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Genetic deletion and pharmacological inhibition of Akt1 isoform attenuates bladder cancer cell proliferation, motility and invasion

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Abstract

Isoform specific expression, intracellular localization and function of Akt in bladder cancer are not known. In the current study, we identified Akt1, followed by Akt2 and Akt3 as the predominant Akt isoform in human T24 and UN-UC-3 metastatic bladder cancer cells. Whereas Akt1 is localized at the membrane, cytoplasm and nucleus, Akt2 is solely cytoplasmic and Akt3 is mostly localized in the nucleus in T24 cells. ShRNA-mediated Akt1 knockdown resulted in impaired T24 cell survival, proliferation, colony formation, migration and microinvasion. Whereas pharmacological inhibition of Akt1 resulted in impaired T24 and UN-UC-3 cell motility, viability and proliferation, effect of pharmacological inhibition by Akt2 inhibitor was limited to proliferation in T24, but not UM-UC-3 cells. Our data provide important clues on the therapeutic benefits of targeting Akt1 for bladder cancer therapy.

Keywords

Akt1; Akt2, Akt3; bladder cancer; proliferation; invasion

1. Introduction

Bladder cancer is the fourth most common cancer among men in the Western countries (Siegel et al., 2014). Lack of tools for the early detection and therapeutic strategies for metastatic bladder cancer are the major problems. Candidate genes regulating bladder cancer

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are still under investigation. Recent studies indicate that de-regulation catalytic domain of PI3-Kinase (p110 α), phosphatase and tensin homolog (PTEN), Akt, p53, and fibroblast growth factor receptor (FGFR) can lead to the development and growth of bladder cancer (Aveyard et al., 1999; Cairns et al., 1998; Cappellen et al., 1997; Junker et al., 2008; Knowles et al., 2009; Lopez-Knowles et al., 2006). High-grade non-invasive and invasive bladder cancers are linked with loss of PTEN and p53 activity (Ching and Hansel, 2010; Puzio-Kuter et al., 2009). Mutations or loss of PTEN has been linked to many cancers (Cairns et al., 1997; Coleman et al., 2014; Huang et al., 2014; Kim et al., 1998; Li et al., 2014; Risinger et al., 1997; Sakurada et al., 1997), including bladder cancer (Aveyard et al., 1999; Cordon-Cardo, 2008; Kanda et al., 2009) thus generating research interest in PTEN/Akt pathway in bladder cancer (Saal et al., 2007).

Akt (protein kinase B) is a serine-threonine kinase that exists in three different isoforms namely Akt1, Akt2 and Akt3 (Somanath et al., 2006). Although Akt has been demonstrated in bladder cancer (Mundhenk et al., 2011), the predominant isoform expressed in bladder cancer and its effect on oncogenic response is not yet clear. Mutation in Akt1 E17K has been reported in 2/44 (4.8%) bladder cancer cell lines and 5/184 (2.7%) bladder tumors (Askham et al., 2010). In addition, significantly higher levels of Serine-473 phosphorylated (active) Akt have been reported in primary bladder carcinoma (Harris et al., 2008; Qian et al., 2009; Wu et al., 2004). However, until today, whether Akt1 is the predominant isoform in bladder cancer cells and whether its activation is necessary for bladder cancer progression is not clear. Here, we examined isoform specific expression and subcellular localization of Akt isoforms in metastatic human T24 bladder cancer cells.

