Molecular Analysis of Lung Adenocarcinoma With or Without Chronic Obstructive Pulmonary Disease and Smoking Habit

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We examined the molecular biological differences among LC cases, with and without smoking, and with or without COPD. Formalin-fixed paraffin-embedded blocks from 36 patients of lung adenocarcinoma that had undergone surgical resection from 2006 to 2009 were examined immunohistochemically for six proteins［AChR M3, ChAT, STAT3, survivin, HIF-1α, and IL-6］considered to modulate signal transduction pathways in cancer cells. The expression levels of AChR M3, and ChAT were significantly higher in smokers with COPD than those without COPD. The expression of STAT3 was significantly higher in non-smokers than smokers. Survivin expression tended to be higher in non-smokers than smokers（*P*=0.068）, albeit insignificantly. The results showed differences in the expression of cholinergic signaling pathway and JAK-STAT pathway components among patients with lung adenocarcinoma, in smokers and non-smokers and with/without COPD. These results could contribute to the treatment of recurrence, and also help to identify targets for cancer prevention strategies.

Key words: Lung adenocarcinoma, Chronic Obstructive Pulmonary Disease, Smoking, Immunohistochemistry

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INTRODUCTION

Lung cancer（LC）is the leading cause of cancer death worldwide, causing 1.5 million deaths annually. Limitations in current treatment combined with the high relapse rate and usually late diagnosis result in a generally poor prognosis and 5-year survival rates around 16%［1］. There is a clear need for better understanding of the involved carcinogenesis mechanisms and new treatment strategies. Chronic obstructive pulmonary disease（COPD）is also a significant cause of morbidity and mortality worldwide. It is thought that by 2020, COPD will be the third leading cause of death worldwide［2］. LC and COPD are both attributable to the combined effects of smoking exposure and an underlying genetic susceptibility［3, 4］. Although smoking accounts for between 80-90% of all LC and COPD cases, these lung diseases only affect a minority of smokers.

Several gene mutations have been associated with LC. Point mutations in the K-ras gene have been detected in 15-30% of all lung adenocarcinomas. Such point mutations are generally detected in smokers, causally implicating the carcinogenic agents in cigarettes［5］. LC patients with K-ras gene mutations have significantly poorer survival compared to patients with no such mutations. Mutations in the epidermal growth factor receptor（EGFR）gene are also found in 30% of lung adenocarcinoma cases. Several retrospective studies demonstrated higher rates of these mutations in females, never-smokers, Asians and in patients with adenocarcinoma［6］. Mutations in the EGFR gene were also significantly associated with carcinogenesis and

response to the tyrosine kinase inhibitor, gefitinib [7]. However, the mechanisms of carcinogenesis in patients with no EGFR mutation remain unknown.

The recent discovery that LC synthesize and secrete acetylcholine (ACh) that acts as a growth factor in LC provides a potential new target to inhibit LC growth. A variety of normal lung cell types synthesize ACh and the respective LC types that arise from those cells similarly synthesize and secrete ACh [8]. It has been shown that small cell lung cancer (SCLC) and NSCLC express nicotinic and muscarinic receptors and cholineacetyltransferase (ChAT), activation of ACh receptors stimulates the growth of NSCLC cell lines [9]. In the same model, blockade of the M3 muscarinic receptor (mAChR) by darifenacin prevented nicotinic and muscarinic receptor-induced growth of squamous cell cancer (SCC) and NSCLC cell lines via initiation of mitogen-activated protein kinase (MAPK) [10, 11].

Signal transducer and activator of transcription (STAT) 3 is another molecule which has become of interest in recent times. A role has been found for STAT3 in the prevention of human tumor cell apoptosis, and it has also been shown to be important in angiogenesis. Expression of phosphorylated STAT3 was detected in 51.2% of the adenocarcinoma of lung [12]. Survivin is known as a molecule downstream of STAT and is a recently identified protein of the inhibitor of apoptosis protein (IAP) family, which suppresses programmed cell death (apoptosis) and regulates cell division [13]. Several studies have shown that survivin is a prognostic indicator for poor survival in NSCLC [14].

