Nuclear Y-Box Binding Protein-1 (YB-1), a Predictive Marker of Prognosis, Is Correlated with Expression of HER2/ErbB2 and HER3/ErbB3 in Non-Small Cell Lung Cancer

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ABSTRACT

Introduction: Nuclear expression of Y-box binding protein 1 (YB-1) is closely associated not only with global drug resistance and expression of several growth factor receptors in various human malignancies, but also with overall patient survival.

Methods: The effect of YB-1 knockdown on expression of EGFR family proteins was examined by Western blot using human lung cancer cell lines. Immunohistochemistry was used to evaluate the expression of nuclear YB-1 and EGFR family proteins in NSCLC patients (n = 104).

Results: In the five NSCLC cell lines, expression of EGFR, HER2, HER3 and c-Met in PC-9 cells, of HER2 and c-Met in EBC-1 cells, and of HER3 in QG56 cells was down-regulated by YB-1 knockdown. By immunohistochemical analysis, we observed that HER3 expression was significantly negatively correlated with nuclear YB-1 expression in squamous cell carcinoma ($p = 0.038$). HER2 expression was positively correlated with nuclear YB-1 expression in adenocarcinoma ($p = 0.052$). Nuclear expression of YB-1 correlated with overall survival of all patients ($p = 0.028$) and of adenocarcinoma patients ($p = 0.007$). Furthermore, there was a significant difference in therapeutic efficacies of gefitinib between patients with nuclear YB-1 expression and those with non-nuclear YB-1 expression in NSCLC patients ($p = 0.004$, n = 26), but not between those with high and those with low expression of EGFR, HER2, HER3 and c-Met.

Conclusion: Nuclear YB-1 expression might be essential for the malignant phenotype in lung cancer patients, and might be an important biomarker for development of therapeutic strategy against NSCLC.

Key words: YB-1, NSCLC, HER2, HER3.

INTRODUCTION

The Y-box binding protein 1 (YB-1) whose a cold shock domain is highly conserved plays essential roles in DNA damage repair and in both transcriptional and translational regulation of various genes in nucleus and cytoplasm.[1, 2] In the nucleus, YB-1 recognizes DNA damage induced by cisplatin and radiation and promotes transcription of drug resistance-relevant genes such as MDR1/ABCB1, a representative multidrug resistance-related ATP binding cassette (ABC) transporter, and MVP/LRP, a drug-resistance related vault protein, suggesting the applicability of YB-1 as a global biomarker of drug resistance.[2, 3] Moreover, nuclear expression of YB-1 significantly correlates with the survival of patients with various malignancies, including ovarian cancer,[4, 5] synovial sarcoma and rhabdomyosarcoma,[6, 7] lung cancer,[8] breast cancer[9] and pediatric glioblastoma.[10] Most of these patients show a close association of nuclear YB-1 expression with poor prognosis, irrespective of treatment modality. It is likely that multiple tumor characteristics, including growth and metastasis/invasion as well as acquisition of global drug resistance, cause YB-1 expression to be associated with poor prognosis in cancer patients.

Nuclear expression of YB-1 is promoted through PI3K/Akt signaling in human breast and ovarian cancer cells in response to growth stimulation,[11, 12] and YB-1 knockdown suppresses expression of DNA replication-related and growth/cell cycle-related genes as well as growth factor genes.[12, 13] YB-1 gene knock-in promotes development of breast cancer of various histological types in animal models, suggesting that YB-1 is a breast cancer oncogene.[14] YB-1 knockdown in mice causes embryonic lethality and severe growth retardatio.[15, 16] Furthermore, YB-1 overexpression induces EGF-independent growth through constitutive EGFR activation in human mammary cells in vitro.[17] Wu et al. reported that the introduction of an Akt-activation-insensitive mutation into YB-1 caused a marked decrease in the expression of both EGFR and HER2, suggesting a close linkage between YB-1 and expression of EGFR and HER2 in breast cancer cells in vitro.[18] YB-1 knockdown also results in markedly decreased expression of EGFR and HER2 in some human breast cancer cell lines in culture.9 Taken together, these basic studies in vitro and in vivo strongly suggest that YB-1 is closely involved in EGF/TGFα-dependent and –independent tumor growth and carcinogenesis in cancer.

The clinical study by Janz and colleagues[19] was the first to demonstrate the close association of nuclear YB-1 expression with HER2 expression in primary breast cancers. This correlation with the expression of EGFR and HER2 in breast cancer patients (n = 389) was further supported by array studies with tumor tissue.[18] Of various genes, including EGFR, HER2, ERα, ERβ and CXCR4, that could be affected by YB-1 knockdown in vitro, biostatistical analysis showed that YB-1 nuclear expression was positively associated with the expression of HER2, and negatively associated with the expression of CXCR4 and ERα.[9] A recent study by Stratford et al. also showed the possible involvement of YB-1 in the therapeutic efficacy of an EGFR-targeting drug, gefitinib, in basal-like breast cancer.[20] These findings suggest that YB-1 may play a key role in the expression of cell growth-related genes, including EGFR family genes, in breast cancer cells, and may also modulate the therapeutic efficacy of EGFR family-targeting drugs.

In the present study, we determined the relationship between YB-1 expression and that of several growth factor receptors, EGFR, HER2, HER3, c-Met and IGF-1R, in human lung cancer cell lines in culture. Moreover, we determined whether nuclear YB-1 expression was correlated with the expression of EGFR, HER2, HER3, c-Met and pAkt in tumor tissue from patients with NSCLC, and also whether therapeutic efficacy of gefitinib was correlated with nuclear YB-1 expression. We discuss the clinical and immunohistochemical characteristics of NSCLC with particular reference to the absence or presence of nuclear YB-1 expression and the expression of EGFR family proteins.

MATERIALS AND METHODS

Cell Lines and Reagents

PC-9, QG56 and 11_18 were cultured in RPMI supplemented with 10% fetal bovine serum (FBS). A549 and EBC-1 were cultured in DMEM supplemented with 10% FBS. Anti-YB-1 was generated as described previously.[21] Anti-EGFR, IGF-1R, Akt, pAkt, Erk and pErk antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-HER2 was purchased from Upstate, Inc. (Lake Placid, NY). Anti-c-Met and anti-HER3 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GAPDH was purchased from TREVIGEN (Gaithersburg, MD).

