Phosphorylated GSK3β-ser^9 and EGFR are Good Prognostic Factors for Lung Carcinomas

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Abstract. *Background: Glycogen synthase kinase-3β (GSK3β), serine/threonine protein kinase, has been reported to repress the Wnt/β-catenin pathway and regulate the balance between cellular proliferation and apoptosis. Epithelial growth factor receptor (EGFR) might phosphorylate GSK3β into inactive phosphorylated GSK3β-ser^9 (P-GSK3β-ser^9). Patients and Methods: P-GSK3β-ser^9 and EGFR expressions were examined on tissue microarrays (TMA) of lung carcinoma (n=154) by immunohistochemistry and compared with clinicopathological parameters of the tumors, including the expression of Ki-67 and phosphatase and tensin homology deleted from human chromosome 10 (PTEN), as well as survival data. Results: The P-GSK3β-ser^9 expression was highest in adenocarcinoma (AD) and lowest in small cell carcinomas (SCC), compared with other types of lung carcinoma (p<0.05). Its expression was negatively correlated with PTEN and Ki-67 expression (p<0.05), but not with age, gender, pleural invasion, lymphatic or venous invasion, lymph node metastasis or Union Internationale Contre le Cancer (UICC) staging (p>0.05). EGFR was strongly expressed in squamous carcinomas (SQ), compared with other types of lung carcinomas. Its expression was also positively correlated with lymphatic and venous invasion and P-GSK3β-ser^9 expression, and negatively with the PTEN expression of the tumors (p<0.05), but not with the age, gender, pleural invasion, lymph node metastasis, or UICC staging (p>0.05). Kaplan-Meier analysis indicated that P-GSK3β-ser^9 and EGFR expressions were negatively linked to survival of lung carcinoma patients when stratified according to histological type (p<0.05). Conclusion: P-GSK3β-ser^9 and EGFR are involved in the histogenesis of different lung carcinomas. Their overexpression might result from PTEN loss and can be considered as markers of worse prognosis in lung carcinoma patients.*

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Key Words: P-GSK3β-ser^9, epithelial growth factor receptor, lung carcinoma, progression, prognosis.

Glycogen synthase kinase-3β (GSK3β) is a highly conserved serine/threonine protein kinase, composed of 482 amino acids with a molecular weight of 46.7 kDa (1, 2). Although GSK3β was originally isolated from skeletal muscle, the enzyme is widely expressed in all tissues with particularly abundant levels in the brain (1). It may be involved in protein synthesis, cell proliferation, cell differentiation, microtubule dynamics and cell motility by phosphorylating initiation factors, components of the cell-division cycle, transcription factors, and proteins involved in microtubule function and cell adhesion (3). Acevedo *et al.* (4) found that inhibition of GSK3β activity significantly led to alterations in the timing in the pronuclear membrane breakdown and mitosis initiation, nuclear development, and cytokinesis, as well as abnormal chromatin segregation, evidenced by incomplete karyokinesis and micronuclei formation. In particular, GSK3β simultaneously mediated signal transduction in the insulin and Wnt pathways with no apparent cross-talk (4). The activity of GSK3β was inhibited *via* phosphorylation of ser-9 by p70 S6 kinase, p90Rsk and Akt (2, 5). In contrast, the activating modulation of GSK3β depended on tyr-216 phosphorylation. Active GSK3β not only suppressed the glycogen synthase, but also inhibited β-catenin translocation into the nucleus through phosphorylation of β-catenin, APC (adenomatous polyposis coli associated protein) and Axin. Additionally, oncogenic transcription factors (such as c-Jun) and proto-oncoprotein (such as Gli protein) are putative GSK3β substrates for phosphorylation-dependent inactivation (1, 5-7). GSK3β knockout mice died *in uteros* from the apoptosis of hepatocytes and GSK3β–/– cells displayed low transcriptional activity of nuclear factor κB (NF-κB) because of the reduced binding of NF-κB to DNA in response to stimuli (such as

tumor necrosis factor α and interleukin 1), indicating that GSK3β might regulate cellular apoptosis (8, 9). Therefore, its inactivation has been hypothesized to interfere with cellular malignant transformation and tumor development.

The epithelial growth factor receptor (EGFR, also known as ErbB1 or HER) was the first receptor identified in the ErbB family of receptor tyrosine kinases (RTK), whose proteins subsequently included four types, including EGFR-1, HER-2, HER-3 and HER-4. EGFR overexpression or dysfunction has been associated with a variety of human malignancies *via* homo-, or hetero-dimerising with other family members (10). Truncated EGFR variant, single nucleotide somatic missense mutations, as well as small in-frame deletions and insertions have recently been documented in the EGFR tyrosine kinase domain and these somatic mutations also appear to be oncogenic in nature, and may play a potential role in altered EGFR signaling pathways in tumorigenesis (11, 12). Once bound to their ligands, such as EGF, transformation growth factor α, betacellulin (BTC), epiregulin (EPR), tomoregulin and neuregulin (NRG) or heregulin (HRG), the EGFR would be activated, which would cause the recruitment of Shc, Grb2, Crk, Grb7, PLCγ, phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and so forth (10, 13, 14). It was well known that the last two molecules can inactivate protein kinase C (PKC) and Akt respectively, which are involved in the GSK3β-ser^9 phosphorylation as positive regulators. PTEN (phosphatase and tensin homology deleted from human chromosome 10) acts as a phospholipid phosphatase with PIP3 as a substrate and one down-stream target of PIP3, protein kinase (Akt/protein kinase B), is continually activated by phosphorylation in cells lacking functional PTEN (5, 15). EGFR dysfunction is frequently found in malignant transformation and aggressive progression, including overexpression and activating mutations (10).

