in uMtCK protein expression was related to the mitochondrial apoptosis pathway in crowded BCa cells, we assessed the expression of cyt c and clevated (activated) caspase 9 proteins by Western blot analysis. A remarkable decrease in cyt c and activated caspase 9 was noted in the two MDA-MB-231 clones with uMtCK stable transfection (Fig. 3C).

3.4. Overexpression of uMtCK and tumor progression and apoptosis of MDA-MB-231 tumor cell inoculated nude mice

Tumor cells from MDA-MB-231-uMtCK clone 1 and MDA-MB-231-null were used for inoculation, and each group consisted of

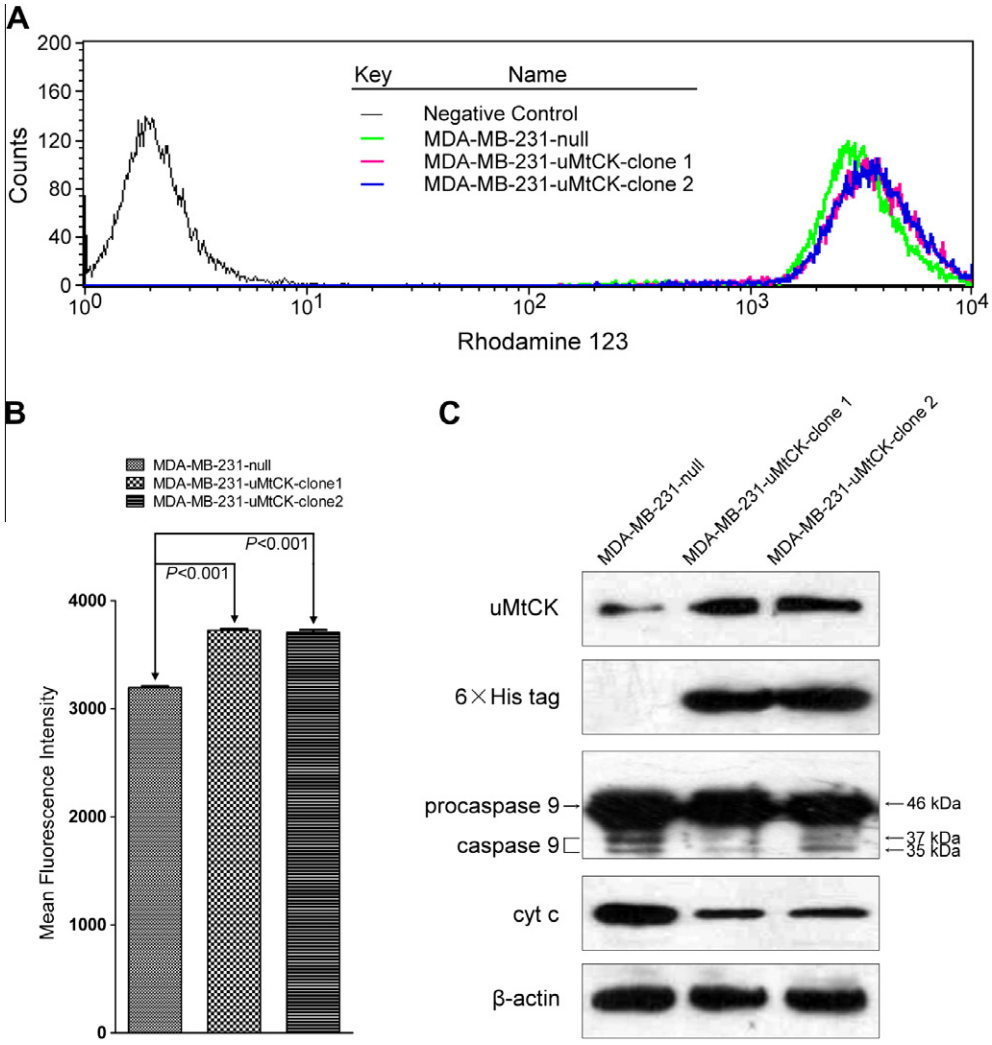

Fig. 3. uMtCK expression and mitochondrial apoptosis pathway when cells were crowded. (A) Flow cytometric analysis shows the Rhodamine-123 staining peak of both uMtCK overexpressed MDA-MB-231 clones with a significant shift to the right compared to that of MDA-MB-231-null when cells were crowded. (B) The mean fluorescent intensity of the former two cell clones was significantly higher than that of the latter clone. (C) Mitochondrial apoptosis pathway-related molecules, cyt c and clevated (activated) caspase 9, were down-regulated in uMtCK overexpressed MDA-MB-231 clones when the cells were crowded.

