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ORIGINAL ARTICLE

NSCLC and the alternative pathway of NF-κB: uncovering an unknown relation

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Abstract Lung cancer is the leading cause of cancer-related deaths worldwide. Although our knowledge on the pathobiology of the disease has increased in the last decades, the prognosis of lung cancer patients has hardly changed. Many signaling pathways are implicated in lung carcinogenesis, but the role of the alternative pathway of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in lung cancer pathogenesis and progression has not been investigated. The aim of our study was to investigate the role of this pathway in non-small cell lung cancer (NSCLC) patients. NF-κB2 and RelB protein expression was retrospectively assessed by immunohistochemistry in tissue samples from 109 NSCLC patients. RelB and NF-κB2 protein levels differed between tumors and adjacent nonneoplastic lung parenchyma. Cytoplasmic immunoreactivity of NF-κB2 and RelB was correlated with tumor stage ($p=0.03$ and $p=0.016$, respectively). In addition, cytoplasmic NF-κB2 levels were related to tumor grade ($p=0.046$). Expression of RelB in the cytoplasm was tumor histologic type-specific, with squamous cell carcinomas having the highest protein levels. Nuclear expression of RelB and NF-κB2 differed between tumor and nonneoplastic tissues, possibly indicating activation of the alternative pathway of NF-κB in cancer cells. Moreover, lymph node metastasis was related to nuclear NF-κB2 expression in tumor cells. The deregulation of the alternative NF-κB pathway in NSCLC could play a role in the development and progression of the disease.

Keywords NF-κB2 · p52 · RelB · Lung cancer · NSCLC

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Introduction

Primary tumors of the lung were rare until the beginning of the twentieth century [1, 2]. At present, lung cancer is the first cause of cancer-related deaths in both sexes (30 % in males, 26 % in females) in the USA and worldwide [3, 4]. Non-small cell lung cancer (NSCLC) and small cell lung cancer are the two major clinical lung cancer subtypes. The majority (85 %) of lung carcinomas are NSCLC (squamous cell carcinoma, adenocarcinoma, and large cell carcinoma) [4]. The current therapeutic approaches, which include surgical resection, platinum-based doublet chemotherapy, radiotherapy, and targeted therapies, rarely cure the disease, and the overall survival rate remains low at approximately 15 % [5].

In the last decades, there has been an expansion of knowledge relating to the pathobiology of malignant epithelial lung tumors, but the prognosis of the disease has little changed.

Although a wide spectrum of genes and proteins has been studied in human lung cancer, it remains unclear whether the alternative nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway can promote tumorigenesis or change the tumor progression. At present, it is well documented that NF-κB has a pleiotropic function by binding to discrete DNA sequences in promoters and enhancers.

The family of NF-κB transcriptional regulators consists of seven members p105/p50 (NF-κB1), p100/p52 (NF-κB2), p65 (RelA), RelB, c-Rel, which are encoded by five genes (NF-κB1, NF-κB2, RELA, RELB, and c-REL) [6, 7]. NF-κB1 and NF-κB2 encode the precursor proteins p105 and p100, which are proteasomaly cleaved to give rise to the functional molecules p50 and p52, respectively [7, 8]. The NF-κB family gives rise to two major signaling pathways, the classical and the alternative. In the alternative pathway, two of the central players are p100/p52 and RelB. As only the Rel family members carry a carboxy-terminal transcriptional activating domain, which is a positive regulator of gene expression, a heterodimer between p100/p52 and RelB must form in order to bind to promoters and enhancers [8, 9].

Until recently, the role of NF-κBs and their signaling pathways has been thought to be limited to coordinating the innate and adaptive immune responses. Their role in cancer development has not been elucidated, although it is now documented that NF-κB is a major factor linking inflammation and carcinogenesis [10, 11]. Moreover, NF-κB is a central player in virus-associated carcinogenesis [12]. In many types of cancer, not only in hematological but also in solid malignancies, e.g., melanoma, breast, prostate, ovarian, pancreatic, colon, head and neck, renal, bladder, liver, astrocytoma, glioblastoma, and thyroid, NF-κB family members exhibit elevated activity [13, 14]. In lung cancer, only p50 and p65 have been studied, while little is known about the role of the alternative pathway of NF-κB in lung carcinogenesis and the progression of the disease [15–17].

