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Aryl hydrocarbon receptor activation and overexpression upregulated fibroblast growth factor-9 in human lung adenocarcinomas

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We had previously reported that aryl hydrocarbon receptors (AhRs) are overexpressed in lung adenocarcinomas. Benzo[a]pyrene (BaP), an AhR agonist, increased FGF-9 expression in human lung adenocarcinoma cells. Similarly, several AhR agonists increased FGF-9 mRNA levels, and BaP-induced FGF-9 expression was prevented by cotreatment with AhR antagonist in human lung adenocarcinoma cells. Furthermore, AhR agonists increased transcriptional activity of FGF-9 promoter. Modulation of AhR expression *via* RNA interference or transient overexpression respectively reduced or increased both constitutive and BaP-induced FGF-9 expression in human lung cells. These results suggested that AhR activation and overexpression increased FGF-9 expression in lung cells. FGF-9 increased growth of lung fibroblasts but not that of lung adenocarcinoma cells. However, conditioned media collected from FGF-9-treated fibroblasts increased cell growth of lung adenocarcinoma cells. Furthermore, lung adenocarcinoma cells expressed FGF receptor 2 and cotreatment with anti-FGF receptor 2 prevented the interaction between fibroblasts and tumor cells. It is likely that FGF-9-stimulated fibroblasts secreted unknown factors, which activated FGF receptor 2 and subsequently promoted growth of lung adenocarcinoma cells. We further compared AhR and FGF-9 expression in 146 non-small cell lung cancer (NSCLC) cases by immunohistochemistry. FGF-9 expression was more common in adenocarcinomas than in squamous cell carcinomas. Furthermore, FGF-9 and AhR expression were well correlated in lung adenocarcinomas. These results suggest that AhR expression correlated positively with FGF-9 expression in lung adenocarcinomas, which might promote tumor growth by modulating communication between tumor cells and fibroblasts. Preventing AhR overexpression or disturbing FGF-9 function may reduce the development of lung adenocarcinomas.

Key words: aryl hydrocarbon receptor; fibroblast growth factor-9; lung adenocarcinoma

Lung cancer is the leading cause of cancer mortality worldwide.[1] Although the incidence of lung adenocarcinoma is growing globally and it has been the most common type of lung cancer in the past 10 years, its etiology remains relatively unclear.[2,3] It has been suggested that the tumor microenvironment, a complex interactive system of epithelial cells, endothelial cells, fibroblasts, and immune cells, among others, is critical for cancer development.[4] Interactions between tumor microenvironment and precancer cells promote tumor progression. Many pro-inflammatory cytokines and growth factors participate in cell–cell interactions. Identifying the changes in the microenvironment of lung adenocarcinomas could facilitate prevention strategies.

Benzo[a]pyrne (BaP) is an animal carcinogen and is present in environmental risk factors for lung cancer, such as cigarette smoke, air pollutants, and cooking oil fumes.[5–7] Recently, it has been reported that BaP treatment increases FGF-9 mRNA levels in human lung adenocarcinoma cells.[8] FGF-9 is an essential regulator of lung development and recovery from hypoxia-induced injury.[9,10] FGF-9 might also play a role in tumor development since it initiates communication between premalignant prostate tumor and stromal cells.[11] Furthermore, FGF-9 promotes neoplastic transformation of epithelial cells, as well as invasion of epithelial and endothelial cells.[12] Therefore, it is important to investigate the regulation of BaP-induced FGF-9 expression in human lung cancer cells and tissues.

