Int J Clin Exp Pathol 2017;10(1):173-185
www.ijcep.com /ISSN:1936-2625/IJCEP0039425

Original Article

Loss of IL-24 expression facilitates invasion in lung adenocarcinoma

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Received September 2, 2016; Accepted November 1, 2016; Epub January 1, 2017; Published January 15, 2017

Abstract: Transduction of oncogenic KRAS and EGFR into immortalized human airway cells induced up-regulation of cytokine genes including CCL3, IL1B, and IL-24 in common. We focused on IL-24 in the present study. IL-24, a secreted protein of IL-10 family, is expressed in the immune system and modulates inflammatory reaction. Since the discovery of the function inducing melanoma cell differentiation, much attention has been paid to its anti-tumor effects. We performed expression analysis of IL-24 in lung cancer cell lines with quantitative real time PCR. IL-24 mRNA expression levels were markedly decreased in adenocarcinoma cell lines. In addition, the expression in some of the cancer cells was restored by 5-azacytidine and trichostatin A treatment. A forced expression of IL-24 inhibited migration in A549 and in PC-9 human lung cancer cells, which had lost the IL-24 expression. Immunohistochemical study with semi-quantitative measurement of 143 lung adenocarcinoma cases revealed invasive predominant subtypes had significantly lower scores of IL-24 expression than a lepidic predominant subtype (P = 0.0240). These results suggested IL-24 could play a role in the common oncogenic pathway, intrinsically suppress invasion early and disappear late in lung adenocarcinoma progression.

Keywords: IL-24, invasion, lung adenocarcinoma

Introduction

Lung cancer research has been changed drastically by combining genetic information and morphology in recent years. Histological subtypes of lung adenocarcinoma were well correlated with driver oncogene abnormality such as *EGFR* and *KRAS* mutation [1]. Molecular targeting agents have been developed for each cancer types [2, 3]. Even though EGFR and KRAS share MAP kinase cascades [4], *EGFR* mutated adenocarcinoma and *KRAS* mutated adenocarcinoma were contrasted and often separately discussed owing to the mutually exclusive relationship [5]. Roles of the genes involved in the common signal pathway to promote lung adenocarcinoma development are still largely unknown.

Interleukin-24 (IL-24) is a secreted cytokine that belongs to the IL-10 gene family. Along with several other IL-10 cytokine family members, it is located on chromosome 1q32-33 in humans and physiologically expressed in the immune system such as thymus, spleen, peripheral blood leukocytes and normal melanocytes to engage in immunomodulatory reaction. Since it was identified as a novel gene that is strongly expressed in human melanoma cells induced to terminally differentiate [6], IL-24 has shown prominent roles in inhibiting tumor growth, invasion, metastasis and angiogenesis in various types of cancer [7].

During the search for common downstream targets modulated by both oncogenic KRAS and EGFR transduction in immortalized human airway cells, we focused on IL-24 in the present study. Moreover, we investigated the potential effects of IL-24 transduction on cell growth and migration activities in both a *KRAS* mutant cell line and an *EGFR* mutant lung cancer cell line. To elucidate its role in lung adenocarcinoma progression, we investigated associations between the levels of IL-24 expression and histopathological parameters in surgically resected primary lung cancers.

Material and methods

Cell lines and cell culture

The cell lines used in this study were provided as follows: An immortalized human airway epithelial cell line (16HBE14o, Simian virus 40 (SV40)-transformed human bronchial epithelial cells) described by Cozens AL et al. [8] was kindly provided by Grunert DC (California Pacific Medical Center Research Institute). A subclone of 16HBE14o cells, described as NHBE-T in this study, was used. Immortalized airway epithelial cell lines (HPL1A and HPL1D, SV40-transformed human small airway epithelial cells) were established by Masuda A et al. [9]. Human lung cancer cell lines (HEK293T, A549, EKVX, H23, H226, H358, H441, H522, H820, H358, H1819, H2087, H460, H1299, HOP62, and HOP92) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Human lung cancer cell line LC2/ad, LCAM1, Lu65, Lu130, Lu135, Lu139, and RERF-LC-KJ were purchased from the Riken Cell Bank (Tsukuba, Japan). Human lung cancer cell lines, PC-3, PC9, and HARA were from Immuno-Biological Laboratories Co. (Gunma, Japan). Human lung cancer cell lines TKB1, TKB2, TKB4, TKB5, TKB6, TKB7, TKB8, TKB9, TKB12, TKB14, TKB15, TBK17, and TKB20 were obtained from Dr. Hiroshi Kamma via Dr. Takuya Yazawa (Dokkyo Medical University School of Medicine, Tochigi, Japan). Primary normal human bronchial epithelial cells (NHBE) and primary small airway epithelial cells (SAEC) were purchased from Lonza (Tokyo, Japan). Cancer cell lines and immortalized cell lines were cultured in either Roswell Park Memorial Institute (RPMI 1640; Wako, Osaka, Japan) or Dulbecco's modified Eagle's medium (DMEM; Wako) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France) and penicillin-streptomycin (Gibco-Invitrogen). SAEC and NHBE were cultured in defined keratinocyte serum free medium (Gibco-Invitrogen). All cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Plasmid construction

The pro-retrovirus vectors bearing KRAS [G12 wild-type (G12) and V12 mutant] [10] and EGFR [wild type (WT), Del (deletion of exon 19: E745-A750), L858R (point mutation in exon 21) [11] were previously described. Complementary DNA (cDNA) coding IL-24 transcript variant 1 (NM_006850.3) was PCR-amplified using cDNA from NHBE cell line and inserted into the pro-retrovirus vector pQCXIP (BD Clontech, Palo Alto, CA, USA). The pro-retrovirus without any cDNA, described as Mock, was used for empty control vector.

Retroviral-mediated gene transfer

The pQCXIP-based expression vectors and the pCL10A1 retrovirus-packaging vector (Imgenex, San Diego, CA) were cotransfected into HEK293T cells with Lipofectamine 2000 reagent (Invitrogen). At 24 hours after the transfection, conditioned medium was recovered as a viral solution. Desired genes were introduced by incubating cells with the viral solution containing 10 g/ml of polybrene (Sigma) for 24 hours. Cells stably expressing desired genes were selected with 2.5 g/ml of puromycin (Invitrogen) for 1 to 3 days. The pooled clones were used for biological analyses and expression profiling.

Microarray analysis

Gene expression in the empty vector (MOCK), EGFR WT, EGFR Del, EGFR L858R transduced NHBE-T cells was comprehensively evaluated with Agilent Array SurePrint G3 Human Gene Expression 8×60 K v2 (Agilent Technologies). Total RNA was extracted from the cells immediately after completing the selection (5 days post-transduction) using the RNA easy kit (Qiagen). Labeling and hybridization were performed according to the manufacturer's recommendations (Agilent Technologies). Probes showing an absence call were excluded. Then, normalization of signal values was performed with computer software (GeneSpring, Agilent technology, Palo Alto, CA). Probes (genes) were screened, the signal values of which were elevated or reduced more than tenfold by both EGFR Del and L858R transduction in comparison with the empty vector transduction. In addition, genes of the signal values fulfilling all the following requirements were selected. The genes up-regulated by oncogenic EGFR transduction should have signal values of: Mock< WT, WT<Del, and WT<L858R. On the contrary, the ones down-regulated should have signal values of: Mock>WT, WT>Del, and WT>L858R.