Small Interfering RNA Transfection and Immunoblotting

The small interfering RNA (siRNA) corresponding to the nucleotide sequence of YB-1 was purchased from QIAGEN.[9] SiRNA duplexes were transfected with Lipofectamine RNAiMAX and Opti-MEM medium (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Forty-eight hours after siRNA transfection, cells were lysed in cold protein extraction reagent (M-PER; PIERCE, Rockford, IL) with protease inhibitors and phosphatase inhibitors. Nuclear and cytoplasmic fractions were prepared as described previously.[12] Lysates were subjected to SDS-PAGE and blotted onto Immobilon membrane (Millipore Corp., Bedford, MA). After transfer, the membrane was incubated with the primary antibody and visualized with secondary antibody coupled to horseradish peroxidase and ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ). For cell proliferation assay, 2.5 x 10^4 cells were seeded in 24-well plates (IWAKI, Tokyo, Japan) and cell number in each well was counted at 96 hours after transfection of siRNA.

Patients and Tumor Samples

We examined 104 patients with primary non-small cell lung cancer whose tumors had been completely surgically removed in the Department of Surgery of Kurume University between 1997 and 2004. Among the 104 patients, 66 patients were diagnosed histologically as having adenocarcinoma, and the other 38 patients were diagnosed as having squamous cell carcinoma. The age of the patients

with non-small cell lung cancer ranged from 41 to 82 years (median 66 years). Of the total number of patients, 67 were men and 37 were women. The median follow-up was 1511.5 days with a range of 159-3801 days. Of these patients, 26 patients received gefitinib against recurrent disease after surgical resection between June 2003 and September 2008 with the median interval between operation and gefitinib treatment 760 days (range: 225-3062). Five patients were treated with gefitinib as initial therapy, and the others were treated with gefitinib as second- or third-line therapy (21 patients, platinum doublets as first line, 5 patients, monotherapy, non-platinum doublets, and platinum doublets as second line).

Immunohistochemistry (IHC)

Paraffin-embedded tissue samples were cut at 4 μm, placed on coated glass slides, and labeled with the following antibodies by the BenchMark XT (Ventana Automated Systems, Inc., Tucson, AZ) or ChemMate ENVISION (DakoCytomation, Glostrup, Denmark) methods: YB-1, EGFR, HER2, HER3, pAkt and c-Met. The BenchMark XT method was used for YB-1, EGFR, HER2, and HER3. This automated system used the streptavidin biotin complex method with DAB as chromogen (Ventana iVIEW DAB Detection Kit). Antigen retrieval of YB-1 and HER2 was performed by heat treatment in CC1 (Ventana, Inc.), and that of EGFR and HER3 was performed by protease treatment (protease K, Ventana, Inc.). The ChemMate ENVISION method was used for pAkt and c-Met. Endogenous peroxidase activity was inhibited by incubating the slides in 3% H_2O_2 for 5 minutes. Each slide was incubated overnight with the antibody at 4°C. For staining detection, DAB was used as chromogen. The samples were viewed using an Olympus BX51 microscope (Olympus, Tokyo, Japan).

Expression of YB-1 protein with variable intensity showed in the nuclei and/or cytoplasm. Only nuclei of cancer cells with strong expression were interpreted as positive. Expression of EGFR and HER2 was classified into four categories: score 0, no staining at all, or weak membrane expression in <10% of cancer cells; score 1+, weak expression in >10% of cancer cells; score 2+, weak to moderate expression on the entire membrane in >10% of the cancer cells; score 3+, strong expression on the entire membrane in >10% of cancer cells. HER3, pAkt and c-Met were classified into the same four categories, but the localization of expression included both membrane and cytoplasm. The scoring of IHC was defined as follows in order that difference of the number of patients in two categories of

negative and positive were small as possible. The expression of HER2 and pAkt were defined as follows: scores of 2+ or 3+ were regarded as positive, and scores of 0 or 1were regarded as negative. The expression of IHC for EGFR, HER3 and c-Met were defined as follows: score of 3+ were regarded as positive, and scores of 0, 1 or 2+ were regarded as negative. All IHC studies were evaluated by two IHC-experienced reviewers who were blind to the clinical status of the patients (A.K. and M. Kage).

Statistical Analysis

Associations between histological type and clinicopathologic findings (age, gender, smoking status, stage, and histological differentiation) and molecular markers (EGFR, HER2, HER3, c-Met, and pAkt) were tested by Fisher's exact test. Associations between YB-1 and clinicopathologic findings and other molecular markers were tested in similar ways. A p value of less than 0.05 was regarded as statistically significant unless indicated. The overall survival was defined as time to death due to any case from the date of surgical operation. The relationships between overall survival and YB-1 expression, as well as other clinicopathologic findings and molecular markers, were examined by the Kaplan-Meier method and the log rank test. Hazard ratios were estimated by Cox regressions. Hazard ratios adjusting for possible confounding factors were also estimated by applying the Cox regression models with the factors as explanatory variables. Twenty six patients were treated by gefitinib after progressive disease. Time to further progressive disease from initiation of gefitinib treatment was evaluated for these patients. The effect of YB-1 on the further progressive disease in the presence of gefitinib was examined in an exploratory matter by the Kaplan-Meier method and the logrank test. Statistical analysis was performed with SAS ver. 9.1 (SAS Institute Inc.) and R ver. 2.7.0.

RESULTS

Knockdown of YB-1 and Expression of the EGFR, HER2, HER3 and c-Met Genes in NSCLC Cell Lines

We examined expression of the growth factor receptors EGFR, HER2, HER3, c-Met and IGF-1R in five NSCLC cell lines. Expression of YB-1 was observed in both total cell fraction and nucleus in all of the five NSCLC cell lines, although expression of YB-1 in both fractions of PC-9 and EBC-1 cells was only about 20% or less of that in the other three lines (Figure 1A and B). Expression levels of EGFR, HER2, HER3, c-Met and IGF-1R varied among the five cell lines. Among the various cell lines, the two lines (PC-9 and EBC-1) with relatively low levels of YB-1 in the nucleus contained much lower amounts of IGF-1R protein than did the other three lines. However, expression of the other receptors (EGFR, HER2, HER3 and c-Met) was not significantly associated with expression levels of YB-1 in nucleus or total cell fraction of any of the cell lines. We next compared protein expression levels in the five NSCLC cell lines after treatment with YB-1 siRNA (Figure 2). Western blot analysis showed that YB-1 siRNA decreased protein levels of YB-1 in all five NSCLC cell lines. YB-1 knockdown resulted in decreased expression of EGFR in PC-9, of HER2 in PC-9 and EBC-1, of HER3 in PC-9 and QG56, and of c-Met in PC-9 and EBC-1. Of the five lines, expression of growth factor receptors was particularly susceptible to siRNA-dependent down-regulation in PC-9 and EBC-1 cells, which contain relatively lower levels of YB-1. However we did not observe decreased expression of growth factor receptor proteins by YB-1 knockdown in 11_18 and A549 cells (Figure 2A and B). Overall, the reduced expression of EGFR family proteins and c-Met protein by YB-1 knockdown in some NSCLC cell lines suggests an association between YB-1 levels and expression of EGFR family proteins or c-Met protein. In addition, proliferation of NSCLC cell lines was markedly suppressed, to a similar extent in all five cell types, by 2 to 50 nmol/L of YB-1 siRNA (Figure 2C).

