Akt activation in renal cell carcinoma: contribution of a decreased PTEN expression and the induction of apoptosis by an Akt inhibitor

S. Hara, M. Oya*, R. Mizuno, A. Horiguchi, K. Marumo & M. Murai

Department of Urology, Keio University School of Medicine, Tokyo, Japan

Received 5 August 2004; accepted 16 January 2005

Background: Akt has been implicated in the oncogenesis of human malignant tumors, because Akt regulates many key effector molecules involved in cell survival. PTEN (phosphatase and tensin homolog deleted on chromosome 10) negatively regulates Akt activation.

Materials and methods: The expression of phosphorylated Akt (p-Akt), total Akt and PTEN was analyzed by Western blotting in 45 renal cell carcinoma (RCC) patients. The Bad and phosphorylated Bad (p-Bad) statuses were analyzed in 20 RCC patients. A phosphatidylinositol ether analog was used as an Akt inhibitor to treat four RCC cell lines, namely Caki-1, KU19-20, SW839 and Caki-2.

Results: The PTEN expression in RCC was observed to decrease and p-Akt expression to increase significantly in comparison with that in the corresponding normal kidney tissue. The PTEN expression inversely correlated with the p-Akt expression. These alterations were specific for clear cell type RCC, but not for papillary or chromophobe type RCC. Alterations in Bad phosphorylation were also specifically observed in clear cell type. The Akt inhibitor induced apoptosis in KU19-20 and Caki-2 cells with a high Akt activity.

Conclusions: A decreased expression of PTEN may be an underlying mechanism for Akt activation. An Akt inhibitor may be a therapeutic option for a subset of RCC with an elevated Akt activity.

Key words: Akt, Bad, PTEN, renal cell carcinoma

Introduction

Akt is a subfamily of the serine/threonine protein kinases and has been implicated to play a crucial role in controlling the balance between cell survival and apoptosis [1]. In various types of human malignant tumors, including colon cancer [2], ovarian cancer [3], endometrial cancer [4], and thyroid cancer [5], high frequency of elevated Akt activation has been demonstrated and its activation has been suggested to play an important role in the carcinogenesis or progression of these tumors. An elevated Akt activation in renal cell carcinoma (RCC) has been reported, however, the underlying mechanism remains to be elucidated [6].

One of the underlying mechanisms for an elevated Akt activation has been demonstrated to be an inactivation of the PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene, which is located on 10q23.3. PTEN is a lipid phosphatase which dephosphorylates the 3-position of phosphatidylinositol 3,4,5-triphosphate (PIP3) [7]. The binding of the pleckstrin homology domain (PH) of Akt to PIP3 on the inner leaflet of the plasma membrane releases the autoinhibitory function of the PH domain, thus allowing phosphoinositide-dependent kinase 1 (PDK1) to phosphorylate Akt. Therefore, PTEN antagonizes the PI3K activity by dephosphorylating PIP3 and thereby negatively regulating Akt.

Phosphorylated and activated Akt modulates the activity of a variety of downstream target proteins that are related to cell survival and proliferation. Proapoptotic protein Bad is known to be a major target protein of activated Akt. Akt phosphorylates and then inactivates Bad, thereby promoting cell survival [8]. Although mutations of the PTEN gene are infrequently observed in RCC [9–12], a decreased PTEN protein expression has been frequently observed [13]. These previous studies suggest that the loss of the PTEN function may be an underlying mechanism of Akt activation in RCC. However, the possible link between Akt activation and PTEN inactivation has not yet been documented in RCC.
In various malignant tumor cell lines, where increased Akt activities were observed, a disruption of Akt activation has been shown to inhibit cell proliferation and therefore has been considered to be a potentially novel therapeutic strategy [14]. Therefore, for RCC with an increased Akt activity, the inhibition of Akt activity can be a potential target for cancer intervention in RCC. However, such a therapeutic approach has never been previously attempted in RCC. At present, there is no effective treatment for advanced RCC with metastasis. Therefore, we attempted to perform a therapeutic approach using Akt inhibitor in RCC cell lines. We herein describe that Akt inhibitor-induced apoptosis in KU19-20 [15] and Caki-2 cells. Constitutive Akt activation in these cell lines was attenuated by the Akt inhibitor in association with downstream Bad dephosphorylation and a reduced Bcl-xL expression.