2. Material and Methods

2.1. Cell Lines, reagents and antibodies

Human T24 (bladder carcinoma) cells, UM-UC-3 (bladder carcinoma) and human dermal micro-vascular endothelial cells (HMEC) were obtained from the ATCC (Manassas, VA). All cell lines were maintained in DMEM (HyClone, Logan, UT) with 10% FBS, 100 Units/ml penicillin, and 100 μ g/ml streptomycin in a 5 % CO₂ atmosphere at 37 °C. Primary antibodies for Akt1 (C73H10 clone of Rabbit mAb Cat #2938), Akt2 (L79B2 clone of Mouse mAb Cat #5239) and Akt3 (L47B1 clone of Mouse mAb Cat #8018) were purchased from Cell Signaling Technology (Danvers, MA). β -actin antibodies (AC74 clone of Mouse mAb Cat #5316) were from Sigma-Aldrich (St. Louis, MO). Inhibitors of Akt1 (A674563) and Akt2 (CCT128930) were obtained from Selleck Chemicals (Houston, TX). HRP-conjugated secondary antibodies (Cat #170-6515 for goat anti-rabbit; Cat #170-6516 for goat anti-mouse) were obtained from Bio-Rad (Hercules, CA) and Alexa Fluor 488 labelled antibodies (Cat #A11008 for goat anti-rabbit; Cat #11001 for goat anti-mouse) was from Invitrogen (Grand Island, NY).

2.2. Preparation of ShControl and ShAkt1 stable T24 cells

ShControl and ShAkt1 Stable cells were developed using lentiviral infection. ShControl (scrambled) and ShAkt1 lentiviral particles were obtained from (Dharmacon, Lafayette, CO). Lentiviral particles at 1 $\times$ 10⁶ pfu along with 8 μ g/ml polybrene (Sigma, MO) were

added to 70-80% confluent T24 cells cultured in 6 well plates. One day later, media with virus was removed and supplemented with fresh medium. After 72 h post virus infection, cells were incubated with 15 µg/ml puromycin (Sigma, MO) for antibiotic selection.

2.3. T24 Cell doubling-time assessment

In each experiment, T24 cell doubling time was determined using the direct cell count and in consideration of logarithmic growth of cancer cells (Kochuparambil et al., 2011). Approximately 20 cells/well were seeded in 200 µl of medium on a 96-well plate in quadruplicates, and counted after 24 h.

2.4. Proliferation assay

Proliferation of T24 and UM-UC-3 cells was determined using the non-radioactive BrdU-based cell proliferation assay (Roche, Switzerland) (Goc et al., 2012b). T24 cells, after lentiviral transfections were seeded in 96-well plates at a density of 5×10^3 cells/well. 5 µM each of A674563 and CCT128930 were used along with cells treated with control vehicle DMSO. After 24 h, cells were subjected to a 5-bromo-2-deoxyuridine assay using the BrdU Labeling and Detection Kit III (Roche, Switzerland). BrdU incorporation into the DNA was determined by measuring the absorbance at both 450 and 690 nm on ELISA plate reader.

2.5. Trypan blue viability assessment

For the pharmacological inhibition studies 5 µM each of A674563 and CCT128930 were used along with T24 and UM-UC-3 cells treated with control vehicle DMSO. After 24 h, cells were collected and re-suspended in 1 x PBS with 0.4% trypan blue solution. Total cells and trypan blue-stained (i.e. nonviable) cells were counted, and the percentage of nonviable cells was calculated (Goc et al., 2011).

2.6. Apoptosis assay

Apoptosis was measured using Cell Death Detection ELISA^{PLUS} kit (Roche, Switzerland) (Goc et al., 2013). T24 cells were plated in 96-well plate at a density of 10^4 cells/well. After 24 h, cells were lysed and centrifuged at 200g for 10 min, and the collected supernatant was subjected to ELISA. The absorbance was measured at 405 nm (reference wavelength, 492 nm).

2.7. Colony formation assay

T24 cells were cultured on 12-well plates until the monolayer was reached. Seven days later, each of the wells was counted for the number of colonies, and ShAkt1 transfected cells were compared with the ShControl-transfected cells. Plates were fixed using 2% paraformaldehyde, briefly stained with crystal violet, and counted visually or by using ImageJ software (Goc et al., 2014).