Association of Nuclear YB-1 Expression with Expression of EGFR, HER2, HER3, c-Met and pAkt in NSCLC

To examine which genes are specifically associated with nuclear YB-1 localization in human NSCLC, we selected five molecular markers: EGFR, HER2, HER3, c-Met and pAkt. Representative immunohistochemical staining patterns are shown in Figure 3. Expression of nuclear YB-1 was detected in 44 of 104 patients. Table 1 shows the results of Fisher's exact tests for association between YB-1 and each of the molecular markers in adenocarcinoma and squamous cell carcinoma. To avoid misinterpretations arising from Simpson's paradox,[22] we put emphasis on analysis by histological differentiation. Such analysis demonstrated that there was significant negative correlation between nuclear expression of YB-1 and expression of HER3 in squamous cell carcinoma patients ($p = 0.038$). There was also a trend to a correlation between nuclear expression of YB-1 and expression of HER2 in adenocarcinoma patients ($p = 0.052$).

Nuclear YB-1 Expression and Survival of NSCLC Patients

The estimated product-limit survival functions of nuclear YB-1 are shown in Figure 4, and the results of log-rank tests and unadjusted hazard ratios are given in Table 2. Survival curves for patients positive for nuclear expression of YB-1 were significantly different from those for patients negative for expression [HR = 1.73 (95% CI: 1.05-2.83), $p = 0.028$]. Further analysis showed that positive nuclear expression of YB-1 significantly affected survival in adenocarcinoma [HR = 2.40 (95% CI: 1.25-4.58), $p = 0.007$] but not in squamous cell carcinoma [HR = 1.50 (95% CI: 0.60-3.72), $p = 0.381$] (Table 2).

Adjusted hazard ratios for patients positive for nuclear YB-1 expression relative to those negative for nuclear YB-1 expression were obtained by applying the Cox regression models with sex, smoking status and histological type as explanatory variables (Table 3). Stage was not adjusted in the Cox regression model, since it might be an intermediate variable between YB-1 expression and overall survival.[23] The adjusted hazard ratio was statistically significantly different from unity [HR = 1.96 (95% CI: 1.13 - 3.38), $p = 0.016$], indicating that nuclear YB-1 expression affects overall survival even after adjustment for possible confounding factors. The Cox regression models with sex and smoking status were also applied separately by histological type to determine the interaction between nuclear YB-1 and histological type. Adjusted hazard ratios for YB-1 expression were similar between

adenocarcinoma and squamous cell carcinoma, although they were statistically significant only for adenocarcinoma patients [HR = 2.19 (95% CI: 1.12 - 4.28), $p = 0.022$ for adenocarcinoma and HR = 2.14 (95% CI: 0.74 - 6.15), $p = 0.158$ for squamous cell carcinoma].

Nuclear YB-1 Expression and Therapeutic Efficacy of Gefitinib

Twenty six patients were administrated by gefitinib after progressive disease. Among 26 patients, 24 patients were histologically diagnosed adenocarcinoma, and other 2 patients were diagnosed squamous cell carcinoma. Eight of them were men and 18 were women. Seven of them were smoker and 19 were non-smoker. The estimated product-limit survival functions of nuclear YB-1 for time to further disease progression from the initiation of gefitinib treatment are shown in Figure 5. Although patients, number for gefitinib treatment was limited, the survival curves for patients with nuclear YB-1 positive expression and those with negative expression are quite distinct with statistical significance ($p = 0.004$).

DUSCUSSION

Wu and colleagues[18] used array studies to establish a close correlation between total YB-1 expression and EGFR and HER2 expression in tumor tissue from breast cancer patients. Our recent immunohistochemical analysis demonstrated that nuclear YB-1 expression is positively correlated with HER2, and negatively correlated with ERα and CXCR4, but not with EGFR, in breast cancer clinical specimens.[9] In the present study, YB-1 knockdown in five NSCLC cell lines caused down-regulation of EGFR, HER2, HER3 and c-Met in PC-9, of HER3 and c-Met in EBC-1, and of HER3 in QG56; there was no change in expression of growth factor receptor family proteins in the other two cell lines. However, cell proliferation was markedly suppressed by YB-1 knockdown in all five cell lines, suggesting that YB-1 siRNA-induced inhibition of cell proliferation might not involve attenuation of these growth factor receptors. The underlying mechanism of YB-1 siRNA-induced growth inhibition in these cell lines remains unknown. Immunohistochemical analysis of clinical samples of NSCLC showed that nuclear YB-1 expression was negatively correlated with HER3 expression in squamous cell carcinoma ($p = 0.038$) and positively correlated with HER2 expression in adenocarcinoma ($p = 0.052$). However, nuclear YB-1 expression was not significantly correlated with EGFR or c-Met expression in either squamous cell carcinoma or adenocarcinoma.

Overexpression of HER2 in an NSCLC cell line with very low EGFR and high HER3 expression levels sensitizes these cells to growth inhibition by the EGFR-targeting drug gefitinib, and gefitinib abrogated formation of HER2/HER3 heterodimers.[24] Consistent with this report, there is a strong correlation between HER3 expression and sensitivity to gefitinib.[25] Another EGFR-targeting drug, erlotinib, also inhibits HER2 tyrosine kinase, and HER2/HER3 heterodimer formation sensitizes lung cancer cells to growth inhibition by erlotinib.[26] Taken together, expression of HER2 and HER3 coupled with formation of their heterodimers plays a critical role in determining the therapeutic efficacy of the EGFR-targeting drugs gefitinib and erlotinib.[27, 28] The possible link between nuclear YB-1 expression and HER2 expression in adenocarcinoma ($p = 0.052$) might be in part responsible for the correlation between nuclear YB-1 expression and survival of adenocarcinoma NSCLC patients. Nuclear YB-1 expression is positively correlated with HER2 expression in breast cancer patients ($p =$

0.015).[9] HER2 expression could thus be modulated by nuclear YB-1 expression not only in breast cancer[9] but also in adenocarcinoma NSCLC (this study). HER3 expression was inversely correlated with nuclear expression of YB-1 in squamous cell carcinoma ($p = 0.038$), but HER3 expression alone did not correlate with survival of NSCLC patients or of the subset with squamous cell carcinoma. It appears unlikely that the inverse correlation of nuclear YB-1 expression with HER3 expression is a factor in the poor prognosis associated with nuclear YB-1 expression in squamous NSCLC patients.

