

Elevated expression of AKT2 correlates with disease severity and poor prognosis in human osteosarcoma

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Abstract. Members of AKT kinase family are central modulators in numerous signaling cascades, which regulate metabolism, cell proliferation, survival and growth. Previously, the knockdown of AKT2 expression has been demonstrated to enhance the efficacy of chemotherapy in patients with osteosarcoma. However, it is currently unknown whether the aberrant expression of AKT2 has relevance to the progression of osteosarcoma. The aim of the present study was to investigate the clinicopathological and prognostic value of AKT2 in osteosarcoma. Formalin-fixed paraffin embedded osteosarcoma and self-paired non-cancerous bone tissue samples were obtained from 126 patients with osteosarcomas. AKT2 expression was detected by an immunohistochemistry assay. Patient survival rates were determined by the Kaplan-Meier method and log-rank test. Cox regression was adopted for multivariate analysis of the prognostic factors to examine the effect of AKT on event-free survival and overall survival in patients with osteosarcomas. AKT2 expression in osteosarcoma tissues was significantly higher than that in non-cancerous bone tissues (immunostaining score, 6.39 ± 1.62 vs. 3.46 ± 1.03 ; $P < 0.001$). In addition, the elevated expression of AKT2 protein was significantly associated with positive recurrence ($P = 0.023$), the presence of metastasis ($P = 0.006$) and poor response to chemotherapy ($P = 0.015$). Furthermore, patients with high AKT2 expression had significantly shorter event-free survival ($P < 0.001$) and overall survival times ($P < 0.001$) than those with lower expression levels. Multivariate analysis further demonstrated that AKT2 expression ($P = 0.029$ and 0.016 , respectively), the status of recurrence ($P = 0.018$ and 0.012 , respectively) and metastasis ($P = 0.020$ and 0.015 ,

respectively), and the response to chemotherapy ($P = 0.011$ and 0.008 , respectively) were all independent prognostic factors for event-free survival and overall survival time. To the best of our knowledge, these data have supported the findings for the first time, that the elevated expression of AKT2 may be associated with aggressive clinical behavior and poor outcome in patients with osteosarcomas. Therefore, AKT2 may be a candidate marker of unfavorable prognosis in osteosarcoma.

Introduction

Osteosarcoma is one of the most common primary bone malignancies and primarily affects adolescents (< 20 years old) (1). It is characterized by frequent vascular invasion, infiltration to adjacent soft tissue, a high rate of local recurrence and early distant metastasis (2). Although multidisciplinary approaches, including chemotherapy and wide resection surgery, are able to significantly improve the clinical outcome for patients with osteosarcoma, the prognosis of advanced cases with distant metastasis and local recurrence remains poor, even with additional extensive chemotherapy (3). Accumulating studies have indicated that a variety of cellular and molecular events may be implicated in the tumorigenesis of osteosarcoma. Therefore, identification of novel markers is required for the prediction of malignant behavior and prognosis of osteosarcomas, in order to guide treatment strategies and improve the clinical outcome of this disease.

AKT family members encode highly similar serine-threonine protein kinases, which belong to the more general class of AGC kinases (related to AMP/GMP kinase and protein kinase C) (4). There are three members of the AKT kinase family: AKT1 (protein kinase B- α , PKB- α), AKT2 (protein kinase B- β , PKB- β) and AKT3 (protein kinase B- γ , PKB- γ) (5). All of the AKT kinases lie at the center of the phosphoinositide 3-kinase (PI3k)/AKT signal transduction pathway, which regulates multiple cellular processes, including cell proliferation, growth, survival, transformation and differentiation (6). Activation of the AKT kinases, initiated by extracellular stimuli in a PI3k-dependent manner, regulates downstream signaling proteins that are involved in cell survival, growth, cell cycle progression and apoptotic response (7). Previously, several studies of cancer biology have demonstrated the crucial role of AKT kinases in tumorigenesis and that it may be possible to target them therapeutically and/or as a chemopreventative

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strategy (8). Among the three AKT kinases, AKT2 has been demonstrated to be associated with the development of human cancers. For example, in non-small cell lung cancer, AKT2 contributes to cell survival via different mechanisms (9). In breast cancer cells, ablation of AKT2 may induce cell cycle arrest and be differentially involved in regulating cell migration and the epithelial-to-mesenchymal transition (10). In hepatocellular carcinoma, AKT2 expression may be upregulated and be a novel independent predictor for the development and progression of this malignancy (11). Furthermore, Zhang *et al* (12) indicated that knockdown of AKT2 expression may have therapeutic applications in enhancing the efficacy of chemotherapy in patients with osteosarcoma. However, it is currently unknown whether the aberrant expression of AKT2 has relevance to the progression of osteosarcoma. The aim of this study was to investigate the clinicopathological and prognostic value of AKT2 in patients with osteosarcoma.