2.8. Migration assay

T24 and UM-UC-3 cells were grown to confluence in DMEM with 10% FBS. A scratch was made in the monolayer and scratch recovery was determined after 12 h. Microscopic pictures were analyzed using ImageJ software and the percentage recovery was calculated

using the equation $100 \times (1 - T_f/T_0)$, where T_f is the area at the endpoint (i.e. 12 h) and T_0 is the area at the time 0 (Al-Azayzih et al., 2012).

2.9. Invasion (trans-endothelial migration) assay

ShControl and ShAkt1 T24 bladder cancer cells were detached from culture plates using cell dissociation buffer (20mM EDTA in PBS, pH 7.4) prior to invasion assay. Trans-endothelial migration of bladder cancer (T24) cells was measured using Electric Cell-substrate Impedance Sensing (ECIS) equipment with a monolayer of HMEC plated on 8W10E+ array chips (Applied Biophysics, Troy, NY). Assay was performed as previously published from the laboratory (Goc et al., 2012a). Once a monolayer of HMEC is established, ShControl T24 and ShAkt1 cells were directly added onto HMEC monolayers at a density of 5×10^4 cells/well in 50 μ l medium. Real-time measurements on the trans-endothelial migration of T24 cells were recorded by the ECIS in the form of changes in the endothelial-barrier resistance at every 1 h for 5 h.

2.10. Immunofluorescence staining

We subjected human T24 metastatic bladder cancer cells for immunocytochemistry analysis to determine the endogenous expression and localization of three Akt isoforms Akt1, Akt2 and Akt3. T24 cells were plated on cell culture chamber slides (Fisher scientific, Pittsburgh, PA) followed by fixation with 1 % paraformaldehyde in 1 X PBS. Cells were permeabilized with 0.1% triton X-100 in PBS and blocked with 2% BSA for 1 h, and incubated with 1:1000 primary antibodies over-night in 4 °C. Alexa Fluor 488 labeled secondary antibodies were applied for 1 h at room temperature washed and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images were taken by confocal fluorescent microscope (Zeiss Axiovert100M, Zeiss, Germany) and analyzed using NIH Image J software (Al-Husein et al., 2013).

2.11. Western blot analysis

We subjected human T24 and UM-UC-3 bladder cancer cells for Western blot analysis to determine the endogenous expression of three Akt isoforms Akt1, Akt2 and Akt3 using isoform specific antibodies. T24 and UM-UC-3 cell lysates were prepared using lysis buffer [50 mM Tris-HCl, pH 7.4, 1 % Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4 , and 1 $\times$ complete protease inhibitors (Roche Applied Science)]. The protein concentration was measured by the D_L protein assay (Bio-Rad, Hercules, CA) (Goc et al., 2014). All the densitometry analysis was performed using NIH Image J software and the data was normalized to loading control β -actin.

2.12. Statistical analysis

Paired two-tailed student t-test was used to determine the level of significance. A 'P value' less than 0.05 were considered statistically significant. Results were expressed as mean $\pm$ S.D. for at least n=3 experiments.

3. Results

3.1. Akt1 is the predominant Akt isoform in T24 and UM-UC-3 bladder cancer cells

Our results indicated that expression level of Akt1 in T24 and UM-UC-3 cells is 2-fold higher than Akt2 (Figure 1A-B and Figure 2A-B, respectively). Whereas expression level of Akt3 is very low in T24 cells, it was not in the detectable levels in UM-UC-3 cells. Overall, we did not observe any changes in the expression levels of Akt1, Akt2 or Akt3 in T24 and UM-UC-3 cells upon 12 h serum starvation as compared to cells cultured in the presence of 10% FBS or 50 nM EGF (Figure 1A-B and Figure 2A-B, respectively).

Immunocytochemistry analysis revealed that Akt1 is ubiquitously expressed in T24 bladder cancer cells in the nucleus, cytoplasm and at the membrane (Figure 1C). In contrast, Akt2 is mostly localized in the cytoplasm, and little expressed Akt3 is mostly present in the nucleus.

