Increased activated Akt expression in renal cell carcinomas and prognosis

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Abstract

Renal carcinogenesis is promoted by overexpression of the activated serine/ threonine kinase Akt (p-Akt) and supposedly a concomitant reduction in phosphatase and tensin homologue deleted on chromosome 10 tumour suppressor gene (PTEN), which normally inhibits the activation of Akt. Because promising anti-cancer therapies increasingly focus on pathways involving p-Akt and PTEN, the present study evaluated the expression of p-Akt in renal cell carcinomas and compared it with prognosis. P-Akt and PTEN expression were analysed in a tissue microarray (TMA) from renal cell carcinoma ($n = 386$) and adjacent uninvolved renal tissue ($n = 32$) specimens. Increased p-Akt was found more often in the nucleus than in the cytoplasm, and PTEN was concomitantly reduced in about 50% of cases. Neither tumour grade nor stage influenced p-Akt expression, whereas the clear cell and papillary subtypes showed increased p-Akt more often than did the chromophobe or sarcomatoid types. Increased cytoplasmic and nuclear p-Akt levels were independent prognostic factors for diminishing patient survival. The present study found significantly increased nuclear but also cytoplasmic p-Akt expression in renal cell carcinoma subtypes. Increased nuclear and cytoplasmic p-Akt was an independent prognostic factor for diminishing patient survival. The considerable number of high-grade and high-stage RCC showing increased p-Akt and reduced PTEN would justify further evaluation of therapeutic concepts based on inhibitors of the PI3K/p-Akt/mTOR pathway.

Keywords: renal cell carcinoma • p-Akt • PTEN • prognosis • patient survival

Introduction

The activated serine/ threonine kinase Akt (p-Akt), also called protein kinase B, controls proteins involved in apoptosis and cell proliferation. In detail, p-Akt inhibits the pro-apoptotic activity of BAD, caspase-9 and the forkhead family, and activates several anti-apoptotic substrates [1, 2]. Furthermore, p-Akt promotes cell cycle progression through the G₁/S phase by [1] inhibiting the glycogen synthase kinase-3 (GSK-3) [2], decreasing the proteolytic degradation of cyclin D₁ and [3] modulating the mammalian target of rapamycin (mTOR) receptor [3–6].

Overexpression of p-Akt may therefore contribute to the development and progression of malignancies (*e.g.* prostate, breast, ovarian, endometrial, pancreatic ductal, thyroid and squamous cell carcinomas and multiple myeloma) and have a negative impact on prognosis [7–14].

The PTEN (phosphatase and tensin homologue deleted on chromosome 10) tumour suppressor gene inhibits the phosphatidylinositol-3-kinase (PI3K)-dependent activation of Akt. Inactivation of PTEN causes p-Akt overexpression [15].

Promising anti-cancer therapies increasingly focus on pathways involving p-Akt and PTEN [6, 16]. In this context, little is known about the p-Akt expression of renal cell carcinomas, which on progression become essentially unresponsive to conventional treatment strategies [17].

Therefore, the present tissue microarray (TMA) study evaluated in renal cell carcinomas the expression of p-Akt and correlated it with that of PTEN and patient survival rate.

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Materials and methods

A TMA was constructed from paraffin-embedded renal cell carcinoma ($n = 440$) and adjacent uninvolved renal tissue ($n = 32$) specimens. In detail, from representative tumour-positive and -negative regions four cores per case (diameter 0.6 mm) were arrayed on nine recipient blocs. Each recipient bloc hosted three to four uninvolved cores, which were randomly distributed between tumour-positive ones, and other uninvolved cores in the last line of tissue cores.

P-Akt staining

Deparaffinized TMA sections (4 μm) were incubated for 15 min. in a methanol solution containing 3% H_2O_2 to block endogenous peroxidase activity. Following antigen retrieval with a 10 mM citrate buffer, the TMA sections were incubated overnight at 4°C with a polyclonal rabbit antibody (1:100) specific for p-Akt (Ser 473) (Abcam, Cambridge, UK). After rinsing in citrate buffer a biotinylated goat anti-rabbit antibody (1:500) labelled with streptavidin and chromogen as substrate was added (UltraVision Detection System Anti-Polyvalent, HRP/AEC, LabVision Corporation, Fremont, CA, USA). Breast cancer specimens served as positive control.