The association of LC with COPD is frequent, as cigarette smoke is the principal causal agent in both diseases. Thus, it is important to examine the molecular and biological differences between LC with smoking or not, and between with COPD or not. We examined whether COPD and smoking can explain the difference in molecular expression in resected lung adenocarcinoma samples.

METHODS

Patients and samples

We used formalin-fixed paraffin-embedded blocks from 36 cases of lung adenocarcinoma that had undergone surgical resection from 2006 to 2009, and were retrieved from Shimane University Hospital. All available clinical information was obtained from the medical records and reports of the referring physicians. Surgical or pathological staging was performed according to the TNM classification of the International Union Against Cancer criteria (6.0th edition). Each patient gave written informed consent before surgical resection. The protocol was approved by the institutional review board of Shimane University Hospital.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tumor samples were sectioned at 4-μm thickness. Tissue samples were routinely deparaffinized in xylene and rehydrated through a series of graded alcohols. Antigen retrieval were carried out using an appropriate heat-induction procedure, and samples were immunostained using a BioGenex AutoStainer i6000® automated staining system, with antibodies against AChR M3 (H-210, Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1: 200), ChAT (MONX10745, Monosan, Netherlands, dilution 1: 200), STAT3 (AP3261a, Abgent, CA, dilution 1: 500), survivin (D-8, Santa Cruz Biotechnology, dilution 1: 100), hypoxia inducible factor-1α (HIF-1α)(H1α67, Santa Cruz Biotechnology, dilution 1: 50), and interleukin 6 (IL-6)(GTX26672, Gene Tex, San Antonio, TX, dilution 1: 200). After buffer washes, samples were visualized using a Dako Envision System based on horseradish peroxidase (Dako, Inc., Carpinteria, CA). Finally, the sections were lightly counterstained with Mayer's hematoxylin, dehydrated through alcohols of increasing concentration, and coverslipped using Permount.

Evaluation of immunostaining

All immunostained sections were evaluated by three authors (T.S, Y.T and T.I) who were blinded to the clinical diagnosis. ChAT, AChR M3, and IL-6 staining apparent in the cytoplasm was assessed semiquantitatively according to three indices: (1) the percentage of area stained (<10%, 25%, 50%, 75%, and 100%); (2) intensity of staining

（none, 0; weak, +1: moderate, +2: and strong, +3）; and, （3） the final score （product of area and intensity, named the histological （H） score）. In evaluating the staining for STAT3, survivin, and HIF-1α, the intensity of nucleic staining was graded as weak, +1; moderate, +2; or strong, +3.

DNA extraction

Formalin-fixed, paraffin-embedded tumor samples were cut into 4-μm-thick sections and placed in a 1.5-ml microfuge tubes. The excised lesions were deparaffinized and the genomic DNA was extracted using a DNA Isolator PS-Rapid Reagent （Wako Pure Chemicals, Osaka, Japan） DNA extraction kit, according to the instructions provided by the manufacturer. DNA amounts were quantified after extraction.

Detection of EGFR gene and k-ras gene mutations

DNA extracted from the tumor tissue was further analyzed using the peptide nucleic acid-locked nucleic acid （PNA-LNA） PCR reaction for EGFR gene mutation [15]. K-ras codon 12 mutations were analyzed using the PCR-restriction fragment length polymorphism （RFLP） method [16].

Statistical analysis

Data were expressed as mean±SD. The Statcel2 statistical software program （OMS, Japan） was used for all analyses. Student's t-test, simple regression analysis, or the Kruskal-Wallis test was applied to assess immunoreactivity. A P value <0.05 denoted the presence of statistical significance.