Methods

Tissue specimens

This study was carried out according to the principles and after the approval of the Committee on Research and Ethics and the Scientific Committee of the University Hospital of Patras, Greece. The study comprised 109 formalin-fixed paraffin-embedded (FFPE) lung tissue specimens of invasive NSCLCs including adjacent nonneoplastic lung parenchyma, retrieved from the archives of the Laboratory of Pathology of the University Hospital of Patras. All cases were surgically managed at the University Hospital of Patras Medical School between 2005 and 2008.

Clinicopathological information was obtained from the pathology reports and is given in Table 1. Sixty (n=60) samples were squamous cell carcinomas, 38 (n=38) were adenocarcinomas, and 8 (n=8) were large cell carcinomas. The majority of patients were men (90.9 %) with a mean age of 67 years (range, 46–81 years). The pathological stage was defined according to the primary pathology reports. All stages were represented with the majority of samples equally distributed between stages I, II, and III. The tumor grade was (2 %) grade 1, (49 %) grade 2, and (43 %) grade 3, respectively. Lymph node status was known for 102 patients. Fifty percent of them had lymph node metastatic disease. First- and second-year survival rates were available in 101 of the 109 patients. Moreover, the regional relapse status in the 2-year observational period was known for 29 patients (11 relapsed).

Table 1 Clinicopathological characteristics of NSCLC patients

Age (years): median (range)	67 (46–81)
Gender: no. (%)	109
Male	99 (90.8 %)
Female	10 (9.2 %)
Site	109
Left lung	51 (46.8 %)
Right lung	58 (53.2 %)
Histology: no. (%)	106
Squamous	60 (56.6 %)
Adenocarcinoma	38 (35.8 %)
Large carcinoma	8 (7.5 %)
Stage: no. (%)	103
0	1 (1.0 %)
I	35 (34.0 %)
II	32 (31.1 %)
III	31 (30.1 %)
IV	4 (3.9 %)
Grade: no. (%)	100
I	2 (2.0 %)
II	49 (49.0 %)
III	43 (43.0 %)
IV	6 (6.0 %)
Maximum diameter (cm): mean (range)	104
	5.39 (1.50–21)
Lymph node infiltration: no. (%)	102
No	51 (50.0 %)
Yes	51 (50.0 %)
Survival (2 years): no. (%)	101
Death	35 (31.8 %)
Alive	66 (60.0 %)
Relapse (2 years): no. (%)	29
No	18 (62.1 %)
Yes	11 (37.9 %)

Immunohistochemical analysis

Four-micrometer sections from FFPE tissue specimens were deparaffinised in xylene and rehydrated in a series of graded alcohols. The sections were then pre-treated in a microwave oven, and peroxidase activity was blocked with 3 % hydrogen peroxide for 20 min, followed by incubation with an appropriate protein blocking solution. Primary antibodies used were mouse monoclonal anti-NF-κB2 (dilution 1:500, clone: C-4, sc-7386, Santa Cruz) and rabbit polyclonal antibody against ReB (dilution 1:100, clone: C-19, sc-226, Santa Cruz). Detection was performed using the Envision detection kit (DAKO) according to the manufacturer's instructions. Diaminobenzidine was used as the chromogen for visualization. Sections were counterstained with Harris' hematoxylin solution, dehydrated, and mounted. To test for specificity, the procedure was repeated in consecutive sections substituting the anti-NF-κB2 and anti-RelB antibodies with protein blocking solution.

Evaluation of immunohistochemistry

All slides were assessed by one pathologist (H.P.) and one investigator (F.D.) independently and blinded to the case. The histological type and tumor grade were confirmed according to the 2004 WHO classification of lung tumors [18]. Cases with staining in >10 % of cells were considered positive. Immunohistochemical reactivity was graded on a scale of 0–3 according to the intensity of the staining, and the percentage of immunopositive cells as follows: 0, no staining or <10 % positive cells; 1, weak staining in >10 % of cells or moderate staining in 10–70 % of cells; 2, moderate staining in >70 % of cells or strong staining in 10–70 % of cells; and 3, strong staining in >70 % of cells. NFkB2 and RelB expression in cancer cells was categorized in three groups (high vs medium vs low) using as a cutoff point the 33rd and 66th percentiles [19]. The intensity and distribution of the NF-κB2 and RelB signal were the parameters used to estimate NF-κB2 and RelB expression. The total score for each slide was the sum of the intensity and distribution (between 0 and 6). Per tissue section, the more representative areas were selected using low-power fields (magnification ×10). The accurate quantification was performed on high-power fields (magnification ×40). Microphotographs were obtained using a Nikon DXM 1200C digital camera mounted on a Nikon Eclipse 80i microscope and ACT-1C software (Nikon Instruments Inc., Melville, NY, USA).

Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences version 17 (SPSS, Chicago, IL, USA). Possible associations between NF-κB and RelB protein expression with the clinicopathological parameters of the tumors were evaluated with the Kruskal–Wallis or the Mann–Whitney tests for ordinal variables and χ^2 test for nominal variables. Spearman's correlations were used to assess associations between variables. Survival rates were estimated using the Kaplan–Meier method and then compared with the log rank test. Cox regression analysis was used to assess the role of the two NF-κB molecules as prognostic factors. For all comparisons, statistical significance was defined as $p<0.05$.

Results

RelB and NF-κB2 protein levels differ between NSCLC and control tissue

The association of NF-κB2 and RelB expression with the clinicopathological characteristics is listed in Tables 2 and 3, respectively. As shown in Fig. 1a–f, immunoreactivity of NF-κB2 and RelB in epithelial cells was cytoplasmic and nuclear in all NSCLC histological subtypes. Cytoplasmic expression of NF-κB2 and RelB was observed in 96.3 and 81.7 % of the tumor specimens, respectively, in contrast to 10 % of tumor-adjacent nonneoplastic tissue (Fig. 2a–b). Moreover, expression levels were higher in tumor cells than in nonneoplastic tissue ($p<0.001$ for both molecules). Regarding nuclear staining, it was noted exclusively in tumor specimens. Approximately half (48.6 %) of the tumor specimens were positive for RelB and 15.7 % for NF-κB2. However, concurrent expression of these molecules was detected only in 12.1 % of cases.

NF-κB2 and RelB expression is correlated with the tumor stage

Cytoplasmic NF-κB2 expression in tumor cells was related to disease stage ($p=0.03$). More specifically, NF-κB2 levels were higher in stages II and III and lower in stages I and IV, with the metastatic stage having the lowest score. Likewise, cytoplasmic RelB levels were lower at stage IV compared to stages I to III ($p=0.016$). In contrast, nuclear NF-κB2 (p52) and RelB expression was similar across stages ($p=0.178$ and $p=0.10$, respectively).

Regional lymph node infiltration is associated with NF-κB2 nuclear expression levels

Lymph node metastasis was found to be related to NF-κB2 expression in the nucleus of primary site tumor cells. That is, the nuclear NF-κB2 protein levels were higher in tumor cells of patients without lymph node metastasis ($p=0.044$). However, no association was detected between NF-κB2 cytoplasmic expression and lymph node positivity. RelB expression, whether cytoplasmic or nuclear, was independent of lymph node metastatic frequency.