PTEN staining

Deparaffinized TMA sections (4 μm) were autoclaved and placed in a citrate buffer (10 mM, pH 6.0) before being incubated with a rabbit anti-PTEN antibody (1:50) ab2979 (Abcam) and subsequently with a secondary anti-rabbit antibody (1:500) (Dako, Glostrup, Denmark). Each incubation step took 30 min. at room temperature. Subsequently, immunohistological staining was performed with a commercially available kit (ABC-Kit® Vectastain, Vector, Burlingame, CA, USA) using haematoxylin counterstaining. Prostate carcinoma tissue sections served as positive control.

Scoring of p-Akt expression

For each core nuclear and cytoplasmic p-Akt expression was separately scored for *staining intensity* (e.g. (0) negative; (1) low; (2) moderate; (3) strong) and *percentage of stained tumour cells* (e.g. (0) 0%; (1) 1% to 10%; (2) 11% to 50%; (3) 51% to 75%; (4) 76% to 100%). In uninvolved renal tissue, the total (e.g. nuclear plus cytoplasmic) p-Akt expression scored from '0' to '6'. In tumour-positive cases, the total (e.g. nuclear plus cytoplasmic) p-Akt expression was classified as either 'normal' (e.g. score from '0' to '6') or 'increased' (e.g. score from '7' to '12').

Scoring of PTEN expression

Cytoplasmic PTEN expression was scored as '1' when comparable to uninvolved renal tissue and '0' when decreased or absent as previously reported [18, 19].

Scoring was done by two independent, blinded pathologists (H.H., K.R.). All renal cell carcinomas included were graded [20] and TNM-staged [21]. Subtyping of renal cell carcinomas followed the WHO tumour classification [22]. Despite the fact that the highly aggressive sarcomatoid renal

cell carcinomas can derive from all subtypes of renal cell carcinomas and are therefore no longer considered a distinct histological entity [23, 24], we present them as their own class.

Overall survival was defined as the time between the date of diagnosis (e.g. date of nephrectomy) and death.

Statistical analysis

Correlations were calculated using the Spearman correlation coefficient. P-Akt expression and PTEN expression in the various tumour subtypes were compared by means of the Mann–Whitney U Test. Kaplan–Meier survival curves were calculated and prognostic factors were analysed pairwise using the log-rank test. For multivariate analysis the Cox regression analysis was used. A $P < 0.05$ was considered statistically significant. Statistical analysis employed the statistics software package SPSS 14.0.0® (SPSS Inc., Chicago, IL, USA).

Results

P-Akt and PTEN expression were analysed in a TMA from renal cell carcinoma ($n = 440$) and adjacent uninvolved renal tissue ($n = 32$) specimens.

Because of technical problems (e.g. core folding or loss, necrotic, hemorrhagic or too little tumour tissue) 12.3% (54/440) of TMA-arrayed renal cell carcinoma cases had to be excluded. Of the remaining cases ($n = 386$) 218 stemmed from male (62.8 ± 11.9 years) and 168 from female (65.5 ± 11.2 years) patients with a mean follow-up time of 91.8 ± 47.5 (male: 90.2 ± 46.9 ; female: 94.0 ± 48.3) months.

Activated Akt (p-Akt) was found more frequently in tumour-positive than in uninvolved tissue (Fig. 1). Furthermore, increased p-Akt was more often located in the nucleus than in the cytoplasm. Nuclear and cytoplasmic p-Akt expression, however, were positively correlated. Irrespective of gender, especially on a nuclear and to a lesser degree on a cytoplasmic level, an increase in p-Akt alone occurred as frequently as it did in combination with a reduction in PTEN (Tables 1 and 2). In contrast, an increase in total (e.g. cytoplasmic, nuclear and cytoplasmic) p-Akt in combination with reduced PTEN (45.6%) occurred approx. five times more often than it did when PTEN was normal (8.3%) (Table 2).

The clear cell and papillary types of renal cell carcinoma showed increased p-Akt (alone and in combination with reduced PTEN) more often than did the chromophobe or sarcomatoid types (Tables 1 and 2). Patient survival for chromophobe, clear cell and papillary types of renal cell carcinoma was better than for the sarcomatoid subtype. This effect tended to be more pronounced for increased p-Akt.

Approximately 50% of high-grade (grades 3 + 4) and high-stage (stages 3 + 4) RCC showed increased p-Akt and reduced PTEN expression (Table 2). Tumour grade and stage had no significant influence on p-Akt expression (alone or in combination with reduced PTEN) (Tables 1 and 2). Similarly, angiogenesis and lymph node invasion occurred irrespective of p-Akt or PTEN expression (Tables 1 and 2).