Quantitative reverse transcription-PCR

First-strand cDNA was synthesized from total RNA of cell lines with the SuperScript First-Strand Synthesis System according to the protocols of the manufacturer (Invitrogen). The cDNA generated was used as a template in

real-time PCR with SYBR Premix EXTaq (TaKaRa Bio, Otsu, Shiga, Japan) and ran on a Thermal Cycler DICE real-time PCR system (Takara). The primer sets used for the detection of IL-24 were purchased from TAKARA Perfect Real Time support system. The means and standard deviations of the copy number of the gene normalized to the value for GAPDH mRNA were statistically obtained from triplicate reactions.

Treatment with 5-azacytidine and trichostatin A

Cells were treated either with 10 mM of 5-azacytidine (Sigma, St. Louis, MO) for 72 hours by exchanging the medium every day or with 300 ng/ml of trichostatin A (Wako, Osaka, Japan) for 24 hours. Cells were also treated with 5-azacytidine for 48 hours and then with a combination of 5-azacytidine and trichostatin A for an additional 24 hours.

Western blotting

The cells grown to sub-confluence were solved with extraction buffer. After centrifugation, supernatants were recovered as protein extracts. The extracts were mixed with equal volumes of SDS buffer, and then boiled. The samples were subjected to SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membrane (Amersham). The membranes were incubated with 1.0% non-fat dry milk in 0.01 M/L Tris-buffered saline containing 0.1% Tween-20 to block non-immunospecific protein binding, and then with 0.1 g/ml of primary antibody against either EGFR (Cell Signaling Technology, Beverly, MA) or IL-24 (GenHunter, Nashville, TN) or β-actin (Sigma). After washing with 0.01 M/L Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with animal- matched horseradish peroxidase-conjugated secondary antibodies (Amersham). Immunoreactivity was visualized with the enhanced chemiluminescence system (ECL, Amersham).

Cell growth assay

Cells (2.5×10^5) were seeded onto a 10-cm culture dish (Iwaki, Tokyo, Japan), and grown to a semiconfluent state for 5 to 7 days. The cells were counted, and 2.5×10^5 cells were seeded again onto a 10-cm dish. Several passages were repeated in the same manner. The sum of population doublings (PDLs) at each point, was calculated by the formula $\Sigma PDLn = \log2 (countn/2.5\times10^5) + \Sigma PDLn-1$.

Colony formation assay

Cells (2.5×10^4) were seeded onto a 10-cm culture dish (Iwaki), and grown for an optimal period (8 to 14 days). The cells were fixed with methanol and Giemsa stained, and colonies visible in scanned photographs were counted.

Cell migration assays

Trans-well chambers (24 wells, 8 μm pore size, Becton Dickinson, Franklin Lakes, and NJ) were used for the assay. The lower chambers were filled with 0.7 ml of DMEM or RPMI containing 10% FBS, and the cells (5.0×10^4/0.2 ml of DMEM or RPMI without fetal bovine serum) were seeded onto the filters of the upper chambers. After 24 hours of incubation, non-migrated cells on the upper side of the filter membranes were removed. The cells migrating through were fixed with methanol, and stained with Giemsa (Merck, Darmstadt, Germany). The cells in three fields of 1.0 mm^2 on filter membranes were counted.

Primary lung adenocarcinoma

A total of 143 adenocarcinomas were removed by radical surgical resection at Kanagawa Prefectural Cardiovascular and Respiratory Center (Yokohama, Japan). This study was approved by the ethics committees of Yokohama City University and Kanagawa Prefectural Cardiovascular Respiratory Center Hospital. Informed consent for research use of the resected materials was obtained from all the subjects. Histological types and disease stages were determined according to WHO Classification of Tumours of Lung, Pleura, Thymus & Heart (4th edition) [12] and International TNM Classification System (7th edition of the UICC) [13]. According to the 4th edition of WHO classification, papillary, acinar, solid and mucinous subtypes were gathered as an invasive predominant group. Although a micropapillary subtype should be included in the group, none of our cases had micropapillary "predominant" histology. The rest of the major adenocarcinoma subtype was a lepidic predominant subtype including adenocarcinoma in situ, minimally invasive adenocarcinoma and lepidic predominant invasive adenocarcinoma, which represented a non-invasive predominant group.

Search for EGFR and KRAS mutations

The tumorous part was dissected from formalin-fixed, paraffin-embedded tissue sections.

The sample DNA was extracted using Nucleospin DNA kit (Takara). A fragment of the coding exons of oncogenic EGFR (exon 18, 19, 20 and 21) and of oncogenic KRAS (exons 2 and 3) was PCR-amplified using HotStar-TaqDNA polymerase (Stratagene) according to the manufacturer's instruction. PCR products were purified with EXoStar (Amersham), and subjected to a dye-terminator reaction using a Big Dye version 3.1 kit (Applied BioSystems, Foster City, CA). Primers used for PCR and DNA sequencing were referred from the previous reports [14, 15].

Immunohistochemistry

Tumor sections were cut from formalin-fixed, paraffin-embedded tissue blocks. Contents of the analyzed cases were described in the result table. Sections were deparaffinized, rehydrated, and incubated with 3% hydrogen peroxide, followed by 5% goat serum to block endogenous peroxidase activities and non-immunospecific protein binding. Sections were boiled in citrate buffer (0.01 M, pH 6.0) for 15 minutes to retrieve masked epitopes and then incubated with a primary antibody against IL-24 (GenHunter). IL-24 antibody specificity was confirmed with western blotting. Immunoreactivity was visualized using an Envision Detection System (DAKO), and nuclei were counterstained with hematoxylin. IL-24 immunohistochemical expression levels were described as follows. The faint level was defined as weaker than the level in the bronchioles but not negative. The modest level was defined as a level equivalent to that in the bronchioles. The strong level was defined as an unequivocally stronger level than that in the bronchioles. The immunohistochemical (IHC) expression score was determined as an average level in the maximal tumor section (if 30%, 10%, 50%, and 10% of neoplastic cells in the maximal section of tumor were negative, faint, modest, and strong levels, respectively, the average level was calculated as "1.4 = 0.3×0+0.1×1+0.5×2+0.1×3").

Statistical analysis

Statistical analyses were conducted using JMP version 9.02 for Windows (SAS, Cary, NC). Error bars in graphical data represent mean ± standard deviation. Statistical significance was determined using Student's t-test for two variables and one-way ANOVA for three or more variables. All reported *P* values are two-tailed, and for all analyses, $P \leq 0.05$ is considered statistically significant.

Results

Downstream molecules whose expressions modulated by the oncogenes

Downstream molecules of oncogenic KRAS gene were comprehensively searched in the preceding study using microarray analysis, in which the mutant KRAS gene was transduced into immortalized airway cells (NHBE-T cells) [16]. Similarly, those of oncogenic EGFR genes were searched. cDNAs of wild type EGFR (WT), EGFR exon 19 deletion (Del) and EGFR exon 21 L858R point mutation (L858R) were transduced into NHBE-T cells by retroviral-mediated gene transfer. Oncogenic EGFR genes caused growth suppressive effect and cytological atypia (**Figure 1**). The down-regulated genes and up-regulated genes were listed (**Tables 1**, **2**). These two microarray analyses elucidated that some cytokine genes, CCL3, IL1B, and IL-24, were commonly up-regulated in the two oncogenes-transduced cells. They may be involved in a shared oncogenic pathway of lung adenocarcinoma. We highlighted IL-24 to proceed to further analyses.

