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Diverse involvement of isoforms and gene aberrations of Akt in human lung carcinomas

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Key words

Akt, gene amplification, isoforms, lung carcinoma, lymph node metastasis

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Emerging evidence confirms a central role of Akt in cancer. To evaluate the relative contribution of deregulated Akt and their clinicopathological significance in lung carcinomas, overexpression, activation of Akt and *AKT* gene increases were investigated. Immunohistochemical staining for 108 cases revealed overexpression of total Akt, Akt1, Akt2 and Akt3 in 61.1, 47.2, 40.7 and 23.1%, respectively, and phosphorylated Akt in 42.6% of cases. Expression of total Akt, Akt2 and Akt3 were frequently observed in small cell carcinoma, but phosphorylated Akt and Akt1 were more frequently observed in squamous cell carcinoma. FISH analysis to evaluate gene increases of *AKT1-3* revealed amplification of *AKT1* in 4.2% and *AKT1* increase by polysomy of chromosome 14 in 27.3% of cases. For *AKT2*, amplification was observed in 3.2% and polysomy of chromosome 19 in 26.3% of cases. *AKT3* increase was observed in 40.0% of cases only by polysomy of chromosome 1. Although "FISH-positive" *AKT1* and *AKT2* gene increases (amplification/high-level polysomy) were found exclusively in the cases overexpressing total Akt, Akt1 or Akt2, respectively, *AKT3* increase was irrelevant of Akt3 expression. Statistically, expressions of Akt2, p-Akt and cytoplasmic-p-Akt were correlated with lymph node metastasis ($P = 0.0479$, $P = 0.0371$ and $P = 0.0310$, respectively). Although *AKT1* and *AKT2* gene increase showed positive correlation with, or trend towards a positive correlation with tumor size ($P = 0.0430$, $P = 0.0590$, respectively), *AKT3* did not. In conclusion, Akt isoforms are differentially involved in the pathological phenotype of lung carcinoma in a diverse manner. Because abnormality of Akt1/*AKT1* and Akt2/*AKT2* correlated with clinicopathological profiles, Akt1/2-specific targeting may open a novel therapeutic window for the group showing Akt deregulation.

Over the past few decades, there has been much promise in the development of tailored therapies for lung cancer, especially given the progress in targeting specific molecules in different types of cancer.[1,2] Therefore, the notion of supplementing conventional chemotherapeutic agents with novel targeted agents has garnered further attention.

The phosphoinositide-3 kinase (PI3K)/Akt pathway is instrumental in both physiological and pathological processes through orchestrating many signaling pathways downstream.[1,3,4] Among effectors in this PI3K/Akt cascade, Akt, a Ser/Thr kinase that belongs to the AGC family (AMP/GMP kinases and protein kinase C) has emerged as a central node of complex signaling pathways, and, thus, has been intensively explored in oncology research.[1,3,4] These family proteins are encoded by three related genes (*AKT1*/PKBα, *AKT2*/PKBβ and *AKT3*/PKBγ), located on chromosomes 14q32, 19q13 and 1q44, respectively.[4,5] Encoded 56-kDa proteins Akt1, 2 and 3 show an 80% amino acid identity, but distinct tissue distributions and functions.[3] While Akt1 is expressed ubiquitously at a high level, Akt2 is expressed particularly in insulin-responsive tissues such as fat, skeletal muscle and the liver.[3] Akt3 is predominately expressed in the brain, the embryonic heart, the testis and the kidney.[4,5]

In response to upstream signals, Akt activation is initiated through phosphorylation at Thr308 by PI3K-dependent kinase-1 (PDK1), and at Ser473 by PDK2. Subsequently, Akt translocates to specific subcellular compartments and exerts their own functions.[3,4]

Akt activation by phosphorylation and/or gene increases has been frequently reported in many types of human cancers. Akt1 overexpression/activation has been found in gastric, ovarian and breast carcinomas,[1,3,6] and *AKT1* amplification has been found in sporadic cases of lung, gastric, breast and prostatic carcinomas.[3,5,7,8] Akt2 is overexpressed in colorectal, ovarian, pancreatic and breast carcinomas.[1,3,6] Amplification of *AKT2* has been more frequently observed compared with *AKT1*, comprising up to 3% of breast, 14% of ovarian, and 15% of pancreatic carcinomas.[3,5,9] *AKT2* increase correlates with the tumor size in soft tissue sarcoma, and amplification correlates with a poor prognosis in ovarian carcinoma.[9,10] Therefore, Akt1 and Akt2 are activated not only via signaling pathways, but also by their own genetic alterations. Subsequent Akt activation is prevalent

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in high grade, advanced tumors, and is associated with metastasis, radioresistance and worse prognosis.[3,11]

Selective activation of Akt3, and not of Akt1 or Akt2, has been observed in several kinds of malignancies, including melanomas in up to 60% of cases, and the Akt3 expression increases during melanoma progression and metastasis.[12] Amplification of *AKT3* has been found at lower frequencies in breast carcinomas, gliomas, hepatocellular carcinomas and melanomas.[2,5,13] However, the clinicopathological significance of Akt3 overexpression in other human carcinomas is still unclear.

In our previous study on lung carcinoma, Flourescence *in situ* hybridization (FISH) analysis revealed amplification of *AKT1* and/or *AKT2* in approximately 7% of cases.[8] However, detailed clarification regarding the contributions of each isoform and alterations in three *AKT* genes has not been attempted in a series of study. Herein, we conduct such comprehensive analysis.

Materials and Methods

Cases and classification. The current study comprised 108 cases of primary lung carcinoma, including 48 cases of adenocarcinoma (AC), 37 squamous cell carcinoma (SCC), 5 large cell carcinoma (LCC) and 18 cases of small cell carcinoma (SCLC), obtained from surgeries at Jichi Medical University and Yamanashi University (Table 1). This study was approved by the Institutional Ethical Review Board and written informed consent was obtained from each patient. Histopathological diagnoses were made according to World Health Organization Classifications,[14] and all cases were categorized by UICC TNM classification.[15] None of the patients had received preoperative chemotherapy.