Table 2 NF-κB2 protein expression and clinicopathological parameters

Parameters		*N*	NFkB2 protein expression in:									
			Cytoplasm				*p*	Nucleus				*p*
			0–3, *n* (%)	4, *n* (%)	5, *n* (%)	6, *n* (%)		0–3, *n* (%)	4, *n* (%)	5, *n* (%)	6, *n* (%)	
Tissue	Neoplastic	108	11 (10.2)	18 (16.7)	23 (21.3)	56 (51.9)		105 (97.2)	3 (2.8)	0 (0)	0 (0)	
Gender	Male	98	9 (9.2)	16 (16.3)	20 (20.4)	53 (54.1)	0.139	95 (96.9)	3 (3.1)	0 (0)	0 (0)	0.839
	Female	10	2 (20.0)	2 (20.0)	3 (33.3)	3 (33.3)		10 (100.0)	0 (0)	0 (0)	0 (0)	
Primary site	Left lung	45	6(13.3)	8 (17.8)	7 (15.6)	24 (53.3)	0.197	42 (93.3)	3 (6.7)	0 (0)	0 (0)	0.307
	Right lung	51	2 (3.9)	7 (13.7)	13 (25.5)	29 (56.9)		51 (100)	0 (0)	0 (0)	0 (0)	
Stage	I	31	3 (9.7)	6 (19.4)	7 (22.6)	15 (48.4)	0.030	30 (96.8)	1 (3.2)	0 (0)	0 (0)	0.178
	II	28	2 (7.1)	1 (3.6)	7 (25.0)	18 (64.3)		27 (93.2)	1 (3.6)	0 (0)	0 (0)	
	III	26	1 (3.8)	4 (15.4)	4 (15.4)	17 (65.4)		26 (100)	0 (0)	0 (0)	0 (0)	
	IV	3	1 (33.3)	1 (33.3)	1 (33.3)	0 (0)		3 (100)	0 (0)	0 (0)	0 (0)	
Lymph nodes	Positive	45	3 (6.7)	6 (13.3)	10 (22.2)	26 (57.8)	0.680	45 (100)	0 (0)	0 (0)	0 (0)	0.044
	Negative	46	4 (8.7)	8 (17.4)	10 (21.7)	24 (52.2)		44 (95.7)	2 (4.3)	0 (0)	0 (0)	
Maximum diameter	< Median	45	3 (6.8)	6 (13.3)	10 (22.2)	26 (57.8)	0.700	44 (97.8)	1 (2.2)	0 (0)	0 (0)	0.260
	≥Median	46	4 (0)	8 (4.0)	10 (36.0)	24 (60.0)		45 (97.8)	1 (2.2)	0 (0)	0 (0)	
Age	<Median	53	5 (9.4)	10 (18.9)	11 (20.1)	27 (51.9)	0.826	51 (98.1)	2 (1.9)	0 (0)	0 (0)	0.284
	≥Median	54	5 (9.3)	8 (14.8)	12 (22.2)	29 (53.7)		53 (98.1)	1 (1.9)	0 (0)	0 (0)	
Histology	Squamous	48	2 (4.2)	8 (16.7)	7 (14.6)	31 (64.6)	0.489	47 (97.9)	1 (2.1)	0 (0)	0 (0)	0.261
	Adenocarcinoma	38	9 (23.7)	3 (7.9)	12 (31.6)	14 (36.8)		38 (100.0)	0 (0)	0 (0)	0 (0)	
	Large cell	8	0 (0)	2 (25.0)	2 (25.0)	4 (50.0)		7 (87.5)	1 (12.5)	0 (0)	0 (0)	
Smoking	≤80 pack-years	10	1 (10.0)	0 (0)	3 (30.0)	6 (60.0)	0.892	10 (100)	0 (0)	0 (0)	0 (0)	0.424
	>80 pack-years	9	2 (22.2)	1 (11.1)	2 (22.2)	4 (44.4)		9 (100.0)	0 (0)	0 (0)	0 (0)	
Relapse	No	18	2 (11.1)	1 (5.6)	3 (16.7)	12 (66.6)	0.348	18 (100.0)	0 (0)	0 (0)	0 (0)	0.201
	Yes	11	1 (9.1)	1 (9.1)	4 (36.4)	5 (45.4)		11 (100.0)	0 (0)	0 (0)	0 (0)	
Survival 2 years	Dead	35	4 (11.4)	5 (14.3)	7 (20.0)	19 (54.3)	0.416	34 (97.2)	1 (2.8)	0 (0)	0 (0)	0.493
	Alive	65	7 (10.8)	10 (15.4)	14 (21.5)	34 (52.3)		64 (98.5)	1 (1.5)	0 (0)	0 (0)	
Differentiation	Well	2	0 (0)	0 (0)	0 (0)	2 (100.0)	0.168	2 (100.0)	0 (0)	0 (0)	0 (0)	0.115
	Moderate	47	2 (4.3)	9 (19.2)	7 (14.9)	29 (61.6)		46 (97.8)	1 (2.2)	0 (0)	0 (0)	
	Poor	49	6 (12.2)	8 (16.3)	15 (30.6)	20 (40.8)		48 (98.0)	1 (2.0)	0 (0)	0 (0)	