Materials and methods

Reagents

The Akt inhibitor is a phosphatidylidyinositol ether analog (1,2-diacylglycerol-3-chloro-1,2-dihydroxy-sn-glycerol-3-phosphate) (Calbiochem, LaJolla, CA) which can interfere with the interaction between Akt and PIP2 [16]. As to the specificity of this drug, the IC50 value for Akt inhibition is 5.0±1.7 μM, while that for PI3K inhibition is 83.0±21.0 μM. In order to totally inhibit Akt activation and to avoid any non-specific effect regarding the inhibition of PI3K, a cell viability test was performed at concentrations between 10 and 40 μM. The drug is solubilized in dimethylsulfoxide (DMSO) and is stored in the dark at −80 °C until use.

Rabbit polyclonal antibodies against Akt, phospho-specific Akt at Ser473 (p-Akt), and Bcl-xL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against Bad and the mouse monoclonal antibodies against PTEN were from Cell Signaling Technology (Beversly, MA). A mouse monoclonal antibody against β-actin came from Sigma (St Louis, MO).

Preparation of tissue extracts

Primary renal cancer tissue specimens were surgically resected from 45 patients with RCC at Keio University Hospital. Forty cases were clear cell type, three were papillary and two were chromophobe used. As for the evaluation of Bad and phosphorylated Bad (p-Bad), 20 cases including 15 patients with RCC at Keio University Hospital. Forty cases were clear cell type, three papillary and two chromophobe were used. As for the evaluation of Bad and phosphorylated Bad (p-Bad), 20 cases including 15 patients with RCC at Keio University Hospital.

Preparation of tissue extracts

Primary renal cancer tissue specimens were surgically resected from 45 patients with RCC at Keio University Hospital. Forty cases were clear cell type, three were papillary and two were chromophobe cell type. For the evaluation of Bad and phosphorylated Bad (p-Bad), 20 cases including 15 clear cell type, three papillary and two chromophobe were used. As for controls, normal renal tissue specimens were obtained from the surrounding tumor free tissues. These tissue specimens were stored at −80 °C until use. The tissue specimens were homogenized in 400 μl of ice-cold TNE buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40 (Roch Diagnostics, Mannheim, Germany), 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF; Boehringer Mannheim, Mannheim, Germany), 10 μg/ml aprotinin and 10 μg/ml leupeptin (Roche Diagnostics)]. The homogenates were then centrifuged at 4 °C for 10 min at 12 000 g and the protein concentrations in the supernatants were determined by the dye-binding method according to the manufacturer’s instructions (Bio-Rad, Hercules, CA).

Western blotting

Supernatants of the homogenates from RCC, control renal tissues and cell lysates were electrophoresed by SDS–PAGE (12% acrylamide) and transferred onto nitrocellulose membranes (Bio-Rad). Non-specific binding was blocked in Tris-buffered saline (TBS) containing 5% non-fat dry milk before incubation with primary antibodies. The concentration of the primary antibodies was 0.5 μg/ml for PTEN, Akt, p-Akt, Bcl-xl and β-actin, 1.0 μg/ml for Bad and p-Bad. The primary antibodies were reacted overnight and second antibodies for 1 h. Immunoreactive bands were visualized by the amplified alkaline phosphatase system according to the manufacturer’s instructions (Bio-Rad). The bands were scanned and quantitatively analyzed using the NIH Image 1.62 program (NIH, Bethesda, MD). The normalization of western blot analyses was performed using β-actin. Namely, the ratio of Akt, p-Akt and PTEN to β-actin was calculated. An analysis for β-actin was done using another membrane. The same lysates used for PTEN, Akt, p-Akt, Bcl-xl, Bad and p-Bad were loaded onto another gel for β-actin as an internal control.