Materials and methods

Patients and tissue samples. This study was approved by the Research Ethics Committee of Shanghai Pudong Hospital, (Shanghai, China). Written informed consent was obtained from all patients. All specimens were anonymous and handled according to the ethical and legal standards.

Formalin-fixed paraffin embedded osteosarcoma and self-paired non-cancerous bone tissue samples were obtained from 126 patients with osteosarcoma at Shanghai Pudong Hospital between 1998 and 2008. The patients ranged from 12–66 years of age (median, 19 years; mean, 25.2 years). The study cohort included 78 males and 48 females. Clinicopathological parameters, including age, gender, tumor size, tumor site, histological subtype, local recurrence, distant metastasis and response to the neoadjuvant chemotherapy are summarized in Table I. All samples were obtained from primary lesions. The biopsies were performed prior to chemotherapy or radiotherapy for diagnostic purposes.

Follow-up was conducted for all 126 patients with osteosarcoma. Patients were monitored with computed tomography (CT), which was performed every three months during the first three years following chemotherapy, every four months during years four and five, and every six months thereafter. The development of local recurrence and distant metastases were detected by CT scans or magnetic resonance imaging. All cases were independently reviewed by two pathologists and discrepancies resolved by consensus review. Of 126 patients with osteosarcoma, 48 (38.10%) experienced local recurrence and 52 (41.27%) patients had succumbed to the disease by the last follow-up date. The median follow-up time was 42 months (range, 2–116 months). Overall survival time was calculated from the date of the initial surgical operation to death. Event-free survival was calculated from the date of the initial surgical operation to the date of secondary cancer, tumor recurrence, distant metastases or mortality from any cause.

Immunohistochemistry analysis. Expression patterns of AKT2 proteins in 126 formalin-fixed paraffin embedded osteosarcomas and self-paired non-cancerous bone tissue samples were detected by immunohistochemical staining. Briefly, all tissue samples were retrieved and divided into 3- μ m sections and

mounted on pre-coated slides. Following deparaffinizing in xylene and washing in a graded series of ethanol, the sections were submerged into EDTA antigenic retrieval buffer (ab93680, Abcam, Cambridge, UK). and microwaved for antigenic retrieval. The slides were incubated with the primary antibody raised against AKT2 (dilution, 1:50, mouse polyclonal antibody; Cell Signaling Technology, Inc., Beverly, MA, USA). All incubations with primary antibody were conducted overnight at 4°C. Following washing in Tris-buffered saline (Sigma, Munich, Germany), the tissue sections were treated with biotinylated rabbit anti-mouse secondary antibody (Zymed Laboratories, Inc., San Francisco, CA, USA), followed by further incubation with streptavidin-horseradish peroxidase complex (Zymed Laboratories, Inc.). The tissue sections were immersed in 3-amino-9-ethyl carbazole and counterstained with 10% Mayer's hematoxylin, then dehydrated and mounted in Crystal Mount. Negative control staining was conducted by substituting non-immune rabbit IgG and phosphate-buffered saline for the primary antibodies.

Following hematoxylin counterstaining, immunostaining was scored by two independent observers, who were blinded to the clinicopathological parameters and clinical outcomes of the patients. The scores of the two observers were compared and any discrepancies were trained through re-examining the stainings by the two pathologists to achieve a consensus score. The number of positive-staining cells exhibiting immunoreactivity on the cytoplasm in ten representative microscopic fields was counted and the percentage of positive cells was calculated. The frequency of AKT2 immunoreactivity in the tissue sections was evaluated as negative (0) when no positive cells were observed within the tumor; weak (1) when <30% of the tumor cells were positive; moderate (2) when 30–60% of the tumor cells were positive and strong (3) when >60% of tumor cells were positive. The intensity of staining was evaluated as 0, 1, 2 and 3 for no staining, weak staining, medium staining and strong staining, respectively. An immunostaining score (IRS) was obtained by multiplying the frequency and intensity score for stained cells (range, 0–9). The cut-off value for IRS of AKT2 protein was selected on the basis of a measure of heterogeneity with the log-rank test statistic, with respect to overall survival. An optimal cut-off value was identified. An IRS of ≥ 4.5 was used to classify the tumors with high expression and <4.5 IRS classified the tumors with low expression of AKT2 protein.

