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Original Article

Silenced Expression of NFKBIA in Lung Adenocarcinoma Patients with a Never-smoking History

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***Nuclear factor of κ-light polypeptide gene enhancer in B cells inhibitor α* (*NFKBIA*), which is a tumor suppressor gene, was found to be silenced in lung adenocarcinomas. We examined NFKBIA expression, mutations in the *EGFR* and *K-ras* genes, and *EML4-ALK* fusion in 101 resected lung adenocarcinoma samples from never-smokers. NFKBIA expression was evaluated using immunohistochemistry. NFKBIA expression was negative in 16 of the 101 samples (15.8%). *EGFR* and *K-ras* mutations and *EML4-ALK* fusion were detected in 61 (60.5%), 1 (1.0%), and 2 (2.0%) of the 101 samples, respectively, in a completely mutually exclusive manner. Negative NFKBIA expression was observed significantly more frequently among the tumors with none of the three genetic alterations compared to those with such alterations ($p = 0.009$). In addition, negative NFKBIA expression was significantly more frequent among the *EGFR*-wild type samples compared to the *EGFR*-mutant samples ($p = 0.013$). In conclusion, NFKBIA expression was silenced in adenocarcinomas without *EGFR*/*K-ras* mutations or *EML4-ALK* fusion, suggesting that the silencing of NFKBIA may play an important role in the carcinogenesis of adenocarcinomas independent of *EGFR*/*K-ras* mutations or *EML4-ALK* fusion.**

Key words: never-smoker, lung cancer, adenocarcinoma, nuclear factor of κ-light polypeptide gene enhancer in B cells inhibitor α, epidermal growth factor receptor

Lung cancer is a leading cause of cancer-related deaths worldwide [1]. Although tobacco smoking is an important risk factor for lung cancer, never-smokers can also develop lung cancer. Actually, lung cancer among never-smokers is the seventh most common cause of cancer-related deaths [2]. Various molecular alterations in lung cancer have been reported [3], and recent studies have focused intensively on driver alterations, such as *K-ras* and *epidermal growth factor receptor* (*EGFR*) mutations and *EML4-ALK* fusion. The latter 2 alterations are known to be frequent among lung adenocarcinomas arising in never-smokers. In addition, the presence and success of effective molecular targeting drugs for patients with these alterations make the identification of causative alterations important and useful.

Considerable evidence shows that nuclear factor of kappa light chain gene enhancer in B cells (NF-κB) is constitutively activated in a variety of solid tumors, including prostate, breast, cervical, pancreatic, and lung cancers [4]. NF-κB is a transcription factor activated by the EGFR pathway and is repressed by NF-κB inhibitor α (NFKBIA). It is encoded by *NFKBIA* located at 14q13 [5]. Hence, *NFKBIA* is

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considered a tumor suppressor gene. *NFKBIA* is reportedly deleted in glioblastomas. The deletion of *NFKBIA* and the amplification of *EGFR* show a pattern of mutual exclusivity [6].

In the present study, we determined the expression status of NFKBIA using immunohistochemistry (IHC) staining, and we examined the incidence of *EGFR* and *K-ras* mutations and *EML4-ALK* fusion to investigate the inter-relationship among these genetic alterations and clinical factors in never-smokers with lung adenocarcinoma.

Materials and Methods

Patients. We obtained 101 lung adenocarcinoma samples from never-smokers who underwent surgical resections at our hospital between September 2004 and June 2008. Never-smokers were defined as patients who had smoked <100 cigarettes in their lifetime. Smoking history was obtained by a direct interview. The patient characteristics are summarized in Table 1. The institutional review board approved this study, and informed consent was obtained from all the patients.

Table 1 Patient characteristics

Variables		No. of patients (%) (n = 101)
Age		
Mean (range)		67.0 (39–89)
	<67 years	49 (48.5)
	≥67 years	52 (51.5)
Sex		
	Male	16 (15.8)
	Female	85 (84.2)
Pathological stage		
	I	87 (86.1)
	II	4 (4.0)
	III	8 (7.9)
	IV	2 (2.0)
NFKBIA IHC		
	Positive	85 (84.2)
	Negative	16 (15.8)
EGFR mutation		
	Mutant	61 (60.4)
	WT	40 (39.6)
K-ras mutation		
	Mutant	1 (1.0)
	WT	100 (99.0)
EML4-ALK fusion gene		
	Positive	2 (2.0)
	Negative	99 (98.0)

IHC, immunohistochemistry; WT, wild type.