RESULTS

Study population

Table 1 summarizes the characteristics of the patients, tumors, and surgery. Non-smokers comprised 13 of 36 cases, with 11 females and 2 males. All smokers （23 cases） were males, and 14 of those had COPD （60.8%）, diagnosed on the basis of FEV1.0% of <70% by spirometry, in addition to clinical features and radiographic findings. All tumors were characterized histologically as adenocarcinoma and subclassified as follows: of the 13 non-smokers, 7 cases had mixed subtypes and 6 cases had bronchioalveolar carcinoma; of smokers with COPD, 12 had mixed subtypes, 1 had bronchioalveolar carcinoma, and 1 had a papillary-type tumor; and, of smokers without COPD, 5 had mixed tumor

Table 1. General patient characteristics

		Non-smokers	Smokers	
			with COPD	without COPD
Subjects (n=36)		13	14	9
Age (years)[a]		74 (±7.6)	71.7 (±9.9)	63.8 (±15.3)
Male/Female (n)		2/11	14/0	9/0
Smoking (pack-years)[a]		0	54.7 (±15.4)	43.8 (±45.6)
FEV1.0% (%)[a]		78.69 (±9.0)	59.26 (±14.4)	77.86 (±4.87)
FEV1/FVC (%pred.)[a]		102.52 (±19.69)	79.0 (±23.18)	95.14 (±12.01)
Pathological stage (n)	I A	10	10	5
	I B	3	3	3
	II B	0	0	1
	III A	0	1	0

COPD   Chronic obstructive pulmonary disease
[a]Mean (SD)

subtypes and 4 had bronchioalveolar carcinoma. Table 1 also details the smoking index (pack-years), FEV1.0%, FEV1.0-%predicted, and pathological stage.

Immunostaining

Representative micrographs for the immunohistochemistry results are presented in Figure 1. The mean histological score and staining score in each group are detailed in Table 2 and 3. First, we divided smokers into two groups, with COPD and without COPD, and evaluated the difference in degree of staining between these groups (Table 2). Among 23 smokers, there were 14 patients with COPD and 9 patients without COPD. Smokers with COPD showed significantly higher expression of AChR M3, ChAT, and survivin compared to smokers without COPD. There was no statistically significant difference in the expression of STAT3, HIF1-α, and IL-6 between the groups. We then divided all patients into the two groups of smokers and non-smokers, and similarly analyzed the immu-
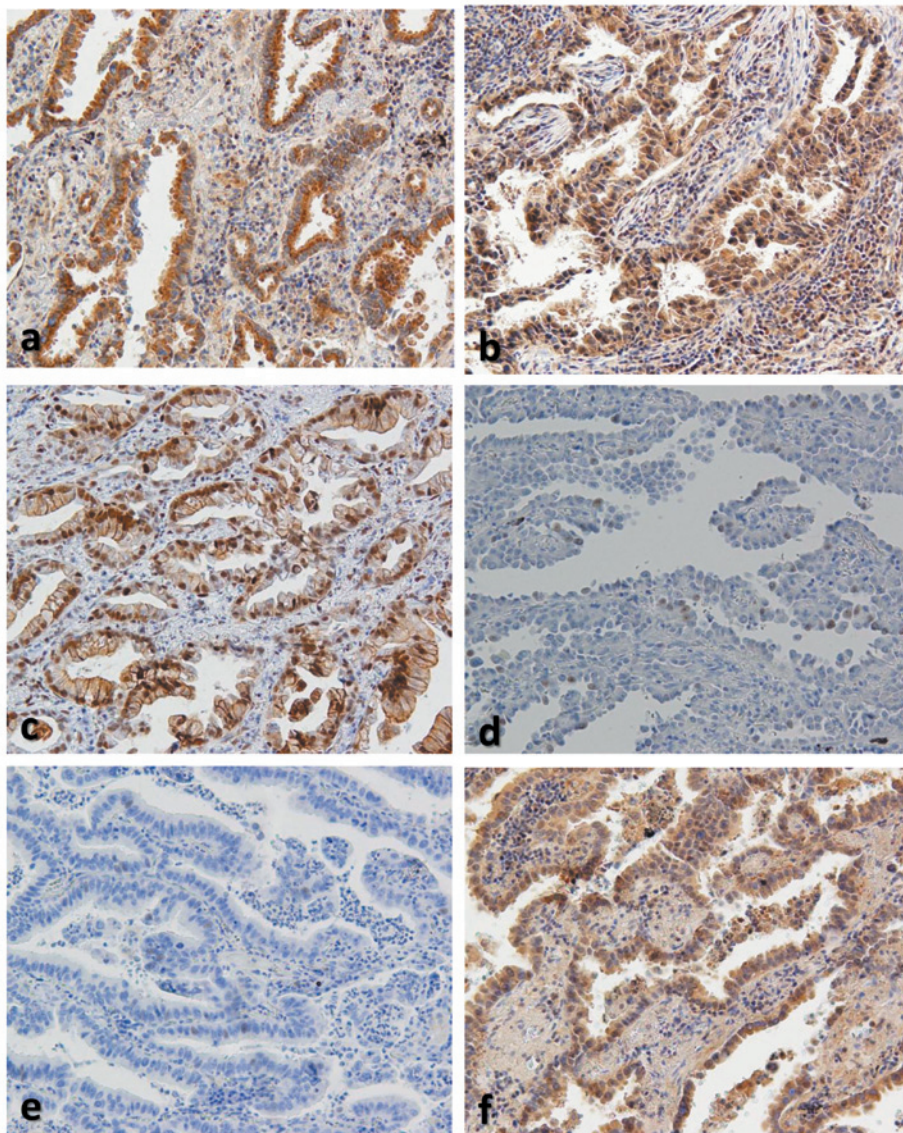