BaP is an AhR agonist and increases tumor incidence in an AhR-dependent manner.[13] Recently, we had reported that AhR is overexpressed in bronchioloalveolar carcinomas (a noninvasive neoplasia that eventually progresses to invasive adenocarcinoma) and adenocarcinomas and that AhR overexpression is more prevalent in adenocarcinomas than in squamous cell carcinoma.[14] AhR is a ligand-activated transcription factor and upon activation, it upregulates expression of a number of genes, including cytochrome P450 family 1 (CYP1A1, CYP1B1) and growth factors.[15,16] However, in the absence of exogenous AhR agonists, AhR overexpression also upregulated constitutive expression of cytochrome P4501B1 in lung adenocarcinoma cells.[14] Similarly, AhR and CYP1B1 overexpression in adenocarcinoma tissues was independent of cigarette smoking, which is known to activate AhR.[14] The objective of the present study was to investigate whether FGF-9 expression is regulated by AhR activation and/or overexpression in human lung adenocarcinoma cells and tissues.

Material and methods

Chemicals

BaP, DBaA, BkF, BaA, NAC and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). TCDD and PCB126 were purchased from ULTRA Scientific (Kingston, RI). PCB39 was purchased from AccuStandard Inc. (New Haven, CT). PCDD and

Abbreviations: AhR, aryl hydrocarbon receptor; BaA, benz[a]anthracene; BaP, benzo[a]pyrene; BkF, benzo[k]fluoranthene; cAMP, cyclic adenosine monophosphate; cDNA, complementary deoxyribonucleic acid; CRE, cAMP-responsive elements; CYP1A1, cytochrome P450 superfamily 1A1; CYP1B1, cytochrome P450 superfamily 1B1; DBaA, dibenz[a]anthracene; DMF, 3′,4′-dimethoxyflavone; DMSO, dimethylsulfoxide; DRE, dioxin response element; EGF, epidermal growth factor; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAH, halogenated aromatic hydrocarbons; mRNA, messenger ribonucleic acid; NAC, N-acetylcysteine; NSCLC, non-small cell lung cancer; PAH, polycyclic aromatic hydrocarbons; PCB39, 3,4′,5-trichlorobiphenyl; PCB126, 3,3′4,4′5′-pentachlorobiphenyl; PCDD, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin; PCDF, 2,3,4,7,8-pentachlorodibenzofuran; RNA, ribonucleic acid; RNAi, ribonucleic acid interference; RT-PCR, reverse transcription polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorobenzo-*p*-dioxin.

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TABLE I – SEQUENCES OF PRIMERS USED AND PCR PRODUCT SIZES OF FGFR2b, FGFR2c, FGFR3b AND FGFR3c

Gene	Primers	Sequences	PCR length (bp)	GeneBank no.
FGFR2b	R2b-F	5′-TGGTCGGAGGAGACGTAGAG-3′	292	NM_000141
	R2b-R	5′-CTTGCTGTTTTGGCAGGAC-3′		
FGFR2c	R2c-F	5′-TGGTCGGAGGAGACGTAGAG-3′	195	NM_022970
	R2c-R	5′-AAAGTTACATTCCGAATATAGAGAACC-3′		
FGFR3b	R3b-F	5′-GGAGTTCCACTGCAAGGTGT-3′	256	NM_022965
	R3b-R	5′GTGAACGCTCAGCCAAAAG-3′		
FGFR3c	R3c-F	5′-GGAGTTCCACTGCAAGGTGT-3′	437	NM_000142
	R3c-R	5′-AAGCGGGAGATCTTGTGC-3′		

PCDF were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). DMF was purchased from INDOFINE Chemical Company, Inc. (Hillsborough, NJ). RPMI1640 medium, penicillin G and streptomycin were obtained from GIBCO BRL (Gaithersburg, MD).

Cell culture and transient transfection

Six human lung adenocarcinoma cell lines, A549, CL1-0, CL5, H23, H1299 and H1355, 2 human lung squamous cell carcinoma cell lines, CH27 and H226, 1 human lung fibroblast cell line, WI-38, and 1 human bronchial epithelial cell line, BEAS-2B were used in this study. H1355 and CL5 cells are gifts from Dr. C-M. Tsai (Veterans General Hospital-Taipei, Taiwan, ROC), were maintained in RPMI1640 medium containing 10% fetal bovine serum (FBS). AhR-interfered cell lines (si1414-6, si1414-7) isolated from H1355 cells have been described previously.[14] The human bronchial epithelial cell line BEAS-2B cells immortalized with SV40 (American Type Culture Collection, Manassas, VA) were maintained in serum-free Laboratory of Human Carcinogenesis-9 (LHC-9) medium (Invitrogen, Carlsbad, CA). WI-38 cells, a gift from Dr T.-H. Ueng (National Taiwan University, Taiwan, ROC), were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% FBS. All cells were incubated in a 37°C incubator under a humidified atmosphere of 5% CO_2 and 95% air.