Table 3 RelB protein expression and clinicopathological parameters

Parameters		*N*	RelB protein expression in:								
			Cytoplasm				*p*	Nucleus			*p*
			0–3, *n* (%)	4, *n* (%)	5, *n* (%)	6, *n* (%)		0–3, *n* (%)	4, *n* (%)	5, *n* (%)	6, *n* (%)	
Tissue	Neoplastic	109	45 (41.3)	41 (37.6)	17 (15.6)	6 (5.5)		96 (88.1)	9 (8.3)	4 (3.7)	0 (0)	
Gender	Male	100	40 (40.0)	38 (38.0)	17 (17.0)	5 (5.0)	0.640	88 (88.0)	7 (7.0)	4 (4.0)	0 (0)	0.481
	Female	9	5 (55.6)	3 (33.3)	0 (0)	1 (11.1)		7 (77.8)	2 (22.2)	0 (0)	0 (0)	
Primary site	Left lung	47	18 (38.3)	20 (42.6)	6 (12.8)	3 (6.4)	0.776	40 (85.1)	6 (12.8)	1 (2.1)	0 (0)	0.556
	Right lung	51	25 (49.0)	14 (27.5)	9 (17.5)	3 (5.9)		46 (90.2)	2 (3.9)	3 (5.9)	0 (0)	
Stage	I	32	15 (46.9)	9 (28.1)	5 (15.6)	3 (9.4)	0.016	26 (81.2)	4 (12.5)	2 (6.3)	0 (0)	0.101
	II	29	15 (51.7)	12 (41.4)	1 (3.4)	1 (3.4)		27 (93.2)	1 (3.4)	1 (3.4)	0 (0)	
	III	26	6 (23.1)	9 (34.6)	9 (34.6)	2 (7.7)		24 (92.3)	2 (7.7)	0 (0)	0 (0)	
	IV	3	2 (66.7)	1 (33.3)	0 (0)	0 (0)		1 (33.3)	1 (33.3)	1 (33.3)	0 (0)	
Lymph nodes	Positive	46	19 (41.3)	18 (39.1)	7 (15.2)	2 (4.3)	0.947	41 (89.1)	4 (8.7)	1 (2.2)	0 (0)	0.959
	Negative	47	20 (42.6)	16 (34.0)	7 (14.9)	4 (8.5)		40 (85.1)	4 (8.5)	3 (6.4)	0 (0)	
Maximum diameter	≤Median	46	15 (32.6)	21 (45.7)	6 (13.0)	4 (8.7)	0.394	40 (87.0)	4 (8.7)	2 (4.3)	0 (0)	0.891
	>Median	46	25 (54.3)	11 (23.9)	8 (17.4)	2 (4.3)		44 (95.7)	3 (6.5)	1 (2.2)	0 (0)	
Age	<Median	54	22 (40.8)	22 (40.8)	9 (16.7)	1 (1.9)	0.259	49 (90.1)	2 (3.8)	3 (5.6)	0 (0)	0.842
	≥Median	53	23 (43.4)	17 (32.1)	8 (15.1)	5 (9.4)		45 (84.0)	7 (13.2)	1 (1.9)	0 (0)	
Histology	Squamous	52	14 (26.9)	18 (34.6)	13 (25.0)	6 (11.6)	0.008	48 (92.4)	2 (3.8)	2 (3.8)	0 (0)	0.066
	Adenocarcinoma	38	23 (60.5)	14 (36.8)	1 (2.6)	0 (0)		32 (84.2)	4 (10.4)	2 (0)	0 (0)	
	Large cell	8	4 (50.0)	2 (25.0)	1 (12.5)	1 (12.5)		8 (100)	0 (0)	0 (0)	0 (0)	
Smoking	≤80 pack-years	9	1 (11.1)	3 (33.3)	4 (44.4)	1 (11.1)	0.415	8 (0)	1 (0.0)	0 (0)	0 (0)	0.809
	>80 pack-years	10	3 (30.0)	5 (50.0)	2 (20.0)	0 (0)		9 (90.0)	1 (10.0)	0 (0)	0 (0)	
Relapse	No	18	4 (22.2)	9 (50.0)	3 (16.6)	2 (11.2)	0.379	16 (88.9)	2 (11.1)	0 (0)	0 (0)	0.352
	Yes	11	6 (54.5)	3 (22.3)	2 (18.2)	0 (0)		11 (100.0)	0 (0)	0 (0)	0 (0)	
Survival 2 years	Dead	35	15 (42.9)	8 (22.9)	9 (25.7)	3 (8.5)	0.101	32 (91.4)	3 (8.6)	0 (0)	0 (0)	0.608
	Alive	65	24 (36.9)	30 (46.2)	8 (12.3)	3 (4.6)		57 (87.7)	5 (7.7)	3 (4.6)	0 (0)	
Differentiation	Well	2	1 (50.0)	0 (0)	1 (50.0)	0 (0)	0.813	2 (100.0)	0 (0)	0 (0)	0 (0)	0.896
	Moderate	49	17 (34.6)	22 (44.9)	5 (10.2)	4 (8.2)		44 (89.8)	3 (6.1)	2 (4.1)	0 (0)	
	Poor	48	19 (39.6)	15 (31.25)	7 (14.6)	3 (6.25)		41 (85.4)	5 (10.4)	2 (4.2)	0 (0)	

