Potential biomarkers involving IKK/RelA signal in early stage non-small cell lung cancer

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The clinical relevance of nuclear factor κB (NF-κB) and its regulatory molecules on prognosis of patient with early stages of non-small cell lung cancer (NSCLC), remains unclear. Therefore, we conducted biomarker analyses with survival in patients with stages I and II NSCLC. Tumor samples were collected from 88 patients with early-stage NSCLC (stages I, II). A minimum follow-up period of 5 years was required. RelA, phosphorylated IκB (pIκBα), pIKKα/β were detected by immunostaining. NF-κB DNA binding activity was assessed by *electrophoretic mobility shift assay*. Association of clinical and pathologic variables (e.g. sex, age, pathologic stage) with relevant molecules was determined by Pearson's χ^2 test or Fisher's exact test. Survival analysis based on single expression of RelA, pIκBα, pIKKα/β as well as composite expressions were evaluated using Cox proportional hazards regression models, and log rank test followed Kaplan-Meier estimates. RelA, pIκBα, pIKKα/β were observed as increased expression in NSCLC tissues compared with adjacent normal tissues and normal lung tissues. These molecules were associated with tumor-node-metastasis stages, T stages and histological status, respectively. Among the molecules analyzed, RelA and pIκBα-positive were statistically significant predictors of patient death in the entire patient population adjusted by age, gender and smoking status; furthermore both RelA and pIκBα-positive was the strongest prognostic indicators of poor prognosis by univariate and multivariate analyses. Borderline positive correlations were observed between RelA and pIκBα or pIKKα/β expression. In this cohort of early-stage NSCLC patients, molecular markers, especially composite application of multiple biomarkers (both nuclear RelA and cytoplasmic pIκB-α expression) that independently predict overall survival have been identified. (*Cancer Sci* 2008; 99: 582–589)

As the leading cause of cancer deaths, lung cancer is the focus of intensive research concerning its treatment, diagnosis, prognosis and so on. Recently, there has been rapid progress in treatment, but the prognosis of patients with lung cancer has improved only minimally. Histologically, non-small cell lung cancer (NSCLC) accounts for approximately 80% of lung cancer cases. Although early-stage NSCLC patients have a relatively favorable prognosis, the risk of disease recurrence and death remains substantial; approximately 40% of these patients will die from recurrent disease.[1–3] At present, no prognostic factors have consistently demonstrated the ability to predict those early stage NSCLC patients who are at increased risk of death. Identification of reliable prognostic factors for disease recurrence and death could have significant clinical importance.[4,5]

One area of intense research is the identification of molecular markers to complement tumor-node-metastasis (TNM) staging to fully assess the prognosis of patients and to define innovative strategies. It has been revealed that activation and inactivation of specific molecular markers may be used in addition to staging for assigning NSCLC patients to subgroups for clinical trials and more intense adjuvant therapy. However, expanded use of these markers for the diagnosis and individualization of therapy has been hampered by lack of universal acceptance of their prognostic significance.[6,7]

The transcription factor nuclear factor-κB (NF-κB) is known to be involved in the regulation of transcription of various genes involved in cell proliferation, angiogenesis, metastasis, survival and suppression of apoptosis. NF-κB proteins are sequestered in the cytoplasm in an inactive state in complex with inhibitor-κB (I-κB). Degradation of I-κB by I-κB kinase (IKK) mediated phosphorylation process releases NF-κB translocate to the nucleus to transcript active target genes, mediating its biological activity.[8,9] A series of studies reported that aberrant expression of NF-κB is related to the development and progression of cancer malignant processes. Activation of NF-κB is verified as a significant mechanism contributing to tumorigenic processes and cancer survival in many kinds of tumors, including pancreatic cancer, breast cancer, gastric carcinoma and prostate cancer.[10–13] Thus, any molecular markers mediated with signal transduction of NF-κB may be useful in evaluation of clinicopathological features.[14]

Although a few reports[15,16] have shown a correlation between NF-κB activation and the clinicopathological features of lung cancer, these studies were limited by either consisting of a heterogeneous group of patients from stage I through stage IV or small size samples measured and potential selection biases. There is no definitive data available to accurately predict long-term outcome in patients and to facilitate early diagnosis of NSCLC. Furthermore, IKK activation is the key step to regulate NF-κB activity in many types of cancer cells. Overactivation of IKK in cancer cells is found to correlate with activity of NF-κB. This suggests that IKK may contribute to aggressive characteristic features. However, there is no direct evidence to support its prognostic value. Also, there is no attempt to combine these multifactors to enhance their diagnostic and prognostic sensitivity.

Taken together, we have chosen to focus on evaluation of the role of NF-κB and its coherent molecules as risk factors in patients with early-stage NSCLC features, to provide further information on NF-κB activation involved in the neoplastic process and biologic behavior of the tumor. The present study, to our knowledge, is the first to combine molecular analysis of NF-κB and its related molecules I-κB and IKK, in association with clinicopathological features, as well as survival in a human early-stage NSCLC population, in a large molecular epidemiological study. This information may be particularly useful as a comparison with new candidate potential markers.

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Materials and Methods

Patients and tissue samples. The 88 patients in the study had pathologically proven stage I and II NSCLC, with either surgical removal or biopsy, but without receiving any therapy regimen prior to surgery or biopsy. Patients were consecutively recruited from January 1996 to July 2001, at the Linyi Municipal Hospital, Daing Hospital and Affiliated Hospital of Qingdao University with detailed clinical data; all subjects were ethnic Chinese. In all cases, paired adjacent normal lung tissue specimens were available (patients undergoing radiotherapy or chemotherapy were excluded from the cohort). The tumor differentiation stages, including N stage or T stage, were evaluated according to operative and pathological findings on the basis of TNM classification. A control group consisted of 50 patients with normal lung tissue, obtained from resection or biopsy of non-malignant lung disease. The samples were snap-frozen in liquid nitrogen immediately after surgical removal or biopsy and stored at –80°C, then subjected to immunoassay with immunohistochemistry, and in some surgical removal cases with *electrophoretic mobility shift assay* (EMSA). H&E-stained slides from each case were reviewed for histologic diagnosis, with the approval of the Ethics Committee and Institutional Review Board of the Chongqing Medical University, in accordance with the ethical standards prescribed by the Helsinki Declaration of the World Medical Association.