Human AhR cDNA, a generous gift from Dr. C.A. Bradfield (Northwestern University Medical School, Chicago, IL), was inserted into pcDNA3 with 3 FLAG sequences 99 bp upstream of the start site. BEAS-2B cells were seeded in to 6-well plates and cultivated until cells reached 60% confluency (24 hr). AhR cDNA was transfected into BEAS-2B cells with the JetPEI cationic polymer transfection reagent (Polyplus-transfection, Inc., New York, NY). After 48 hr incubation, transfected BEAS-2B cells were collected for quantification of CYP1B1 and FGF-9 mRNA and AhR protein levels. Transcriptional activity of human FGF-9 promoter induced by AhR agonists was examined by the pGL3-FGF-9 (a kind gift from Dr. H. Sunny Sun, National Cheng Kung University, Taiwan), which carries a human FGF-9 promoter.[17] H1355 cells were transiently cotransfected with pGL3 or pGL-3-FGF-9 with pCMV-β-galactocidase using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and treated with 0.1% DMSO, 1 nM TCDD or 1 μM BaP for 48 hr. Transfected cells were then lysated by lysis buffer (Promega, Madison, WI). Expression of firefly luciferase was analyzed by luciferase analysis kit (Promega, Madison, WI). Luciferase activity normalized with β-galactocidase represents FGF-9 promoter activity. Induction of FGF-9 promoter activity was normalized by its activity in pGL-3 transfected cells which served as the vehicle control.

Evaulation for cell growth of CL5 and WI-38 cells

CL5 or WI-38 cells were incubated with 10 ng/ml FGF-9 (R&D Systems, Minneapolis, MN) in RPMI1640 medium or DMEM containing 1% FBS for 3 days respectively. To obtain FGF-9-conditioned medium, WI-38 cells were incubated with or without 10 ng/ml FGF-9 in DMEM containing 1% FBS for 1 day. After 1 day incubation, conditioned media were collected and mixed with an equal volume of RPMI1640 medium containing 1% FBS. CL5 cells were then cultivated with this conditioned medium with or without 1 μg/ml antihuman FGF receptor 2 (FGFR2) (R&D Systems, Minneapolis, MN) for 3 days. According to manufacturer's instruction, this anti-human FGFR2 neutralized both FGFR2b and 2c and the neutralization efficiency for FGFR2b is 5 to 10 fold higher than for FGFR2c. Cell growth of CL5 and WI-38 cells were measured with 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Enzyme-linked immunosorbent assay

CL5 cells were treated with 0.1% DMSO, 1 μM BaP or 1 nM TCDD for 1 day and the media were collected for quantifying FGF-9 concentration by using the enzyme-linked immunosorbent assay (ELISA) method. Assays were performed in an anti-FGF-9 coated 96-well plate (human FGF-9 ELISA Kit, Raybio, Inc, Norcross, GA). Briefly, 100 μl of media were added into each well in duplicate, and then the plate was incubated at 4°C for overnight. Assays were performed according to manufacturer's instruction. The absorbance at 450 nm was measured.