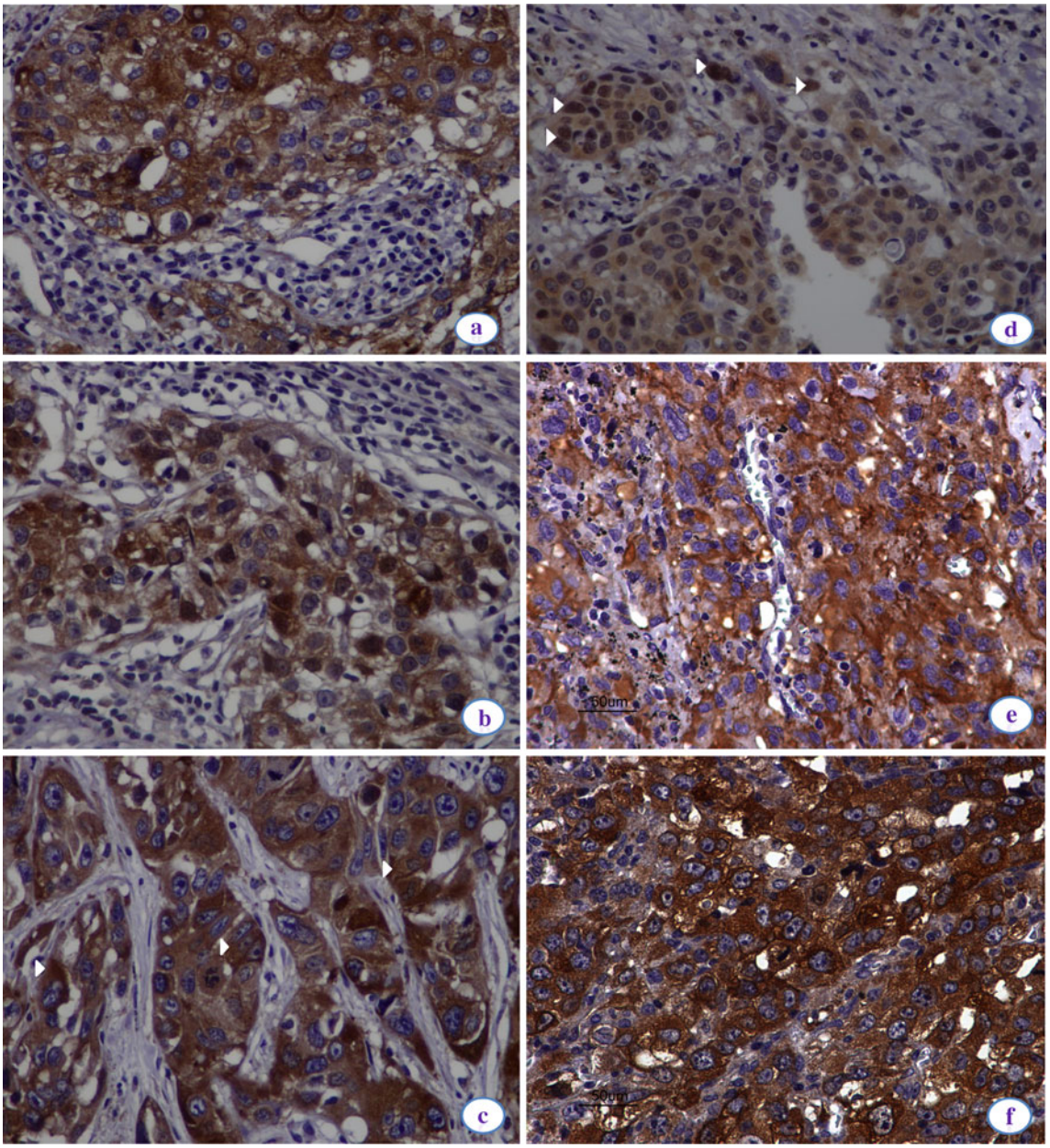

Fig. 1 **a** Representative histologic section of lung adenocarcinoma showing strong cytoplasmic immunopositivity for NF-κB2 (×40). **b** Strong nuclear and cytoplasmic staining for RelB in a representative histologic section of lung adenocarcinoma (×4O). **c** Strong diffuse cytoplasmic immunopositivity of NF-κB2 protein in invasive squamous cell carcinoma of the lung (×40). **d** Representative histologic of lung squamous cell carcinoma showing strong nuclear immunostaining and moderate cytoplasmic immunopositivity for RelB (×40)**. e** Strong cytoplasmic NF-κB2 expression in a representative section of human lung large cell carcinoma (×40). **f** Representative section of human lung large cell carcinoma with strong cytoplasmic RelB expression (×40)

NF-κB2 cytoplasmic expression is correlated with tumor grade

Using the two-tier grading system, we found that NF-κB2 levels in the cytoplasm were related to the grade of the tumor. Low-grade tumors had higher NF-κB2 levels compared to high-grade tumors ($p=0.046$). However, p52 in the nucleus was unrelated to grade. Moreover, RelB protein levels, independently of subcellular location, remained unchanged across grades.