Table 1. Patients and tumor characteristics

Characteristics	Number (108 cases)
Gender	
Male	57
Female	51
Smoking history†	
No	14
Yes	94
Histology	
AC	48
SCC	37
LCC	5
SCLC	18
T factor (NSCLC, 90 cases)	
T1	41
T2	32
T3	17
N factor (NSCLC, 90 cases)	
N0	52
N1	25
N2	13
N3	0
Stage (NSCLC, 90 cases)	
I	41
II	31
IIIa	18

†Brinkman index: 0–3000, average 956, median 850. AC, adenocarcinoma; LCC, large cell carcinoma; NSCLC, non-small cell lung carcinoma. SCC, squamous cell carcinoma; SCLC, small cell carcinoma.

This cohort was partially used in our previous work.[8] Excluding the cases in which FISH was not succeeded in the previous series (12 cases), informed consent for this extended study was not obtained from the patients (nine cases) and the cases in which the amount of tissue left was small (28 cases), 86 cases left. Those cases were reevaluated, except for the analysis of *EGFR* mutation, together with newly obtained samples (22 cases), and all tissue samples were further used for other additional analyses performed in the present study. Following reevaluation of previous results, several minor changes in the staining score were made: for total-Akt (T-Akt) stain, "1+" was changed to "2+" in four cases, "2+" to "1+" in one case, and for phosphorylated-Akt (p-Akt), "1+" was changed to "2+" in three cases, and "2+" to "1+" in one case. The FISH results were identical in the reevaluation.

Immunohistochemistry (IHC). Paraffin-embedded tissues were serially sectioned and stained with primary antibodies: T-Akt (polyclonal; Cell Signaling Technology [CST], Beverly, MA, USA) 1:300; Akt1 (monoclonal, C73H10, CST) 1:50; Akt2 (polyclonal; Abcam, Cambridge, UK) 1:150; Akt3 (polyclonal; Abcam) 1:120; and phosphorylated-Akt (p-Akt^{Ser473}, monoclonal, D9E, CST) 1:50. The sensitivity and the specificity of the antibodies has been validated.[8,16–18] Visualization was performed using a CSAII kit (Catalyzed Signal Amplification System 2; Dako, Glostrup, Denmark).

Immunohistochemical expression was evaluated by two observers (YD and AO). "Positive" was defined as staining that was more intense than occasional faint staining seen in non-neoplastic cells.[10,16] IHC score was quantitatively evaluated by the fraction of "positive" cells: negative, <10%; 1+, 10%≤, <50%; 2+, 50%≤.[10,19] Discordance was resolved by discussion. Because in our previous analysis, none of the IHC-negative cases exhibited gene amplification or high-level polysomy,[8,10] we regarded 1+ and 2+ as "overexpression," and they were combined as "IHC positive" when scores were categorized into two groups for statistical analysis.

FISH. Gene copy increases were analyzed by FISH as described previously.[8,10,20] Target and respective reference probes for *AKT1* and *AKT2* were prepared as previously described.[8,10] For *AKT3*, a bacterial artificial chromosome (BAC) clone, RP11-269F20, which contains a part of the *AKT3* gene at 1q44 (http://www.ncbi.nlm.nih.gov), and a pUC1.77 plasmid as a centromere probe for chromosome 1 were used.[21] The specificity and localization of these probes were confirmed on metaphase spreads of normal lymphocytes (Abbott, Green Oaks, IL, USA).[8,10]

Gene copy increase was evaluated as the ratio of the total number of *AKT* signals over the reference signals by two observers (YD and AO). Results were classified into four categories: (i) disomy (≤2 copies in >90% of cancer cells); (ii) low-level polysomy (≥3 copies of target accompanied by an equivalent number of reference genes in ≥10% and <40%, without amplification); (iii) high-level polysomy (polysomy in ≥40%, without amplification); and (iv) amplification (presence of tight clusters, average target/reference gene ratio of ≥2 or ≥15 copies of target per cell in ≥10% of cells).[8,10,20,22] We found in previous studies that two groups of tumors harboring *AKT* increase by high level or low level polysomy had distinct pathobiological profiles:[8,18,20,22] (i) although the former always exhibited Akt overexpression and activation, the latter did not; and (ii) there was a reciprocal relationship between *AKT* gains by amplification or high-level polysomy and epidermal growth

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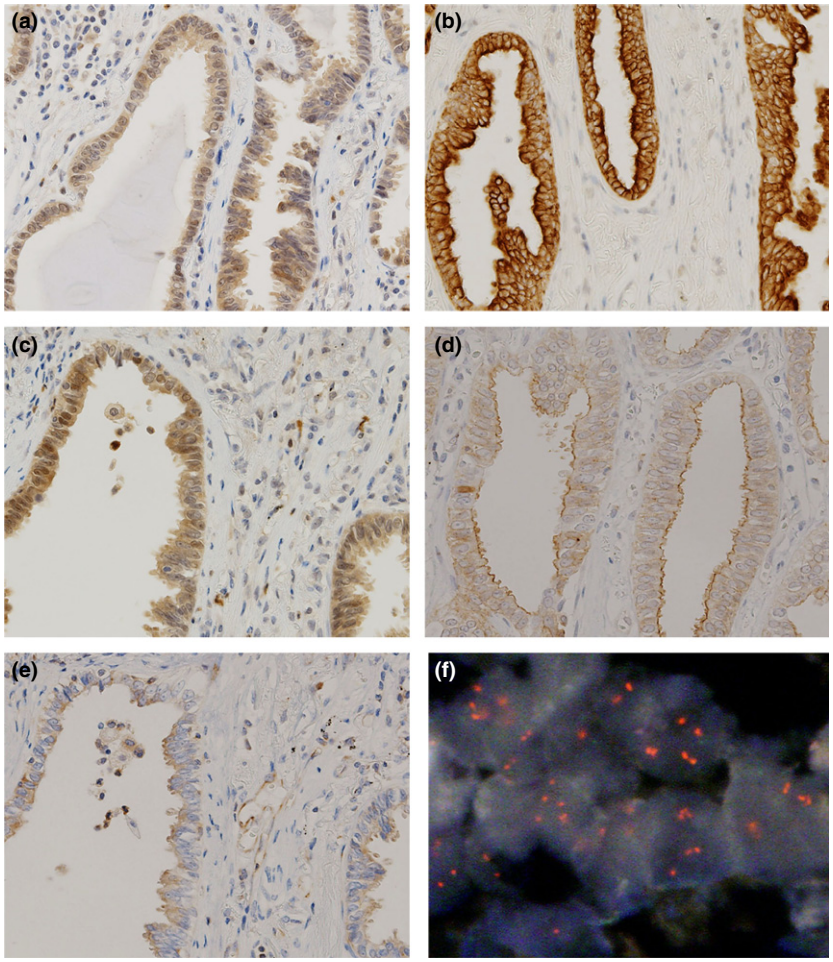