Lung cancer is one of the most common malignancies and greatest causes of cancer-related death in Japan and worldwide despite the increased survival of cancer patients who receive advanced chemotherapy (16, 17). Histologically, the proportion and incidence of lung adenocarcinoma (AD) has been increasing markedly over the past two decades, surpassing squamous cell carcinoma (SQ) as the most common histological subtype of lung cancer in many countries (18). In the present study, phosphorylated GSK3β-ser^9 (P-GSK3β-ser^9) and EGFR expression was examined in lung carcinomas and compared with the clinicopathological parameters of the tumors, including PTEN expression, as well as survival time to explore their roles in the stepwise development of lung carcinoma.

Patients and Methods

Subjects. Lung carcinomas (n=154) were collected in our affiliated hospital and in Kouseiren Takaoka Hospital, Japan, between 1993 to 2006 from 104 men and 50 women (aged 40-85 years, mean= 69.6 years). Among them, 26 were complicated by pleural invasion and 53 by lymph node metastasis. All patients provided consent for use of tumor tissue for clinical research and our University Ethical Committee approved the research protocol. One hundred and forty six were followed up by consulting their case documents and telephoning.

Pathology. All tissues were fixed in 4% neutralized formaldehyde, embedded in paraffin and sectioned at 4 μm. The sections were stained with hematoxylin and eosin (HE) to confirm their histological diagnosis according to World Health Organization (WHO) criteria (18). The staging for each lung carcinoma was evaluated according to the Union Internationale Contre le Cancer (UICC) system for the extent of tumor spread (19). Lymphatic and venous invasion were also determined.

Tissue microarray (TMA). The HE-stained sections of the selected tumor cases were examined and representative areas of the solid tumor were identified for sampling. Two 2 mm diameter tissue cores per donor block were punched off and transferred to a recipient block carrying a maximum of 48 cores using a Tissue Microarrayer (AZUMAYA KIN-1, Tokyo, Japan). Four μm thick sections were consecutively incised from each recipient block and transferred to poly-lysine-coated glass slides. HE staining was performed on the TMA for confirmation of tumor tissues (Figure 1a).

Immunohistochemistry. Consecutive sections were deparaffinized with xylene, dehydrated with alcohol and subjected to antigen retrieval by irradiating in target retrieval solution (TRS, DAKO, Carpinteria, CA, USA) for 5 min in a microwave oven (Oriental Rotor Ltd. Co., Tokyo, Japan). Five percent bovine serum albumin was then applied for 1 min to prevent non-specific binding. The sections were incubated with primary antibodies for 15 min, then treated with anti-mouse or anti-rabbit Envison-PO (DAKO) antibodies for 15 min. All the incubations were performed in a microwave oven to allow intermittent irradiation as described previously (20). After each treatment, the slides were washed with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) three times for 1 min. Rabbit phosphorylated anti-P-GSK3β-ser^9 (Signal Antibody Technology, USA; 1:300), mouse anti-EGFR (NovoCastra, Newcastle upon Tyne, UK; 1:100), rabbit anti-Ki-67 (DAKO; 1:25) and mouse anti-PTEN (NovoCastra; 1:150) antibodies were used to examine the respective proteins. Anti-rabbit or anti-mouse Envison-PO (DAKO) was employed as the secondary antibody in our study. Omission of the primary antibody was used as a negative control.

Evaluation of immunohistochemistry. The immunoreactivity to P-GSK3β-ser^9 was localized in the cytoplasm (Figures 1b-d), to EGFR in the cytoplasm and membrane (Figures 1e-g), while to PTEN and Ki-67 in the nucleus (Figures 1h, i). One hundred cells were randomly selected and counted from 5 representative fields. The percentage of positive cells was graded semi-quantitatively with a four-tier scoring system: negative (–), less than 5%; weakly positive (+), 6-25%; moderately positive (++), 26-50% and strongly positive (+++), more than 51%. The evaluation was performed blindly by two independent observers (Y. Takano and H. Zheng).