Clinical outcomes were followed from the date of surgery to either the date of death or December 31, 2006. Among the 88 patients analyzed, the length of follow-up was 39–62 months (median: 51 months). In this group 53 patients died during the observation period to December 31, 2006, with follow-up ranging 39–60 months (median: 48 months). In the group of 35 surviving patients the follow-up time was 60 months. All patients were traced every 3 months by clinical examination and basic laboratory tests during the first 3 years from diagnosis and then every 6 months. For survival analysis, the outcomes were divided into three categories: (i) death caused by lung cancer; (ii) death from other causes without signs of relapse; and (iii) still alive on December 31, 2006. Survival was calculated at the time from follow-up to death from any cause for overall survival. For overall survival, all deaths, irrespective of cause, were considered events. Data concerning the patients who died from other causes or who were still alive at the end of our study were censored.

Immunohistochemistry. Immunostaining was performed as described previously[17] but with a slight modification. Briefly, sections were incubated with anti-RelA against the p65 (RelA) unit (p65; 1:150; sc-109, Santa Cruz Biotechnology, Santa Cruz, CA, USA), antiphosphorylated IκB-α that recognizes an epitope Ser 32 (1:40; sc-8404, Santa Cruz Biotechnology), anti-IκBα (1:100; sc-1643, Santa Cruz Biotechnology), antiphospho-IKKα/β(Ser176/180) (1:100; #2697S, Cell Signaling Technology, Beverly, MA), anti-IKKα (1:100; sc-7183, Santa Cruz Biotechnology) and incubated with secondary antibodies (goat antimouse immunoglobulins, Shanghai Sangon Biotech, Shanghai, China). After development of the color with diaminobenzidine, the slides were counterstained with hematoxylin. Negative control slides were run with every batch, which included an isotype-matched immunoglobulin at the same concentration as that of the primary antibody. Under standard light microscopy at ×400, single neoplastic cell-positive cytoplasmic and nuclear staining was defined as described previously.[17] Cytoplasmic-positive cells of average 20 fields per tumor from typical section were counted and submitted to positive rates. If the positive rate exceed 5%, immunostaining results were considered positive for this patient, whereas cases with any pattern of nuclear staining were considered positive for nuclear NF-κB.

Nuclear extracts and EMSA. Nuclear proteins of tissue specimens were extracted using an NE-PER Nuclear and Cytoplasmic Extract kit (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's instructions. EMSA was performed using Gel Shift Assay System kit (Promega, Madison, WI, USA) as described previously.[11] Briefly, probes of double-stranded oligonucleotides (NF-κB 5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were labeled by a kinase reaction with T4 polynucleotide kinase and γ-^{32}P adenosine trisphosphate (ATP) (Yahui Biotech Inc., Beijing, China); 10 μg of protein extract was incubated with 2×10^4 c.p.m. of oligonucleotide probe along with 1 μg poly(dI-dC) (Sigma, St. Louis, MO, USA) in binding buffer for 15 min at room temperature. Equal loading of nuclear extracts was monitored by Oct–1 binding. In competition experiments, a 50-fold unlabeled oligonucleotide was included in the binding reaction. For supershift assays, 2 μL of polyclonal antibodies to p65, p50 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in the presence or absence of their neutralizing peptides was preincubated for 30 min with the nuclear extracts before the probe was added. The probe was allowed to bind for 20 min at room temperature. The resulting protein-DNA complexes were analyzed on 4% non-denaturing polyacrylamide gels in 0.5 × Tris-borate-EDTA (edetic acid) buffer and then subjected to autoradiograph.

Statistical analysis. Statistical comparisons of the two protein stainings were compared using McNemar χ^2 test. Pearson's χ^2 test or Fisher's exact test was used to examine correlations between the RelA nuclear staining, pIκB-α staining percentages and the clinicopathologic features. Univariate and multivariate Cox proportional hazards regression models were used to examine prognostic value of single or multiple analysis of RelA nuclear, pIκBα, and pIKKα/β adjusted by age, gender and smoking status. The relative optical density value of autoradiograms from EMSA were processed by the Wilcoxon Mann–Whitney *U*-test. Survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. Statistical analyses were carried out using SPSS software for Windows (SPSS, Inc., Chicago, IL, USA). Taking into account the fact that multiple comparisons were made, a level of significance of $P < 0.01$ was adopted.

Results

Staining pattern of RelA in NSCLC tissues. To ascertain if NF-κB activity changes with human early-stage NSCLC clinicopathological features, RelA/NF-κB expression analysis was performed using immunohistochemistry. The cells were accounted and subjected to calculate the percentage of positively stained cells, which represent the active form. Fig. 1 shows the representative pattern of increased RelA/NF-κB staining in both the nuclei (arrow) and cytoplasm (arrow head). As expected, RelA nuclear staining was significantly increased in tumor cells in comparison to that of adjacent normal epithelial cells. The percentages of tumor tissues, adjacent normal tissues and normal epithelial cells with nuclear staining of RelA/NF-κB was 46.6%, 29.5% and 24.0% (Table 1, $P = 0.029$, 0.011, Pearson's χ^2 test), respectively.

Concomitant activation of NF-κB-binding activity and RelA expression in NSCLC tissues. To further confirm whether nuclear staining is the relevant staining with prognosis significance, EMSA was performed to show the increased nuclear translocation of RelA representing the activated form of NF-κB. Extracts of nuclear proteins from patients and controls were analyzed for NF-κB binding activity. Oct–1 DNA binding was equivalent in all of these samples. Supershift assays revealed that the proteins in the shifted complex are the p50 and p65 isoforms of NF-κB. The shift in p65 appears as decreased NF-κB-DNA binding complexes because of conformational changes in the p65 subunit induced by antibody binding, that preclude DNA binding (Fig. 2). There