In addition to the effect of altering the status of EGFR family proteins on the therapeutic efficacy of EGFR-targeting drugs, amplification of c-Met has been identified as another acquired drug resistance mechanism.[29] c-Met modifies drug sensitivity to gefitinib through HER3-dependent activation of the PI3K/Akt pathway.[25] Moreover, Cappuzzo et al. [30] have reported that gefitinib-treated patients with a high HER3 gene copy number had significantly higher response rates and times to progression, although with no improvement in overall survival. Nuclear expression of YB-1 also affected expression of c-Met in two NSCLC cell lines in culture, but we could not observe any significant correlation between nuclear YB-1 expression and c-Met expression in NSCLC. This suggests that it is unlikely that nuclear YB-1 expression determines c-Met expression in NSCLC patients.

Nuclear YB-1 expression is often associated with poor prognosis in various human malignancies, including breast cancer.[4-10] In NSCLC, our previous IHC study on 196 patients demonstrated that nuclear YB-1 expression is significantly associated with poor prognosis in all patients ($p = 0.0424$) and in patients with squamous cell carcinoma ($p = 0.0313$), but not in patients with adenocarcinoma ($p = 0.2015$).[8] Our present study demonstrated a close association of nuclear YB-1 expression with poor prognosis in all patients ($p = 0.028$) and in patients with adenocarcinoma ($p = 0.007$), but not in patients with squamous cell carcinoma ($p = 0.381$). This inconsistency may result from the small number of patients with squamous cell carcinoma in the present study. The results of the Cox regression analysis given in Table 3 provide similar estimates of the hazard ratio of nuclear YB-1 expression in adenocarcinoma and squamous cell carcinoma, although they were not necessarily statistically significant. Further studies with more definitive clinicopathological characterization of the patients included are required to establish the types of NSCLC in which nuclear expression of YB-1

predicts poor prognosis.

Of NSCLC patients (n = 104) tested in this study, we examined whether therapeutic efficacies of gefitinib were associated with expression levels of nuclear YB-1 expression when recurrent 26 patients (24 adenocarcinoma, 2 squamous cell carcinoma) were treated by gefitinib. Although number of treated patients was limited, the absence or presence of nuclear YB-1 expression shows a significant correlation with differences in the survival curves after progression disease. Concerning EGFR mutations that are highly susceptible to the therapeutic efficacy by gefitinib, we also observed statistically significant differences between patients with mutant EGFR and these with wild-type EGFR (Azuma et. al., unpublished data). Nuclear YB-1 expression might have a significant predictive value for progression disease in NSCLC patients when treated with EGFR-targeting drug. Further study is in progress whether nuclear expression of YB- 1 could be associated with gefitinib-susceptible EGFR mutations.

In conclusion, our results show that nuclear YB-1 expression is strongly associated with overall survival of NSCLC patients (n = 104). Of the EGFR family proteins, nuclear YB-1 expression is associated with expression of HER2 in both histological types of NSCLC and of HER3 in squamous cell carcinoma type of NSCLC. Expression of YB-1 in the nucleus might therefore affect the therapeutic efficacy of EGFR-targeting drugs. Nuclear YB-1 expression is associated with progressive disease survival of NSCLC patients (n = 26) when treated with gefitinib. Together, these observations indicate that nuclear YB-1 is a novel biomarker in NSCLC.

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FIGURE LEGENDS

FIGURE 1. Expression of YB-1, EGFR, HER2, HER3, c-Met and IGF-1R in human lung cancer cells. A, Expression of total and nuclear YB-1, EGFR, HER2, HER3, c-Met and IGF-1R was determined by immunoblotting conducted on protein lysates extracted from these cell lines. Detection of GAPDH served as a loading control. B, Levels of total and nuclear YB-1, EGFR, HER2, HER3, c-Met and IGF-1R expression were measured by densitometry.

FIGURE 2. Effect of YB-1 knockdown on expression of EGFR, HER2, HER3, c-Met and IGF-1R in human lung cancer cells. A, Effect of YB-1 knockdown on expression of EGFR, HER2, HER3, c-Met and IGF-1R was analyzed by immunoblotting. Cells were incubated with control or YB-1 siRNA for 48 hours, and lysates were prepared. B, Levels of YB-1, EGFR, HER2, HER3, c-Met and IGF-1R expression were measured by densitometry. C, Effect of YB-1 knockdown on proliferation of five lung cancer cell lines. Data are expressed as the mean ± SD of triplicate experiments.

FIGURE 3. Histological findings and expression of YB-1, EGFR, HER2, HER3, c-Met and pAkt in human lung cancer. YB-1 expression was assessed as two patterns: nuclear positive or negative. Cancer cells showed strong expression of EGFR, HER2, HER3 and c-Met in the membrane. Moderate-to-strong expression of pAkt was found in the cytoplasm.

FIGURE 4. Kaplan-Meier plots of overall survival according to nuclear YB-1 expression in 104 patients with lung cancer. Total NSCLC patients (n = 104) (A), patients with adenocarcinoma (n = 66) (B), and patients with squamous cell carcinoma (n = 38) (C).

FIGURE 5. Kaplan-Meier estimate for time to further progressive disease from the initiation of gefitinib according to nuclear YB-1 expression in 26 patients with gefitinib treated after progressive disease.