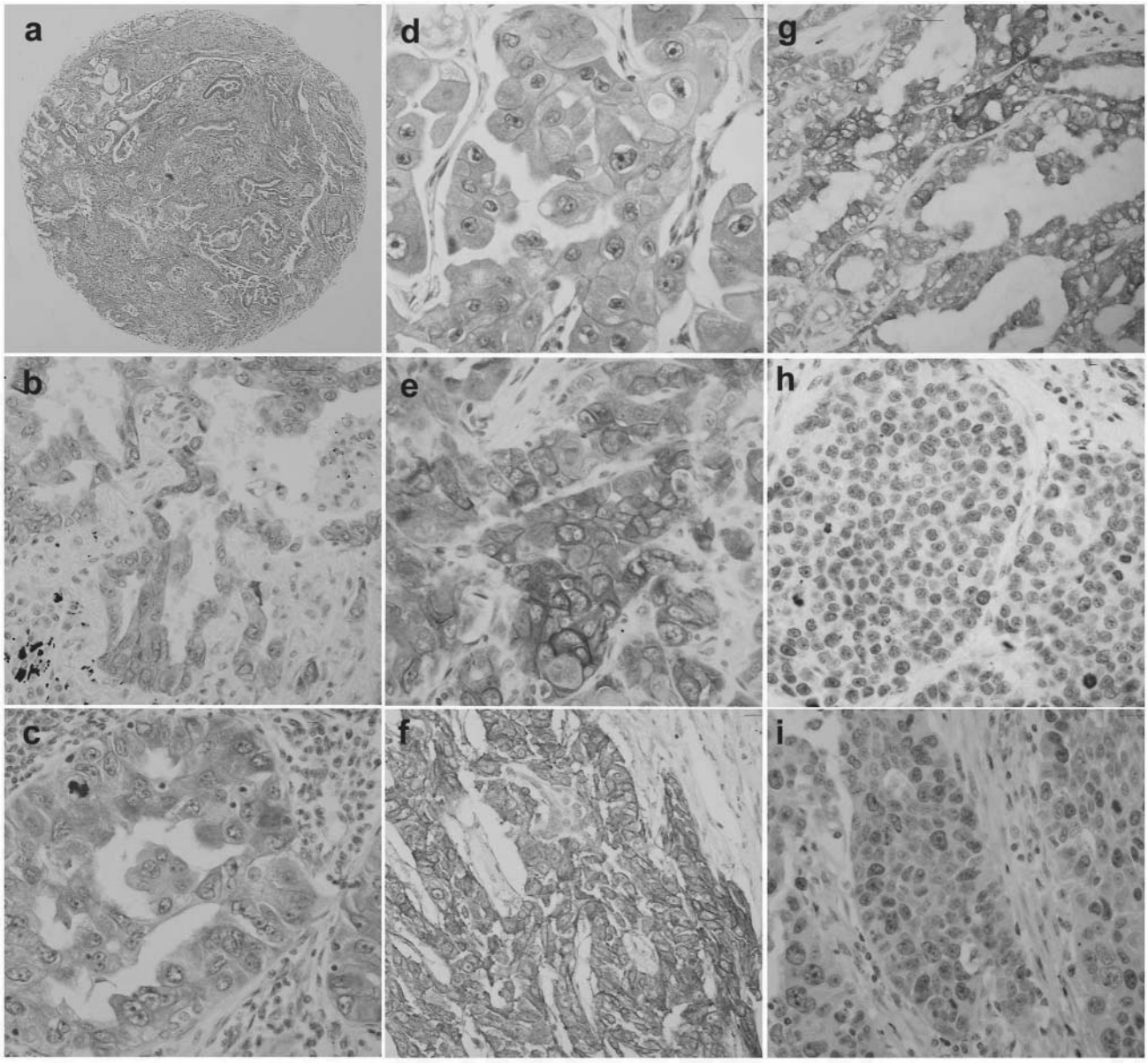

Figure 1. *HE staining and immunostaining of the samples of lung carcinomas. HE staining of TMA of lung carcinoma (a). Immunoreactivity to P-GSK3β-ser^9 was distributed in the cytoplasm, to EGFR in the membrane and cytoplasm, and to PTEN and Ki-67 in the nucleus. Strong expression of P-GSK3β-ser^9 was shown in lung AD and LCC (b-d). There was strong EGFR expression in SQ and AD (e-g). Some lung carcinomas displayed strong expression of PTEN (h) and Ki-67 (i).*

Statistical analysis. Statistical evaluation was performed using the Spearman correlation test to analyze the rank data. Kaplan-Meier survival plots were generated and comparisons between survival curves were made with the log-rank test. All the tests were two-sided and $p<0.05$ was considered as statistically significant. SPSS 10.0 (SPSS Inc, Chicago, IL, USA) software was employed to analyze all data.

Results

As summarized in Table I, P-GSK3β-ser^9 expression was highest in AD and lowest in small cell carcinomas (SCC), compared with other types of lung carcinomas ($p<0.05$). Its expression was negatively correlated with PTEN and Ki-67 expression ($p<0.05$), but not with age, sex, pleural invasion, lymphatic or venous invasion, lymph node metastasis or UICC staging ($p>0.05$). Higher EGFR expression was observed in the male carcinoma patients, compared with the female ones and further analysis indicated the statistical data were near to significance. In contrast to P-GSK3β-ser^9, EGFR was more strongly expressed in SQ than in other types of lung carcinoma. Its expression was also positively

Table I. *Relationship between P-GSK3β-ser^9 expression and clinicopathological parameters of lung carcinoma.*