NFKBIA Immunohistochemistry. For the IHC, 4-μm sections were cut from paraffin-embedded tissue specimens and placed on MAS-GP type A coated glass slides (Matsunami Glass, Osaka, Japan). The slides were then deparaffinized in xylene and rehydrated in a graded series of ethanol (100%, 100%, 90%, 70%, and 50%). After the antigens were revealed with 10 mM sodium citrate (pH 6.0), the slides were incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase. To inhibit nonspecific binding, the samples were incubated in diluted normal horse serum for 30 min. After blocking, the slides were incubated with NFKBIA antibody (Abcam, Cambridge, UK; diluted 1 : 50 in PBS) at 4℃ overnight. The slides were washed in PBS for 5 min and incubated in secondary antibody (ImmPRESS Anti-Rabbit Ig peroxidase Polymer Detection Kit; Vector Laboratories, Peterborough, UK) for 30 min at room temperature. The slides were stained with 3, 3'-diaminobenzidine (DAB Substrate Kit, Vector Laboratories) and were counterstained with Mayer's hematoxylin [7, 8].

Two investigators (MF and KI) who were unaware of the clinical data independently evaluated the marker staining under a light microscope at $\times 400$ magnification. We defined positive NFKBIA protein expression as $\geq 10\%$ of the tumor cells exhibiting cytoplasmic or membranous staining according to our previous IHC study regarding EGFR protein [9] (Fig. 1).

DNA and RNA extractions. Genomic DNA was obtained from primary tumors using standard phenol-chloroform (1 : 1) extraction, followed by ethanol precipitation, or by using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Total RNA was extracted from the primary tumors using the RNeasy mini Kit (Qiagen) according to the manufacturer's protocol. Oligo(dT)-primed cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with DNase treatment.

Detection of EGFR and K-ras mutations and EML4-ALK fusion events. The *EGFR* mutational status was determined using a mutant nonenriched PCR assay, as reported [10]. Briefly, the common deletions of exon 19 were distinguished from the wild type based on PCR product length polymorphisms using 12% polyacrylamide gel electrophoresis

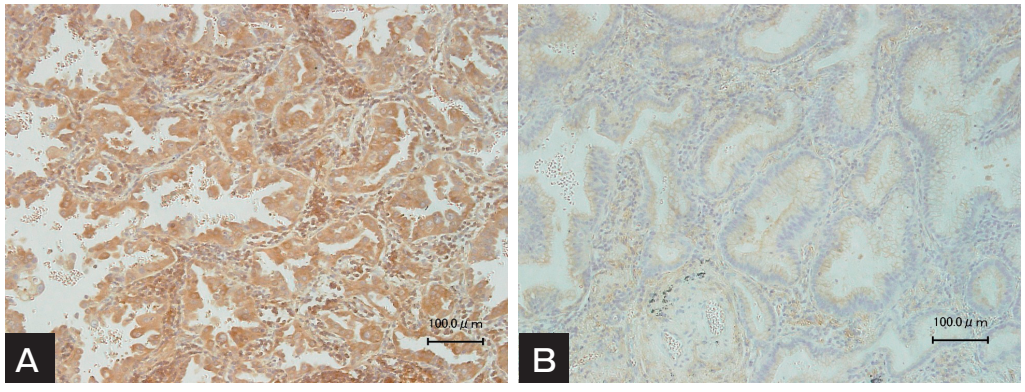

Fig. 1 Representative results of NFKBIA IHC and expression levels. **A**, Positive staining for NFKBIA; **B**, Negative staining for NFKBIA.

(PAGE) via ethidium bromide staining. For the exon 21 L858R mutation, Sau96I digestion, which specifically digests the mutant type, was performed prior to 12% PAGE. The *K-ras* mutations in codons 12 and 13 were examined using PCR-based direct sequencing on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems), as reported [11–13]. Screening for *EML4-ALK* fusion was performed using ALK IHC and was confirmed using a reverse transcriptional (RT)-PCR assay.