Cell lines and cultures

Four different renal cancer cell lines, Caki-1, KU19-20, SW-839 and Caki-2 were cultured in an RPMI 1640 medium with 10% fetal bovine serum and streptomycin (100 μg/ml). KU19-20 is a renal cancer cell line established at our institute [15]. Caki-1, SW-839 and Caki-2 were purchased from the American Tissue Culture Collection (Rockville, MD). Prior to treatment with chemicals, they were serum-starved overnight.

Caki-1 was derived from skin metastases of clear cell RCC. KU19-20 was obtained from subcutaneous metastases of clear cell RCC. SW-839 was from clear cell RCC. Caki-2 was from a primary lesion of clear cell RCC.

Cell viability assay

For testing the sensitivity to Akt inhibitor, 200 μl aliquots of cell suspension (5 × 10^5 cells/ml) were seeded in flat-bottom 96-well plates. After 24 h, the medium was replaced with a serum-free medium containing the indicated reagents and then was incubated for 24, 48 and 72 h. Cell viability was determined by an Alamar Blue assay (Iwaki Glass, Chiba, Japan). Fluorescence of the reduced Alamar Blue was measured on a fluoroscan (Titerk Fluoroskan II; Labsystems, Tokyo, Japan) at 590 nm by excitation at 544 nm. Each experiment was performed in triplicate.

Detection of apoptosis using the flow cytometric TUNEL method

After treatment with the Akt inhibitor (20 μM) for 48 h, adherent and non-adherent cells were pooled and fixed in 1% paraformaldehyde, permeabilized in 70% ethanol and stored at −20 °C. Breaks at 3'-OH DNA were detected by the TUNEL technique using the Apoptosis Fluorescein (FITC) kit (Oncore, Gaithersburg, MD) according to the supplier’s instructions. FITC-labeled cells were stained with propidium iodide (PI) and analyzed by flow cytometry using an EPICS ALTRA flow cyrometer (Beckman Coulter, Fullerton, CA).

Statistical analysis

The unpaired-t test was used to determine the differences of Akt, p-Akt and PTEN expression between RCC and corresponding normal kidney tissue. The growth inhibitory effect was evaluated by two-way ANOVA. A Spearman’s rank correlation test was performed to examine the relationship.

Results

Akt activation is inversely correlated with PTEN expression in RCC

Akt is well known to be activated by phosphorylation at Ser 473. We therefore examined the Akt activation status in RCC by western blotting using phosphorylated Akt (p-Akt)
antibody, which recognizes only phosphorylated Akt at Ser 473. We evaluated the expressed amount of total Akt protein, p-Akt protein and the expressed amount of PTEN protein in 45 samples of RCC and corresponding normal kidney tissue (Figure 1A). The expressed amount of total Akt protein in RCC and the corresponding kidney tissue was almost the same level. In contrast, the p-Akt expression in RCC was observed to increase significantly in comparison with that in the corresponding normal kidney tissue ($P < 0.0001$) (Figure 1B). The PTEN expression in RCC was observed to decrease significantly in comparison with that in the corresponding normal kidney tissue ($P < 0.0001$) (Figure 1C). The p-Akt expression inversely correlated with the PTEN expression ($r = -0.313; n = 45; P = 0.0360$) (Figure 1D).

**Alterations in the Akt and Bad signal pathway were specifically observed in clear cell type, but not in papillary and chromophobe cell type RCC**

In order to investigate the tumor type specific mechanism in Akt activation, alterations in Akt activation and PTEN expression were investigated according to the tumor cell type. As a result, Akt activation and a decreased PTEN expression were observed only in clear cell type, but not in papillary or chromophobe type (Figure 1A). The proapoptotic protein Bad is known to be a major target protein of activated Akt [8]. The Bad status was investigated using 20 RCC tissue samples. Alterations in Bad phosphorylation were also observed in clear cell type, but not in papillary type or chromophobe type (Figure 1A). Taken together, Akt activation, decreased PTEN expression and Bad phosphorylation are considered to be special events for clear cell type, but not for papillary type or chromophobe type RCC.