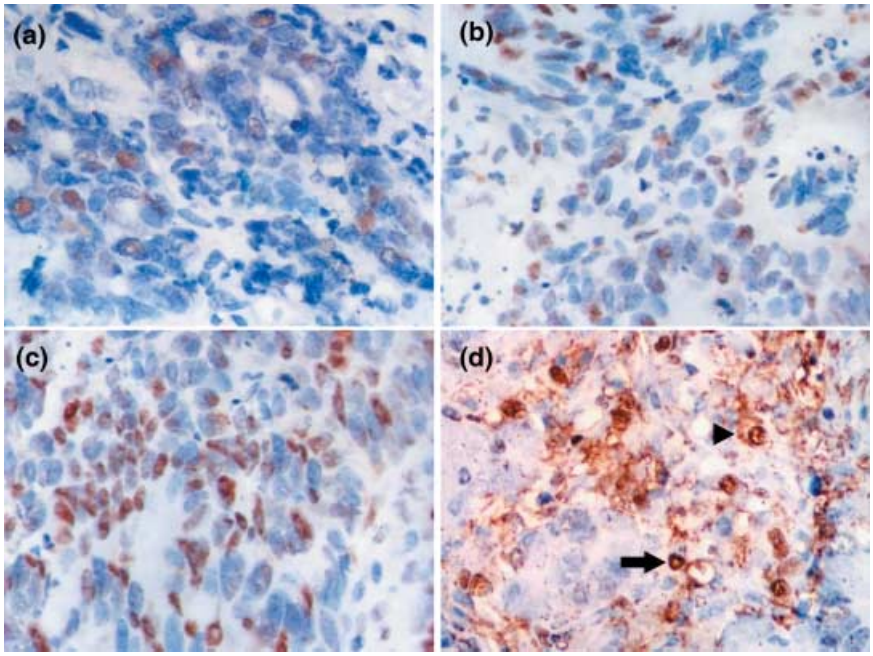

Fig. 1. Immunohistochemical detection of RelA/NF-κB (nuclear factor-κB) in representative tissue specimens and paired normal lung tissue. The subsequent analysis was carried out as described in the Material and Methods section. Tumor tissues show either cytoplasmic (arrow lead) or nuclear (arrow) staining. (a) RelA/NF-κB was expressed in the adjacent normal lung tissues next to cancer ×400). (b) RelA/NF-κB staining in the normal lung tissues without cancer (×400). (c) Expression pattern of RelA in human non-small cell lung cancer (NSCLC) tissue specimens (×400). (d) Heavy expression of RelA/NF-κB a in 66-year-old patient with stage II NSCLC that recurred at 11 months and progressed to chemotherapy and radiotherapy-refractory metastatic disease (×400). Average from at least two independent experiments counted in duplicate. Representative fields are shown above.

Table 1. Associations of nuclear RelA and cytoplasmic pIκB-α staining status in cases, adjacent tissues and control

Variables	Cases (n = 88) (%)	Adjacent tissues (n = 88) (%)	P	Control (n = 50) (%)	P
Nuclear RelA status					
Nuclear (+)	41 (46.6)	26 (29.5)	0.029	12 (24.0)	0.011
Nuclear RelA(–)	47 (53.4)	62 (70.5)		38 (76.0)	
Cytoplasmic pIκB-α status					
Cytoplasmic pIκB-α(+)	27 (30.7)	12 (13.6)	0.010	5 (10.0)	0.003
Cytoplasmic pIκB-α(–)	61 (69.3)	76 (86.4)		45 (90.0)	
Total IκB-α					
Total IκB-α(+)	36 (40.9)	29 (32.9)	0.349	22 (44.0)	0.724
Total IκB-α(–)	52 (59.1)	59 (67.1)		28 (56.0)	

is a significant relationship of NF-κB-DNA binding activity with RelA nuclear expression (data not shown). Fig. 2 illustrates the increased NF-κB-binding activity was frequently detected in human NSCLC tissues with RelA immunostaining nuclear positive tissues as compared with nuclear negative tissues and matched non-neoplastic tissue or adjacent normal tissue. Our results showed that NF-κB was activated in NSCLC tissues but not in normal lung tissues and adjacent tumor tissues.

Phosphorylated and total IκBα protein staining analysis. Generally, NF-κB is activated by phosphorylating its inhibitor I-κB. To further determine whether induction of phosphorylated IκB-α was also companied with activation form of RelA. Then, we further performed *in vitro* experiments to detect phosphorylated and total IκB-α protein levels. As expected, I-κB staining showed a different pattern from that of RelA, with most positive staining localized within the cytoplasm (Fig. 3). The expression of phosphorylated IκB-α protein in the cytoplasm also increased in tumor tissues compared with that in the adjacent and normal lung tissues (Table 1, $P = 0.010$, 0.003, Pearson's χ^2 test). The level of total IκBα was maintained at a high level, even similar to that of adjacent and normal lung tissues, representing the resting condition (Table 1, $P = 0.349$, 0.724). So, according to the current data, we can see that enhanced IκBα phosphorylation is indeed present in NSCLC tissues, which is consistent with induction of RelA/NF-κB activity as measured above. It was further verified by a significant correlation between the expressions of the two proteins of RelA/NF-κB and pIκBα within NSCLC cases (Table 2, $P = 0.029$, McNemar χ^2 test).

NF-κB activation and pIKKα/β expression in NSCLC tissues. Because IκB was thought to be phosphorylated to degradate by IKK and pIKKα/β represent the active subunit (or form) to mediate NF-κB activation through I-RB phosphorylation. Next, we further investigated the correlation between NF-κB activation and pIKKα/β expression by immunohistochemical analysis. When stained with antiphospho-IKKα/β(Ser176/180), anti-IKKα antibody, a cytoplasmic staining pattern was observed and cytoplasmic positivity was noted in tumor cells (Fig. 4). The cytoplasmic positivity rate for the subset was 30.7% (27 of 88) for pIKKα/β

Table 2. Nuclear RelA in relation to status of cytoplasmic pIκBα and pIKKα/β (McNemar χ^2 test)

	RelA (+)	RelA (–)
pIκB-α status*		
pIκB-α(+)	16	11
pIκB-α(–)	25	36
pIKKα/β status**		
pIKKα/β(+)	15	12
pIKKα/β(–)	26	35

*$P = 0.029$, **$P = 0.034$.