IL-24 mRNA expression in the lung cancer and non-cancer cell lines

The oncogenic EGFRs and KRAS transduced NHBE-T cells showed higher levels of IL-24 compared to the wild type genes or empty vector transduced NHBE-T cells, which confirmed the microarray analyses (**Figure 2A**). We also measured IL-24 mRNA levels in lung cancer cell lines, immortalized human airway cell lines, a normal human bronchial cell line and a human small airway cell line. IL-24 mRNA levels were decreased in adenocarcinoma cell lines and small cell carcinoma cell lines compared to other subtypes of lung cancer cell lines and normal human cells (**Figure 2B**). Notably, the levels were under detection limit in H441, H522, PC-3, TKB20, H460, and TKB12 cells.

Effects of DNA methyltransferase inhibitor and histone acetyltransferase inhibitor on IL-24 mRNA expression in lung cancer cell lines

To evaluate the expression regulation of IL-24 in human lung cancer cells, treatment with an inhibitor for a DNA methyl-transferase (5-AZA-dc) in combination with an inhibitor for histone deacetylase (trichostatin A, TSA) was performed. It restored IL-24 expression in A549, H460, RERF-LC-KJ, TKB-5, and H1819 human

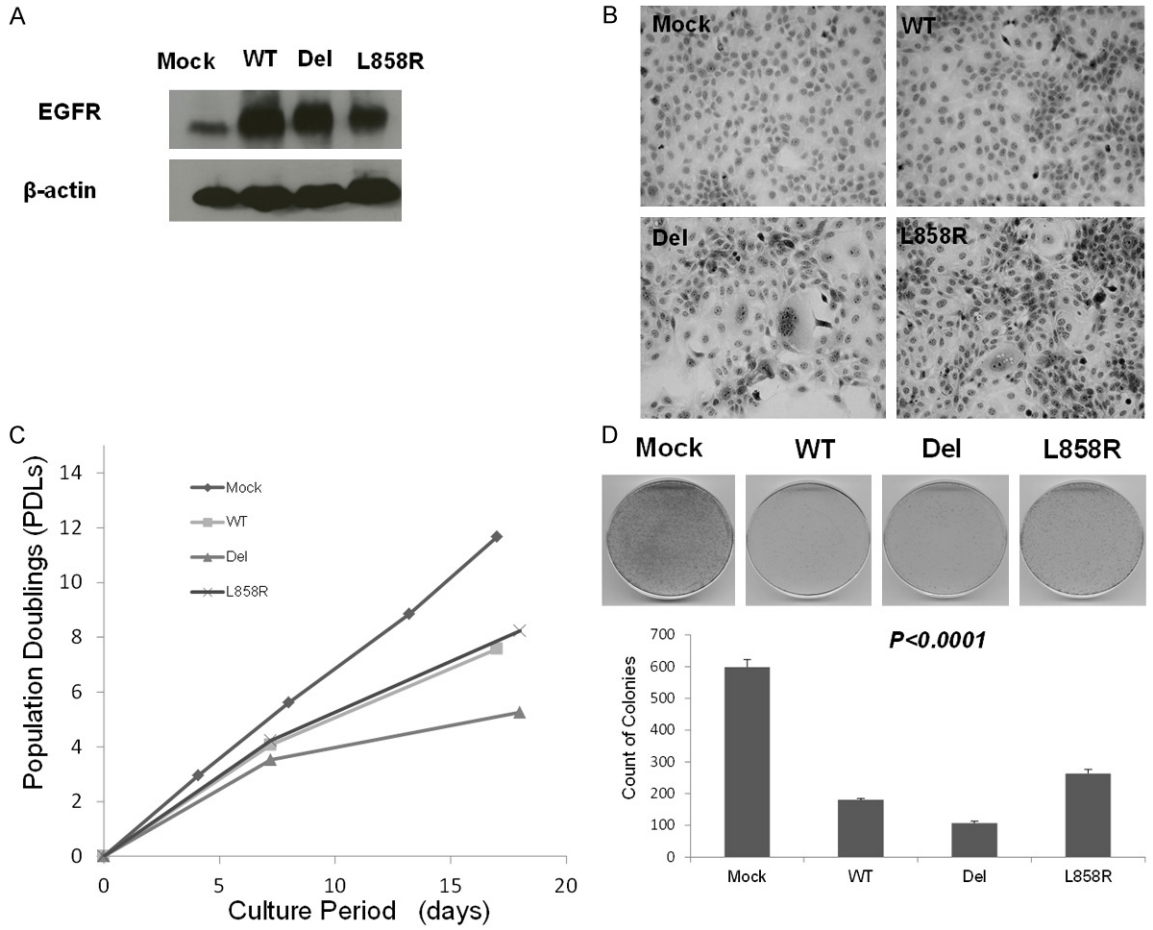

Figure 1. Biological effects of oncogenic EGFR on NHBE-T cells. Empty vector (pQCXIP; MOCK), Wild type EGFR (WT), EGFR exon 19 deletion (Del), or EGFR exon 21 L858R point mutation (L858R) were transduced into NHBE-T cells. The cells harvested immediately after the selection process were examined for the expression of EGFR and β-actin by Western blotting (A). After the selection cells (2.5×10^4) were re-seeded onto chamber slides (LAB-TEK, Electron Microscopy Science; Hatfield, PA) and cultured for 48 hours. They were then fixed with ethanol, and stained with the Papanicolaou method (B). Cells selected were grown and passed several times. Cumulated population doublings are presented (C). The surviving populations were harvested and counted, and 2.5×10^4 cells were reseeded onto a 10-cm dish. After 10 days, the cells were fixed and stained with Giemsa. The means and standard deviations (error bars) of colony counts from triplicate experiments are presented (D).