Fig. 1. Representative micrograph of immunohistochemistry showing lung adenocarcinoma expression of AChR M3, ChAT, STAT3, survivin, HIF-1α, and IL-6.

Dark brown staining in cytoplasm indicates the AChR M3, ChAT, and IL-6 immunoreactivity with counterstained nuclei in blue (a, b, f). Histological scores were evaluated at +9, +9, and +8.7 in figure (a), (b), and (f), respectively. The brown nuclear staining indicates the STAT3, survivin, and HIF-1α immunoreactivity (c, d, e). The intensity of staining in these images was scored at +4. Magnification, ×100.
Figure (a) and (f) are in non-smokers. Other figures are in smokers with COPD.

Table 2. Results of immunohistochemical and gene mutation analysis of lung

adenocarcinomas in Smokers (n=23)

	Smokers		
	With COPD (n=14)	Without COPD (n=9)	p-value
Histological score－mean			
AChR M3	7.74	6.59	0.003
ChAT	7.5	6.52	0.042
IL-6	7.14	6.70	0.487
Intensity of staining score－mean			
STAT3	2.83	2.78	0.890
survivin	2.36	1.56	0.018
HIF-1α	2.64	1.74	0.076
Gene mutation			
EGFR gene mutation +/- (n)	1/11*	1/8	
K-ras gene mutation +/- (n)	6/8	2/7	

AChR M3, M3 muscarinic acetylcholine receptor, ChAT: cholineacetyltransferase,
IL-6: interleukin 6, STAT3: Signal transducer and activator of transcription3, HIF1-a:
hypoxia inducible factor-1α, EGFR: epidermal growth factor receptor, Histological
score: product of the percentage of area stained and intensity of staining.
* DNA was not amplified in 2 cases of 14 smokers with COPD.

Table 3. Results of immunohistochemical and gene mutation analysis of lung

adenocarcinomas in Smokers and Non-smokers (n=36)

	Smokers (n=23)	Non-smokers (n=13)	p-value
Histological score－mean			
AChR M3	7.29	7.95	0.060
ChAT	7.12	7.72	0.146
IL-6	6.90	6.59	0.474
Intensity of staining score－mean			
STAT3	2.81	3.69	0.004
survivin	2.04	2.59	0.068
HIF-1α	2.29	2.74	0.230
Gene mutation			
EGFR gene mutation +/- (n)	2/19*	2/11	
K-ras gene mutation +/- (n)	8/15	5/8	

* DNA was not amplified in 2 cases of 23 smokers.