Fig. 1. Results of immunohistochemical staining and FISH analysis. A case of adenocarcinoma that exhibited nuclear/cytoplasmic positive staining for total-Akt (a) and Akt1 (c), and cytoplasmic staining for phosphorylated-Akt (b), Akt2 (d), Akt3 (e). FISH revealed amplification of *AKT1* (f).

factor receptor (*EGFR*) mutation or *EGFR* increase, but no relationship was found between low-level polysomy and *EGFR* aberrations. Therefore, we categorized numerical *AKT* status into two groups for the statistical analysis: *AKT*-"FISH-positive" (gene amplification/high-level polysomy) and *AKT*-"FISH-negative" (low-level polysomy/disomy).[20,22]

Nucleotide sequence analysis. Among 48 cases of AC, *EGFR* mutations had already been examined in 24 cases in the previous study. Among the remaining 24 cases, the hot spots of *EGFR* mutations between exon 18 and exon 21 were investigated using the peptide nucleic acid-locked nucleic acid-PCR clamp reaction method in 20 cases in which plenty of tissues were available in the paraffin blocks.[8,18]

Statistical analysis. Observers' accordance in the evaluation of IHC results was analyzed by kappa (κ) statistics. Other statistical analyses were performed using the JMP software package (version 11; SAS Institute Inc., Cary, NC, USA). Differences in the rate of positive immunostaining between two categories were analyzed by Fisher's test. The Mann–Whitney test was also used to analyze two categories. Log-rank analysis using the Kaplan–Meier method was applied for the correlation between the variables and the patients' survival. In the multivariate analysis, Cox proportional-hazards regression analysis was used. A two-sided *P*-value of <0.05 was used to determine statistical significance.

Results

Immunohistochemistry. Results of IHC are presented in Figures 1–4 and Table 2. Inter-observer agreement was "almost perfect" (κ: T-Akt = 0.87; Akt1 = 0.84; Akt2 = 0.78; Akt3 = 0.95; p-Akt = 0.88).

T-Akt. In non-neoplastic tissues, weak staining was observed in the cytoplasm of bronchial epithelial cells, lymphocytes and endothelial cells. In tumors, positive staining was observed in 66 cases (61.1%) predominantly in the cytoplasm and less commonly in the nucleus (45 *vs* 27 cases); these constitute 25 cases of AC (52.1%), 24 of SCC (64.9%), 4 of LCC (80.0%) and 13 of SCLC (72.2%). Staining was observed without a specific pattern of heterogeneity and no significant difference in positive rate was found among histological types ($P = 0.220$). The IHC score varied depending on the histological type; 61.5% of the positive cases in SCLC had scores of 2+, but only 40.0% in AC.

p-Akt. Weak p-Akt staining was occasionally observed in the cytoplasm of bronchial epithelial cells. In tumors, it was observed in 46 cases (42.6%) in the cytoplasm and less frequently in the nucleus (36 *vs* 17 cases); these constitute 17 cases of AC (35.4%), 18 of SCC (48.6%), 3 of LCC (60.0%) and 8 of SCLC (44.4%). There were no significant differences among histological types ($p = 0.917$). In regards to IHC score, 50% of the positive cases in SCLC had scores of 2+, but only 35.3% in AC.

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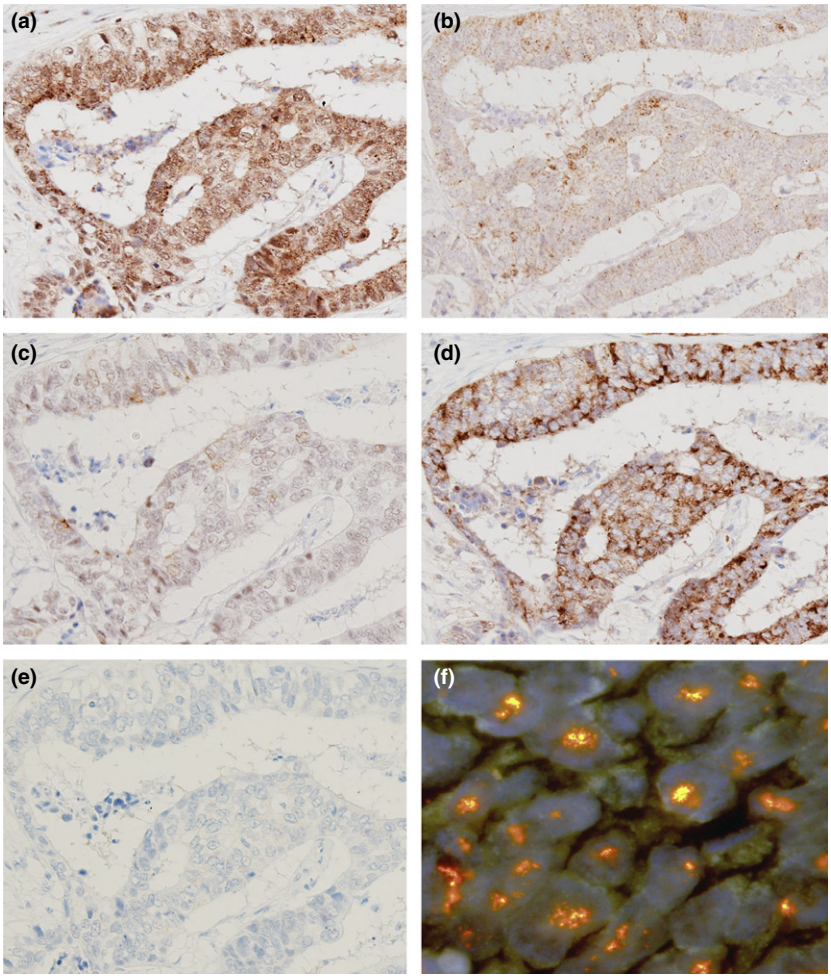

Fig. 2. A case of adenocarcinoma that exhibited nuclear/cytoplasmic positive staining for total-Akt (a), cytoplasmic staining for phosphorylated-Akt (b) and Akt2 (d) and Akt1 staining in the nucleus (c). Akt3 staining was negative (e). FISH revealed cluster-type amplification of *AKT2* (f).