Table 1. Down-regulated genes

			Mock		EGFR WT		EGFR Del		EGFR L858R			
Symbol	Genbank	Map	Signal	Flag	Signal	Flag	Signal	Flag	Signal	Flag	Del/Mock	L858R/Mock
UNC79	NM_020818	14q32.12	-1.592	P	-5.475	A	-6.465	A	-6.368	A	-29.308	-27.407
NEK11	NM_024800	3q22.1	-2.098	P	-5.466	A	-6.514	A	-6.538	A	-21.341	-21.696
SNORD121B	NR_003690	9p13.3	-1.814	P	-5.037	A	-6.149	A	-5.668	A	-20.175	-14.457
SYT12	NM_177963	11q13.2	2.915	P	0.515	P	-0.417	P	-0.585	P	-10.068	-11.310
SMAD6	NM_005585	15q22.31	-1.737	P	-2.928	P	-5.099	A	-5.166	A	-10.287	-10.775

MOCK, empty vector-transduced NHBE-T; WT, wild-type EGFR transduced NHBE-T; Del, oncogenic mutant of EGFR exon 19 deletion-transduced NHBE-T; L858R, oncogenic mutant of EGFR L858R point mutation-transduced NHBE-T cells; Symbol, gene name; Map, Chromosomal locus; Genbank, gene bank accession number. Flags indicate whether gene expression is present (P) or absent (A).

lung cancer cells, whereas non-neoplastic airway epithelial cells (SAEC) demonstrated no restoration of IL-24 expression (**Figure 3**). These data suggested epigenetic modification

Table 2. Up-regulated genes

			Mock		EGFR WT		EGFR Del		EGFR L858R			
Symbol	Genbank	Map	Signal	Flag	Signal	Flag	Signal	Flag	Signal	Flag	Del/Mock	L858R/Mock
CCL3	NM_002983	17q12	-5.087	A	-4.322	A	-1.148	P	5.237	P	15.337	1282.385
IL24	NM_001185156	1q32	-6.073	A	-0.359	P	1.490	P	2.791	P	189.044	466.051
CSF3	NM_000759	17q11.2-q12	-5.882	A	-2.623	P	-0.041	P	2.087	P	57.343	250.639
IL24	NM_001185156	1q32	-1.725	P	2.589	P	4.423	P	5.768	P	70.913	180.157
KRT6A	NM_005554	12q13.13	-5.150	A	-1.736	P	-0.428	P	1.694	P	26.403	114.895
SCG5	NM_003020	15q13-q14	-6.068	A	-3.953	P	-2.269	P	0.636	P	13.913	104.271
IL1B	NM_000576	2q14	0.563	P	2.844	P	5.080	P	6.935	P	22.887	82.827
KRT6C	NM_173086	12q13.13	-6.433	A	-4.895	A	-2.688	P	-0.557	P	13.404	58.718
RSU1	NM_012425	10p13	-4.759	A	-0.041	P	0.101	P	0.541	P	29.050	39.399
KRT6B	NM_005555	12q13.13	-2.795	P	-0.452	P	1.151	P	1.501	P	15.407	19.637
IL1A	NM_000575	2q14	-0.105	P	2.164	P	3.280	P	4.093	P	10.446	18.351
HSD3B1	NM_000862	1p13.1	-2.312	P	-1.209	P	1.773	P	1.745	P	16.971	16.640
MMP1	NM_002421	11q22.3	-0.573	P	1.302	P	2.801	P	3.376	P	10.367	15.445
NLRP3	NM_001243133	1q44	-5.303	A	-4.579	A	-1.933	P	-1.374	P	10.344	15.240
MMP3	NM_002422	11q22.3	-1.293	P	2.087	P	3.059	P	2.388	P	20.431	12.827
CLIC5	NM_016929	6p12.3	-6.206	A	-5.840	A	-2.837	P	-2.817	P	10.329	10.474

MOCK, empty vector-transduced NHBE-T; WT, wild-type EGFR transduced NHBE-T; Del, oncogenic mutant of EGFR exon 19 deletion-transduced NHBE-T; L858R, oncogenic mutant of EGFR L858R point mutation-transduced NHBE-T cells; Symbol, gene name; Map, Chromosomal locus; Genbank, gene bank accession number. Flags indicate whether gene expression is present (P) or absent (A).

might affect IL-24 expression in the human lung cancer cells.

Biological effects of IL-24 on cell lines with KRAS or EGFR mutations

To characterize IL-24 function in lung cancer *in vitro*, cDNA of IL-24 was introduced into A549 and PC-9 cells using retrovirus transfection. Over expression of IL-24 was confirmed by western blot (**Figure 4A**, **4E**). Neither of the cells showed any change in growth activity evaluated by growth curve analysis and colony formation assay (**Figure 4B**, **4C**, **4F** and **4G**). Migration assay revealed the number of the IL-24 overexpressed cancer cells infiltrating through the membrane was significantly smaller than that of the control cells (**Figure 4D** and **4H**).

Immunohistochemical expression of IL-24 in primary lung cancers regarding pathological factors

Finally, to elucidate the role of IL-24 expression in lung cancer tissue, immunohistochemistry of IL-24 was performed. The expression was localized in cytoplasm of bronchiole cells and cancer cells (**Figure 5A**). The expression status was semi-quantitatively scored according to the intensity and the proportion of the positive cells. On the basis of cell line expression profiles of IL-24 and current extensive research with driver oncogenes such as *EGFR* and *KRAS*, we targeted adenocarcinoma cases. The associations between the scores with histological types/subtypes, vascular involvement or lymphatic canal involvement, pathological stages, Ki-67 growth activity, and oncogenic mutations, were analyzed (**Table 3**). On the whole, the immunohistochemical scores of well differentiated carcinomas were higher than those of poorly differentiated carcinomas. In addition, the invasive predominant group (papillary, acinar, solid or mucinous predominant subtypes) had significantly lower immunohistochemical scores than the non-invasive predominant group (lepidic predominant subtype) (**Figure 5B**). Stage I adenocarcinoma cases showed significantly higher scores than Stage II or more advanced cases. No significant association between the IL-24 levels and oncogenic mutations in adenocarcinomas (**Table 3**).

Discussion

The aim of the present study is to identify essential factors involved in a common signal transduction promoting carcinogenesis of the lung. We focused on IL-24, through a comprehensive search for downstream targets of the two major driver oncogenes, *EGFR* and *KRAS*.