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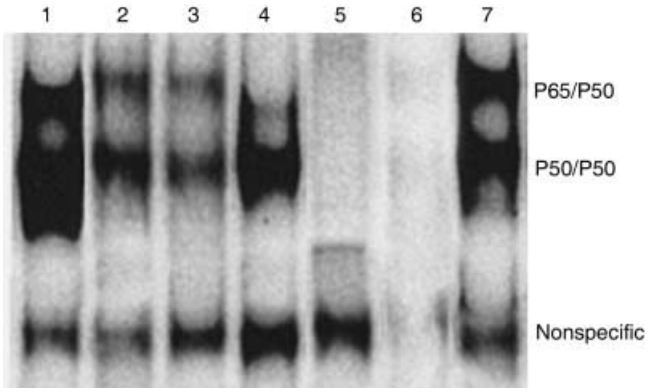

Fig. 2. Nuclear factor-κB (NF-κB) DNA binding activity was assayed by EMSA in nuclear extracts isolated from tumor samples, its paired adjacent normal tissue and non-cancer lung tissues. Nuclear extracts (50 μg) were used to determine the NF-κB DNA binding activity in Human NSCLC tissue (RelA+) (line 1*)* (RelA-) (line 2*)* and adjacent lung tissue (line 3*)*. Supershift analysis was performed to confirm the specificity of NF-κB DNA binding with antibodies specific for RelA (p65) (recognizes RelA/p50 heterodimer, so supershifted RelA/p50) (line 4), NF-κB1 (p50) (recognizes p50 homodimer and RelA/p50 heterodimer, so supershifted RelA/p50 and p50/p50) (line 5) and a competitive study with a 50-fold excess of unlabeled oligonucleotide (diminished the intensity of both RelA/p50 and p50/p50) (line 6), 50-fold excess of another oligonucleotide AP-1 do not decrease its binding activity, proven specificity of current NF-κB DNA binding activity assay (line 7). Data from one representative experiment are shown. Oct –1 was selected as a constitutively expressed control and showed equivalent DNA binding activity in all of these samples (data not shown).

and 36.4% (32 of 88) for IKKα, respectively, and the tumor cell immunoreactivity was more than or equal to that of the benign cells, no statistical significance was attained. Correlation analysis between NF-κB activation and pIKKα/β expression with McNemar χ^2 test showed a positive relation (Table 2, $P = 0.034$). Representative images showing simultaneous cytoplasmic expression of IKKα and pIKKα/β staining are presented in Fig. 4.

Association between clinicopathologic features and potential prognostic factors staining. Correlation between the clinicopathologic features of the 88 NSCLC cases with their RelA and/or IκB-α staining data are summarized as described in 'Materials and Methods'. As shown in Table 3, RelA/NF-κB overexpression was found in 46.6% of tumors and it appeared to correlate marginally with tumor TNM stages (Table 3, $P = 0.018$, Pearson's χ^2 test), age ($P = 0.012$) and smoking status ($P = 0.011$). For cytoplasmic pIκBα (+) staining, it was more likely associated with T stages ($P = 0.033$), and histological status ($P = 0.014$), respectively. Moreover, in composite analysis of both RelA and pIκBα, it showed that both positive expression of the two proteins was correlated with histological types ($P = 0.002$), TNM stages ($P = 0.001$) and T stages ($P = 0.000$). No association was found between pIKKα/β staining and clinicopathologic features (data not shown).

Survival analysis based on RelA and/or IκB-α expression. In order to analyze whether the nuclear RelA, cytoplasmic pIκBα and pIKKα/β expression are correlated with NSCLC cancer patient survival, first, the survival analyses were performed using the log rank test followed by Kaplan-Meier estimates. Sex, histological type and smoking status of the tumor patients were also included in the analyses. The overall Kaplan-Meier survival curves for RelA (+) and pIκBα (+) staining expression are shown in Fig. 5. The results of the tests for sex and histological type were non-significant (result not shown), while RelA (+) ($P = 0.016$, log rank test) and pIκBα (+) ($P = 0.002$, log rank test) was linked to poor overall survival, but pIKKα/β (–) gave no survival dominance ($P = 0.063$, log rank test). Combined evaluation of both nuclear RelA and IκB-α expression showed very poor survival rates ($P = 0.000$, log rank test). We further performed stratified log rank test with strata defined by TNM stages (I, II). The results for the pIκBα (+) test ($P = 0.003$) and the RelA (+) and pIκBα (+) test ($P = 0.000$) were statistically significant, which were in accordance with the outcome of the univariate analysis. However, the results for the RelA (+) tests became marginally non-significant ($P = 0.049$). Thus, for those two tests, the adjustment for the effect of TNM stage qualitatively changed the conclusion obtained from the unstratified analysis.

Univariate analyses and multivariate analysis of overall survival using the Cox proportional hazards model was further performed to determine the prognostic value of single or bis-analysis of RelA, pIκBα and pIKKα/β expression when adjusted with other prognostic factors including age, gender and smoking status. Results shown in Table 4 indicate that after adjustment for effects of age, stage, and smoking history, the prognostic effect of RelA (multivariate HR = 2.961, 95% CI = 1.292–6.787, $P = 0.010$) or pIκBα (multivariate HR = 3.132, 95% CI = 1.442–6.807, $P = 0.004$) was enhanced. Both RelA and pIκBα (+) showed significant association with higher risk of death compared to other expression patterns of both the two biomarkers (multivariate HR = 18.242, 95% CI = 6.087–54.667, $P = 0.000$) (Table 4). Survival curves also confirmed this result. So these results indicate a significant inverse relationship of NF-κB activation with a poor prognosis, and combined RelA and IκB-α analysis provide more valuable prognosis information.

Discussion

It is important to establish prognostic factors that will define subgroups of patients with early-stage NSCLC to choose more suitable treatments. Using NF-κB as a prognostic factor, it was found to be correlated with poor prognosis in several kinds of cancers, including gastric, breast and pancreatic cancers.[10–14] However, others reported that NF-κB activation positively correlates with better patient outcome.[18] A precise explanation for this pattern is lacking; perhaps the role of NF-κB results

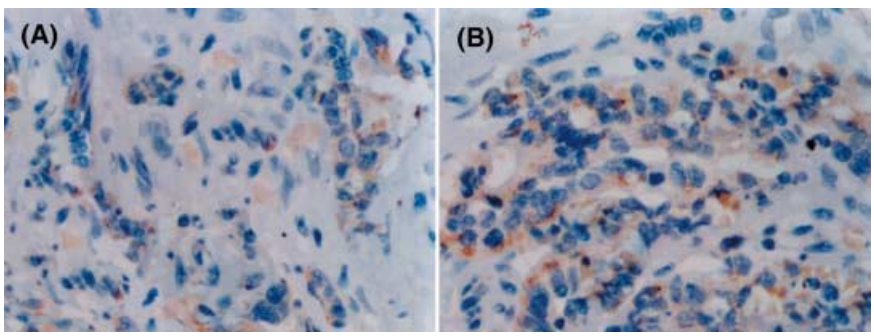

Fig. 3. Immunostaining of phosphorylated and total IκBα protein level with antibody-specific for phosphorylated or total IκBα protein, respectively. Note the strong cytoplasmic staining in human NSCLC. A, pIκBα staining in the lung NSCLC tissue. B, paired total IκBα staining in the same patient. Immunostained cells were counted using a light microscope and positive rate were determined as defined in material and methods.