Clinicopathological features	n	P-GSK3β-ser^9 expression					
		–	+	++	+++	PR (%)	*p*-value
Gender							>0.05
Female	50	8	12	8	22	84.0	
Male	104	26	22	30	26	75.0	
Age(years)							>0.05
<65	51	13	10	12	16	74.5	
≥65	103	21	24	26	32	79.6	
Histological classification							<0.05
Squamous cell carcinoma	37	9	10	10	8	75.7	
Adenocarcinoma	85	11	16	21	37	87.1*	
Large cell carcinoma	14	4	5	4	1	71.4	
Small cell carcinoma	18	10	3	3	2	44.4†	
Pleural invasion							>0.05
–	128	30	28	30	40	76.6	
+	26	4	6	8	8	84.6	
Lymphatic invasion							>0.05
–	117	26	29	28	33	77.8	
+	38	8	5	10	15	78.9	
Venous invasion							>0.05
–	127	30	32	29	36	76.4	
+	27	4	2	9	12	85.2	
Lymph node metastasis							>0.05
–	101	21	21	28	31	79.2	
+	53	13	13	10	17	75.5	
UICC staging							>0.05
I/II	115	24	25	29	37	79.1	
III/IV	37	9	8	9	11	75.7	
PTEN expression							<0.05
–	72	15	8	17	32	79.2	
+	30	6	7	8	9	80.0	
++	28	4	13	7	4	85.7	
+++	23	8	6	6	3	65.2	
Ki-67 expression							<0.05
–	66	16	15	20	15	75.8	
+	22	1	9	5	7	95.5	
++	22	10	3	4	5	54.5	
+++	27	7	7	9	4	74.1	

*, †Compared to other histological subtypes. PR, positive rate.

correlated with lymphatic and venous invasion, and P-GSK3β-ser^9 expression, and negatively correlated with the PTEN expression of the tumors ($p<0.05$), but not correlated with pleural invasion, lymph node metastasis, or UICC staging ($p>0.05$, Table II).

Follow-up information was available on 146 patients with lung carcinomas for periods ranging from 1 month to 12 years (mean=20.2 months). Figures 2 and 3 show survival curves stratified according to P-GSK3β-ser^9 and EGFR expression. Univariate analysis using the Kaplan-Meier method indicated that P-GSK3β-ser^9 expression was not a good prognostic marker for lung carcinomas overall ($p>0.05$), but EGFR was. When lung carcinomas were classified according to histological types, both P-GSK3β-ser^9 and EGFR were markers for a worse prognosis of the lung carcinoma patients ($p<0.05$).

Discussion

GSK3β reportedly has three main biological functions, regulating glycogen metabolism, repressing Wnt signaling, and maintaining the balance between cellular proliferation and apoptosis, which therefore has implications for the pathogenesis and development of malignancies (5, 6). Here, for the first time, the inactive form of GSK3β, P-GSK3β-ser^9 was found to be the most strongly expressed in AD and least in SCC. It was well known clinically that SCC grows very rapidly and metastasizes easily, so it needs more energy

Table II. *Relationship between EGFR expression and clinicopathological parameters of lung carcinoma.*

Clinicopathological features	n	EGFR expression					
		–	+	++	+++	PR (%)	*p*-value
Gender							>0.05
Female	50	33	5	1	11	34.0	
Male	104	49	15	12	28	52.9	
Age(years)							>0.05
<65	51	26	6	5	14	49.0	
≥65	103	56	14	8	25	45.6	
Histological classification							<0.05
Squamous cell carcinoma	37	8	6	6	17	78.4*	
Adenocarcinoma	85	54	10	3	18	36.5	
Large cell carcinoma	14	4	4	3	3	71.4	
Small cell carcinoma	18	16	0	1	1	11.1	
Pleural invasion							>0.05
–	128	63	16	11	38	50.8	
+	26	19	4	2	1	26.9	
Lymphatic invasion							<0.05
–	116	67	16	9	24	42.2	
+	38	15	4	4	15	60.5	
Venous invasion							<0.05
–	128	73	16	9	30	43.0	
+	26	9	4	4	9	65.4	
Lymph node metastasis							>0.05
–	101	53	15	8	25	47.5	
+	53	29	5	5	14	45.3	
UICC staging							<0.05
I/II	115	59	17	8	31	48.7	
III/IV	37	22	3	5	7	40.5	
PTEN expression							<0.05
–	72	29	12	4	27	59.7	
+	30	22	2	2	4	26.7	
++	28	18	3	1	6	35.7	
+++	23	13	3	6	1	43.5	
P-GSK3β-ser^9 expression							<0.05
–	34	20	7	1	6	41.2	
+	34	21	4	1	8	38.2	
++	38	21	4	5	8	44.7	
+++	48	20	5	6	17	58.3	

*Compared to other histological subtypes. PR, positive rate.

for synthesis and metabolism. The first recognized function of active GSK3β was its involvement in glucose metabolism by inhibiting glycogen synthase activity, causing a relative increase in glucose metabolism. It is believed that tumors generally depend on anaerobic pathways to catalyze glucose to ATP even in conditions of abundant oxygen, and tumor cells retain ATP production for the requirements of immortal survival (15). Therefore, the low expression of inactive GSK3β might contribute to the high supply of energy through inhibition of glucose synthesis in SCC. However, the association might be weaker in AD. P-GSK3β-ser^9 has also been reported to be up-regulated in colorectal carcinoma, but down-regulated in hepatocellular carcinoma (6, 21, 22). Taken together, it appears that P-GSK3β-ser^9 expression displays organ specificity in different carcinomas, possibly due to different regulatory signaling pathways and subsequent biological functions.