nostaining results（Table 3）. Of 23 smokers and 13 non-smokers, the non-smokers showed significantly higher expression of STAT3 compared to smokers. Although not a significant difference, the expression of survivin tended to be higher in non-smokers compared to smokers（$P=0.068$）. There was no statistically significant difference in the expression of AChR，ChAT，HIF1-α，or IL-6 between smokers and non-smokers. Finally, we found that the expression of AChR M3 correlated with ChAT in tissues of smokers（$P=0.008$, $R=0.538$; Figure 2）.
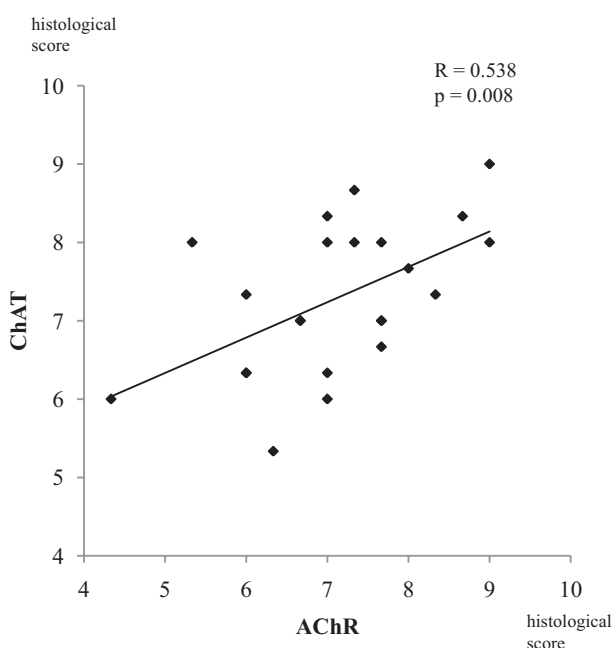

Fig. 2. Dispersed distribution of the histological score of AChR and ChAT expression in smokers (n=23)

Analysis of distribution showed coexpression of staining in samples from smokers (n=14 smokers with COPD; n=9 smokers without COPD).

EGFR gene and k-ras gene mutations

EGFR gene mutations were detected in 2 of the 13 non-smokers, in 1 of the 14 smokers with COPD, and in 1 of the 9 smokers without COPD. Two of the non-smokers had a point mutation in exon21（L858R）, one smoker with COPD showed deletion of exon19, and one smoker without COPD had point mutation of exon21（L858R）. K-ras gene mutations were detected in 5 of the 13 non-smokers, 6 of the 14 smokers with COPD, and 2 of the 9 smokers without COPD.

DISCUSSION

To our knowledge, this is the first report of pulmonary adenocarcinoma with a background of COPD having a significantly higher incidence of AChR and ChAT expression compared to pulmonary adenocarcinoma without COPD. In addition, the expression of these two proteins in tissues correlated with smoking habit, in agreement with previous reports［10, 11］.

Expression of mAChR and ChAT was detected previously in LC cell lines and in resected LC tissues［10, 11］. The proposed mechanism for such expression was AChR activation promoting the phosphorylation of MAPK and Akt, and thus involving proliferation of cancer cells and inhibition of apoptosis. Our current result further revealed that the expression of AChR and ChAT was higher in the lung carcinoma than in the preexisting lung tissue, and was also significantly higher in patients with COPD compared to those without COPD exposed to similar smoking levels. These new findings implicated the cholinergic signal transduction pathway in the carcinogenesis of LC associated with COPD. In smokers exposed to nicotine, the manifestation of AChR and ChAT was significantly facilitated in LC complicated with COPD, compared to LC not complicated with COPD. Various studies have shown that the activation of mAChR due to nicotine promotes the proliferation of LC. Therefore, cholinergic signal transduction is activated in the lungs of patients with complications of COPD due to nicotine stimulation, not only inducing carcinogenesis, but also possibly being involved in the generation of COPD. Future studies should analyze staining intensities for these proteins in the normal lungs of patients with COPD.

It has also been shown that the phosphorylation of MAPK and Akt due to the activation of AChR in LC was inhibited by selective M3 receptor antagonists［10, 11］. Tiotropium is widely used clinically in the treatment of COPD and is known to increase long-term survival rate; it also acts as a selective M3 receptor antagonist［17］. Tiotropium also suppresses the proliferation of LC cells *in vitro*［18］. Since LC is the primary cause of death in COPD patients, tiotropium might help to limit the LC, and thus reduce mortality in patients with

COPD.