Figure 1

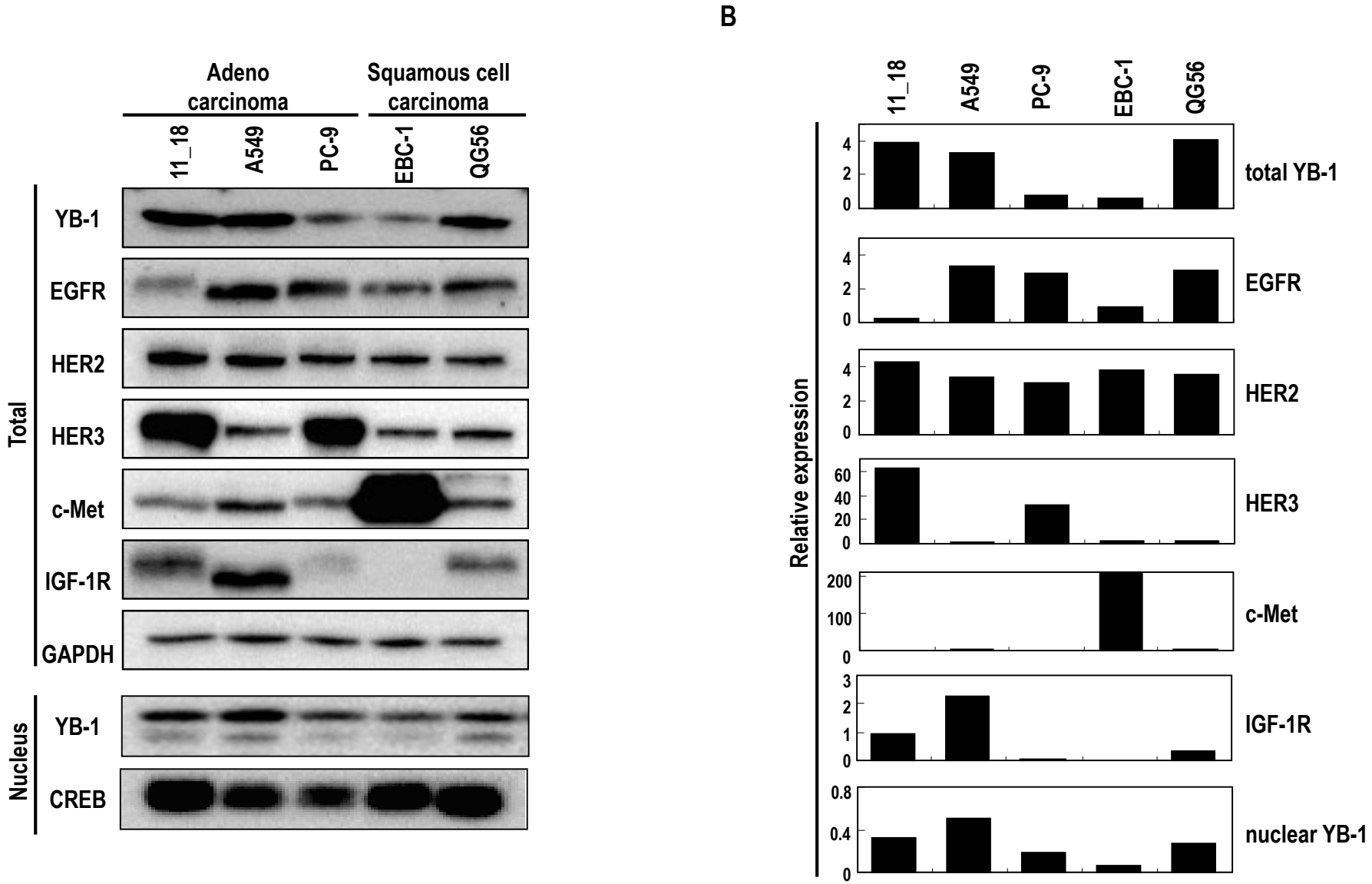

A
Adeno carcinoma
Squamous cell carcinoma
11_18
A549
PC-9
EBC-1
QG56
Total
YB-1
EGFR
HER2
HER3
c-Met
IGF-1R
GAPDH
Nucleus
YB-1
CREB
B
Relative expression
total YB-1
EGFR
HER2
HER3
c-Met
IGF-1R
nuclear YB-1