Table 3. Association between RelA, pIκB-α staining and pathological characteristics of non-small cell lung cancer patients (Pearson's χ^2 test)

Prognostic factors	RelA (+) *n* (%)	*P*	pIκB-α (+) *n* (%)	*P*	RelA (+) and pIκB-α (+)	*P*
Age(year) (*n*)						
Under 55.1 (45)	18 (40.0)	0.012	18 (40.0)	0.336	7 (15.5)	0.260
Over 55.1 (33)	23 (69.6)		9 (27.2)		9 (27.2)	
Gender						
Female (39)	14 (35.9)	0.405	10 (25.6)	0.819	7 (17.9)	0.784
Male (59)	27 (45.8)		17 (28.8)		9 (15.3)	
Histology						
Squamous carcinoma (40)	17 (42.5)	0.198	6 (15.0)	0.014	1 (2.5)	0.002
Adenocarcinoma (23)	13 (56.5)		10 (43.5)		7 (30.4)	
Others (25)	11 (44.0)		11 (44.0)		8 (32.0)	
Smoking status						
=27 pack-years (17)	9 (52.9)	0.011	6 (35.3)	0.878	3 (17.6)	0.351
<27 pack-years (42)	25 (59.5)		12 (28.6)		10 (23.8)	
Nonsmoker (29)	7 (24.1)		9 (31.0)		3 (10.3)	
TNM stages						
Stages I (45)	15 (33.3)	0.018	10 (22.2)	0.106	2 (4.4)	0.001
Stages II (43)	26 (60.5)		17 (39.5)		14 (32.6)	
T stages						
T1 (39)	16 (41.0)	0.199	10 (25.6)	0.033	4 (10.3)	0.000
T2 (36)	16 (44.4)		9 (25.0)		4 (11.1)	
T3 (13)	9 (69.2)		8 (61.5)		8 (61.5)	
N stages						
N0 (58)	24 (41.4)	0.186	20 (34.5)	0.336	10 (17.2)	0.776
N (130)	17 (56.7)		7 (23.3)		6 (20.0)	

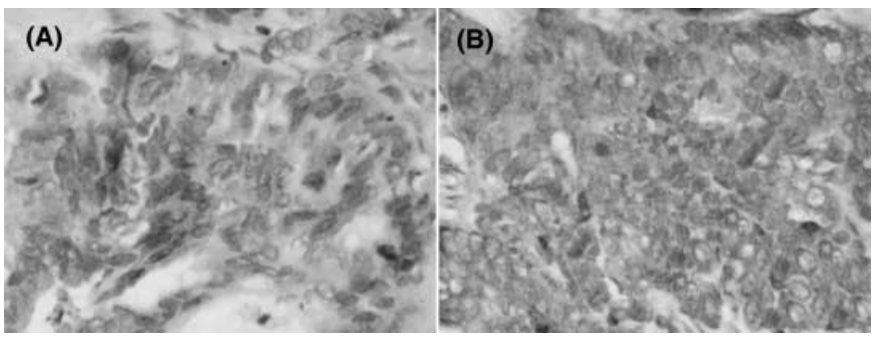

Fig. 4. Immunostaining of pIKKα/β and IKKα with antibodies of antiphospho-IKKα/β, anti-IKKα, respectively. The positive stain is also located within cytoplasmic staining in human NSCLC. A, pIKKα/β staining in the lung NSCLC tissue. B, paired IKKα staining in the same patient.

Table 4. Univariate and multivariate analysis of potential molecular markers for overall survival of early stage non-small cell lung cancer

	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)†	*P*
Nuclear RelA (+)				
(+ *vs.* –)	2.264 (1.135–4.514)	0.020	2.961 (1.292–6.787)	0.010
Cytoplasmic pIκBα (+)				
(+ *vs.* –)	2.943 (1.450–5.975)	0.003	3.132 (1.442–6.807)	0.004
pIκBα (+)				
(+ *vs.* –)	1.654 (0.830–3.297)	0.152	1.779 (0.859–3.684)	0.121
RelA and pIκBα				
+/– or –/+ *vs.* +/+	13.013 (4.986–33.966)	0.000	18.242 (6.087–54.667)	0.000
–/– *vs.* +/– or –/+	2.353 (1.013–5.470)	0.047	2.452 (1.016–5.917)	0.046
pIKKα/β				
(+ *vs.* –)	1.926 (0.943–3.930)	0.072	2.106 (0.974–4.554)	0.058
IKKα				
(+ *vs.* –)	1.831 (0.916–3.661)	0.087	2.216 (1.057–4.647)	0.035

†Adjusted for age, gender and smoking history. HR, hazard ratio.

from activity of other oncogenes like p53, Myc, etc. Our findings presented here, which varies significantly from the data reported by other investigators[15,16] and other cancer types[19,20] further extend knowledge of NF-κB as a molecular prognostic factor, limited to patients with early-stage NSCLC. Based on previous findings that nuclear RelA may be a strong and independent prognostic factor in lung cancer, consistent with its role in carcinogenesis, we further identified 88 patients of