**Induction of apoptosis by an Akt inhibitor in RCC cell lines**

Akt activation is considered to be a frequent event in RCC [6]. We next developed a therapeutic approach targeting Akt activation in RCC cell lines. An Akt inhibitor, a phosphatidylinositol ether analog, was used to block the Akt activation. KU19-20 and Caki-2 have an activated Akt status whereas Caki-1 and SW839 have relatively low Akt activity. Inversely, KU19-20 and Caki-2 have a low PTEN expression, whereas Caki-1 and SW839 have a relatively high PTEN expression. Akt inhibitor decreased the cell viability in a dose-dependent manner (Figure 2A). The effect was prominent in KU19-20, moderate in Caki-2, whereas the effect was minimal in Caki-1.

---

**Figure 1.** (A) Representative samples of western blotting for PTEN, total Akt, p-Akt, total Bad and p-Bad in renal cell carcinoma (RCC) tissue (T) and the corresponding non-neoplastic kidney tissue (N). #1–#3 are clear cell type, #39 and #40 are papillary cell type and #41 is chromophobe cell type. β-actin is used for an internal control. (B) The ratio of p-Akt to β-actin in 45 samples. The p-Akt expression in RCC increases significantly in comparison with that in the corresponding normal kidney tissue ($P < 0.0001$). (C) PTEN expression in RCC was observed to decrease significantly more than that in kidney tissue ($P < 0.0001$). (D) Correlation between the p-Akt expression and PTEN expression ($r = -0.313; n = 45; P = 0.0360$).
Figure 2. (A) Dose-dependent growth inhibition of Akt inhibitor on four renal cell carcinoma cell lines, Caki-1, KU19-20, SW839 and Caki-2. A growth inhibition was prominent in KU19-20 and Caki-2 by the Akt inhibitor at 48 h. Bars: S.D.; *P<0.05, **P<0.01. (B) Detection of apoptosis by the TUNEL method using a flow cytometer. A prominent shift of the cells with a DNA double-strand break was observed in KU19-20 cells treated by a 20 μM Akt inhibitor. The shift was also observed in Caki-2 cells, but not in Caki-1 and SW839. The cells with log fluorescence intensity >101 were considered to be positive for apoptosis. (C) The effects of an Akt inhibitor on the expression of PTEN, Akt, p-Akt, Bad, p-Bad and Bcl-xL determined by western blotting. Note the prominent downregulation of p-Akt and p-Bad in the KU19-20 and Caki-2 cells. (D) The bands in (C) are quantified and the ratio of p-Akt to the total Akt expression and the ratio of p-Bad to the total Bad expression are shown as graphs. Bad dephosphorylation was correlated with the Akt dephosphorylation time-dependently in KU19-20 and Caki-2 cells.
and SW839. The Akt inhibitor (20μM, 48 h treatment) induced apoptosis in KU19-20 and Caki-2 cells as determined by a flowcytometric TUNEL assay (Figure 2B).

Bad dephosphorylation is a downstream target of apoptosis via Akt inactivation

Bad has been shown to be a downstream target for Akt activation. Bad is known to play an anti-apoptotic role in a phosphorylated protein Bad, thus inactivating Bad and protecting cells undergoing apoptosis. Indeed, KU19-20 and Caki-2 cells with activated Akt show a prominent phosphorylation of Bad (Figure 2C). The Akt inhibitor decreased Akt phosphorylation and Bad phosphorylation in KU19-20 and Caki-2 cells (Figure 2D). This attenuated phosphorylation of proteins may be an underlying mechanism of Akt inhibition. Furthermore, Akt inhibitor induced a decreased expression of Bcl-xL. This may also contribute to the apoptosis-inducing effect of Akt inhibitor, because Bcl-xL is an anti-apoptotic protein implicated to play a role in protecting cells undergoing apoptosis.