ALK Immunohistochemistry. Unstained paraffin-embedded sections were depleted of paraffin with xylene, rehydrated with a graded series of ethanol solutions, and then subjected to heat-induced antigen retrieval with Target Retrieval Solution (pH 9.0) (Dako, Glostrup, Denmark) before IHC staining with a mouse monoclonal antibody to ALK (ALK1, 1 : 25 dilution; Dako). Immune complexes were detected using a DAB system (Dako) with minor modifications.

RT-PCR. The primers that we used to identify the *EML4-ALK* fusion transcript were chosen to enable the detection of all possible in-frame fusions of *EML4* to exon 20 of *ALK* in which the kinase domain of ALK would be preserved. The forward primers used were EML4 72F (5'-GTCAGCTCTTGAGTCACGAGTT-3') and Fusion-RT-S (5'-GTGCAGTGTTTAGCATTCTTGGGG-3'), and the reverse primer was ALK 3078RR (5'-ATCCAGTTCGTCCTGTTCAGAGC-3') [14]. PCR was performed for *EML4-ALK* under the following conditions: 94℃ for 10 min, followed by 35 cycles of denaturation at 94℃ for 1 min, annealing at 64℃ for 1 min, and polymerization at 72℃ for 1 min, with a final extension step at 72℃ for 7 min. RT-PCR for GAPDH expression was performed under the same conditions in each tumor sample as an internal control.

Statistical analysis. Differences between the 2 groups were assessed using the χ^2 test or Fisher's exact test, as appropriate. All of the data were analyzed using JMP, version 9.0.0 (SAS Institute Inc., Cary, NC, USA). For each analysis, probability values of less than 0.05 were considered significant.

Results

NFKBIA expression. NFKBIA expression was silenced in 16 (15.8%) of the 101 patients. Examples of the IHC staining patterns are illustrated in Fig. 1. The relationships between clinical factors and the NFKBIA expression status are detailed in Table 2. No significant associations were observed between the NFKBIA expression status and any of the clinical factors that were examined.

EGFR and K-ras mutations and EML4-ALK fusion. *EGFR* mutations were detected in 61 (60.4%) of the 101 patients; 29 patients had an exon 19 deletion, and 32 had an exon 21 mutation (L858R). By contrast, *K-ras* mutation and *EML4-ALK* fusion were observed in 1 (1%) and 2 (2%) of the 101 patients, respectively. The relationships between the clinical factors that were examined and the *EGFR* and *K-ras* mutation and *EML4-ALK* fusion statuses are shown in Table 2. The incidences of *EGFR* mutations according to age or gender were not significantly different.

Inter-relationships among the molecular alterations. Negative NFKBIA expression was found to be significantly more frequent among the *EGFR* wild-type patients compared to the *EGFR* mutant patients ($p = 0.013$), whereas no correlation was observed between the NFKBIA expression level and the *K-ras* mutation or *EML4-ALK* fusion status (Table 3). Negative NFKBIA expression appeared significantly more frequently in the tumors without any of these three genetic alterations compared to those with a genetic alteration ($p = 0.009$).

Discussion

In this study, NFKBIA expression was repressed in some subsets of the lung adenocarcinomas. NFKBIA can be inactivated through several mechanisms. Although the deletion of *NFKBIA* has been identified, 14q13 — which is the locus of NFKBIA — is not frequently deleted in non-small cell lung cancer (NSCLC) [15]. In cancer epigenetics, aberrant CpG methylation in the promoter region is a key mechanism for gene inactivation, resulting in tumorigenesis in human malignancies including lung cancer [16, 17]. Thus, we analyzed the methylation of *NFKBIA* using lung cancer cell lines in which NFKBIA expression was repressed. However, we did not detect methylation in the cell lines that were tested (data not shown).

Although a mutation of *NFKBIA* has been reported [18], such mutations are rare. Epidermal growth factor (EGF) induces NFKBIA degradation by phosphorylation at tyrosine 42, resulting in the activation of NF-κB. Interestingly, the HCC827 and H3255 cell lines, which have *EGFR* mutations causing the constitutive activation of the EGFR pathway, exhibit the EGF-independent activation of NF-κB [19].

Our findings indicate that the NFKBIA silencing tended to occur more frequently in *EGFR* wild-type adenocarcinomas, which is similar to the results regarding glioblastoma reported by Bredel *et al.* [6].