3.2. Akt1 deficiency impairs T24 bladder cancer cell functions in vitro

To determine the role of predominant Akt isoform in bladder cancer cells, we generated stable T24 cells deficient in Akt1 via lenti-viral-mediated ShRNA silencing (Figure 3A). Silencing Akt1 gene resulted in impaired colony formation by T24 cells ($P<0.01$) (Fig. 3B). Our data revealed that Akt1 deficiency results in 30 % increase in the doubling time ($P<0.001$) (Fig. 3C) and 25% decrease in proliferation ($P<0.05$) (Fig. 3D) when cultured in 10 % FBS conditions or in 12 h serum deprived conditions (Suppl. Fig. 1). Lack of Akt1 also resulted in significantly increased number of non-viable and apoptotic T24 cells compared to control cells ($P<0.05$) (Fig. 3E and 3F; Suppl. Fig. 1).

3.3. Akt1 is important for T24 bladder cancer cell migration and invasion

To determine the effect of Akt1 deficiency on cell migration and invasion, we subjected ShControl and ShAkt1 T24 cells for migration assay using a monolayer scratch assay. Reduction in Akt1 in T24 bladder cancer cells significantly inhibited their migration ($P<0.05$) (Figure 4A). Using a highly sensitive *in vitro* ECIS (Electric Cell-substrate Impedance Sensing) technology, we tested if Akt1 isoform might mediate T24 cell transendothelial migration. Silencing Akt1 in T24 cells significantly abolished their invasive potential (Figure 4B).

3.4. Akt2 regulates proliferation but not viability and motility of T24 cells

We determined the efficacy of pharmacological inhibition of Akt1 isoform in bladder cancer cells on viability, motility and proliferation as compared to pharmacological inhibition of Akt2, the next predominant isoform expressed in bladder cancer cells. Pre-treatment of T24 and UM-UC-3 cells with Akt1 inhibitor A674563, but not Akt2 inhibitor CCT128930 resulted in a dose-dependent inhibition of cell migration ($P<0.001$ and $P<0.05$, respectively) (Figure 5A and 6A). Interestingly, a dose of 5 μM (but not 10 and 20 μM) dose of Akt2 inhibitor was observed to enhance the motility of T24 cells. Whereas pretreatment of T24 and UM-UC-3 cells with Akt1 inhibitor resulted in 4-fold increase in the number of non-viable cells, pretreatment with Akt2 inhibitor had no significant effect on cell viability ($P<0.0001$ and $P<0.05$) (Figure 5B and 6B, respectively). Interestingly, pharmacological inhibition of both Akt1 and Akt2 resulted in a robust and significant inhibition of T24

bladder cancer cell proliferation, respectively ($P < 0.001$ and $P < 0.05$, respectively) (Figure 5C and 6C). However, effect of Akt2 inhibitor on proliferation was not observed in UM-UC-3 cells.

4. Discussion

The PI3K/Akt signaling is one of the most frequently de-regulated pathways in human cancers (Grant, 2008; Knowles et al., 2009; Mitra et al., 2006; Sun et al., 2011; Szanto et al., 2009). Since Akt is closely involved in a variety of cellular hallmarks of cancer such as survival, proliferation, migration and invasion, it has become a potential therapeutic target (Cuconati et al., 2013; Jazirehi et al., 2012; Madhunapantula et al., 2011). Despite these advances, specific functions of Akt in metastatic bladder cancer cells have not yet been investigated. Since Akt exists in three different isoforms, it is not known which isoform is predominantly expressed and plays a major role in invasive bladder cancer cells. In the current report, we present the evidence that Akt1, followed by Akt2 and Akt3, is the predominantly expressed Akt isoform, which is responsible for the bladder cancer cell tumorigenic and metastatic phenotype. Moreover, our findings emphasize that specific pharmacological inhibition of Akt1 can be a potential strategy for bladder cancer therapy.