Discussion

This is the first report evaluating both Akt activation and PTEN expression simultaneously in RCC. We herein demonstrate that the total Akt expression did not differ substantially between RCC and kidney tissue. However, Akt was activated significantly more in RCC than in kidney tissue, presumably due to the decreased PTEN expression because p-Akt expression and PTEN expression were inversely correlated.

In various types of human malignant tumors, an elevated Akt activation has been demonstrated and the mechanisms for Akt activation have been shown to depend on the types of tumors. For example, ovarian carcinomas and endometrial carcinoma have shown an increased Akt activation due to a high frequency of PTEN inactivation [3, 4]. On the other hand, thyroid cancer, in which PTEN inactivation is uncommon, has shown an elevated Akt activation due to an overexpression of Akt [5]. Recent studies have demonstrated that a somatic PTEN mutation in primary RCC and renal cancer cell lines is found at a relatively lower incidence than in other malignant tumors, such as glioblastoma, melanoma, prostate cancer and endometrial carcinoma [17]. A PTEN mutation in RCC was thus found especially in high grade and advanced clear cell type RCC with distant metastases or renal vein tumor invasions, thus suggesting that PTEN mutations occur as a late-stage event and may contribute to an invasive and metastatic tumor phenotype [12]. On the other hand, Brenner et al. [13] demonstrated that a markedly reduced expression of PTEN was found in clear cell type RCC regardless of pathological grade and thus concluded that the PTEN expression was lost during early renal carcinogenesis. The discrepancy between the frequency of PTEN mutation and that of PTEN expression loss may be due to additional mechanisms for PTEN inactivation other than gene deletion and mutations, including promoter methylation or translational modification. Our results have shown that a decreased PTEN expression may predispose to a constitutive Akt activation in RCC. Notably, these alterations, including Akt activation, decreased PTEN expression and Bad phosphorylation, were specifically observed in clear cell type, but not in papillary or chromophobe type RCC.

The Akt inhibitor induced apoptosis in KU19-20 and Caki-2 cells with a high Akt activity, whereas the Akt inhibitor had a minimal effect on the proliferation of Caki-1 and SW839 cells with a lower Akt activity. The Akt activity in KU19-20 and Caki-2 cells appears to be closely related to cancer cell survival, whereas in Caki-1 and SW839 cells with a low Akt activity, this signal pathway does not seem to play any role in cancer cell survival. These results suggest that the pre-treatment status of Akt activity may play a decisive role in the effectiveness of Akt inhibitor.

RCC, found asymptotically in the early stages, can be treated by surgery with a favorable prognosis. Advanced RCC with metastasis has a poor prognosis because RCC is resistant to chemotherapy and radiotherapy, while immunotherapy, such as interferon and interleukin-2, the current treatment option, has only between a 15 and 20% efficacy [18]. As a result, a novel therapy for these patients is currently needed. To assess the functional relevance of Akt activation in cell survival, we used an Akt inhibitor and evaluated the inhibition of cell proliferation in parallel with the downregulation of Akt phosphorylation. In a subset of RCC with elevated Akt activation, Akt inhibitor therefore has a therapeutic potential for patients in whom there is no efficient therapy at present.

Interestingly, a prominent phosphorylation of Bad was observed in KU19-20 and Caki-2 cells, thus suggesting that proapoptotic Bad protein was inactivated. It is conceivable that phosphorylated Bad may play a role in the survival of KU19-20 and Caki-2 cells and that dephosphorylation by Akt inhibitor induces the cancer cells to undergo apoptosis. Bad phosphorylation may be closely related to aberrant survival, namely, carcinogenesis of RCC. In contrast, a proliferation of Caki-1 and SW839 cells had minimal effect by the Akt inhibitor. Notably, Caki-1 and SW839 cells had only a slight degree of Bad phosphorylation, therefore, Bad phosphorylation does not seem to contribute to the carcinogenesis of Caki-1 and SW839 cells. Targeting Akt signal cascades may have a therapeutic potential for clear cell type RCC with an elevated Akt activity.

Acknowledgements

We authors wish to thank Mr Hiroshi Nakazawa and Ms Azusa Yamanouchi for their technical help. This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References