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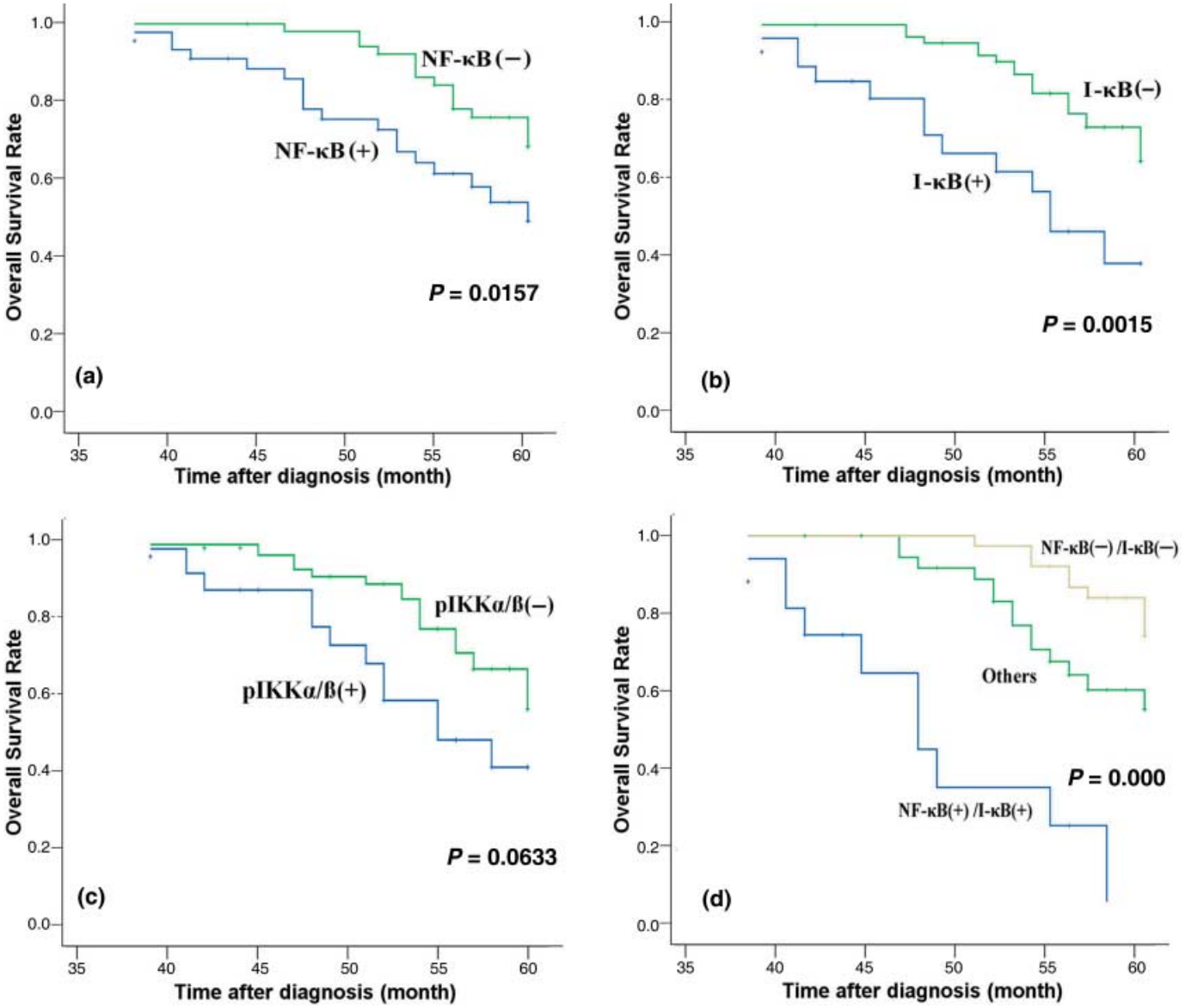

Fig. 5. Overall survival of the patient stratified by (a) nuclear factor-κB (NF-κB) status, patients with nuclear NF-κB(+) showed poor survival just attain marginal statistic significance ($P = 0.0157$, log-rank test) (b) IκBα status, patients with IκBα(+) pattern showed poorer outcome than the remainder of the population ($P = 0.0015$). (c) pIKKα/β status, patients with patients with pIKKα/β(–) showed no survival dominance ($P = 0.0633$) (d) combined NF-κB(–)/IκBα expression, patients with NSCLC expressing NF-κB(+)/IκBα(+) suffered significant poor survival compared with those expressed remaining expression patterns of NF-κB and IκBα ($P = 0.000$).

early-stage NSCLC with complete information for IKK functional expression information. Here, it indicated that NSCLC patients who were positive for nuclear RelA and cytoplasmic pIκBα showed poorer prognosis than those who were negative for nuclear RelA and cytoplasmic IκB-α, which was verified with the evaluation of pIKKα/β, the functional subunit of IKK. In this study, restricted to patients with complete information for all biomarkers involved, it is difficult to speculate on potential biases affecting our results. It is possible, for example, that small tumors with limited tissue availability, such as biopsy, were under-represented in this cohort.

We only accounted for the number of the cells staining for nuclear RelA, which have proven to be the active form in our previous study, to estimate NF-κB activation levels in cancer tissue involved, while for pIκBα, total IκB and pIKKα/β, the positive stains were mainly located within the cytoplasm, which is consistent with their physiological dynamic localization.[21–23] To better understand the effect of NF-κB, we performed another assay of NF-κB, NF-κB DNA binding activity by EMSA in lung cancer; an assay of NF-κB DNA binding activity in nuclear protein can provide functional analysis of components of activation complex for achieving finer distinctions within the neoplastic continuum.[24,25] Here we demonstrated that NF-κB-binding activity was consistently associated with nuclear staining of RelA, which further indicated that the nuclear staining is the relevant staining.

In addition to conventional NF-κB evaluation, other genes that mediate activation of NF-κB preventing apoptotic cell death may also play a critical role in carcinogenesis. Phosphorylation of cytoplasmic IκBα could facilitate releasing of NF-κB from the cytoplasm into the nucleus. Research of NF-κB pathways have shown that a non-phosphorylatable form of I-κB can significantly prevent cell viability in cancer cells[26] and an increased I-κB mRNA and protein expression paralleling that of

NF-κB positivity in human pancreatic cell lines[24] and oral cavity squamous cell cancers[27], respectively, have been reported. So it is a common belief that phosphorylation of I-κB should be positively related to levels of nuclear NF-κB.[28–30]

In this study, the expression of NF-κB and its relative molecules IκB, IKK in NSCLC was also taken into account. We selected monoclonal antibodies that bind only to phosphorylated IκBα to perform immunohistochemistry. Highly expressed phosphorylation of the IκBα protein was detected in NSCLC but not in normal lung tissues, and it reached statistical significance, consistent with its biological role indicated *in vitro*.[31–33] The positive staining of total IκBα was also detected to corroborate the immunohistochemical staining data. Interestingly, there is no obvious discriminant of total IκBα expression among the cases measured, including NSCLC, normal lung tissues; and furthermore the expression level maintained at relative high level response to that of pIκB-α. These findings were not confirmed by others. This pattern demonstrated that phosphorylation of IκB-α from total IκBα is the active process to facilitate NF-κB translocation.[34] In the present study, the authors also found a borderline statistically significant relationship between NF-κB and pIκBα in early-stage NSCLC, which has not been reported previously.