Statistical analysis. SPSS version 16.0 software for Windows (SPSS, Inc., Chicago, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC, USA) were used for statistical analysis. Continuous variables were expressed as the mean \pm standard deviation. Associations between the expression of AKT2 and clinicopathological parameters were assessed using a χ^2 test. Patient survival and their differences were determined by Kaplan-Meier method and a log-rank test. Cox regression (proportional hazard model) was adopted for multivariate analysis of prognostic factors. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Elevated expression of AKT2 in human osteosarcoma tissues. Expression patterns and subcellular localization of

Table I. Association between AKT2 expression and different clinicopathological features of osteosarcoma patients.

Clinicopathological feature	No. of cases	AKT2 expression		P-value
		High (n, %)	Low (n, %)	
Age				
<20	80	45 (56.25)	35 (43.75)	NS
≥20	46	25 (54.35)	21 (45.65)	
Gender				
Male	82	47 (57.32)	35 (42.68)	NS
Female	44	23 (52.27)	21 (47.73)	
Tumor size (cm)				
≥5.0	76	40 (52.63)	36 (47.37)	NS
<5.0	50	30 (60.00)	20 (40.00)	
Tumor site				
Femur	66	37 (56.06)	29 (43.94)	NS
Tibia	50	28 (56.00)	22 (43.00)	
Other	10	5 (50.00)	5 (50.00)	
Histological type				
Osteoblastic	58	27 (46.55)	31 (53.45)	NS
Chondroblastic	30	20 (66.67)	10 (33.33)	
Fibroblastic	22	13 (59.09)	9 (40.91)	
Other	16	10 (62.50)	6 (37.50)	
Recurrence				
Negative	55	22 (40.00)	33 (60.00)	0.028
Positive	71	48 (67.61)	23 (32.39)	
Metastasis				
Negative	57	18 (31.58)	39 (68.42)	0.006
Positive	69	52 (75.36)	17 (24.64)	
Response to chemotherapy				
Good	66	28 (42.42)	32 (57.58)	0.015
Poor	60	42 (70.00)	24 (30.00)	

NS, not significant.

AKT2 protein in 126 pairs of osteosarcoma and matched non-cancerous bone tissues were detected by an immunohistochemical assay. As demonstrated in Fig. 1, strongly positive immunostaining of AKT2 protein was detected in the nucleus and/or cytoplasm of tumor cells in osteosarcoma tissues with a uniform or interspersed granular pattern, while weakly positive or negative immunostaining was detected in non-cancerous bone tissues. In addition, the statistical analysis demonstrated that the expression levels of AKT2 protein in osteosarcoma tissues were significantly higher than in corresponding non-cancerous bone tissues (IRS, 6.39 ± 1.62 vs. 3.46 ± 1.03 ; $P < 0.001$). Of 126 patients with osteosarcomas, 70 (55.56%) and 56 (44.44%) belonged to the high and low AKT2 expression groups, respectively.

Elevated expression of AKT2 associates with tumor progression of osteosarcoma tissues. Table I summarizes the association of AKT2 expression with the clinicopathological features of the osteosarcoma patients. The elevated expression

of AKT2 protein was significantly associated with positive recurrence ($P = 0.028$), the presence of metastasis ($P = 0.006$) and poor response to chemotherapy ($P = 0.015$). However, the expression of AKT2 protein was not correlated with other factors of patients including age, gender, tumor size, tumor site and histological subtype (all $P > 0.05$).

Elevated expression of AKT2 associates with poor prognosis in osteosarcoma patients. The prognostic value of AKT2 expression in human osteosarcoma was further investigated by Kaplan-Meier analysis and a log-rank test. As demonstrated in Fig. 2, osteosarcoma tissues with high AKT2 expression were correlated with a shorter overall survival (Fig. 2A; $P < 0.001$) and shorter event-free survival time (Fig. 2B; $P < 0.001$). Univariate analysis revealed that the positive recurrence and metastasis, the high expression of AKT2 protein and the poor response to chemotherapy were all significantly correlated with poor overall survival ($P = 0.006$, $P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively; Table II) and event-free survival

Table II. Univariate analysis of prognostic parameters in patients with osteosarcoma by Cox regression analysis.