Akt1. Weak Akt1 staining was occasionally observed in the cytoplasm of bronchial epithelial cells and lymphocytes. In tumors, it was observed in 51 cases (47.2%), including 22 of AC (45.8%), 18 of SCC (48.6%), 4 of LCC (80.0%) and 7 SCLC (38.9%), and was more prevalent in the nucleus than in the cytoplasm (34 *vs* 25 cases). It was found more frequently compared to Akt2 in AC and in SCC.

Akt2. In non-neoplastic tissues, significant staining was not observed. In tumors, Akt2 staining was observed in 44 cases (40.7%), including 16 AC (33.3%), 16 SCC (43.2%), 2 LCC (40.0%) and 10 SCLC (55.6%). Compared with Akt1, Akt2 was more frequently expressed in SCLC. Akt2 was found to be localized predominantly in the cytoplasm compared with the nucleus (41 *vs* 8 cases).

Akt3. Weak Akt3 staining was observed in endothelial cells. In tumors, it was observed in 25 cases (23.1%), including 9 AC (18.8%), 7 SCC (18.9%), 2 LCC (40.0%) and 7 SCLC (38.9%). Its localization was predominantly in the cytoplasm in all histological types (23 cytoplasmic *vs* two cases with nuclear).

FISH. FISH for *AKT1*, *AKT2* and *AKT3* was performed and the signals were successfully visualized in 95 cases, of which 60 cases were "T-Akt-positive" (Table 2). These 95 cases include 43 cases of AC (including 23 T-Akt-positive cases), 33 of SCC (21 T-Akt-positive cases), 5 of LCC (4 T-Akt-positive cases) and 14 of SCLC (12 T-Akt-positive cases). The regions exhibiting *AKT1* and *AKT2*, but not *AKT3* increases, were overlapped with respective isoform-positive regions on IHC. Overall results are presented in Figures 1–4 and Table 2.

AKT1 was amplified in four cases (4.2%): one case each of AC (*AKT1*/reference signal ratio [*AKT1* ratio] of 2.25, Fig. 1), SCC (*AKT1* ratio, 2.51), LCC (*AKT1* ratio, 2.87) and SCLC (*AKT1* ratio, 3.48). *AKT1* increase with high-level polysomy of chromosome 14 was found in eight cases (8.4%). Sixty-five cases (68.4%) exhibited disomy.

AKT2 was amplified in three cases (3.2%): one case of AC with a clustered signal (Fig. 2), 1 SCC (*AKT2*-ratio, 3.05) and one case of SCLC with a clustered signal. *AKT2* increase with high-level polysomy of chromosome 19 was detected in 11 T-Akt-expressing cases (11.6%). Sixty-seven cases (70.5%) exhibited disomy.

For *AKT3*, no amplification was found, but *AKT3* increase with polysomy of chromosome 1 was detected in 38 cases: 28 cases (including eight cases of high-level polysomy) were T-Akt-positive (Fig. 3) and 10 cases (including two cases of high-level polysomy) were T-Akt-negative (Fig. 4). Therefore, 57 cases (60.0%) exhibited disomy.

Although co-amplification of *AKT* was not observed, all seven cases showing amplification harbored polysomy of other chromosome(s).

Collectively, amplification was found in 7.4% of total cases and FISH-positive-gene increase (amplification/high-level polysomy) of one or more *AKT* was observed in 27 cases (28.4%).

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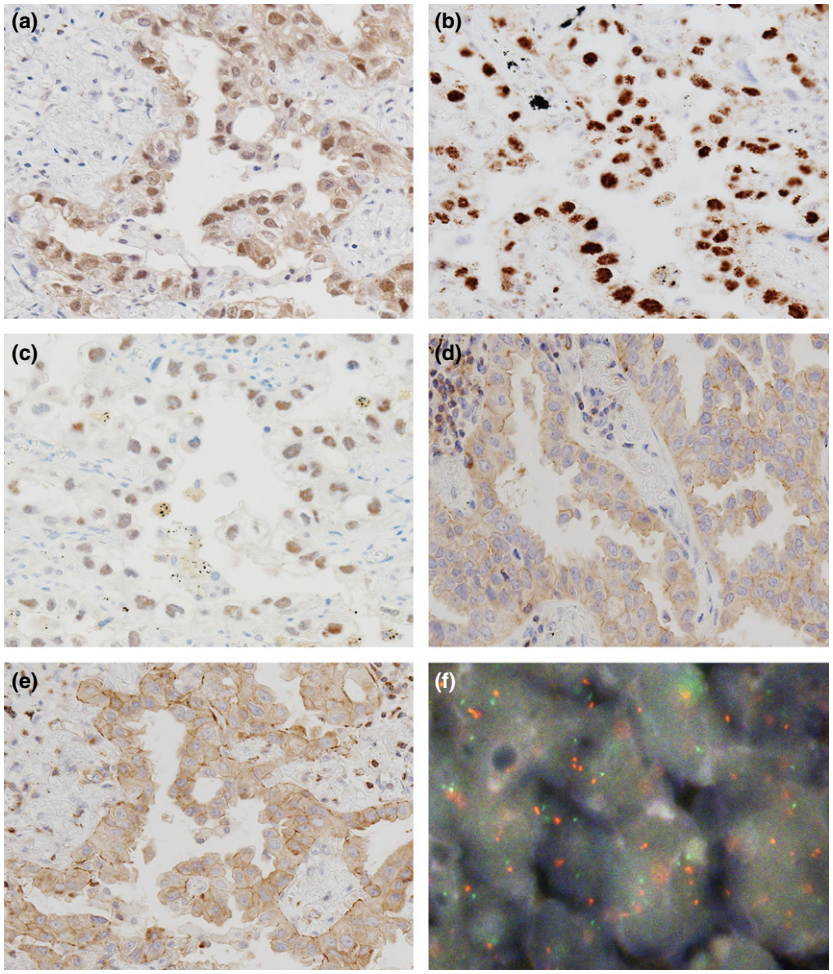