Fig. 1 The figure illustrates p-AKT expression in tubular epithelial cells of uninvolved renal tissue (**A**), increased nuclear (**B**) and cytoplasmic (**C**) p-AKT expression in clear cell (B, C) and simultaneously increased nuclear and cytoplasmic p-AKT expression in chromophobe renal cell carcinoma (**D**) (magnification 400 $\times$).

Furthermore, total, cytoplasmic and cytoplasmic-nuclear p-Akt expression was positively, but only nuclear p-Akt expression was negatively, correlated with PTEN expression.

Alone and in combination with reduced PTEN an increase solely in cytoplasmic p-Akt tended to shorten patient survival more than did a solely nuclear p-Akt increase (Figs. 2 and 3). Furthermore, elevated cytoplasmic and nuclear p-Akt levels were independent prognostic factors for patient survival.

Discussion

Activated Akt (p-Akt) was found more frequently in tumour-positive than in uninvolved tissue. On a nuclear and to a lesser

degree on a cytoplasmic level an increase in p-Akt alone occurred as frequently as it did in combination with a reduction in PTEN. Increased cytoplasmic and nuclear p-Akt levels were independent prognostic factors for decreased patient survival.

The activated serine/ threonine kinase Akt (p-Akt) controls proteins involved in apoptosis and cell proliferation [1]. Overexpression of p-Akt, however, may contribute to the development and progression of various malignancies and consequently have a negative impact on prognosis [7–14]. Moreover, concomitantly reduced PTEN, which normally inhibits the phosphatidylinositol-3-kinase (PI3K)-dependent activation of AKT, further facilitates p-Akt overexpression [15]. Because promising anti-cancer therapies increasingly focus on pathways involving p-Akt and PTEN [6, 16], the present study focused on p-Akt and PTEN expression of renal cell carcinomas, which on progression become essentially unresponsive to conventional treatment strategies [17].

Table 1 Increased p-Akt expression in renal cell carcinoma in comparison to uninvolved renal tissue

	Total		Increased p-Akt		
		Total	Cytoplasmic	Nuclear	Simultaneously nuclear and cytoplasmic
	<i>n</i>	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
Gender					
Male	218	115 (52.8)	11 (5)	67 (30.7)	37 (17)
Female	168	95 (56.5)	13 (7.7)	58 (34.5)	24 (14.3)
Renal cell carcinoma	386	210 (54.4)	24 (6.2)	125 (32.4)	61 (15.8)
Type					
Clear cell	309	161 (52.1) ¹	17 (5.5) ²	111 (35.9) ²	33 (10.7) ¹
Papillary	40	38 (95)	3 (7.5)	8 (20)	27 (67.5)
Chromophobe	20	6 (30) ¹	4 (20)	1 (5)	1 (5) ¹
Sarcomatoid	17	5 (29.4) ¹	0 (0) ²	5 (29.4) ²	0 (0) ¹
Grade					
1	60	29 (48.3)	1 (1.7)	25 (41.7)	3 (5)
2	164	89 (54.3)	12 (7.3)	47 (28.7)	30 (18.3)
3	135	79 (58.5)	9 (6.7)	44 (32.6)	26 (19.3)
4	27	13 (48.1)	2 (7.4)	9 (33.3)	2 (7.4)
TNM stage					
1	237	129 (54.4)	12 (5.1)	74 (31.2) ⁷	43 (18.1)
2	65	31 (47.7)	5 (7.7)	19 (29.2) ⁷	7 (10.8)
3	82	48 (58.5)	7 (8.5)	30 (36.6) ⁷	11 (13.4)
4	2	2 (100)	0 (0)	2 (100)	0 (0)
PTEN		3	4	5	6
Reduced	344	176 (51.2)	18 (5.2)	117 (34.0)	41 (11.9)
Normal	42	34 (81)	6 (14.3)	8 (19)	20 (47.6)
Angioinvasion					
Positive	93	52 (55.9)	6 (6.5)	36 (38.7)	10 (10.8)
Negative	293	158 (53.9)	18 (6.1)	89 (30.4)	51 (17.4)
Lymph node invasion					
Positive	11	7 (63.6)	0 (0)	5 (45.5)	2 (18.2)
Negative	152	82 (53.9)	8 (5.3)	48 (31.6)	26 (17.1)

¹ Significant to papillary renal cell carcinoma ($P < 0.005$).² Significant to chromophobe renal cell carcinoma ($P < 0.05$).³ Correlation of PTEN with total p-Akt ($P < 0.005$, $R = 0.19$).⁴ Correlation of PTEN with cytoplasmic p-Akt ($P = 0.017$, $R = 0.13$).⁵ Correlation of PTEN with nuclear p-Akt ($P = 0.01$, $P = -0.14$).⁶ Correlation of PTEN with simultaneous nuclear and cytoplasmic p-Akt ($P < 0.005$, $R = 0.315$).⁷ Significant to TNM stage 4 ($P < 0.05$).