EGFR is a receptor tyrosine kinase highly expressed in epithelial tumors, whose activation has been shown to cause autophosphorylation of specific tyrosine residues in the intracellular domain which then initiate a multitude of effects including cell proliferation, cell differentiation, angiogenesis, metastasis and anti-apoptosis (10). Although most investigators have found frequent EGFR mutation or

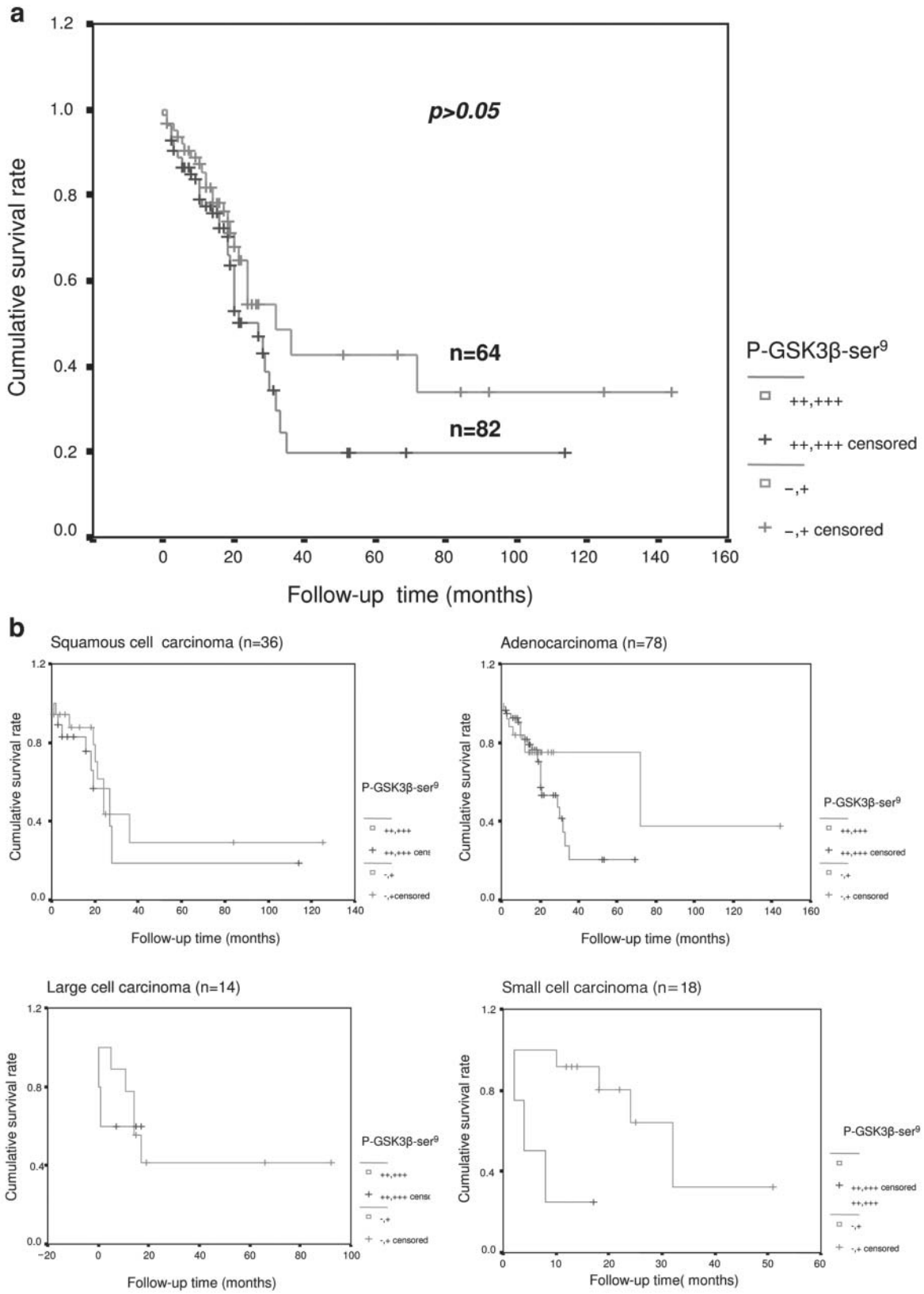

Figure 2. *Correlation between P-GSK3β-ser^9 status and prognosis of the lung carcinoma patients. Kaplan-Meier curves for cumulative survival rate of patients with all lung carcinomas according to P-GSK3β-ser^9 expression (a) and further stratified by histological type (b).*