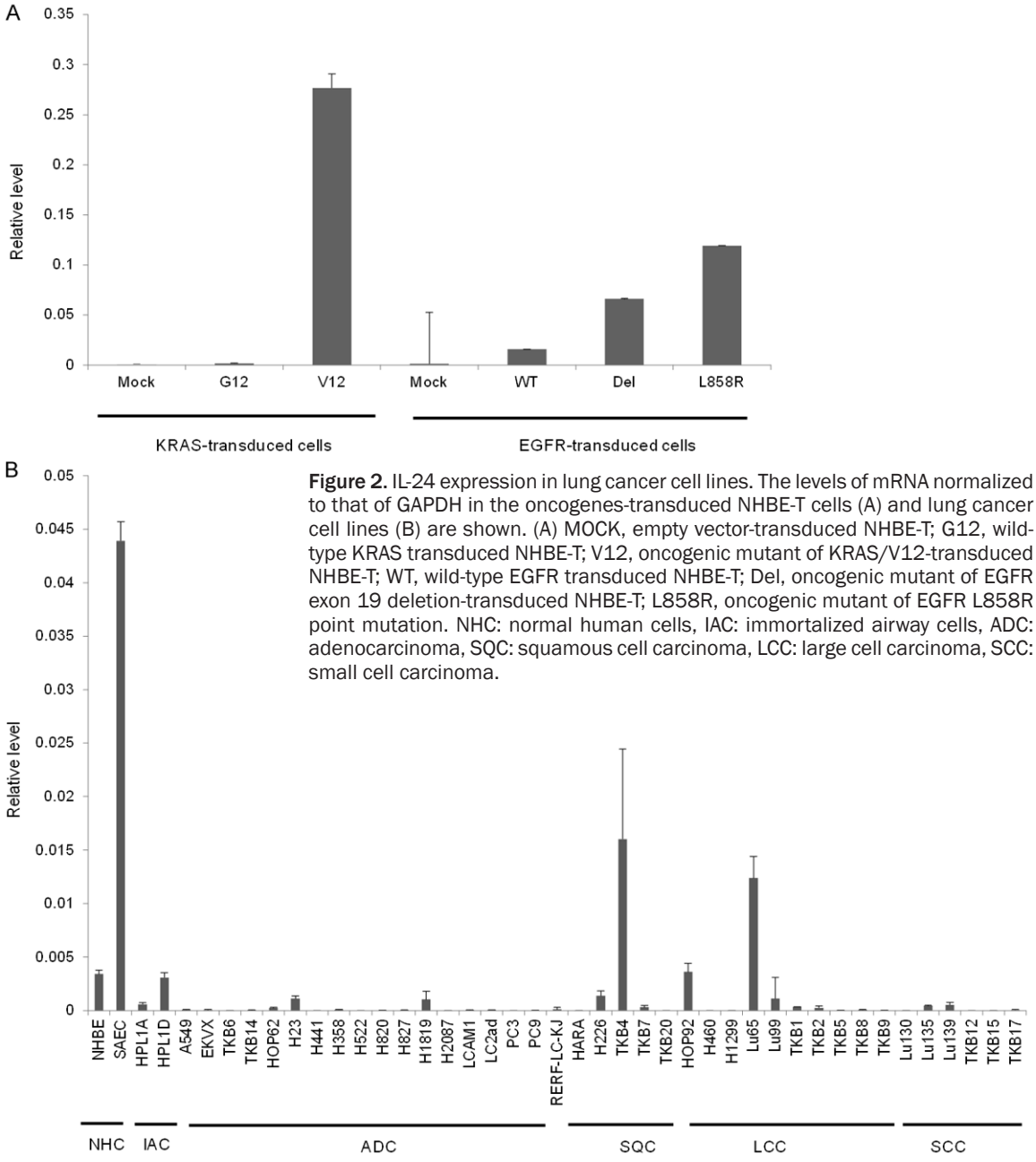

Figure 2. IL-24 expression in lung cancer cell lines. The levels of mRNA normalized to that of GAPDH in the oncogenes-transduced NHBE-T cells (A) and lung cancer cell lines (B) are shown. (A) MOCK, empty vector-transduced NHBE-T; G12, wild-type KRAS transduced NHBE-T; V12, oncogenic mutant of KRAS/V12-transduced NHBE-T; WT, wild-type EGFR transduced NHBE-T; Del, oncogenic mutant of EGFR exon 19 deletion-transduced NHBE-T; L858R, oncogenic mutant of EGFR L858R point mutation. NHC: normal human cells, IAC: immortalized airway cells, ADC: adenocarcinoma, SQC: squamous cell carcinoma, LCC: large cell carcinoma, SCC: small cell carcinoma.

Oncogenic EGFR transduction in NHBE-T cells induced marked growth suppression and cytological atypia. This phenomenon had been observed in our oncogenic KRAS transduction as well [16]. A small subset of mutant EGFR overexpressed immortalized esophageal epithelial cells exhibited proliferative arrest and morphology compatible with senescence in the past report [17]. While one might define it as oncogene-induced senescence [18], we considered the effect in immortalized human airway cells to be an initiating model of oncogenic EGFR/KRAS-mediated carcinogenesis to advance further analyses.

Immunohistochemical expression level of IL-24 was stronger in early stages of lung cancer, while it was weaker in advanced stages. Most of the lung cancer cell lines, which generally derived from advanced cancers, lost its expression. Stronger expression of IL-24 was linked with better postoperative outcome in adenocarcinomas at Stage I (data not shown), which was consistent with the previous clinical report

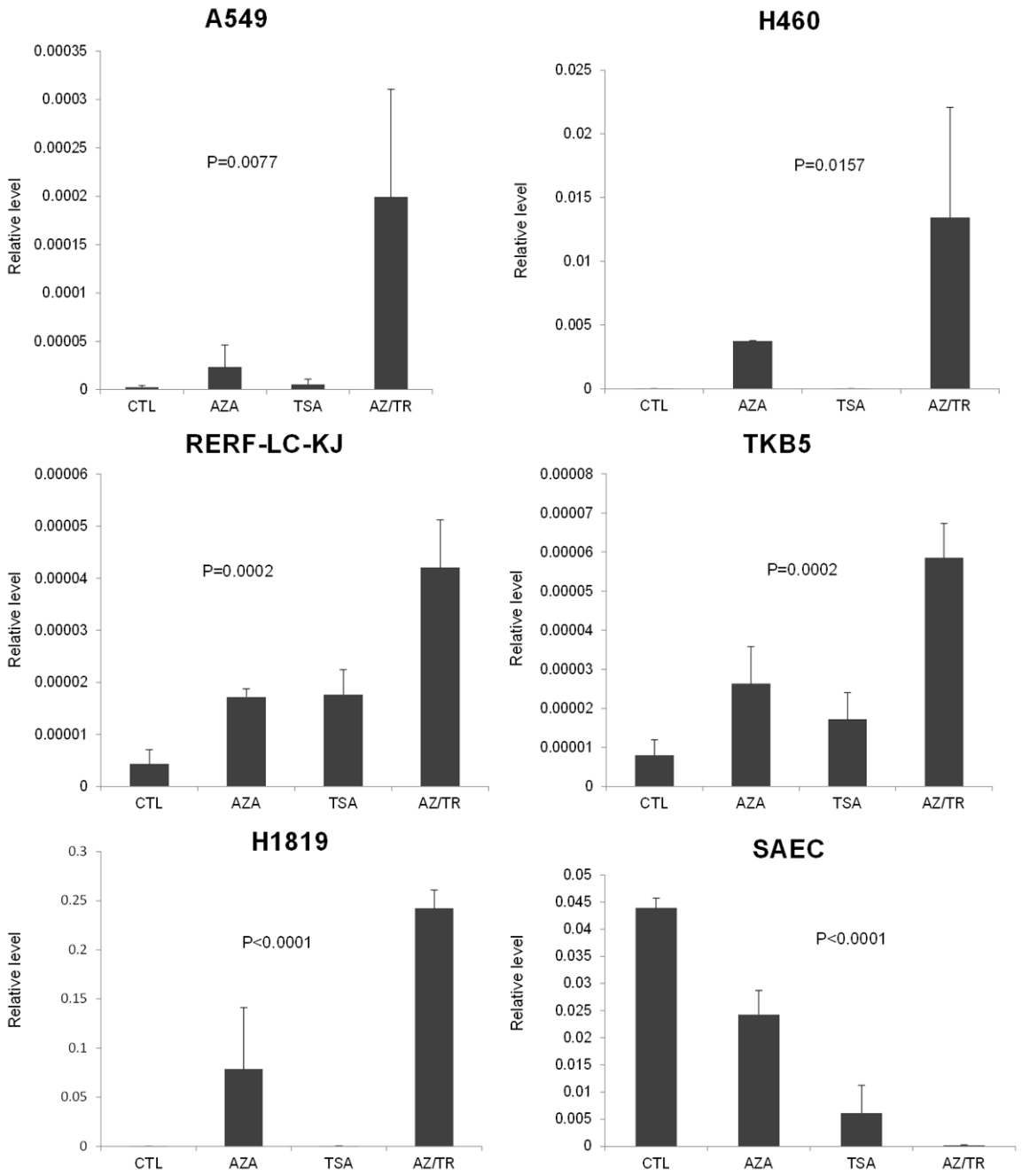

Figure 3. Effects of DNA methyltransferase inhibitor (5-azacytidine (AZA)) and histone acetyltransferase inhibitor (trichostatin A [TSA]) on IL-24 mRNA expression in lung cancer cell lines. The cells treated with vehicle control (CTL), AZA, TSA, or a combination of AZA and TSA (AZ/TR) were examined for the restoration of IL-24 mRNA expression. The relative level of IL-24 normalized to those of GAPDH was calculated. Means and standard deviations (error bars) from triplicate experiments are shown.