Fig. 3. A case of adenocarcinoma that exhibited nuclear/cytoplasmic staining for total-Akt (a), phosphorylated-Akt (b), Akt1 (c) and cytoplasmic staining for Akt2 (d) as well as Akt3 (e). FISH revealed an increase in gene-specific signals (orange fluorescence) and reference probe signals (green fluorescence), indicating *AKT3* increase with high-level polysomy (f).

The linkage between FISH-positive *AKT1* and *AKT2* increases were correlated ($P = 0.0147$). However, between *AKT1* and *AKT3* ($P = 0.6097$), or between *AKT2* and *AKT3* ($P = 0.1630$), no correlation was found.

Epidermal growth factor receptor-mutation spectrum and correlation with immunohistochemical results. In our previous study, mutations of the *EGFR* were found in 10 of 24 AC cases: leucine to arginine substitution at codon 858 (L858R) in five cases, in-frame deletions from 746 through 753 within exon 19 (del.ex19) in four cases and glycine to serine substitution at codon 719 (G719S) in one case. In the current study, mutation analysis was performed in the remaining 20 cases, which revealed nine cases of mutation. Collectively, the *EGFR* mutations was found in 19 cases (43.2%): L858R in 10 cases, del.ex19 in seven cases, leucine to glutamine substitution at codon 861 (L861Q) and glycine to serine substitution at codon 719 (G719S) in one case each. One of the cases revealing del.ex19 was accompanied by threonine to methionine substitution at codon 790 (T790M). Although the correlations between the IHC score of T-Akt, p-Akt and Akt isoforms and *EGFR* status were analyzed, the difference between the groups of wild-type and mutated-*EGFR* was not statistically significant (T-Akt, $P = 0.3313$; p-Akt, $P = 0.0756$; Akt1, $P = 0.1966$; Akt2, $P = 0.8497$; Akt3, $P = 0.6441$).

Comparison of immunohistochemistry and FISH results. The results of IHC and FISH were compared for possible correlations (Table 3).

First, overexpression of each protein was mutually correlated ($P \leq 0.0252$).

Second, all the tumors exhibiting FISH-positive *AKT1* and *AKT2*, but not *AKT3* increases, revealed T-Akt and p-Akt overexpression. The difference in the frequencies of T-Akt or p-Akt overexpression between FISH-positive and FISH-negative groups was statistically significant, except for *AKT3*. Consistently, the IHC score of T-Akt or p-Akt and FISH-positive *AKT1* and *AKT2*, but not *AKT3* increases, were significantly correlated.

Third, all the cases harboring FISH-positive *AKT* increases revealed overexpression of respective isoforms, except two cases harboring *AKT3* increases. The differences in the frequencies of isoform overexpression between FISH-positive and FISH-negative groups were statistically significant. However, the IHC scores of only Akt1 ($P = 0.0345$) and Akt2 ($P < 0.0001$), but not of Akt3 ($P = 0.1960$), were correlated with FISH-positive gene increases. Consistently, among 10 cases harboring *AKT3* increase, only five cases revealed 2+ expression of Akt3.

Finally, viewed from the subcellular localization, that of T-Akt and p-Akt was correlated ($P = 0.0141$) and nuclear T-Akt was correlated with Akt1 overexpression ($P = 0.0355$), suggesting that overexpression of Akt1 represents nuclear accumulation of T-Akt and subsequent higher Akt activity in the nucleus. In contrast, cytoplasmic p-Akt was correlated with Akt2 ($P = 0.0475$). Therefore, Akt activity in the cytoplasm is positively regulated by Akt2.

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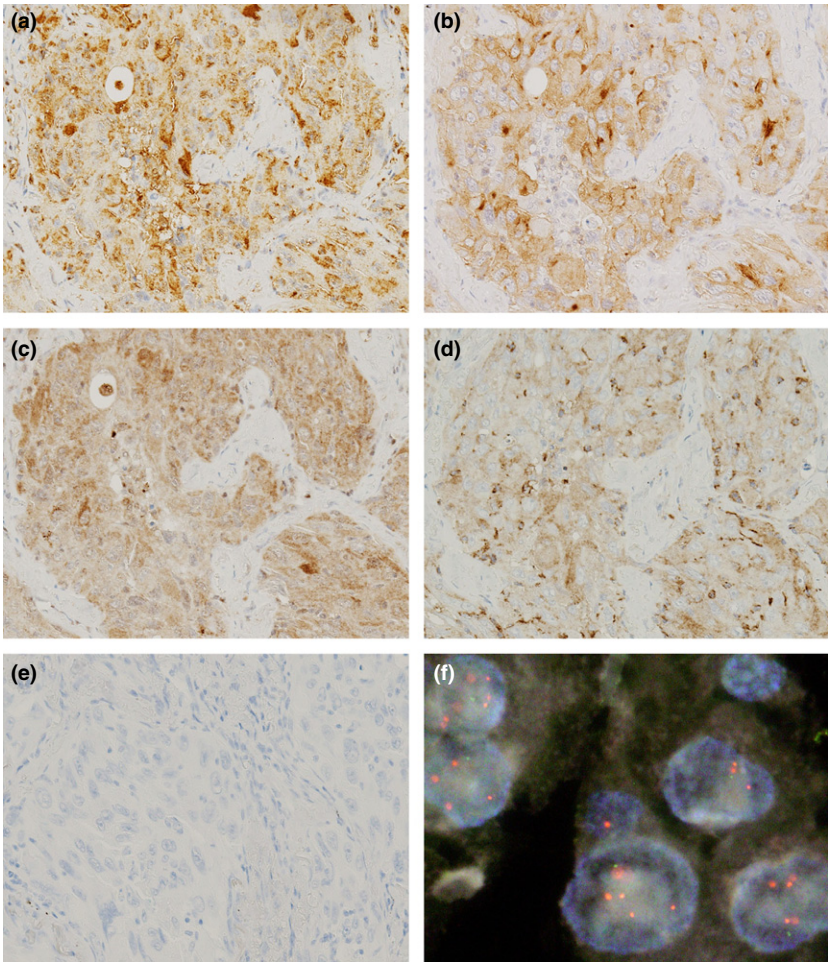