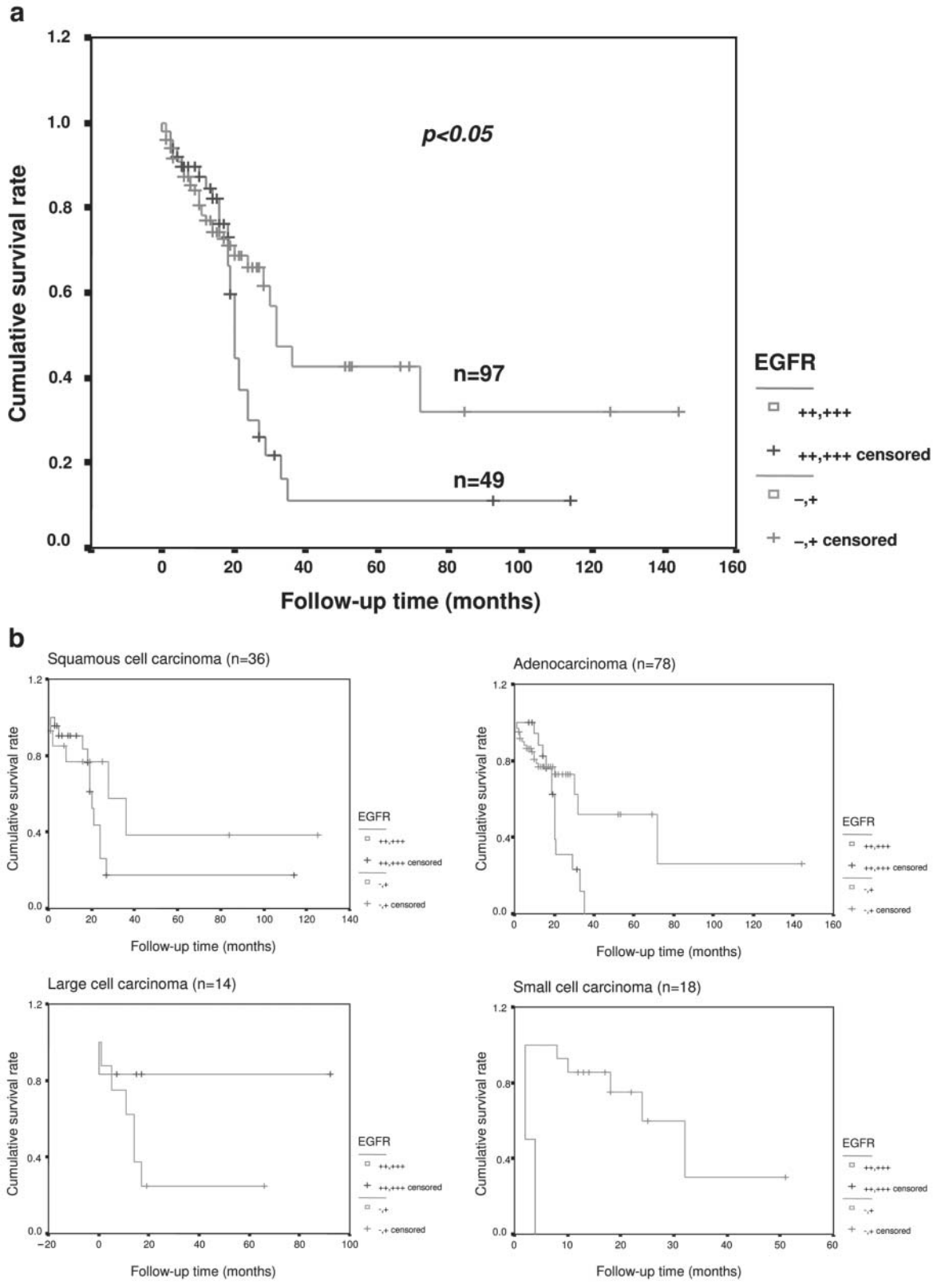

Figure 3. *Correlation between EGFR status and prognosis of the lung carcinoma patients. Kaplan-Meier curves for cumulative survival rate of patients with all lung carcinomas according to EGFR expression (a) and further stratified by histological types (b).*

amplification in lung carcinomas, including the AD subtype, which positively correlated with its protein expression, the functions of their encoding proteins were also unclear in lung carcinomas (23-26). The observed higher expression of EGFR in SQ than in the other types of lung carcinoma in the present study was in line with other reports (26-28). EGFR was more frequently expressed in lung carcinomas with lymphatic and venous invasion, which was consistent with the data of Fontanini *et al.* (29). Xue *et al.* (30) have provided *in vitro* evidence that EGFR overexpression results in increased tumor cell motility *in vivo* coordinately with enhanced intravasation and metastasis. These findings have indicated that EGFR overexpression contributed to the histogenesis and local invasion of lung carcinomas. We have also found EGFR immunoreactivity in a few cases of gastrointestinal AD (data not shown). Mammano *et al.* (31) did not find any mutation of EGFR in gastric AD and their immunostaining data were similar to ours. These findings indicate that EGFR plays more important role in SQ and gastrointestinal AD might be different from lung AD, possibly due to the cellular origin or the EGFR mutation status.

To further elucidate the molecular mechanisms of P-GSK3β-ser^9 and EGFR expression in lung carcinoma, the expression of PTEN and Ki-67 were also examined. It is generally well-known that Ki-67 is present in the G_1-, S- and G_2-phases of the cell cycle, as well as in mitosis and that Akt, down-stream of PTEN, is responsible for the phosphorylation of GSK3β induced by EGFR (2, 15, 32). In the present study, the lung carcinomas with high P-GSK3β-ser^9 expression exhibited weak expression of Ki-67 and PTEN, but strong expression of EGFR. These data have provided *ex vivo* evidence that EGFR might inactivate the GSK3β into the P-GSK3β-ser^9 form, causing low proliferation of lung carcinoma cells. Activated Akt has been shown to display high activity for phosphorylating GSK3β without PTEN inhibition (2).