Currently, the major issues in targeting Akt for cancer therapy are (1) that there are 3 isoforms of Akt expressed from three different chromosomes (Somanath et al., 2006), which may have various cellular function, and (2) that Akt inhibitors used in cancer clinical trials are broad spectrum and not isoform specific (Berndt et al., 2010; Yang et al., 2004). Apart from this, although predominant isoform control major cellular functions, there are instances the least expressed is the rate limiting isoform. For example, the eukaryotic initiation factor-4E (eIF4E), which is least expressed in mammalian cells is the rate limiting factor in protein synthesis cascade downstream of mammalian target of rapamycin (mTOR) (Mamane et al., 2004). Furthermore, reports have also indicated a pro-apoptotic role of nuclear Akt in MCF7, PC3 and NIH-3T3 fibroblasts (Maddika et al., 2008; Maddika et al., 2009). This demands identification of the predominant Akt isoform in bladder cancer cells to study the effect of its genetic deletion on bladder cancer cell functions, determine their intracellular localization and investigate the effects of pharmacological inhibition of major isoform as compared to that of the least expressed isoform.

Our study identifies Akt1 as the predominant Akt isoform in bladder cancer cells with its presence both in the cytoplasm and the nucleus, and that genetic deletion or pharmacological inhibition of Akt1 results in the inhibition of bladder cancer cell functions. Our study revealed that Akt1, the predominant isoform of Akt expressed in T24 and UM-UC-3 bladder cancer cells. Akt1 controls all the major cellular function such as proliferation, survival, colony formation, migration and invasion. Interestingly, whereas pharmacological inhibition of Akt2 have not had any effect on T24 and UM-UC-3 bladder cancer cell survival and migration, it did significantly inhibit T24 (but not UM-UC-3) cell proliferation suggesting that both Akt1 and Akt2 may be involved in different pathways in the regulation of T24 cell proliferation at specific stages of bladder cancer.

In summary, Akt1 isoform appears to be robustly involved in the tumorigenesis and invasion of bladder cancer cells. Although translational significance of these effects has to be confirmed using animal models *in vivo*, our study however provides supportive evidence that pharmacological inhibition of Akt1 may have tumor suppressive effects in invasive bladder cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Akt1 is the predominant isoform in T24 bladder cancer cells. A) Human T24 cells were cultured in the conditions of serum starvation, and in the presence of 50 nM EGF or 10% FBS. Figure shows Western blot analysis of the cell lysates probed with Akt1, Akt2, and Akt3 antibodies. B) Band densitometry analysis of the Western blots showing abundant presence of Akt1 isoform in T24 bladder cancer cells in the presence of serum and EGF. C) Figure showing immunofluorescent staining of Akt1, Akt2 and Akt3 in T24 bladder cancer cells in serum starved conditions (upper panel). Lower panel shows the co-localization of specific Akt isoforms with DAPI as analyzed using Image J Software (63X Magnification). The data are presented as mean $\pm$ S.D. (n=3) of triplicate experiments; * $P < 0.001$, # $P < 0.05$ compared to control experiments within the same group.

Figure 2.

Akt1 is the predominant isoform in UM-UC-3 bladder cancer cells. A) Human UM-UC-3 cells were cultured in the conditions of serum starvation, and in the presence of 50 nM EGF or 10% FBS. Figure shows Western blot analysis of the cell lysates probed with Akt1, Akt2, and Akt3 antibodies. B) Band densitometry analysis of the Western blots showing abundant presence of Akt1 isoform in UM-UC-3 bladder cancer cells in the presence of serum and EGF. The data are presented as mean $\pm$ S.D. (n=3) of triplicate experiments; # P<0.05 compared to control experiments within the same group.

Figure 3.