We also found that LC tissue from non-smokers showed significantly higher expression of STAT3, a STAT-family transcription factor, compared to that from smokers, and a non-significant tendency for higher expression of survivin, which acts downstream of STAT3. Janus kinase (JAK) is activated by the interactions of IL-6 family cytokines or growth factors such as epidermal growth factor with such receptors, and STAT3 is activated by the activated JAK phosphotyrosine 705 of STAT3 (JAK-STAT pathway). Transcription is initiated by activated STAT3 binding to the target DNA, which is believed to function in the proliferation of LC cells and inhibition of apoptosis [19].

Moreover, EGFR mutations have been detected in 30% of LC from non-smoking women [6], with k-ras gene mutations were detected in 15-30% of lung adenocarcinoma. STAT3 is thought to act downstream of EGFR and ras signal transduction [19], and previous results indicated increased expression of STAT3 in cell lines expressing an EGFR mutation and showing sensitivity to gefitinib, compared to cell lines without such genetic mutation [20]. In addition, the incidence of EGFR mutation is seemingly higher in non-smokers than smokers [6], and the expression of downstream STAT3 might be accentuated due to the activation of EGFR in non-smokers. However, this study found no significant correlation between the genetic mutations in EGFR/k-ras and the expression intensity of STAT3 (P=0.706, 0.654, respectively; data not shown). Similar studies in larger populations are clearly needed.

Based on these findings, we propose that STAT3 can play a role in LC carcinogenesis. The STAT3 pathway has been implicated in the mechanism of lung adenocarcinoma in non-smokers, with the exception of EGFR and aplastic lymphoma kinase-echinoderm-microtubule-associated protein like4 (ALK-EML4), which are clearly present. In past reports, survivin was described as an anti-apoptotic protein belonging to the IAP family that contributes to survival rate by its varied expression levels in various types of cancer [14].

We examined the expression intensity of IL-6, which stimulates JAK-STAT signaling in association with the expression of STAT3, but found no significant difference in IL-6 expression between smokers and non-smokers in this study. It was therefore proposed that other molecules might participate greatly in the upregulation of JAK-STAT pathway.

This study has certain limitations. Most immunohistochemical biomarkers were far from being homogeneously distributed in tumor bulk. There is also the problem of specific antibody availability, and their indication for use in paraffinized material.

Conclusion

It became evident that differential expression of the cholinergic signal transduction pathway and the STAT-type signal transduction pathway was caused by the presence of complications with COPD and the presence of smoking in lung adenocarcinoma. The personalization of treatment for non-small-cell lung cancer has advanced, and EGFR tyrosine kinase inhibitor (EGFR-TKI) is used for patients positive for the genetic mutation EGFR, with ALK inhibitor being used for cases that express the ALK-EML4 fusion gene. For tissue types, the efficacy of pemetrexed is evident in non-squamous cell carcinoma. In the investigation of excisable NSCLC performed in this study, it was revealed that the molecular biological background differs between smokers/non-smokers and with/without complications of COPD. Not only are these investigations applicable to the treatment of postoperative relapse, they also contribute to the specification of the target factor regarding cancer chemoprevention. Studies in patients with advanced LC will therefore be necessary in the future.

List of Abbreviations

LC: lung cancer; COPD: chronic obstructive pulmonary disease; AChR M3: M3 muscarinic acetylcholine receptor; ChAT: cholineacetyltransferase; STAT: Signal transducer and activator of transcription; JAK: janus kinases; HIF-1α: hypoxia inducible factor-1α; IL-6: interleukin 6; EGFR: epidermal growth factor receptor; SCLC: small cell lung cancer; SCC: squamous cell cancer; MAPK: mitogen-activated protein kinase; IAP: inhibitor of apoptosis protein; PNA-LNA: peptide nucleic acid-locked nucleic acid; RFLP: restriction fragment length poly-

morphism; ALK-EML4: aplastic lymphoma kinase-echinoderm-microtubule-associated protein like4; EGFR-TKI: EGFR tyrosine kinase inhibitor.

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