[19]. These data suggested IL-24 could emerge as a suppresser in the early stage and disappear in the advanced stage of lung cancer.

No significant difference in the IL-24 levels was found between adenocarcinomas with *EGFR* mutations and with *KRAS* mutations. The result supports our notion that IL-24 is a common player in the KRAS and EGFR-mediated oncogenic pathway. Also, the levels did not differ between the adenocarcinomas with and without those mutations. The other driver onco-

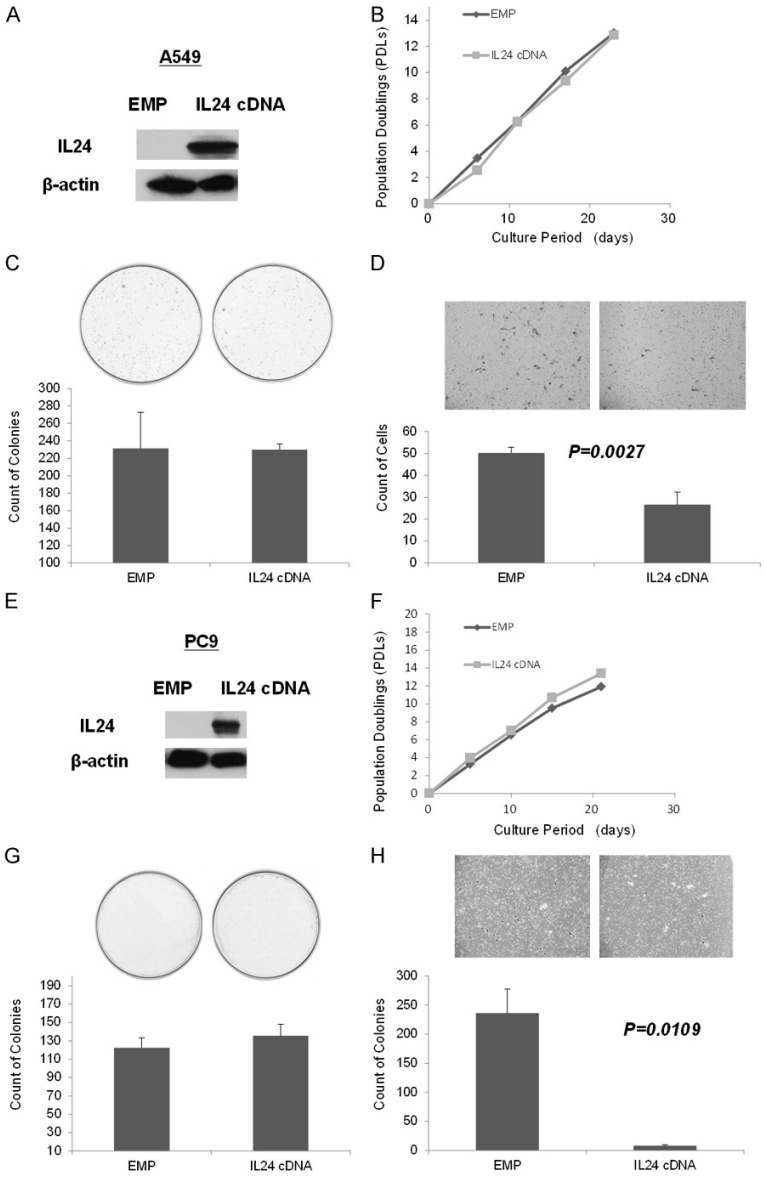

Figure 4. The effects of forced expression of IL-24 on cell growth and migration. The empty vector (EMP), and IL-24 transcriptional variant 1 (IL24 cDNA) were transduced into A549 cells (*KRAS*-mutated cell line) (A-D) and PC9 (*EGFR*-mutated cell line) (E-H). Cells harvested immediately after the selection were examined for the expression of IL-24 and β-actin by Western blotting (A, E). Cells selected were grown and passed several times. Cumulated population doublings are presented as a line chart (B, F). The surviving cells were harvested and counted, and 2.0×10^4 cells were re-seeded on a 10 cm dish. After 10 days, cells were methanol-fixed and Giemsa-stained. The means and standard deviations (error bars) of colony counts from triplicate experiments are presented (C, E). The cells that migrated through the pores of the trans-well membranes were stained with hematoxylin; magnification, ×100 (D). (H) The results of migration assays. Three independent assays were performed. The means and standard deviations (error bars) of migrated cells are counted.

genes, such as *ALK, RET* and *ROS*, might share the common pathway to induce IL-24 expression.

In inflammatory state, IL-24 is preferentially expressed by T_H2 cells where STAT6, GATA-binding protein 3 (GATA3), and JUN are suggested to participate in transcriptional regulation [20-22]. Since STAT6, GATA3 and JUN are known to be in downstream of the EGFR-RAS-ERK pathway, these transcriptional factors seem plausible enough to be engaged in up-regulation of IL-24 likewise in response to the oncogenic stimuli such as mutations of *KRAS* and *EGFR*. In fact, IL-24 was activated by RAS signaling [23]. On the other hand, the exact molecular mechanism of altered IL-24 expression in the tumor pathogenesis has been currently unclear. It was revealed that treatment with an inhibitor for a DNA methyl-transferase (5-AZA-dc) in combination with an inhibitor for histone deacetylase (trichostatin A, TSA) restored IL-24 expression in several lung cancer cell lines including A549 cells, but not in non-neoplastic airway epithelial cells. The result coincided with a previous observation, which suggested epigenetic modification could alter IL-24 expression in neoplastic cells [24]. Taken together, it is of great interest to investigate the DNA methylation and acetylation in the IL-24 gene promoter region, especially binding sites for STAT6, GATA3 and JUN.