Phosphorylation of IκBα and IκBβ is achieved by the IKK, IKK therefore represents a major regulator of the NF-κB through I-κB pathway. Others have reported targeting IKK could effectively inhibit NF-κB activity[29,35] so IKK/phosphorylation of I-κB could represent states of proliferation of cancer cells. Hyperactivated IKK were also measured in certain cancer cell lines.[30,36] To elucidate whether IKK was also involved in patients with early-stage NSCLC, we measured pIKKα/β immunostaining level, the active form of IKK. In this study it was found that although immunohistochemical IKK demonstrated high expression levels in nuclear NF-κB and I-κB positive cases, it was just marginally non-significant ($P = 0.02$) within any clinicopathological parameter. This phenomenon may partly further verify the contribution of IKK/NF-κB signal axis to the NSCLC phenotype, although the clinical value of IKK needs further confirmation with additional investigation.

Until now, there is still considerable controversy as to exactly how NF-κB alterations affect the outcome of patients with cancer. Our previous study suggested that NF-κB was an independent unfavorable prognostic marker in patients with NSCLC. Tang[15] and Zhang *et al*.[16] have also associated NF-κB with major aggressiveness in NSCLC patients. But a comprehensive multivariable analysis is not feasible in these studies because the majority of studies analyzed only include a single or few biomarkers at a time. Also the cases analyzed consist of a heterogeneous group of patients. Furthermore, as the number of potential molecular markers increases, it has become more difficult to assess which prognostic factors are likely to be clinically relevant. In this regard, the development of integration of molecular marker assessment represents a significant methodologic advance. In the present study, when analyzed separately, RelA/NF-κB and pIκBα were associated with each other and with poor overall survival. This observation is in line with previous studies,[15,16] and further, evaluated in combination, the positive result of both the NF-κB and/or pIκBα test remained and showed more definite unfavorable prognostic factors for survival in the multivariate analysis. This study suggests that NF-κB activity and pIκBα may be used as prognostic biomarkers of NSCLC, and it is more reliable than single-use of nuclear NF-κB activity. In spite of its variability, it is easily accessible, so it may be helpful for diagnosis and predicting early-stage cancer in patients before radical surgery or in patients who cannot undergo surgery.[37,38] Thus, combined evaluation of patients with NF-κB/pIκBα and TNM staging may predict the clinical prognosis of NSCLC cancer patients more correctly.

Taken together, molecular analysis of potential markers involved in IKK/NF-κB signal yielded two independent predictors of poorer disease-specific survival: RelA/NF-κB and pIκBα expression in patients with stages I and II NSCLC. The integration of molecular marker assessments implied that both RelA/NF-κB and pIκBα positivity may be useful in the diagnosis and treatment of lung cancer as a decision-making biomarker for predicting treatment efficacy. Hopefully, these technologies will eventually provide the clinician with a reliable, validated molecular staging system to improve diagnosis and treatment strategies for NSCLC. Prospective studies in a larger population including very early stage patients should be carried out to investigate the integration of molecular marker assessment to further define their roles in tumor proliferation and metastasis.[39] We also anticipate that the NF-κB signal pathway will continue to be a significant target in investigation of cancer.

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References

1 Hu Y, McDermott MP, Ahrendt SA. The p53 codon 72 proline allele is associated with p53 gene mutations in non-small cell lung cancer. *Clin Cancer Res* 2005; **11** (7): 2502–9.
2 Kim JS, Kim JW, Han J, Shim YM, Park J, Kim DH. Cohypermethylation of p16 and FHIT promoters as a prognostic factor of recurrence in surgically resected stage I non-small cell lung cancer. *Cancer Res* 2006; **66** (8): 4049–54.
3 Lu C, Soria JC, Tang X *et al*. Prognostic factors in resected stage I non-small-cell lung cancer: a multivariate analysis of six molecular markers. *J Clin Oncol* 2004; **22** (22): 4575–83.
4 Singhal S, Vachani A, Antin-Ozerkis D, Kaiser LR, Albelda SM. Prognostic implications of cell cycle, apoptosis, and angiogenesis biomarkers in non-small cell lung cancer: a review. *Clin Cancer Res* 2005; **11** (11): 3974–86.
5 Esposito V, Baldi A, Tonini G *et al*. Analysis of cell cycle regulator proteins in non-small cell lung cancer. *J Clin Pathol* 2004; **57** (1): 58–63.
6 Le QT, Chen E, Salim A *et al*. An evaluation of tumor oxygenation and gene expression in patients with early stage non-small cell lung cancers. *Clin Cancer Res* 2006; **12** (5): 1507–14.
7 Birim O, Kappetein AP, van Klaveren RJ, Bogers AJ. Prognostic factors in non-small cell lung cancer surgery. *Eur J Surg Oncol* 2006; **32** (1): 12–23.
8 Kapahi P, Takahashi T, Natoli G *et al*. Inhibition of NF-kappa B activation by arsenite through reaction with a critical cysteine in the activation loop of Ikappa B kinase. *J Biol Chem* 2000; **275** (46): 36062–6.
9 Huang S, Pettaway CA, Uehara H, Bucana CD, Fidler IJ. Blockade of NF-kappaB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. *Oncogene* 2001; **20** (31): 4188–97.
10 Ross JS, Kallakury BV, Sheehan CE *et al*. Expression of nuclear factor-kappa B and I kappa B alpha proteins in prostatic adenocarcinomas: correlation of nuclear factor-kappa B immunoreactivity with disease recurrence. *Clin Cancer Res* 2004; **10** (7): 2466–72.
11 Sasaki N, Morisaki T, Hashizume K *et al*. Nuclear factor-kappaB p65 (RelA) transcription factor is constitutively activated in human gastric carcinoma tissue. *Clin Cancer Res* 2001; **7** (12): 4136–42.
12 El-Rayes BF, Ali S, Ali IF, Philip PA, Abbruzzese J, Sarkar FH. Potentiation of the effect of erlotinib by genistein in pancreatic cancer: the role Akt nuclear factor-{kappa}B. *Cancer Res* 2006; **66** (21): 10553–9.
13 Huber MA, Azoitei N, Baumann B *et al*. NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. *J Clin Invest* 2004; **114** (4): 569–81.
14 Van Waes C. Nuclear factor-kappaB in development, prevention, and therapy of cancer. *Clin Cancer Res* 2007; **13** (4): 1076–82.
15 Zhang Z, Ma J, Li N, Sun N, Wang C. Expression of nuclear factor-kappaB and its clinical significance in nonsmall-cell lung cancer. *Ann Thorac Surg* 2006; **82** (1): 243–8.