Table 2 Relationships among clinical factors and genetic alterations

Variables	NFKBIA +/−	*P*	*EGFR* +/−	*P*	*K-ras* +/−	*P*	*EML4-ALK* +/−	*P*
Age								
<67 years (n = 49)	40 / 9	0.7	27 / 22	0.4	1 / 48	0.5	2 / 47	0.2
≥67 years (n = 52)	45 / 7		34 / 18		0 / 52		0 / 52	
Sex								
Male (n = 16)	12 / 4	0.5	11 / 5	0.6	0 / 16	1.0	0 / 16	1.0
Female (n = 85)	73 / 12		50 / 35		1 / 84		2 / 83	
Pathological stage								
I (n = 87)	71 / 16	0.2	51 / 36	0.5	1 / 86	1.0	2 / 85	1.0
II–IV (n = 14)	14 / 0		10 / 4		0 / 14		0 / 14	

Table 3 Inter-relationships among the 4 genetic alterations

Variables		NFKBIA +/−	*P*	*EGFR* +/−	*P*	*K-ras* +/−	*P*	*EML4-ALK* +/−	*P*
NFKBIA	+			56 / 29	0.013	1 / 84	1	2 / 83	1
	−			5 / 11		0 / 16		0 / 16	
EGFR	+	56 / 5	0.013			0 / 61	0.4	0 / 61	1
	−	29 / 11				1 / 39		2 / 38	
K-ras	+	1 / 0	1	0 / 1	0.4			0 / 1	1
	−	84 / 16		61 / 39				2 / 98	
EML4-ALK	+	2 / 0	1	0 / 2	0.15	0 / 2	1		
	−	83 / 16		61 / 38		1 / 98			

Negative NFKBIA expression occurred significantly more frequently in tumors without any of the 3 genetic alterations than in those with an alteration ($p = 0.009$). In addition, negative NFKBIA expression was observed significantly more frequently among *EGFR* wild-type patients than among *EGFR* mutant patients ($p = 0.013$).

Moreover, negative NFKBIA expression was significantly more frequent in the tumors without *EGFR*, *K-ras* or *EML4-ALK* genetic alterations than in those with such a genetic alteration. These findings indicate that the silencing of NFKBIA may play a pivotal role in the lung adenocarcinomas of never-smokers without *EGFR*, *K-ras*, or *EML4-ALK* genetic alterations.

Both chemotherapeutics and radiation induce NF-κB activation in cancer cells, contributing to the development of resistance to these therapies [20]. Considering the above-mentioned knowledge and our present findings, targeting the NF-κB pathway—including NFKBIA—is a potential therapeutic option for the treatment of NSCLC patients, especially for those without *EGFR*, *K-ras*, or *EML4-ALK* alterations.

Although we observed negative NFKBIA expression more frequently in the *EGFR* wild-type adenocarcinomas, we also detected negative NFKBIA expression in five *EGFR*-mutant cases. Bivona *et al.* reported that NF-κB activation through the downregulation of NFKBIA confers EGFR- tyrosine kinase inhibitor (TKI) resistance in *EGFR*-mutant lung cancers with EGFR-TKI treatment [21]. Regarding the above-mentioned five cases, no data of EGFR-TKI sensitivity were available because no recurrence has been observed after the patients' surgery. We sometimes encounter patients who are resistant to EGFR-TKI although they have TKI-sensitive *EGFR* mutations without T790M mutation, *MET* amplification or other known resistance-related alterations. In such cases, it may be preferable to examine the alteration of NFKBIA.

A limitation of this study is that the evaluation of NFKBIA expression was done only by IHC. We may have to confirm the expression status of NFKBIA by other methods such as western blotting, although there is a technical difficulty because of the contamination of normal cells in clinical tumor samples. In addition, we did not perform an *in vitro* assay to examine the functional importance of NFKBIA loss in *EGFR* wild-type lung cancer. Further investigation is needed to clarify the impact of silenced NFKBIA expression in lung cancer.

In conclusion, our findings suggest that the loss of NFKBIA expression is a frequent event in lung adenocarcinomas of never-smokers and may mediate an important non-tobacco carcinogenic pathway, particularly in lung adenocarcinomas with wild-type *EGFR*.

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