Fig. 4. A case of squamous cell carcinoma that exhibited cytoplasmic staining for total Akt (a), phosphorylated-Akt (b), Akt1 (c) and Akt2 (d). Akt3 staining was negative (e), but FISH showed *AKT3* increase with high-level polysomy (f).

Clinicopathological analysis. Overall, data was statistically analyzed for their correlation with clinicopathological profiles (Table 3).

When expression and activation of Akt was analyzed in correlation with smoking history by Brinkman index, none showed statistically significant correlation (T-Akt, $P = 0.3002$; p-Akt, $P = 0.8515$; Akt1, $P = 0.6537$; Akt2, $P = 0.7033$; Akt3, $P = 0.1749$).

In all cases, *AKT* increase was not correlated with specific histological types in non-small cell carcinoma (NSCLC) (*AKT1*, $P = 0.1820$; *AKT2*, $P = 0.2022$; *AKT3*, $P = 0.4088$). FISH-positive-*AKT1* increase was correlated with tumor size (pT, $P = 0.0430$) and *AKT2* increase also showed the same trend, but not significant ($P = 0.0590$). Moreover, nodal status (pN) in NSCLC was correlated with p-Akt ($P = 0.0371$), cytoplasmic p-Akt ($P = 0.0310$) and with Akt2 ($P = 0.0479$). Although *AKT2* increase was correlated with p-Akt ($P = 0.0014$), it revealed only a trend toward correlation with pN without statistical significance ($P = 0.0645$). *AKT3* increase revealed no correlation with pT or pN.

When analyzed with respect to overall survival (OS), a significant correlation was observed with pT ($P = 0.0483$) and pN ($P = 0.0182$) (Fig. 5). Expression of T-Akt, isoforms, p-Akt or *AKT* increases did not reach statistical significance. In multivariate analysis, only pN had a statistical impact in all cases of NSCLC ($P = 0.0332$) and in AC ($P = 0.0415$). Mutation of *EGFR* in AC did not show statistical impact on prognosis ($P = 0.0792$).

Discussion

Based on our current knowledge regarding the pathobiology of human cancer, Akt has been interpreted as a high-priority therapeutic target. However, the participation profiles of each Akt isoform and its clinicopathological significance have not been explored comprehensively.

In this study, several pathobiological profiles were found in each category defined by the *AKT*-gene status.

First, we identified amplification of *AKT1* and *AKT2*, but not of *AKT3*. However, gene increases with high-level polysomy were observed in all *AKT*. With regards to the heterogeneity of gene increase, because *AKT1* and *AKT2* increase was found in IHC-positive areas, heterogeneity in the FISH-results may be equivalent to the heterogeneity in the IHC results. *AKT3* gene increase occurred regardless of Akt3 protein expression; thus, we performed FISH for *AKT3* in a broader area. As a result, heterogeneity of *AKT* gains was present, but the particular distribution pattern was not identified histologically (data not shown).

Second, all *AKT1*-FISH-positive or *AKT2*-FISH-positive tumors exhibited overexpression of T-Akt, each isoform and p-Akt. However, *AKT3* increases were not always accompanied by overexpression of those proteins. Thus, *AKT*3 gene increases may occasionally be nonpathogenic "passengers."

Finally, consistent with a previous study reporting that *AKT2*-transfected cells were more metastatic *in vivo* due to promotion of cell motility by Akt2,[3,23] Akt2 expression was correlated with pN. Although *AKT2* increase did not show

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Table 2. Results of immunohistochemical and FISH analyses

	Histology (cases)					
	AC (48)	SCC (37)	LCC (5)	NSCLC (90)	SCLC (18)	Total (108)
	Positive cases (2+)					
IHC						
T-Akt	25 (10)	24 (11)	4 (3)	53 (24)	13 (8)	66 (32)
p-Akt	17 (6)	18 (8)	3 (2)	38 (16)	8 (4)	46 (20)
Akt1	22 (8)	18 (7)	4 (2)	44 (17)	7 (2)	51 (19)
Akt2	16 (6)	16 (5)	2 (1)	34 (12)	10 (5)	44 (17)
Akt3	9 (1)	7 (0)	2 (0)	18 (1)	7 (1)	25 (2)
FISH						
AKT1						
A	1	1	1	3	1	4
H	2	4	1	7	1	8
L	8	6	2	16	2	18
AKT2						
A	1	1	0	2	1	3
H	2	5	1	8	3	11
L	4	6	1	11	3	14
AKT3						
A	0	0	0	0	0	0
H	3	3	1	7	3	10
L	9	11	2	22	6	28

AC, adenocarcinoma; *AKT1, AKT2, AKT3*, numerical status of genes; A, amplification; H, high-level polysomy; L, low-level polysomy; LCC, large cell carcinoma; NSCLC, non-small cell lung carcinoma; p-Akt, phosphorylated-Ak; SCC, Squamous cell carcinoma; SCLC, small cell lung carcinoma; T-Akt, total-Akt.

significant correlation with pN, it showed correlation with p-Akt, which was correlated with pN. Thus, *AKT2* increase may also play a part, even indirectly, during the process of nodal metastasis.