RelB cytoplasmic expression is higher in squamous cell carcinoma

RelB cytoplasmic expression differed between NSCLC types. In particular, RelB expression in the cytoplasm of

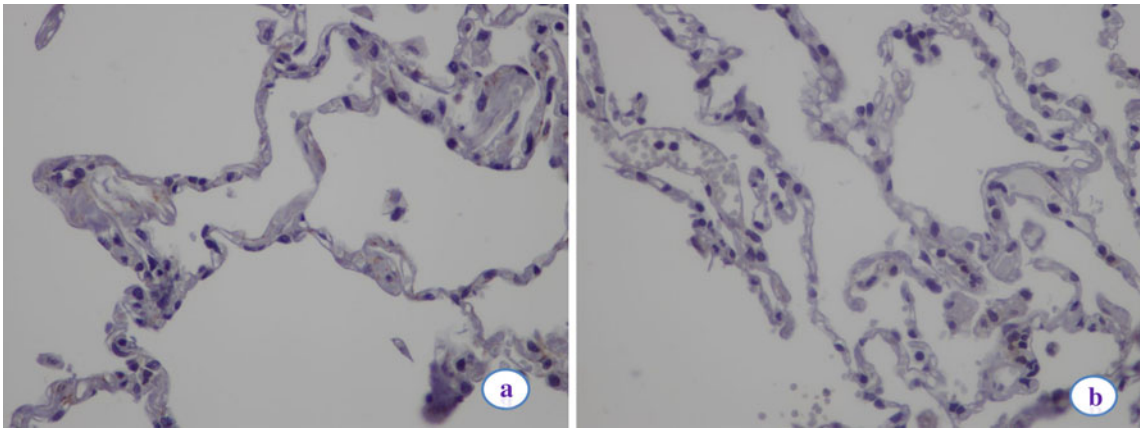

Fig. 2 **a** Negative immunostaining for NF-κB2 in alveolar epithelium and interstitium of adjacent nonneoplastic lung parenchyma (×40). **b** Negative immunostaining for RelB in alveolar epithelium and interstitium of adjacent nonneoplastic lung parenchyma (×40)

squamous cell carcinomas was higher than in the other NSCLC histological types ($p=0.008$). Moreover, expression levels were lower in adenocarcinomas than large cell carcinomas, although the difference was not significant. In addition, we observed a trend of higher nuclear RelB expression in the squamous cell subtype compared to other subtypes ($p=0.066$), although no differences were observed between adenocarcinomas and large cell carcinomas ($p=1.00$). Furthermore, NF-κB2 levels, independently of localization, did not differ between tumor types.

Correlation of protein levels between RelB and NF-κB2

NF-κB2 and RelB levels (cytoplasmic as well as nuclear) were positively correlated ($p=0.029$ and $p=0.004$, respectively). Additionally, RelB cytoplasmic and nuclear protein levels were correlated ($p<0.001$), although no such relation was noted for NF-κB2.

NF-κB2 in the cytoplasm is overexpressed in right lung tumors

In right lung tumors, cytoplasmic NF-κB2 levels were higher compared to left lung tumors. The difference was significant ($p=0.032$) when cytoplasmic expression was divided into three groups (low vs intermediate vs high). NF-κB2 nuclear expression and RelB nuclear or cytoplasmic expression were unrelated to primary site.

No correlation was found with age, sex, smoking, maximum tumor diameter, relapse rate, and overall survival

Patients' age was unrelated to the expression levels of NF-κB2 and RelB. Patients of both age groups, over and under the age of 65 (the median of our cohort), had the same NF-κB2 and RelB protein levels. Likewise, smoking, as estimated using the pack-years (median exposure of 80 pack-years), had no impact on the expression pattern of NF-κB2 and RelB. In addition, no significant correlation was found with maximum tumor diameter. Tumors with maximum diameter bigger or smaller than 4.5 cm, which was the median of our group, had similar levels of NF-κB2 and RelB. Furthermore, neither NF-κB2 nor RelB protein expression correlated with the regional disease recurrence and overall survival.