Until now, the prognostic significance of P-GSK3β-ser^9 in malignancies has not been described. Here for the first time, we analyzed the relation of P-GSK3β-ser^9 expression with the survival of 146 patients with lung carcinomas and found a close link between its positive expression and poor survival when patients were stratified to the histological classification. EGFR was positively correlated with a worse prognosis for the carcinoma patients, independent of the histological type, consistent with a previous report (33). Therefore, it is suggested that P-GSK3β-ser^9 and EGFR might be regarded as good biomarkers to indicate a worse prognosis for carcinoma patients in clinicopathological practice.

In conclusion, P-GSK3β-ser^9 and EGFR expression might be involved in lung carcinogenesis and histogenesis. P-GSK3β-ser^9 and EGFR overexpression might result from PTEN loss and be considered as markers of worse prognosis for lung carcinoma patients. This study also supported the opinion that GSK3β might be suppressed through the EGFR pathway.

Acknowledgements

We particularly thank Tokimasa Kumada and Hideki Hatta for their technical help and Yukari Inoue for her secretarial assistance. This work was supported by Grants-in-Aid for Scientific Research 14770072, 15922084 and 18590324 from the Japanese Ministry of Education, Science, Sports and Culture, the 21st Century COE Program in Japan, and the Japanese Smoking Research Foundation.

References

1 Droucheau E, Primot A, Thomas V, Mattei D, Knockaert M, Richardson C, Sallicandro P, Alano P, Jafarshad A, Baratte B, Kunick C, Parzy D, Pearl L, Doerig C and Meijer L: *Plasmodium falciparum* glycogen synthase kinase-3: molecular model, expression, intracellular localisation and selective inhibitors. Biochim Biophys Acta *1697*: 181-196, 2004.

2 Ali A, Hoeflich KP and Woodgett JR: Glycogen synthase kinase-3: properties, functions, and regulation. Chem Rev *101*: 2527-2540, 2001.

3 Frame S and Cohen P: GSK3 takes centre stage more than 20 years after its discovery. Biochem J *359*: 1-16, 2001.

4 Acevedo N, Wang X, Dunn RL and Smith GD: Glycogen synthase kinase-3 regulation of chromatin segregation and cytokinesis in mouse preimplantation embryos. Mol Reprod Dev *74*: 178-188, 2007.

5 Mulholland DJ, Dedhar S, Wu H and Nelson CC: PTEN and GSK3beta: key regulators of progression to androgen-independent prostate cancer. Oncogene *5*: 329-337, 2006.

6 Shakoori A, Ougolkov A, Yu ZW, Zhang B, Modarressi MH, Billadeau DD, Mai M, Takahashi Y and Minamoto T: Deregulated GSK3beta activity in colorectal cancer: its association with tumor cell survival and proliferation. Biochem Biophys Res Commun *334*: 1365-1373, 2005.

7 Patel S, Doble B and Woodgett JR: Glycogen synthase kinase-3 in insulin and Wnt signalling: a double-edged sword? Biochem Soc Trans *32*: 803-808, 2004.

8 Kim L and Kimmel AR: GSK3, a master switch regulating cell-fate specification and tumorigenesis. Curr Opin Genet Dev *10*: 508-514, 2000.

9 Pearl LH and Barford D: Regulation of protein kinases in insulin, growth factor and Wnt signalling. Curr Opin Struct Biol *12*: 761-767, 2002.

10 Sebastian S, Settleman J, Reshkin SJ, Azzariti A, Bellizzi A and Paradiso A: The complexity of targeting EGFR signalling in cancer: from expression to turnover. Biochim Biophys Acta *1766*: 120-139, 2006.

11 Jiang J, Greulich H, Janne PA, Sellers WR, Meyerson M and Griffin JD: Epidermal growth factor-independent transformation of Ba/F3 cells with cancer-derived epidermal growth factor receptor mutants induces gefitinib-sensitive cell cycle progression. Cancer Res *65*: 8968-8974, 2005.

12 Greulich H, Chen TH, Feng W, Janne PA, Alvarez JV, Zappaterra M, Bulmer SE, Frank DA, Hahn WC, Sellers WR and Meyerson M: Oncogenic transformation by inhibitor-sensitive and -resistant EGFR mutants. PLoS Med *2*: e313, 2005.