Variable	Overall survival		Event-free survival	
	P-value	Relative risk	P-value	Relative risk
Age at diagnosis (years)				
<20 vs. \geq 20	0.132	0.982	0.171	1.058
Gender				
Male vs. female	0.162	0.961	0.189	1.146
Tumor size (cm)				
<5.0 vs. \geq 5.0	0.113	1.268	0.115	1.321
Tumor site				
Femur vs. tibia and other	0.207	1.199	0.131	1.087
Histological type				
Osteoblastic vs. other	0.106	1.425	0.129	1.525
Recurrence				
Negative vs. positive	0.006	3.168	0.01	3.032
Metastasis				
Negative vs. positive	<0.001	6.879	<0.001	6.532
Response to chemotherapy				
Good vs. poor	<0.001	8.332	<0.001	7.969
AKT2 expression				
Low vs. high	<0.001	6.501	<0.001	6.089

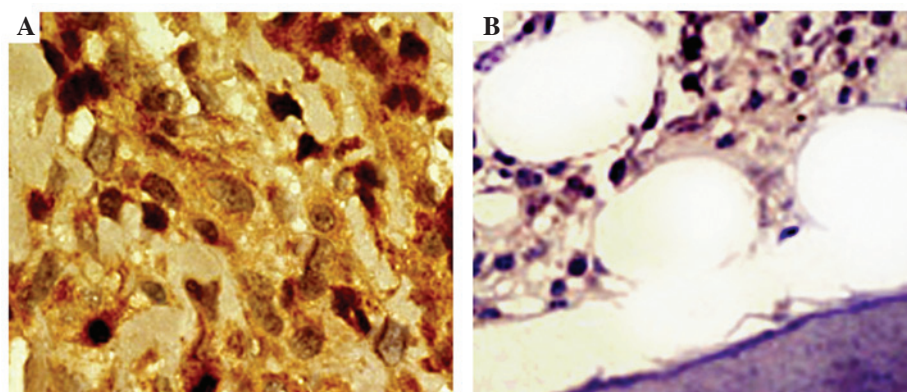


Figure 1. Representative AKT2 protein immunostaining in (A) formalin-fixed paraffin embedded osteosarcoma and (B) self-paired non-cancerous bone tissue samples. Strongly positive immunostaining of AKT2 protein was detected in the nucleus and/or cytoplasm of tumor cells in osteosarcoma tissues with a uniform or interspersed granular pattern, while weakly positive or negative immunostaining were detected in non-cancerous bone tissues (magnification, x400).

($P=0.01$, $P<0.001$, $P<0.001$ and $P<0.001$, respectively; Table II) of osteosarcoma patients. Multivariate analysis, as summarized in Table III, further demonstrated that AKT2 expression ($P=0.029$ and 0.016 , respectively), the status of recurrence ($P=0.018$ and 0.012 , respectively) and metastasis ($P=0.020$ and 0.015 , respectively), and the response to chemotherapy ($P=0.011$ and 0.008 , respectively) were all independent prognostic factors for overall survival and event-free survival time.

Discussion

Despite the advancement of therapeutic strategies, it is extremely difficult to improve the prognosis of patients with

osteosarcoma. Furthermore, as 75% of osteosarcoma cases occur in 10-20-year-old patients, this disease is a significant burden to families and society (13). For these reasons, the screening of efficient molecular markers is of great significance to differentiate the malignant progression, determine the clinicopathological characteristics and predict the clinical outcome of patients with osteosarcoma. In the present study, it was demonstrated that elevated expression of AKT2 may be associated with the progression of osteosarcoma. The results of the immunohistochemical assay reveal that AKT2 is upregulated in primary osteosarcoma specimens. Furthermore, the expression level of AKT2 significantly correlated with aggressive disease severity in the patients. The statistical analyses

Table III. Multivariate analysis of prognostic parameters in patient with osteosarcomas by Cox regression analysis.

Variable	Overall survival		Event-free survival	
	P-value	Relative risk	P-value	Relative risk
Recurrence				
Negative vs. positive	0.018	3.141	0.012	3.226
Metastasis				
Negative vs. positive	0.020	2.817	0.015	3.962
Response to chemotherapy				
Good vs. poor	0.011	3.309	0.008	4.556
AKT2 expression				
Low vs. high	0.029	2.261	0.016	3.198