Table 2 Increased p-Akt and reduced PTEN expression in renal cell carcinoma in comparison to uninvolved renal tissue

	Total	Reduced PTEN/increased p-Akt			
		Total	Cytoplasmic	Nuclear	Simultaneously nuclear and cytoplasmic
	<i>n</i>	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
Gender					
Male	218	101 (46.3)	8 (3.7)	65 (29.8)	28 (12.8)
Female	168	75 (44.6)	10 (6)	53 (31.5)	12 (7.1)
Renal cell carcinoma	386	176 (45.6)	18 (4.7)	118 (30.6)	40 (10.4)
Type					
Clear cell	309	143 (56.3) ²	13 (4.2)	106 (34.3) ²	24 (7.8) ¹
Papillary	40	25 (62.5)	3 (7.5)	6 (15)	16 (40)
Chromophobe	20	3 (15) ¹	2 (10)	1 (5)	0 (0) ¹
Sarcomatoid	17	5 (29.4) ¹	0 (0)	5 (29.4)	0 (0) ¹
Grade					
1	60	26 (43.3)	1 (1.7)	23 (38.3)	2 (3.3)
2	164	73 (44.5)	9 (5.5)	44 (26.8)	20 (12.2)
3	135	65 (48.1)	6 (4.4)	43 (31.9)	16 (11.9)
4	27	12 (44.4)	2 (7.4)	8 (29.6)	2 (7.4)
TNM stage					
1	237	106 (44.7) ³	9 (3.8)	68 (28.7) ³	29 (12.2)
2	65	27 (41.5) ³	3 (4.6)	19 (29.2) ³	5 (7.7)
3	82	41 (50)	6 (7.3)	29 (35.4) ³	6 (7.3)
4	2	2 (100)	0 (0)	2 (100)	0 (0)
Angioinvasion					
Positive	93	45 (48.4)	4 (4.3)	34 (36.6)	7 (7.5)
Negative	293	131 (44.7)	14 (4.8)	84 (28.7)	33 (11.3)
Lymph node invasion					
Positive	11	6 (54.5)	0 (0)	5 (45.5)	1 (9.1)
Negative	152	71 (46.7)	7 (4.6)	46 (30.3)	18 (11.8)

¹ Significant to papillary renal cell carcinoma ($P < 0.005$).² Significant to chromophobe renal cell carcinoma ($P < 0.005$).³ Significant to TNM stage 4 ($P < 0.05$).

It was previously shown that Akt, once activated, translocates from the cytoplasm to the nucleus [25]. The role of this nuclear translocation, the specific nuclear target structures and, above all, the consecutively initiated regulatory processes, which may increase tumour proliferation rate or dedifferentiation or a combination of both, have not been fully clarified so far [26].

In detail, p-Akt expression in the nucleus and the cytoplasm in renal cell carcinoma was positively correlated in the present study, which is in accordance with previous findings [27]. Furthermore, irrespective of gender, especially on a nuclear and to a lesser degree on a cytoplasmic level, an increase in p-Akt alone occurred as frequently as it did in combination with a reduction in PTEN

Fig. 2 Depicts Kaplan–Meier survival curves for patients with renal cell carcinoma with simultaneously increased nuclear and cytoplasmic p-Akt (A), normal p-Akt (B), only nucleus-increased p-Akt (C) and only cytoplasm-increased p-Akt expression (D). (1) Significant to normal (B) and simultaneously increased nuclear and cytoplasmic p-Akt (A) expression ($P < 0.05$).

Fig. 3 Depicts Kaplan–Meier survival curves for patients with renal cell carcinoma with simultaneously increased nuclear and cytoplasmic p-Akt/reduced PTEN (A), normal p-Akt/normal PTEN (B), only nucleus-increased p-Akt/reduced PTEN (C) and only cytoplasm-increased p-Akt/reduced PTEN expression (D). (1) Significant to normal p-Akt/PTEN (B) and simultaneously increased nuclear and cytoplasmic p-Akt/reduced PTEN (A) expression ($P < 0.05$).

(Tables 1, 2). Previously, the overall correlation between p-Akt and PTEN expression in renal cell carcinoma was reported to be negative [28], absent on a cytoplasmic level but also positive on a nuclear level [27]. An additional facet is added to this controversy by the present study, which found the correlation between p-Akt and PTEN expression to be positive on a cytoplasmic but negative on a nuclear level. Interestingly enough, albeit in a small sample size ($n = 44$), a previous analysis also found the co-existence of high levels of PTEN and of p-Akt, but failed to report on cytoplasmic or nuclear correlation between PTEN and p-Akt in renal cell carcinoma [29].