Figure 2

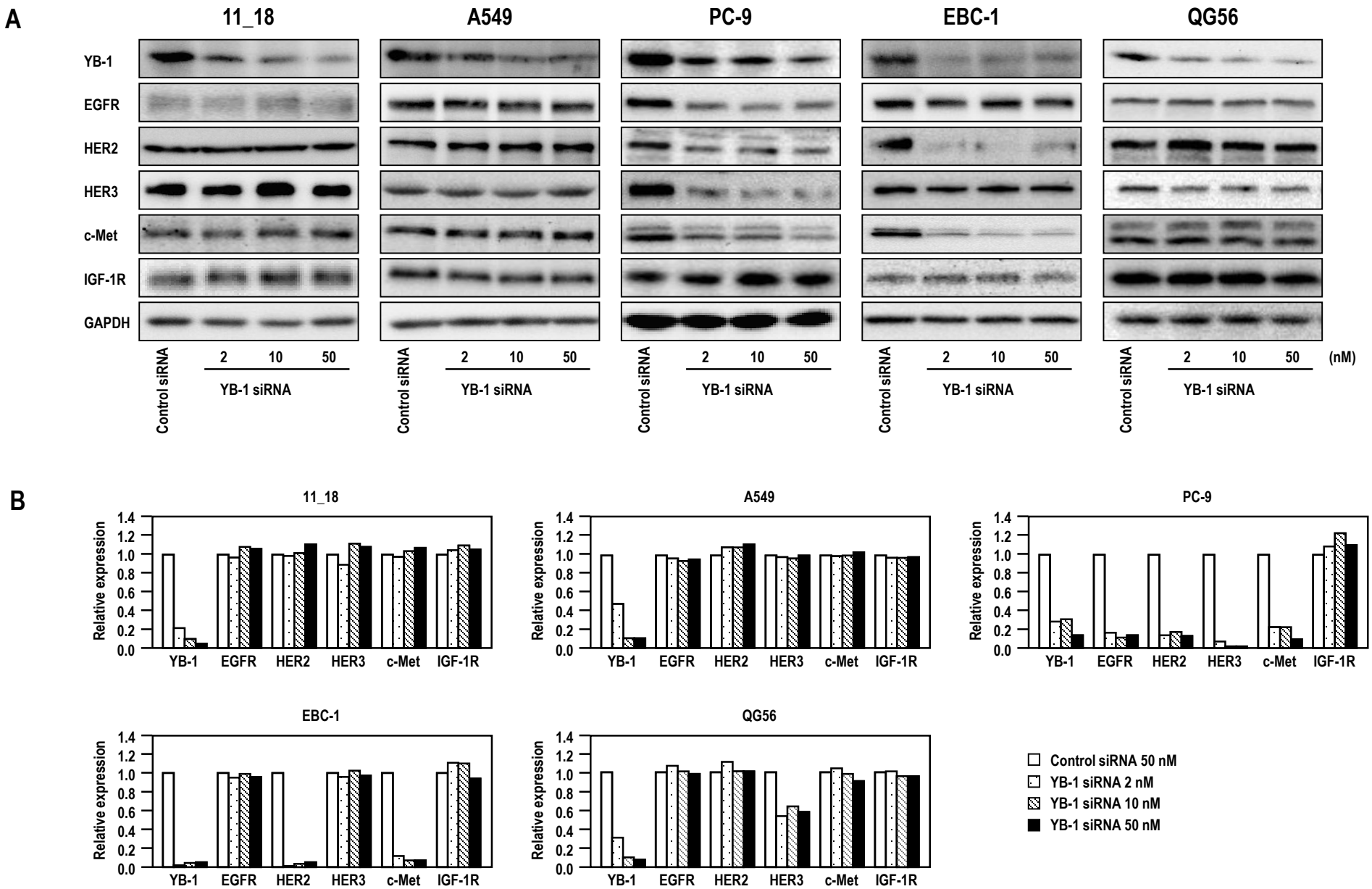

A
11_18
A549
PC-9
EBC-1
QG56
YB-1
EGFR
HER2
HER3
c-Met
IGF-1R
GAPDH
Control siRNA
2
10
50
(nM)
YB-1 siRNA
B
Relative expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
YB-1
EGFR
HER2
HER3
c-Met
IGF-1R
Control siRNA 50 nM
YB-1 siRNA 2 nM
YB-1 siRNA 10 nM
YB-1 siRNA 50 nM

Figure 2

C

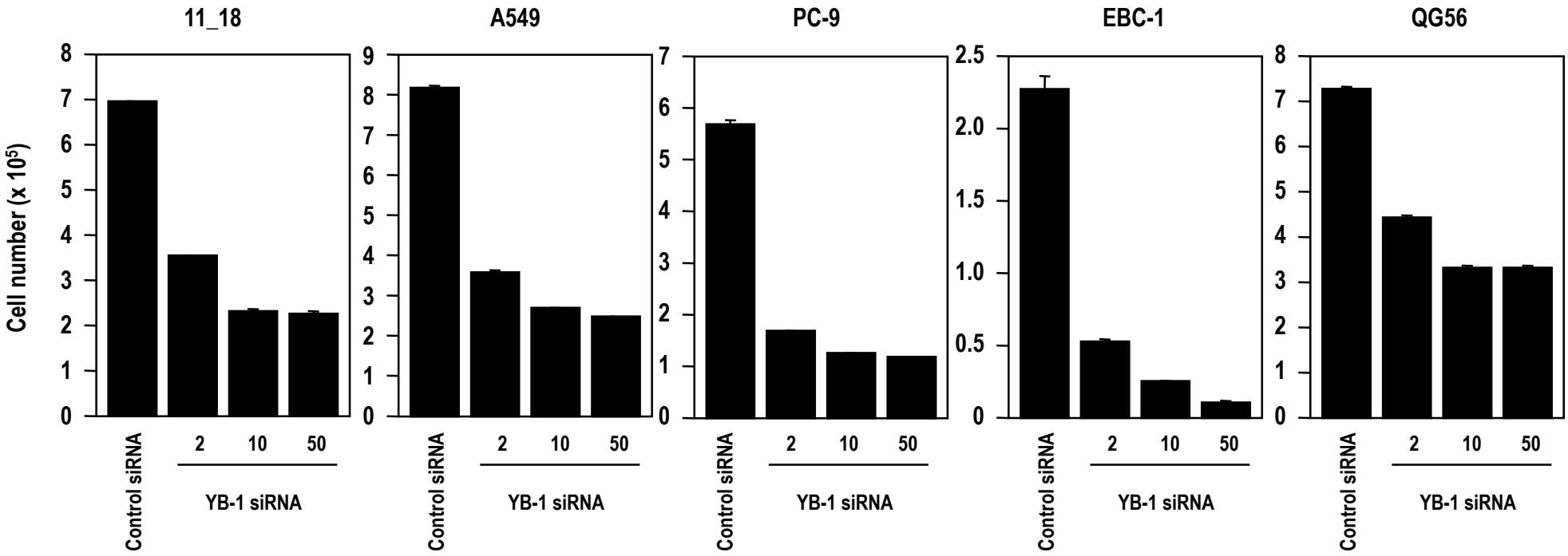
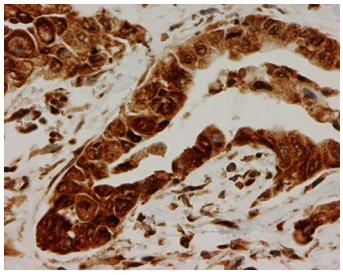
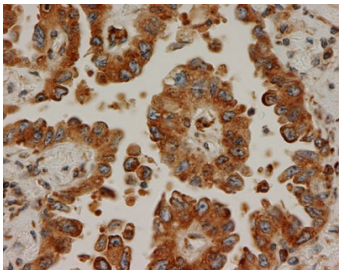

11_18
A549
PC-9
EBC-1
QG56
Cell number (x 10^5)
Control siRNA
2
10
50
YB-1 siRNA

Figure 3

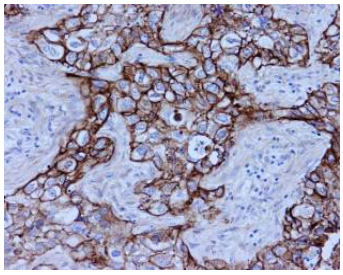
Nuclear YB-1 positive(x400)



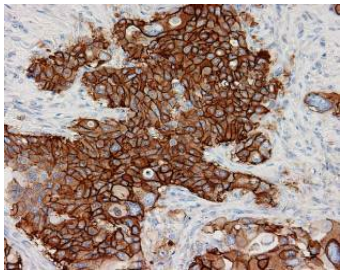
Nuclear YB-1 negative(x400)



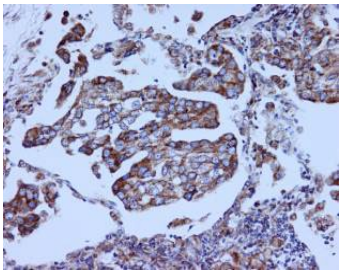
EGFR (x200)



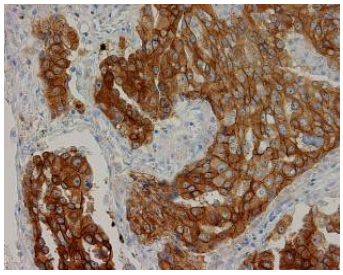
HER2 (x200)