8 nude mice. All injected mice developed visible tumors as early as 14 days. Starting from the fifth week, the size of the MDA-MB-231-uMtCK bearing tumors was larger than the MDA-MB-231-null bearing tumors and after 8 weeks of inoculation, the average tumor size in the former group was 9.40-fold larger than that of the latter control group ($P < 0.05$; Fig. 4A). Meanwhile, the gain in body weight of MDA-MB-231-uMtCK tumor bearing mice was significantly lower than that of MDA-MB-231-null tumor bearing mice beginning at the fourth week and, at the end of 8 weeks after inoculation, the body weight gain of the former group was 38.5% lower than that of the latter control group ($P < 0.05$; Fig. 4B).

TUNEL staining of tumor tissues showed that the MDA-MB-231-uMtCK inoculated tumors had remarkably less apoptosis than the MDA-MB-231-null inoculated tumors (Fig. 4C). The average AI of MDA-MB-231-uMtCK inoculated tumors was 43.7% lower than that of MDA-MB-231-null inoculated tumors ($P < 0.001$; Fig. 4D).

4. Discussion

BCa is a critical type of malignant tumor and the mechanisms by which cancer cells can rapidly grow and jeopardize human life is still under active investigation. Although the role of uMtCK expression has been studied in several tumors, very limited studies on BCa have been performed. This is the first report on the expression of uMtCK being detectable at the protein level in a large proportion of IDC-NOS (85.5%) cases and almost one-third of the 55 tumors (32.7%) showed strong cytoplasmic staining. In addition, follow-up data analysis revealed that uMtCK expression was significantly associated with reduced PFS and OS in this cohort of patients, indicating the potential as a biomarker for a poor prognosis in BCa patients. Additional studies identified that overexpression of uMtCK promoted BCa cell growth in culture and tumor growth in nude mice, suggesting it to be an active player in BCa progression. uMtCK inhibited cell apoptosis when tumor cells became congested in culture, which presumably helped BCa cells to thrive. With identification of these basic facts, we further explored how uMtCK takes part in the cascade of BCa progression.

The mechanisms by which cancer cells escape the growth constraints imposed on normal cells by cell crowding are poorly understood [20]. A complex set of changes occur, including depletion of components of the media and pH changes, when cells become confluent [15]. The mitochondrial pathway involves release of cyt c from the intermembrane space to the cytosol, loss of