Specific deletion of Akt1 inhibits T24 bladder cancer cell functions *in vitro*. A) T24 cells transfected with ShControl or ShAkt1 via lenti-viral infections were subjected to Western blot analysis against Akt1 antibodies, and densitometry analysis performed to confirm lack of protein expression of Akt1 gene with ShRNA. B) Histogram showing the number of colonies developed by the human ShControl and ShAkt1 T24 cells 7 days after plating in 10 % FBS containing medium (n=3). C) Histogram showing the doubling time of human ShControl and ShAkt1 T24 cells in 10 % FBS containing medium (n=6). D) Histogram showing the proliferation of human ShControl and ShAkt1 T24 cells in 10 % FBS containing medium (n=6). E) Histogram showing the number of Trypan Blue stained (non-viable) human ShControl and ShAkt1 T24 cells in 10 % FBS containing medium (n=6). F) Histogram showing the number of apoptotic human ShControl and ShAkt1 T24 cells in 10 % FBS containing medium (n=6). The data are presented as mean $\pm$ S.D.; * P<0.001, P<0.01, # P<0.05 compared to ShControl transfected cells.

Figure 4.

Akt1 gene knockdown inhibits T24 bladder cancer cell migration and microinvasion. A) Histogram showing the degree of migration as detected by the efficiency of the human ShControl and ShAkt1 T24 cells in 10 % FBS containing medium to fill the wound in a scratch assay (n=3). B) Histogram showing trans-endothelial migration (micro-metastasis) of ShControl and ShAkt1 T24 human bladder cancer cells measured using ECIS equipment. T24 cells were detached from the plate by using cell dissociation buffer [20mM EDTA in PBS (pH=7.4)] to avoid receptor loss because of trypsin digestion and directly added onto the endothelial cell monolayer at a density of 5×10^4 cells/well in 50 μ l serum containing DMEM. Real-time measurements on the trans-endothelial migration of PC3 cells were recorded and then analyzed by the ECIS instrument up to 5 h. Data are presented as mean $\pm$ S.D. (n=3); * P<0.001, P<0.01, # P<0.05 compared to control experiments within the same group.

Figure 5.

Pharmacological inhibition of Akt1, but not Akt2 inhibits T24 bladder cancer cell migration and viability. A) Histogram showing the degree of migration as detected by the efficiency of the T24 cells treated with specific inhibitors of Akt1 (A674563) and Akt2 (CCT128930) at 5, 10 and 20 μ M concentrations in 10 % FBS containing medium to fill the wound in a scratch assay (n=3). B) Histogram showing the viability of T24 cells after exclusion of the Trypan blue stained non-viable cells post treatment with specific inhibitors of Akt1 (A674563) and Akt2 (CCT128930) at 5 μ M concentrations in 10 % FBS containing medium (n=3). C) Histogram showing the rate of proliferation of T24 cells post treatment with specific inhibitors of Akt1 (A674563) and Akt2 (CCT128930) at 5 μ M concentrations in 10 % FBS containing medium (n=3); # $P < 0.001$, $P < 0.0001$, * $P < 0.05$ compared to control experiments within the same group.

Figure 6.

Pharmacological inhibition of Akt1, but not Akt2 inhibits UM-UC-3 bladder cancer cell migration and viability. A) Histogram showing the degree of migration as detected by the efficiency of the UM-UC-3 cells treated with specific inhibitors of Akt1 (A674563) and Akt2 (CCT128930) at 5, 10 and 20 μ M concentrations in 10 % FBS containing medium to fill the wound in a scratch assay (n=3). B) Histogram showing the viability of UM-UC-3 cells after exclusion of the Trypan blue stained non-viable cells post treatment with specific inhibitors of Akt1 (A674563) and Akt2 (CCT128930) at 5 μ M concentrations in 10 % FBS containing medium (n=6). C) Histogram showing the rate of proliferation of UM-UC-3 cells post treatment with specific inhibitors of Akt1 (A674563) and Akt2 (CCT128930) at 5 μ M concentrations in 10 % FBS containing medium (n=3); # P<0.05 compared to control experiments within the same group.