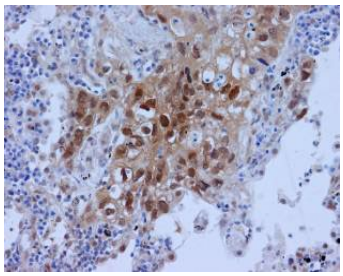
HER3 (x200)



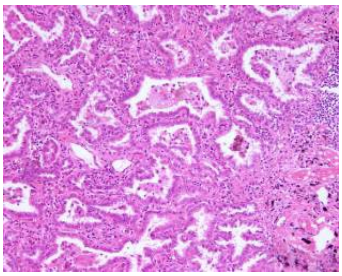
c-Met (x200)



pAkt (x200)



HE/Adeno (x100)



HE/Squamous (x100)

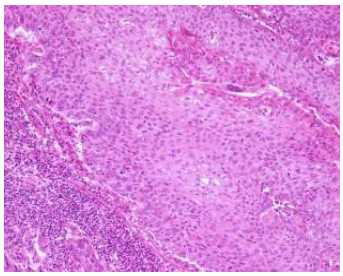
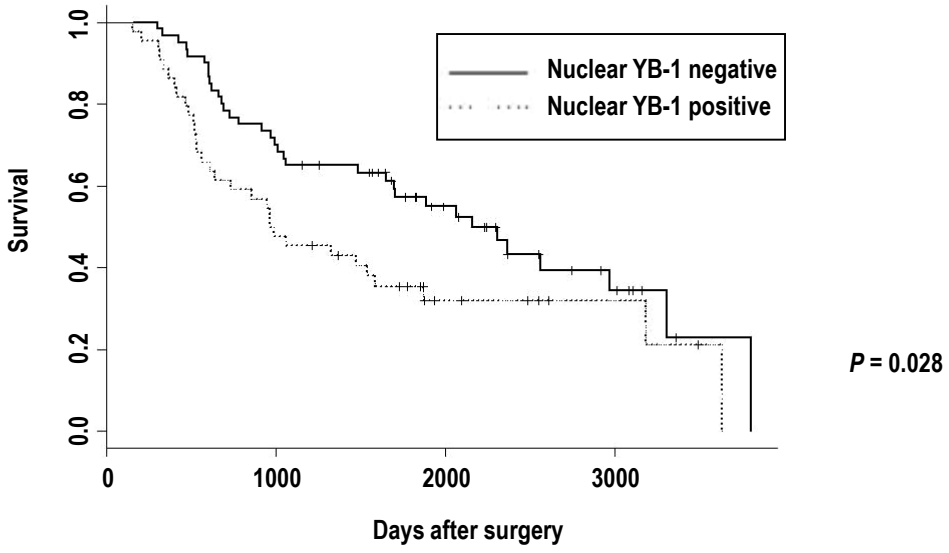
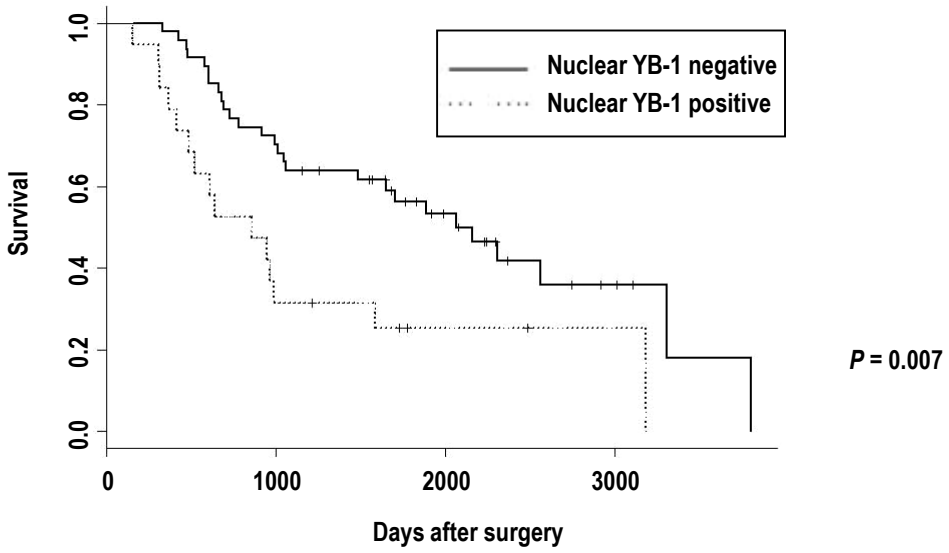


Figure 4

A.


Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
Days after surgery
Nuclear YB-1 negative
Nuclear YB-1 positive
P = 0.028

B.


Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
Days after surgery
Nuclear YB-1 negative
Nuclear YB-1 positive
P = 0.007

C.

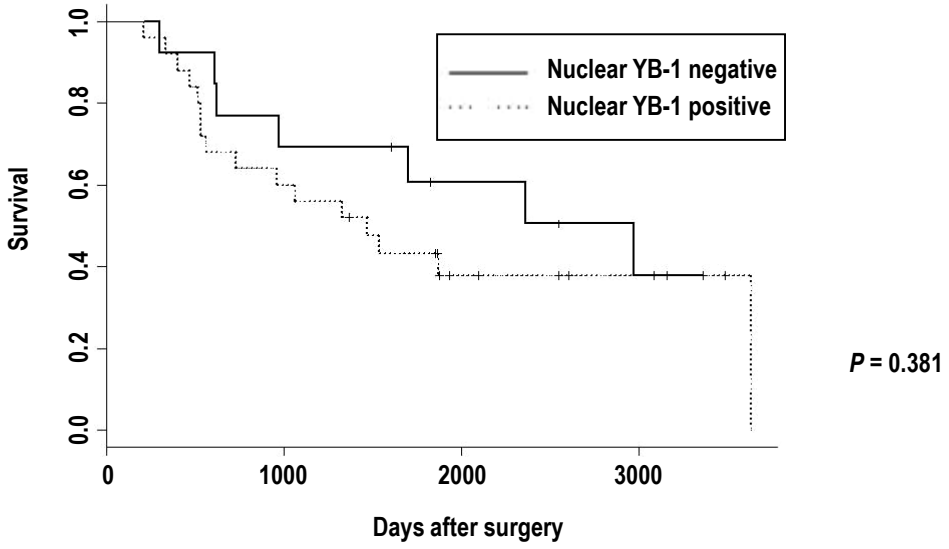

Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
Days after surgery
Nuclear YB-1 negative
Nuclear YB-1 positive
P = 0.381