Discussion

NF-κB is a functionally pleiotropic factor implicated in the pathobiology of a variety of diseases [20]. In the last years, experimental evidence implicates the deregulation of the non-canonical NF-κB pathway in solid tumors, but its role in lung cancer pathogenesis and progression remains unknown [21].

In the present study, the immunohistochemical analysis of NF-κB2 and RelB proteins in NSCLC tissue samples revealed a statistically different expression pattern compared to adjacent nonneoplastic lung parenchyma. Cytoplasmic expression of both proteins was noted more frequently in neoplastic cells compared to adjacent nonneoplastic epithelium, while nuclear expression of both molecules was restricted to tumor tissue. These findings may reflect the activation of the alternative pathway of NF-κB in NSCLC patients. This is consistent with the observation of Lukashev et al. [22] that the TNF receptor family member, lymphotoxin-β receptor, whose activation results in the activation of the alternative pathway of NF-κB, is overexpressed in 87 to 96 % of a wide range of solid tumors, including lung cancer. Similarly, 51.9 and 58.9 % of NSCLC patients were positive for another activator of the alternative NF-κB pathway, CD40 and its ligand, CD154, respectively.

Moreover, CD40 expression in tumors is correlated with a poor prognosis since the activation of CD40 in cancer cells boosts the malignant potential of NCSLC [23, 24].

Furthermore, NF-κB2 and RelB seem to be expressed in the cytoplasm of cancer cells in a stage-dependent manner. NF-κB2 and RelB expression is lowest in patients with metastatic disease. However, previous studies in NK/T lymphomas, prostate, and esophageal tumors reported no relation of NF-κB2 and RelB expression with stage [25–27]. It is possible that the role of these molecules differs between different tumor types. Although our results should be treated with caution because of the small number of stage IV patients, they may reflect the different roles of NF-κB2 and RelB in the different stages of lung cancer progression. Additionally, it is documented that the final effect of NF-κB function may depend on the duration of NF-κB activation and the cellular context, and it can be either tumor suppressive or tumor promoting [28, 29]. This possible dual role of NF-κB during tumor progression needs to be clarified by further studies in NSCLC.

Moreover, supportive to the stage-dependent expression of NF-κB subunits is our finding that nuclear NF-κB (p52) levels are associated with lymph node metastasis. The reduced protein levels of NF-κB in the nucleus of cancer cells seem to be related to lymph node infiltration. In agreement with our findings, the nuclear localization of p65 (another member of the NF-κB family) in primary prostate tumors is a valuable marker for the prediction of lymph node metastasis [30]. It is possible that the activation of the alternative pathway of NF-κB and the subsequent nuclear translocation of NF-κB (p52) may hamper metastasis of the cancer cell. Perhaps the repeal of this brake, in a particular moment of the tumor's physical history, increases the lymph node metastatic potential. In addition, the higher levels of NF-κB2 in the cytoplasm were associated with low-grade tumors. This indicates that NF-κB2 in the cytoplasm may be correlated with an impediment of the dedifferentiation process. In prostate cancer, no relationship was noted between p52 nuclear expression and grade, although RelB was associated with tumor grade [25]. One intriguing point of our finding is that only the cytoplasmic NF-κB2 expression is correlated with tumor grade and not the nuclear.

In this study, for the first time, we found that RelB cytoplasmic expression is higher in squamous cell carcinomas compared to adenocarcinomas and large cell carcinomas. The difference between the histological groups may reflect the different microenvironments of these carcinomas that influence the activation of the NF-κB pathways in a cell type-specific manner. In agreement with our findings is the different molecular biology of the three NSCLC histological types [4].

NF-κB2 and RelB, the pivotal players of the alternative NF-κB pathway, are thought to have an active transcriptional activity when they are localized nuclearly. In our study, both were located mainly in the cytoplasm posing the question of whether cytoplasmic NF-κB2 and RelB maintain a biological function. Moreover, it would be of interest to investigate whether this cellular lodging reflects an escape mechanism of the malignant cell, which may trap p52 and RelB in the cytoplasm being benefitted more from their presence in the cytoplasm rather than of their absence from the nucleus.

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Conflict of interest We declare that we have no conflict of interest.

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