Invasion of lung cancer involves multiple processes such as angiogenesis, degradation of matrix barriers, disruption of cell-cell and cell-matrix adhesion and inducement of cellular motility [25]. Establishment of the experimental model is challenging. Ectopic adenovirus mediated gene transfer of IL-24

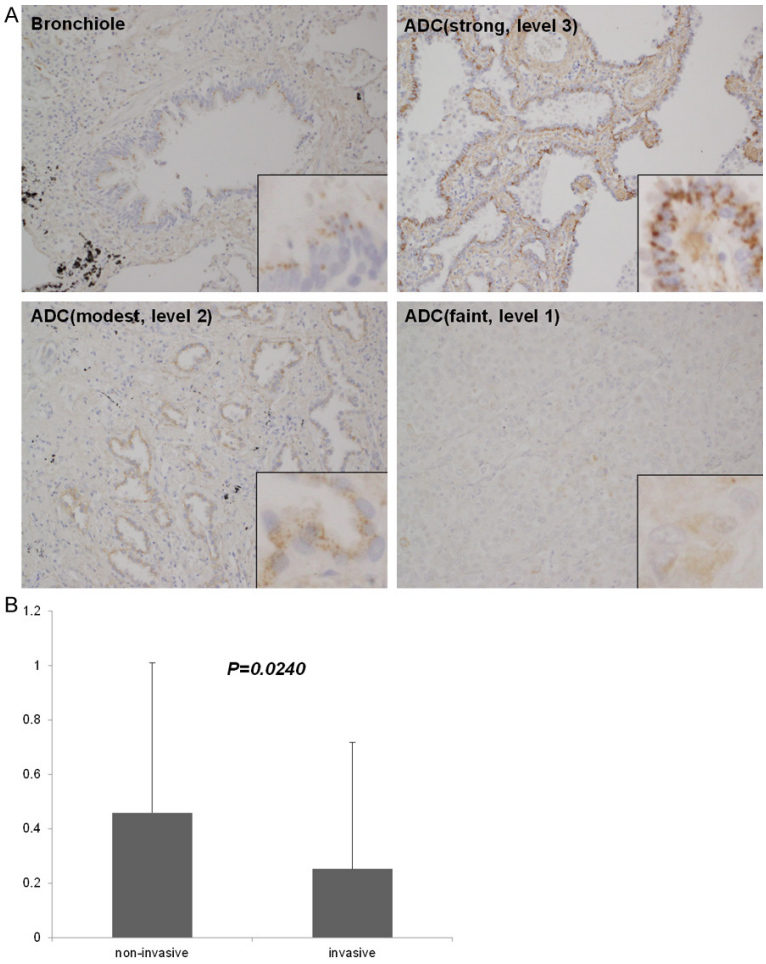

Figure 5. IL-24 immunohistochemistry. A. IL-24 was localized in the cytoplasm of bronchioles. To semi quantitatively measure the expression of IL-24, the faint level was defined as weaker than the level of the bronchioles but not negative. The modest level was defined as a level equivalent to that of the bronchioles. The strong level was defined as an unequivocally stronger level than that of the bronchioles. The immunohistochemical expression score was determined as an average level in the largest tumor section. The insets were high power view of cytoplasm. B. Difference of the levels in non-invasive component-predominant group and invasive component predominant group (non-invasive predominant group; adenocarcinoma in situ, minimally invasive adenocarcinoma, lepidic predominant invasive adenocarcinoma, invasive predominant group; acinar, papillary, solid, and mucinous adenocarcinoma subtypes.) is shown in a bar graph.

inhibited invasion and migration of human lung cancer cells *in vitro* and *in vivo* [26]. Recently, this process was accomplished by disrupting the SDF-1/CXCR4 signaling pathway [27]. Since retrovirus mediated gene transfection have the ability to integrate genes into the host genome in a stable fashion, IL-24 integrated in the lung cancer cells seemed to recapture the endogenous inhibitory function. Additionally, we confirmed using human clinical samples, for the first time, reduced IL-24 expression was associated with invasive histological subtypes. Therefore, which part of the invasion processes does IL-24 interfere with might be the next question.

Accumulating studies have found anti-tumor effects of IL-24 in various types of cancer cells, including gliomas, ovarian, breast, liver, pancreatic, gastric, colorectal, renal, and prostate cancer cells [28]. Lung cancer is no exception [29-32]. Restoration of IL-24 in progressed lung cancer might become a treatment option. If so, examining IL-24 status before treatment would allow oncologists to select cases applicable of IL-24 treatment.

In conclusion, our results indicated that IL-24 could be involved in the common oncogenic pathway of lung adenocarcinoma, intrinsically inhibit invasion in early carcinogenesis, and disappear as the cancer progresses in part by epigenetic modification. These findings could enhance the current development of IL-24 treatment strategy for lung cancer.

Acknowledgements

The authors would like to thank Emi Honda, Misa Otara and Ikuko Nanba (Kanagawa Prefectural Cardiovascular and Respiratory Center Hospital, Yokohama, Japan) for their assistance. This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosure of conflict of interest

None.

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Table 3. Immunohistochemical scores of IL-24 in surgically resected lung cancer specimens

	Expression score (mean ± SD)	*P* value*
ADC (143)	0.314±0.499	
Differentiation		0.0744
Well (56)	0.432±0.568	
Mod (69)	0.235±0.446	
Por (18)	0.250±0.406	
Subtype		0.0525
Lepidic (42)	0.460±0.551	
Acinar (34)	0.350±0.562	
Papillary (35)	0.123±0.256	
Solid (18)	0.250±0.406	
Mucinous (14)	0.350±0.630	
Non-invasive predominance or Invasive predominance		0.0240
Non-invasive (42)	0.459±0.551	
Invasive (101)	0.253±0.465	
Vascular involvement (143)		0.1606
Absent (83)	0.364±0.548	
Present (63)	0.245±0.416	
Lymphatic canal involvement (143)		0.0714
Absent (55)	0.409±0.595	
Present (88)	0.255±0.422	
Stage (143)		0.0252
1 (111)	0.364±0.540	
2, 3 and 4 (32)	0.141±0.259	
Ki-67 labeling index (143)		0.9685
Low level (<10%) (51)	0.312±0.533	
High level (≥10%) (92)	0.315±0.482	
Oncogenic mutation/ADC (85)		0.1636
EGFR (29)	0.524±0.675	
KRAS (12)	0.350±0.392	
None (44)	0.277±0.464	

*Student's t-test for two variables and one-way ANOVA for three or more variables. Abbreviations are ADC, adenocarcinoma; WEL, well differentiated; MOD, moderately differentiated; POR, poorly differentiated carcinomas. "None" stands for cases without *KRAS* or *EGFR* mutations.

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References

[1] Yoshizawa A, Sumiyoshi S, Sonobe M, Kobayashi M, Fujimoto M, Kawakami F, Tsuruyama T, Travis WD, Date H and Haga H. Validation of the IASLC/ATS/ERS lung adenocarcinoma classification for prognosis and association with EGFR and KRAS gene mutations: analysis of 440 Japanese patients. J Thorac Oncol 2013; 8: 52-61.

[2] Jänne PA, Shaw AT, Pereira JR, Jeannin G, Vansteenkiste J, Barrios C, Franke FA, Grinsted L, Zazulina V and Smith P. Selumetinib plus docetaxel for KRAS-mutant advanced non-small-cell lung cancer: a randomised, multicentre, placebo-controlled, phase 2 study. Lancet Oncol 2013; 14: 38-47.

[3] Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, Palmero R, Garcia-Gomez R, Pallares C and Sanchez JM. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2012; 13: 239-246.

[4] Goffin JR and Zbuk K. Epidermal growth factor receptor: pathway, therapies, and pipeline. Clin Ther 2013; 35: 1282-1303.