Figure 5

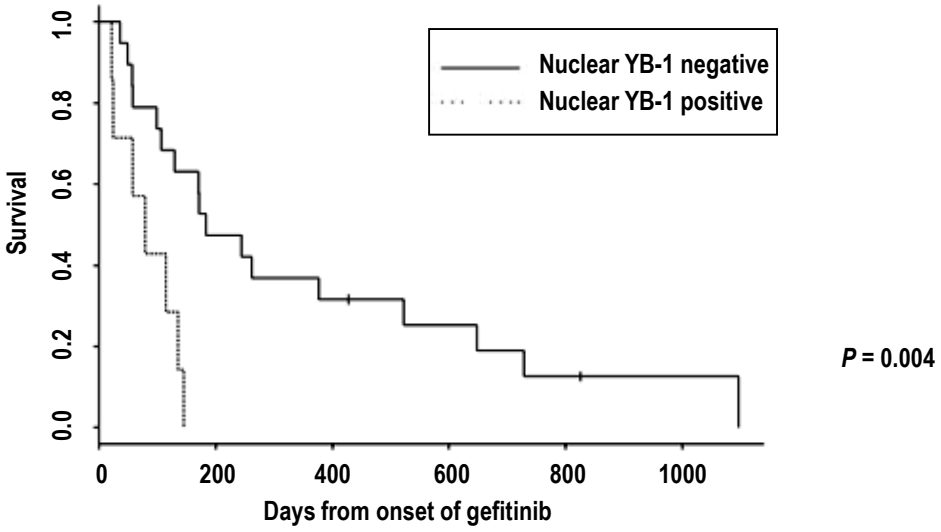

Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
Days from onset of gefitinib
Nuclear YB-1 negative
Nuclear YB-1 positive
P = 0.004

TABLE 1. Correlation Between nuclear YB-1 Expression and Expression of Five Target Genes in Adenocarcinoma and Squamous Cell Carcinoma of NSCLC

	Adenocarcinoma							Squamous cell carcinoma						
			Nuclear YB-1							Nuclear YB-1				
			Negative		Positive					Negative		Positive		
Variables	**n**	**%**	**n**	**%**	**n**	**%**	***p***	**n**	**%**	**n**	**%**	**n**	**%**	***p***
EGFR														
Negative	42	63.6	30	63.8	12	63.2	1.000	25	65.8	10	76.9	15	60.0	0.473
Positive	24	36.4	17	36.2	7	36.8		13	34.2	3	23.1	10	40.0	
HER2														
Negative	60	90.9	45	95.7	15	78.9	0.052	36	94.7	11	84.6	25	100.0	0.111
Positive	6	9.1	2	4.3	4	21.1		2	5.3	2	15.4	0	0.0	
HER3														
Negative	37	56.1	26	55.3	11	57.9	1.000	33	86.8	9	69.2	24	96.0	0.038
Positive	29	43.9	21	44.7	8	42.1		5	13.2	4	30.8	1	4.0	
c-Met														
Negative	47	71.2	34	72.3	13	68.4	0.770	35	92.1	11	84.6	24	96.0	0.265
Positive	19	28.8	13	27.7	6	31.6		3	7.9	2	15.4	1	4.0	
pAkt														
Negative	41	62.1	29	61.7	12	63.2	1.000	27	71.1	9	69.2	18	72.0	1.000
Positive	25	37.9	18	38.3	7	36.8		11	28.9	4	30.8	7	28.0	

TABLE 2. Univariate Analysis of Patients Characteristics and Expression of Nuclear YB-1 and Other Target Genes in Relation to Regarding Overall Survival

	Adenocarcinoma			Squamous cell carcinoma			Total		
		Overall survival			Overall survival			Overall survival	
Variables	**n**	**HR (95% CI)**	***p***	**n**	**HR (95% CI)**	***p***	**n**	**HR (95% CI)**	***p***
YB-1									
Negative	47	1.00	0.007	13	1.00	0.381	60	1.00	0.028
Positive	19	2.40 (1.25-4.58)		25	1.50 (0.60-3.72)		44	1.73 (1.05-2.83)	
EGFR									
Negative	42	1.00	0.098	25	1.00	0.190	67	1.00	0.701
Positive	24	1.71 (0.90-3.23)		13	0.52 (0.19-1.41)		37	1.11 (0.66-1.87)	
HER2									
Negative	60	1.00	0.566	36	1.00	0.005*	96	1.00	0.130
Positive	6	1.36 (0.48-3.86)		2	6.89 (1.45-32.7)		8	1.91 (0.82-4.47)	
HER3									
Negative	37	1.00	0.173	33	1.00	0.468	70	1.00	0.155
Positive	29	0.64 (0.33-1.23)		5	0.59 (0.14-2.52)		34	0.66 (0.38-1.17)	
c-Met									
Negative	47	1.00	0.528	35	1.00	0.645	82	1.00	0.428
Positive	19	1.24 (0.64-2.40)		3	1.41 (0.33-6.12)		22	1.27 (0.71-2.27)	
pAkt									
Negative	41	1.00	0.540	27	1.00	0.087	68	1.00	0.128
Positive	25	0.82 (0.42-1.57)		11	0.40 (0.13-1.19)		36	0.65 (0.38-1.14)	

Abbreviation: 95% CI, 95% confidence interval.

* Significance of HER2 for squamous cell carcinoma may be artefactual obtained since only two HER2 positive patients were observed and they happened to have extremely short overall survival by chance.

TABLE 3. Cox Regression Analysis for Overall Survival

	Adenocarcinoma		Squamous cell carcinoma		Total	
	HR (95% CI)	***P***	**HR (95% CI)**	***p***	**HR (95% CI)**	***p***
YB-1	1.00	0.022	1.00	0.158	1.00	0.016
	2.19 (1.12-4.28)		2.14 (0.74-6.15)		1.96 (1.13-3.38)	
Gender	1.00	0.218	1.00	0.764	1.00	0.380
	1.77 (0.63-5.03)		0.71 (0.17-3.64)		1.51 (0.61-3.75)	
Smoking	1.00	0.726	1.00	0.414	1.00	0.655
	0.83 (0.29-2.39)		0.47 (0.08-2.88)		0.81 (0.31-2.07)	
Histological					1.00	0.248
					1.43 (0.78-2.65)	

Abbreviation: 95% CI, 65% confidence interval.