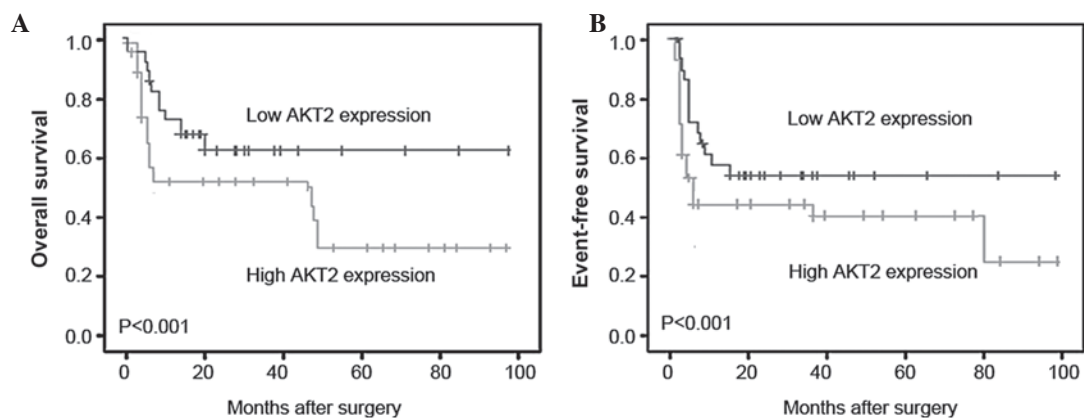


Figure 2. Kaplan-Meier curves for survival time in patients with osteosarcoma are divided according to AKT2 expression. (A) Overall survival and (B) event-free survival of patients with high vs. low AKT2 expression levels are shown. Osteosarcoma patients with high AKT2 expression were correlated with a shorter overall survival ($P<0.001$) and shorter event-free survival time ($P<0.001$).

revealed that the patients with high expression of AKT2 had a poorer prognosis. Based on these findings, it was suggested that the expression of AKT2 may be of value in predicting the progression and prognosis of patients with osteosarcoma.

Activation of specific AKT family members promotes tumor cell cycling, survival and invasiveness, enhances telomerase activity and modulates angiogenesis (14). These processes are all considered to be hallmarks of cancer. In particular, accumulating evidence indicates that alterations in AKT2 are common in numerous types of human cancer. For example, Cheng *et al* (15) identified the amplification and overexpression of AKT2 in human ovarian carcinoma tissues and cell lines and Roy *et al* (16) demonstrated that the overexpression of AKT2 may be an early event during colon tumorigenesis. Furthermore, Cheng *et al* (17) indicated that the overexpression of AKT2 may be capable of transforming NIH3T3 cells, and AKT2 antisense RNA may inhibit the tumorigenic phenotype of cancer cells exhibiting amplified AKT2. In the osteosarcoma literature, the activation of AKT2 has been demonstrated to be capable of promoting a chemoresistant phenotype and the inhibition of this kinase may sensitize chemoresistant cancer cells (12). Consistent with these previous findings, data from the present study demonstrated that the expression of AKT2 was significantly increased in osteosarcoma tissues compared

with non-cancerous bone tissues. Notably, it was identified that the elevated expression of AKT2 protein may be closely associated with the status of recurrence and metastasis, the response to chemotherapy and the clinical outcome of patients with osteosarcoma, implicating that AKT2 protein may have an oncogenic role during the progression of this malignancy.

In previous years, a variety of mechanisms regarding the oncogenic role of AKT2 have been delineated, but not fully elucidated. It is well established that the activation of AKT kinases is a multi-step process that involves membrane translocation and phosphorylation, and is triggered by the engagement of receptor tyrosine kinases by peptide growth factors and cytokines (18,19). AKT kinases belong to the PI3K/AKT signaling pathway, including the upstream PI3K, PTEN and LKB1, and the downstream tuberous sclerosis complex 2 and eukaryotic initiation factor 4E. All these components have been linked to tumorigenesis (20). The critical step in the signaling cascade leading to AKT activation is stimulation of the growth factor receptor-associated PI3K that forms a direct axis with AKT (21). Aside from this, several serine-threonine phosphatases, including protein phosphatase 2A, may also be involved in the inactivation of AKT *in vivo* (22). However, the precise molecular mechanisms for the altered expression of AKT2 in human osteosarcoma remain unclear thus far.

In conclusion, to the best of our knowledge, this is the first study to indicate that the elevated expression of AKT2 may be associated with aggressive clinical behavior and poor outcome in patients with osteosarcoma. Therefore, AKT2 may be a candidate marker of unfavorable prognosis in osteosarcoma. Further studies of the molecular mechanisms by which AKT2 contributes to the initiation and progression of osteosarcoma are warranted to facilitate further clinical application of this evidence.

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