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16 Tang X, Liu D, Shishodia S, Ozburn N *et al*. Nuclear factor-kappaB (NF-kappaB) is frequently expressed in lung cancer and preneoplastic lesions. *Cancer* 2006; **107** (11): 2637–46.
17 Guo CB, Wang S, Deng C, Zhang DL, Wang FL, Jin XQ. Relationship between matrix metalloproteinase 2 and lung cancer progression. *Mol Diagn Ther* 2007; **11** (3): 183–92.
18 Lee BL, Lee HS, Jung J *et al*. Nuclear factor-kappaB activation correlates with better prognosis and Akt activation in human gastric cancer. *Clin Cancer Res* 2005; **11** (7): 2518–25.
19 Yang J, Richmond A. Constitutive IkappaB kinase activity correlates with nuclear factor-kappaB activation in human melanoma cells. *Cancer Res* 2001; **61** (22): 8150–7.
20 Stathopoulos GT, Zhu Z, Everhart MB *et al*. Nuclear factor-kappaB affects tumor progression in a mouse model of malignant pleural effusion. *Am J Respir Cell Mol Biol* 2006; **34** (2): 142–50.
21 Bayon Y, Ortiz MA, Lopez-Hernandez FJ *et al*. Inhibition of IkappaB kinase by a new class of retinoid-related anticancer agents that induce apoptosis. *Mol Cell Biol* 2003; **23** (3): 1061–74.
23 Farhana L, Dawson MI, Fontana JA. Apoptosis induction by a novel retinoid-related molecule requires nuclear factor-kappaB activation. *Cancer Res* 2005; **65** (11): 4909–17.
22 Yu M, Yeh J, Van Waes C. Protein kinase casein kinase 2 mediates inhibitor-kappaB kinase and aberrant nuclear factor-kappaB activation by serum factor (s) in head and neck squamous carcinoma cells. *Cancer Res* 2006; **66** (13): 6722–31.
24 Dolcet X, Llobet D, Encinas M *et al*. Proteasome inhibitors induce death but activate NF-kappaB on endometrial carcinoma cell lines and primary culture explants. *J Biol Chem* 2006; **281** (31): 22118–30.
25 Tergaonkar V, Bottero V, Ikawa M, Li Q, Verma IM. IkappaB kinase-independent IkappaBalpha degradation pathway: functional NF-kappaB activity and implications for cancer therapy. *Mol Cell Biol* 2003; **23** (22): 8070–83.
26 Li Z, Niu J, Uwagawa T, Peng B, Chiao PJ. Function of polo-like kinase 3 in NF-kappaB-mediated proapoptotic response. *J Biol Chem* 2005; **280** (17): 16843–50.
27 Charalambous MP, Maihofner C, Bhambra U, Lightfoot T, Gooderham NJ; Colorectal Cancer Study Group. Upregulation of cyclooxygenase-2 is accompanied by increased expression of nuclear factor-kappa B and I kappa B kinase-alpha in human colorectal cancer epithelial cells. *Br J Cancer* 2003; **88** (10): 1598–604.
28 Baeuerle PA, Baltimore DI kappa B. a specific inhibitor of the NF-kappa B transcription factor. *Science* 1988; **242** (4878): 540–6.
29 Imbert V, Rupec RA, Livolsi A *et al*. Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. *Cell* 1996; **86** (5): 787–98.
30 DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* 1997; **388** (6642): 548–54.
31 O'Connor S, Shumway S, Miyamoto S. Inhibition of IkappaBalpha nuclear export as an approach to abrogate nuclear factor-kappaB-dependent cancer cell survival. *Mol Cancer Res* 2005; **3** (1): 42–9.
32 Mabuchi S, Ohmichi M, Nishio Y *et al*. Inhibition of NFkappaB increases the efficacy of cisplatin in in vitro and in vivo ovarian cancer models. *J Biol Chem* 2004; **279** (22): 23477–85.
33 Babbar N, Hacker A, Huang Y, Casero RA Jr. Tumor necrosis factor alpha induces spermidine/spermine N1-acetyltransferase through nuclear factor kappaB in non-small cell lung cancer cells. *J Biol Chem* 2006; **281** (34): 24182–92.
34 Mabuchi S, Ohmichi M, Nishio Y *et al*. Inhibition of inhibitor of nuclear factor-kappaB phosphorylation increases the efficacy of paclitaxel in in vitro and in vivo ovarian cancer models. *Clin Cancer Res* 2004; **10** (22): 7645–54.
35 Luo JL, Kamata H, Karin M. IKK/NF-kappaB signaling. balancing life and death – a new approach to cancer therapy. *J Clin Invest* 2005; **115** (10): 2625–32.
36 Luo JL, Kamata H, Karin M. The anti-death machinery in IKK/NF-kappaB signaling. *J Clin Immunol* 2005; **25** (6): 541–50.
37 Taillade L, Penault-Llorca F, Boulet T *et al*. Immunohistochemichal expression of biomarkers: a comparative study between diagnostic bronchial biopsies and surgical specimens of non-small-cell lung cancer. *Ann Oncol* 2007; **18** (6): 1043–50.
38 Yanaihara N, Caplen N, Bowman E *et al*. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 2006; **9** (3): 189–98.
39 Fong KM, Sekido Y, Gazdar AF, Minna JD. Lung cancer. 9: molecular biology of lung cancer: clinical implications. *Thorax* 2003; **58** (10): 892–900.