All three isoforms translocate from the cytoplasm to the nucleus in response to a variety of stimuli.[24] In our results, because Akt1 was more frequently observed in the nucleus compared to Akt2 and Akt3, nuclear Akt2 and Akt3 may be unstable in the nucleus or localize in the nucleus more transiently compared with Akt1. One of the possible underlying mechanisms is the effect of differential dephosphorylation of each Akt isoform by specific phosphatases, pleckstrin homology domain leucine-rich repeat protein phosphatases (PHLPPs): PHLPP1 dephosphorylates and inactivates Akt2 and Akt3, while PHLPP2 targets Akt1 and Akt3 in both the nucleus and the cytoplasm.[25,26]

Clinicopathological analyses in lung carcinomas have shown various results regarding Akt.[8,17,27] Several studies found that expression of p-Akt was not associated with prognosis,[8] and was even associated with longer survival,[17] but other studies showed a poorer prognosis.[27] Recently, it was described that cases expressing nuclear p-Akt showed a poorer prognosis.[28] Nonetheless, other investigators have suggested that cancers expressing p-Akt in the nucleus have a better prognosis and responded better to targeted therapy.[19] Analytical methods have recently been further refined due to the development of commercially available isoform-specific or phosphorylated-form-specific antibodies applicable to IHC on paraffin-embedded tissue. One study reveals that high expression of non-phosphorylated Akt2 and low expression of p-Akt Thr^{308} in cancer cells as well as high expression of Akt3 in stromal cells are independent predictors of improved survival.[27] Although the mode of Akt involvement may differ depending on the site of Akt phosphorylation, our current results have shown that p-Akt-Ser^{473} as well as Akt2 expression correlated with pN, and thus, they could potentially be negative prognostic markers despite that these were not significantly correlated with OS.

Although past studies have suggested that amplification of *AKT* in tumors generally indicates a poor prognosis,[3] the prognostic significance of *AKT* was not confirmed in this

Table 3. Results of statistical analyses

	T-Akt (IHC)	p-Akt (IHC)	Akt1 (IHC)	Akt2 (IHC)	*AKT1*	*AKT2*	*AKT3*
p-Akt	$P < 0.0001$†,‡ [n-p-Akt∝n-T-Akt $P = 0.0141$†,‡]						
Akt1	$P < 0.0001$†,‡ [n-T-Akt∝Akt1 $P = 0.0355$†,‡]	$P < 0.0001$†,‡					
Akt2	$P < 0.0001$†,‡	$P < 0.0001$†,‡ [c-p-Akt∝Akt2 $P = 0.0475$†,‡]	$P < 0.0001$†,‡				
Akt3	$P < 0.0001$†,‡	$P = 0.0252$†,‡	$P < 0.0001$†,‡	$P < 0.0001$†,‡			
AKT1	$P = 0.0070$†,§ $P = 0.0008$§,¶,††	$P < 0.0001$†,§ $P = 0.0048$§,¶,††	$P = 0.0002$†,‡ $P = 0.0345$§,¶,††				
AKT2	$P = 0.0017$†,§ $P = 0.0009$§,¶,††	$P < 0.0001$†,§ $P = 0.0014$§,¶,††	$P = 0.6682$†,§	$P < 0.0001$†,§ $P < 0.0001$§,¶,††	$P = 0.0147$†,‡,§		
AKT3	$P = 0.1569$†,§ $P = 0.1311$§,¶,††	$P = 0.5056$†,§ $P = 0.2698$§,¶,††			$P = 0.6097$†,§	$P = 0.1630$†,§	
pT	$P = 0.0933$††,‡‡	$P = 0.0599$††,‡‡	$P = 0.6232$††,‡‡	$P = 0.0541$††,‡‡	$P = 0.0430$††,§§	$P = 0.0590$††,§§	$P = 0.5371$††,§§
pN		$P = 0.0371$††,‡‡ [c-p-Akt∝pN $P = 0.0310$††,‡‡]		$P = 0.0479$††,‡‡ [c-Akt2∝pN $P = 0.0496$††,‡‡]	$P = 0.0637$††,§§	$P = 0.0645$††,§§	$P = 0.1652$††,§§

†Fisher's exact test; ‡n = 108; §n = 95; ¶IHC score versuss FISH-positive/negative; ††Mann-Whitney test; ‡‡*n* = 90; §§n = 81. *AKT1*, *AKT2*, *AKT3*, numerical status of genes; c, cytoplasmic; IHC, immunohistochemical positivity; n, nuclear; p-Akt, phosphorylated-Akt; T-Akt, total Akt.