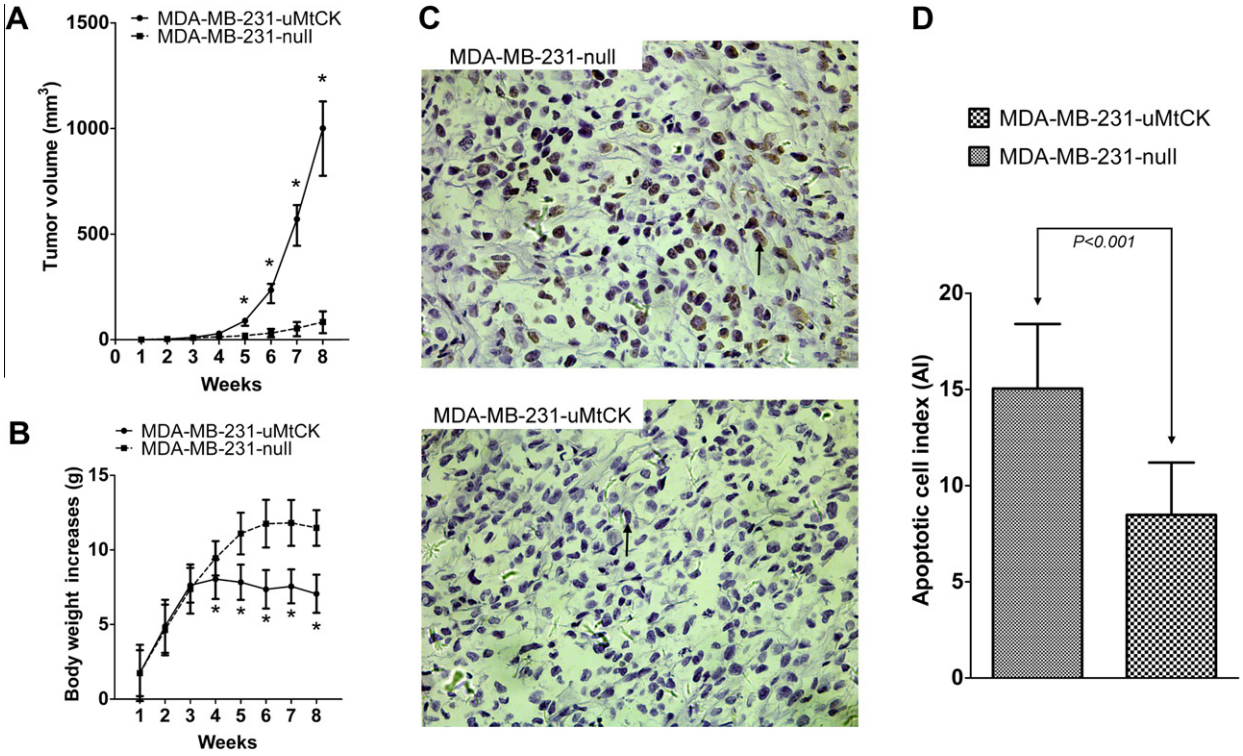

Fig. 4. Effect of uMtCK expression analyzed in BCa cell inoculated nude mice. Overexpression of uMtCK accelerates tumor growth (A) and reduces body weight gain (B) in MDA-MB-231 inoculated nude mice (*$P < 0.05$). MDA-MB-231-uMtCK and MDA-MB-231-null inoculated tumors stained with TUNEL are shown in (C) and the arrows indicate the apoptotic cells. The AI of the MDA-MB-231-uMtCK inoculated tumor is significantly lower than that of the MDA-MB-231-null inoculated tumor (D).

$\Delta\Psi$m, hyperdensity of the matrix, and shrinkage of the organelles [21–23]. Loss of $\Delta\Psi$m results in the release of cyt c, which facilitates the formation of apoptosomes by interacting with apoptotic protease activating factor 1 (Apaf-1). It then recruits and activates procaspase-9 to start the proteolytic cascade that ultimately leads to cell disintegration [24]. It is well-known that $\Delta\Psi$m, an important mitochondrial factor in apoptosis induction, is sustained by the hydrogen ion concentration gradients on both sides of the mitochondrial inner membrane. Hence the complicated changes triggered by cell crowding are likely able to initiate the mitochondrial apoptosis pathway. Lenz et al. discovered that, in Human keratinocyte HaCaT and cancer HeLa cells, uMtCK inhibition led to a reduction in $\Delta\Psi$m and drastic changes in mitochondrial shape and morphology [25]. Based on these studies, we speculate that uMtCK might help to overcome crowding and restore cell proliferation in BCa cells by inhibiting the mitochondrial apoptosis pathway.

In exploring the relationships of cell crowding, uMtCK expression and the mitochondrial apoptosis pathway using the uMtCK-transfected BCa cell lines, our research indicated that overexpression of uMtCK can stabilize $\Delta\Psi$m after cells reach confluence in culture, and therefore the remarkable decrease of cyt c and activated caspase 9 eventually prevents the initiation of cellular apoptosis.

In vivo study to investigate the tumorigenicity of uMtCK-transfected BCa cells in nude mice revealed that uMtCK overexpression significantly increased the growth rate of bearing tumors, with early appearance of cancer cachexia and significant reduction in the number of apoptotic tumor cells.

In the current study, the *in vitro* results of cytobiology, animal models and statistical analysis of patient samples demonstrated a high degree of consistency, which strongly argues for uMtCK expression playing an important role in escaping growth inhibition through the mechanism of apoptosis inhibition. Our data also suggest uMtCK expression is a potential marker for predicting the aggressive behavior of BCa. Further investigation of uMtCK silencing by RNA interference or of blocking its function by specific agents may lead to sufficient intervention and slowing down or even stopping BCa progression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.147.

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