Further studies clearly need to analyse not only the quantity but also the function of PTEN and to investigate additional factors (*e.g.* epidermal, platelet-derived or insulin-like growth factors, but also mitogens and cytokines, *e.g.* IL3) activating PI3K and consecutively p-Akt [3, 29, 30].

In contrast to a previous underpowered study indicating p-Akt expression to be independent of the subtype of renal cell carcinoma [31], the present study's papillary and clear cell renal cell carcinomas showed increased p-Akt expression (alone and in combination with reduced PTEN) more often than did chromophobe or sarcomatoid subtypes.

Prognosis of patients with the sarcomatoid subtype, however, was poorer than that of patients with papillary, clear cell or chromophobe subtype. This effect tended to be even more pronounced in cases of increased p-Akt. It is noteworthy, however, that the sarcomatoid subtype showed increased p-Akt only nuclearly, which was previously also reported for a stronger aggressiveness of prostate cancer [13]. The effect of reduced PTEN alone or in com-

bination with increased p-Akt in this context could not be investigated, because the number of cases was too small.

The correlation between increased p-Akt levels and renal cell carcinoma grade and stage is still a matter of controversy. In detail, Horiguchi *et al.* reported, without referring to subcellular levels, increased p-Akt levels to be positively correlated with higher grade and stage of renal cell carcinomas [31], whereas Pantuck *et al.* found no such correlation [27]. Similarly, the present study showed p-Akt expression (alone and in combination with reduced PTEN) to be independent of grade and stage of renal cell carcinomas. Moreover, angioinvasion and lymph node invasion of renal cell carcinomas were found to occur irrespective of p-Akt and PTEN expression.

Numerous previous studies reported increased p-Akt in various tumour types (*e.g.* endometrial carcinoma, pancreatic ductal carcinoma, oral squamous cell carcinoma, breast cancer) to significantly shorten patient survival and above all to be an independent prognostic factor for survival [12, 14, 32, 33]. With regard to renal cell carcinomas, however, the role of p-Akt expression in respect to patient survival is still a matter of controversy. Horiguchi *et al.* reported that although increased p-Akt shortens patient survival, it is not an independent prognostic factor for it [31]. Pantuck *et al.*, in contrast, reported cytoplasm-increased p-Akt to be an independent prognostic factor for diminished patient survival, whereas nucleus-increased p-Akt even indicated improved survival [27]. The present study ultimately shows that, alone and in combination with reduced PTEN, a solely cytoplasmic

p-Akt increase tends to shorten patient survival more than does a solely nuclear p-Akt increase. Furthermore, increased p-Akt in the cytoplasm and the nucleus was an independent prognostic factor for patient survival.

The discrepancy between the findings of Pantuck *et al.* and our own findings can be attributed at least in part to the fact that Pantuck *et al.* used a deliberately defined cut-off value to classify increased and non-increased p-Akt, whereas the present study classified p-Akt expression in tumour-positive tissue as increased when it was stronger than in uninvolved tissue.

In malignant tumours with p-Akt expression (*e.g.* non-small lung cell cancer, breast cancer [34, 35], but also in advanced and metastatic renal cell carcinoma [16]), a significant resistance to chemo- and radiotherapy was previously shown. For these patients, novel and partly still experimental therapeutic concepts focus on the use of various inhibitors of the PI3K/p-Akt/mTOR pathway [6, 28, 36]. Research on predictors for therapeutic success of these novel concepts identified above all increased p-Akt in combination with low PTEN [6, 37]. Because the present study showed increased p-Akt and reduced PTEN expression in a con-

siderable number of high-grade and high-stage tumours and cytoplasmic and nuclear p-Akt to be independent prognostic factors for diminishing patient survival, the evaluation of therapeutic concepts based on inhibitors of the PI3K/p-Akt/mTOR pathway in these patients is more than justified.

In conclusion, the present study found significantly increased nuclear but also cytoplasmic p-Akt expression in renal cell carcinoma subtypes. Furthermore, increased nuclear and cytoplasmic p-Akt was an independent prognostic factor for diminishing patient survival. The considerable number of high-grade and high-stage RCC showing increased p-Akt and reduced PTEN would justify further evaluation of therapeutic concepts based on inhibitors of the PI3K/p-Akt/mTOR pathway.

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