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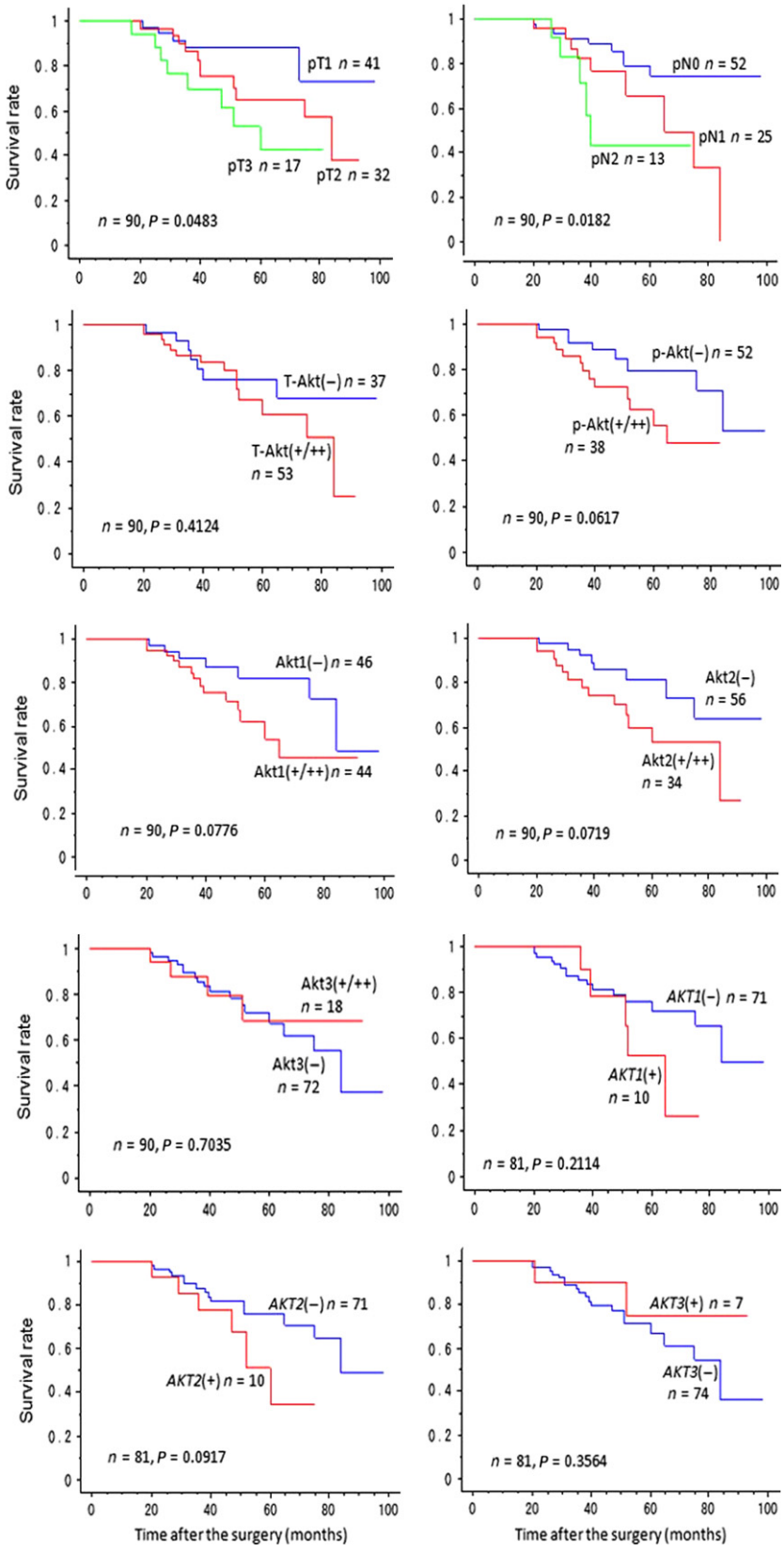

Fig. 5. Kaplan–Meier survival curves for overall survival and the results of the log-rank test. Higher UICC tumor grade (pT) and nodal status (pN) were significantly correlated with survival. *AKT1*(−), disomy/increase by low-level polysomy of *AKT1* gene, *AKT1*(+), amplification/increase with high-level polysomy of *AKT1* gene; T-Akt, total Akt; p-Akt, phosphorylated-Akt; −, immunohistochemical score 0 (negative); +/++, immunohistochemical score 1 or 2 (positive).

study. However, *AKT1* or *AKT2* increases correlated with, or showed a trend towards correlation with tumor size, suggesting their involvement in advanced stage. Moreover, because *AKT2* increase showed a trend toward correlation with pN, increases of those two genes could also be utilized as negative prognostic markers, enabling more restricted targeted therapies. In

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contrast, although *AKT3* increases were found in a larger fraction of cases, we did not find significant correlations with clinicopathological factors.

The mutation status of *EGFR* was not found to be correlated with Akt expression, clinicopathological factors or prognosis in this series, probably due to multiple inputs and complex cross-talk between the *EGFR*/Akt pathway and others.

Each Akt isoform, in particular Akt1 and Akt2, contributes similarly to the phosphorylation of the substrates and could compensate for or compete with one another in a variety of pathobiological events.[4,29] Consistently, combined knockdown of two or more isoforms, but not of individual Akt isoforms, caused significant effects.[29] However, each isoform has a different function in the different types of cells. Past studies have continuously added new layers to the complex mutual function of Akt isoforms: Akt1 promotes carcinogenesis in breast cancer and inhibits cell migration and invasion, whereas Akt2 inhibits carcinogenesis and promotes migration and invasion[6,23] These diverse functions of Akt isoforms are often regulated by their binding and/or modulator proteins, and such isoform-specificity of the substrates is determined by spatial localization of each isoform, and, thus, by the accessibility to specific modulator proteins.[30] In this context, we found that subcellular localization of T-Akt and p-Akt was correlated and that overexpression of Akt1 correlated with nuclear-T-Akt, subsequently leading to higher Akt activity in the nucleus. In contrast, Akt activity in the cytoplasm may be positively regulated by Akt2.

In the current study, expression of T-Akt and isoforms or Akt activation was not correlated with the Brinkman index. This may be partially explained by the notion that Akt is frequently phosphorylated in cancers harboring EGFR-mutation, which are known to be prevalent in AC of non-smokers.[18,31]

The development of inhibitors targeting dysregulated Akt has been shedding light on new modalities of cancer treatment. In lung carcinomas, the sensitivity of NSCLC to gefitinib has been shown to depend on the Akt suppression.[19] Therefore, a combinatorial strategy with conventional agents and Akt-targeted agents may be an efficient strategy for future translational medicine. Given the different functions of isoforms, potential for isoform-specific targeting has been under focus. Because inhibitors for Akt1 and Akt2, but not for Akt3, are promising in lung cancer, targeting Akt1 and Akt2 specifically with dual Akt1/Akt2 inhibitors provides another avenue. MK-2206 is an orally active allosteric Akt inhibitor against colon and breast cancers, presently in clinical trial. While MK-2206 is equally potent against Akt1 and Akt2, it is far less potent against Akt3.[32,33] Another Akt inhibitor Akti1/2, which is an ATP non-competitive inhibitor, also inhibits Akt1 and Akt2.[32,34] In contrast, SBF-1, a synthetic steroidal glycoside, blocks the interaction between PDK1 and Akt3, and, thus, could be applied to target melanoma growth and metastasis.[35]

In conclusion, the present study has revealed the previously undiscovered function of Akt-isoforms and *AKT* genes as potential regulators of pathobiological behavior in lung carcinomas; that is, the critical involvement of Akt1/*AKT1* and Akt2/*AKT2* in tumors harboring "FISH-positive" increase of *AKT1/AKT2* (22.1%), but not of Akt3/*AKT3*.

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Disclosure Statement

The authors have no conflict of interest to declare.

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