[5] Schmid K, Oehl N, Wrba F, Pirker R, Pirker C and Filipits M. EGFR/KRAS/BRAF mutations in primary lung adenocarcinomas and corresponding locoregional lymph node metastases. Clin Cancer Res 2009; 15: 4554-4560.

[6] Fisher PB. Is mda-7/IL-24 a "magic bullet" for cancer? Cancer Res 2005; 65: 10128-10138.

[7] Menezes ME, Bhatia S, Bhoopathi P, Das SK, Emdad L, Dasgupta S, Dent P, Wang XY, Sarkar

D and Fisher PB. MDA-7/IL-24: multifunctional cancer killing cytokine. Adv Exp Med Biol 2014; 818: 127-153.
[8] Cozens A, Yezzi M, Kunzelmann K, Ohrui T, Chin L, Eng K, Finkbeiner W, Widdicombe J and Gruenert D. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. Am J Respir Cell Mol Biol 1994; 10: 38-47.
[9] Masuda A, Kondo M, Saito T, Yatabe Y, Kobayashi T, Okamoto M, Suyama M, Takahashi T and Takahashi T. Establishment of human peripheral lung epithelial cell lines (HPL1) retaining differentiated characteristics and responsiveness to epidermal growth factor, hepatocyte growth factor, and transforming growth factor β1. Cancer Res 1997; 57: 4898-4904.
[10] Okudela K, Yazawa T, Suzuki T, Sugimura H and Kitamura H. Role of 3'-phosphoinositides in oncogenic KRAS-induced modulation of shape and motility of airway epithelial cells. Pathol Int 2009; 59: 28-37.
[11] Okudela K, Suzuki T, Umeda S, Tateishi Y, Mitsui H, Miyagi Y and Ohashi K. A comprehensive search for microRNAs with expression profiles modulated by oncogenic KRAS: potential involvement of miR-31 in lung carcinogenesis. Oncol Rep 2014; 32: 1374-1384.
[12] Travis WD, Brambilla E, Burke A, Marx A, Nicholson AG and Cancer IAfRo. WHO classification of tumours of the lung, pleura, thymus and heart. International Agency for Research on Cancer 2015.
[13] Sobin LH, Gospodarowicz MK and Wittekind C. TNM classification of malignant tumours. John Wiley & Sons 2011.
[14] Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J and Haber DA. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004; 350: 2129-2139.
[15] Woo T, Okudela K, Yazawa T, Wada N, Ogawa N, Ishiwa N, Tajiri M, Rino Y, Kitamura H and Masuda M. Prognostic value of KRAS mutations and Ki-67 expression in stage I lung adenocarcinomas. Lung Cancer 2009; 65: 355-362.
[16] Okudela K, Yazawa T, Woo T, Sakaeda M, Ishii J, Mitsui H, Shimoyamada H, Sato H, Tajiri M, Ogawa N, Masuda M, Takahashi T, Sugimura H and Kitamura H. Down-regulation of DUSP6 expression in lung cancer: its mechanism and potential role in carcinogenesis. Am J Pathol 2009; 175: 867-881.
[17] Ohashi S, Natsuizaka M, Wong GS, Michaylira CZ, Grugan KD, Stairs DB, Kalabis J, Vega ME, Kalman RA, Nakagawa M, Klein-Szanto AJ, Herlyn M, Diehl JA, Rustgi AK and Nakagawa H. Epidermal growth factor receptor and mutant p53 expand an esophageal cellular subpopulation capable of epithelial-to-mesenchymal transition through ZEB transcription factors. Cancer Res 2010; 70: 4174-4184.
[18] Chandeck C and Mooi WJ. Oncogene-induced cellular senescence. Adv Anat Pathol 2010; 17: 42-48.
[19] Ishikawa S, Nakagawa T, Miyahara R, Kawano Y, Takenaka K, Yanagihara K, Otake Y, Katakura H, Wada H and Tanaka F. Expression of MDA-7/IL-24 and its clinical significance in resected non-small cell lung cancer. Clin Cancer Res 2005; 11: 1198-1202.
[20] Sahoo A, Lee CG, Jash A, Son JS, Kim G, Kwon HK, So JS and Im SH. Stat6 and c-Jun mediate Th2 cell-specific IL-24 gene expression. J Immunol 2011; 186: 4098-4109.
[21] Stevens L, Htut TM, White D, Li X, Hanidu A, Stearns C, Labadia ME, Li J, Brown M and Yang J. Involvement of GATA3 in Protein kinase C Stinduced Th2 cytokine expression. Eur J Immunol 2006; 36: 3305-3314.
[22] Schaefer G, Venkataraman C and Schindler U. Cutting edge: FISP (IL-4-induced secreted protein), a novel cytokine-like molecule secreted by Th2 cells. J Immunol 2001; 166: 5859-5863.
[23] Tan Z, Wang M and Liang P. Identification by differential display of IL-24 autocrine loop activated by ras oncogenes. Methods Mol Biol 2006; 317: 207-218.
[24] Sahoo A and Im SH. Molecular mechanisms governing IL-24 gene expression. Immune Network 2012; 12: 1-7.
[25] Perlikos F, Harrington KJ and Syrigos KN. Key molecular mechanisms in lung cancer invasion and metastasis: a comprehensive review. Crit Rev Oncol Hematol 2013; 87: 1-11.
[26] Ramesh R, Ito I, Gopalan B, Saito Y, Mhashilkar AM and Chada S. Ectopic production of MDA-7/IL-24 inhibits invasion and migration of human lung cancer cells. Mol Ther 2004; 9: 510-518.
[27] Panneerselvam J, Jin J, Shanker M, Lauderdale J, Bates J, Wang Q, Zhao YD, Archibald SJ, Hubin TJ and Ramesh R. IL-24 inhibits lung cancer cell migration and invasion by disrupting the SDF-1/CXCR4 signaling axis. PLoS One 2015; 10: e0122439.
[28] Whitaker EL, Filippov VA and Duerksen-Hughes PJ. Interleukin 24: mechanisms and therapeutic potential of an anti-cancer gene. Cytokine Growth Factor Rev 2012; 23: 323-331.
[29] Sieger KA, Mhashilkar AM, Stewart A, Sutton RB, Strube RW, Chen SY, Pataer A, Swisher SG, Grimm EA, Ramesh R and Chada S. The tumor

suppressor activity of MDA-7/IL-24 is mediated by intracellular protein expression in NSCLC cells. Mol Ther 2004; 9: 355-367.

[30] Zhu Y, Lv H, Xie Y, Sheng W, Xiang J and Yang J. Enhanced tumor suppression by an ING4/IL-24 bicistronic adenovirus-mediated gene cotransfer in human non-small cell lung cancer cells. Cancer Gene Ther 2011; 18: 627-636.

[31] Zhong S, Yu D, Wang Y, Qiu S, Wu S and Liu XY. An armed oncolytic adenovirus ZD55-IL-24 combined with ADM or DDP demonstrated enhanced antitumor effect in lung cancer. Acta Oncol 2010; 49: 91-99.

[32] Nishikawa T, Munshi A, Story MD, Ismail S, Stevens C, Chada S and Meyn RE. Adenoviral-mediated mda-7 expression suppresses DNA repair capacity and radiosensitizes non-small-cell lung cancer cells. Oncogene 2004; 23: 7125-7131.