13 Yarden Y and Sliwkowski MX: Untangling the ErbB signalling network. Nat Rev Mol Cell Biol *2*: 127-137, 2001.
14 Normanno N, Bianco C, De Luca A, Maiello MR and Salomon DS: Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment. Endocr Relat Cancer *10*: 1-21, 2003.
15 Grimes CA and Jope RS: The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol *65*: 391-426, 2001.
16 Wang J, Cheng YW, Wu DW, Chen JT, Chen CY, Chou MC and Lee H: Frequent FHIT gene loss of heterozygosity in human papillomavirus-infected non-smoking female lung cancer in Taiwan. Cancer Lett *235*: 18-25, 2006.
17 Akyurek N, Memis L, Ekinci O, Kokturk N and Ozturk C: Survivin expression in pre-invasive lesions and non-small cell lung carcinoma. Virchows Arch *449*: 164-170, 2006.
18 Travis WD, Brambilla E, Muller-Hermelink HK and Harris CC: World Health Organization Classification of Tumors: Pathology & Genetics, Tumors of the Lung, Pleura, Thymus and Heart. IARCPress and Oxford University Press, 2000.
19 Sobin LH and Wittekind CH: TNM Classification of Malignant Tumors. 6th edition. John Wiley & Sons, Hoboken, New Jersey, 2002.
20 Kumada T, Tsuneyama K, Hatta H, Ishizawa S and Takano Y: Improved 1-h rapid immunostaining method using intermittent microwave irradiation: practicability based on 5 years application in Toyama Medical and Pharmaceutical University Hospital. Mod Pathol *17*: 1141-1149, 2004.
21 Ban KC, Singh H, Krishnan R and Seow HF: GSK-3beta phosphorylation and alteration of beta-catenin in hepatocellular carcinoma. Cancer Lett *199*: 201-208, 2003.
22 Desbois-Mouthon C, Blivet-Van Eggelpoel MJ, Beurel E, Boissan M, Delelo R, Cadoret A and Capeau J: Dysregulation of glycogen synthase kinase-3beta signaling in hepatocellular carcinoma cells. Hepatology *36*: 1528-1536, 2002.
23 Sonobe M, Nakagawa M, Takenaka K, Katakura H, Adachi M, Yanagihara K, Otake Y, Wada H and Tanaka F: Influence of epidermal growth factor receptor (EGFR) gene mutations on the expression of EGFR, phosphoryl-Akt, and phosphoryl-MAPK, and on the prognosis of patients with non-small cell lung cancer. J Surg Oncol *95*: 63-69, 2006.
24 Tokumo M, Toyooka S, Ichihara S, Ohashi K, Tsukuda K, Ichimura K, Tabata M, Kiura K, Aoe M, Sano Y, Date H and Shimizu N: Double mutation and gene copy number of *EGFR* in gefitinib refractory non-small-cell lung cancer. Lung Cancer *53*: 117-121, 2006.
25 Takano T, Ohe Y, Sakamoto H, Tsuta K, Matsuno Y, Tateishi U, Yamamoto S, Nokihara H, Yamamoto N, Sekine I, Kunitoh H, Shibata T, Sakiyama T, Yoshida T and Tamura T: Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. J Clin Oncol *23*: 6829-6837, 2005.
26 Dacic S, Flanagan M, Cieply K, Ramalingam S, Luketich J, Belani C and Yousem SA: Significance of EGFR protein expression and gene amplification in non-small cell lung carcinoma. Am J Clin Pathol *125*: 860-865, 2006.
27 Sithanandam G, Smith GT, Masuda A, Takahashi T, Anderson LM and Fornwald LW: Cell cycle activation in lung adenocarcinoma cells by the ErbB3/phosphatidylinositol 3-kinase/Akt pathway. Carcinogenesis *24*: 1581-1592, 2003.
28 Ahn JH, Kim SW, Hong SM, Suh C, Kim WK, Lee IC and Lee JS: Epidermal growth factor receptor (EGFR) expression in operable non-small cell lung carcinoma. J Korean Med Sci *19*: 529-535, 2004.
29 Fontanini G, Vignati S, Bigini D, Mussi A, Lucchi H, Angeletti CA, Pingitore R, Pepe S, Basolo F and Bevilacqua G: Epidermal growth factor receptor (EGFr) expression in non-small cell lung carcinomas correlates with metastatic involvement of hilar and mediastinal lymph nodes in the squamous subtype. Eur J Cancer *31A*: 178-183, 1995.
30 Xue C, Wyckoff J, Liang F, Sidani M, Violini S, Tsai KL, Zhang ZY, Sahai E, Condeelis J and Segall JE: Epidermal growth factor receptor overexpression results in increased tumor cell motility *in vivo* coordinately with enhanced intravasation and metastasis. Cancer Res *66*: 192-197, 2006.
31 Mammano E, Belluco C, Sciro M, Mencarelli R, Agostini M, Michelotto M, Marchet A and Nitti D: Epidermal growth factor receptor (EGFR): mutational and protein expression analysis in gastric cancer. Anticancer Res *26*: 3547-3550, 2006.
32 Zheng H, Tsuneyama K, Cheng C, Takahashi H, Cui Z, Murai Y, Nomoto K and Takano Y: An immunohistochemical study of p53 and Ki-67 in gastrointestinal adenoma and adenocarcinoma using tissue microarray. Anticancer Res *26*: 2353-2360, 2006.
33 Niemiec J, Kolodziejski L and Dyczek S: EGFR LI and Ki-67 LI are independent prognostic parameters influencing survivals of surgically treated squamous cell lung cancer patients. Neoplasma *52*: 231-237, 2005.

Received April 2, 2007
Revised June 1, 2007
Accepted June 14, 2007