Reverse transcription polymerase chain reaction assay

RNA was extracted with chloroform from cells stored in TriReagent (Life Technologies, Rockville, MD). Synthesis of cDNA was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using 1.5 μg of total RNA. The primer sequences for detecting FGFR2b, FGFR2c, FGFR3b and FGFR3c, and expected size of PCR products were listed in Table I. PCR was subjected to 35 cycles of amplification (30 sec denaturation at 95°C, 30 sec annealing at 57°C, and 1 min elongation at 72°C) after a 5 min denaturation at 95°C, and following by final elongation at 72°C for 5 min. These PCR products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide for visualizing PCR products.

Quantitative real-time RT-PCR assay

cDNA was prepared as previously described. Quantitative PCR was carried out using the TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and analyzed on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primer sequences and optimal primer concentrations for AhR, CYP1B1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been described previously.[14,18] Briefly, primers for AhR were 5′-AGAGTCTGGACAAGGAA TTGAA-3′ and 5′-GAAGTGGAGTAGCTATCGCAAA-3′, for GAPDH were 5′-GCACCGTCAAGGCTGAGAAC-3′ and 5′-GC CTTCTCCATGGTGGTGAA-3′, as well as for CYP1B1 5′-CGG CTGGATTTGGAGAACGTA-3′ and 5′-TGATCCAATTCTGC CTGCACT-3′. Probe of AhR was 5′-TTCCCTTGGAAATTCAT TGCCAGAA-3′, of GAPDH was 5′-CCCATCACCATCTTCC AGGAGCGAG-3′, as well as of CYP1B1 5′-TCTTCACCAGGT ATCCTGATGTGCAGACTC-3′. Primers and probes for FGF-9 were purchased from TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA). PCR reactions consisted of an initial step of 2 min at 50°C, a polymerase activation step for 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Quantitative values were obtained from the threshold cycle (C_T) number. Target gene expression levels in each sample were

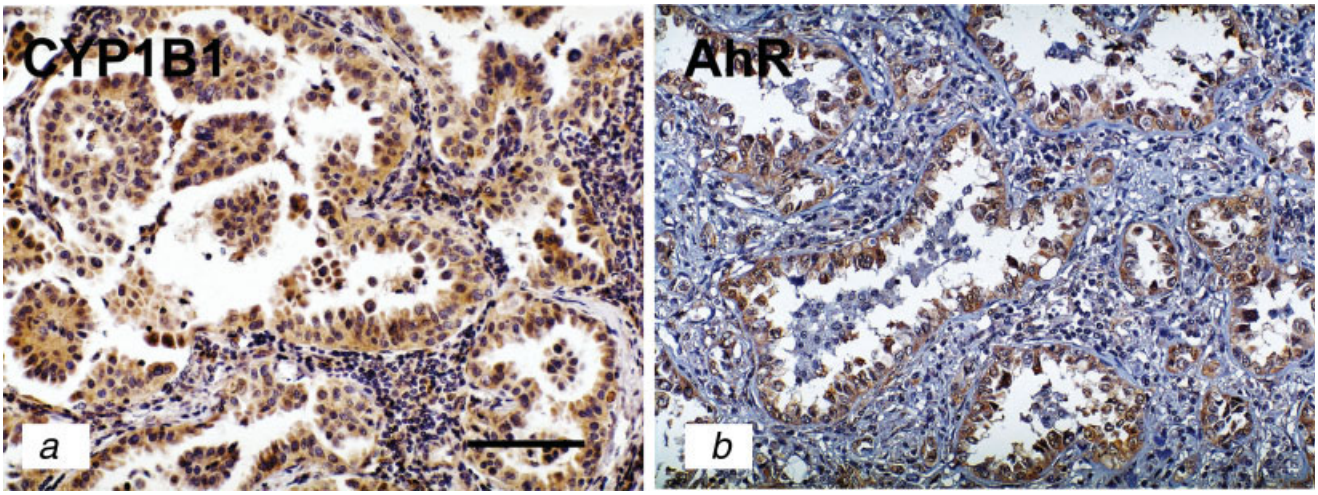

Figure 1 – Immunostainings for CYP1B1 and AhR in human lung adenocarcinoma tissues. Tumor cells appeared to be strongly positive for both CYP1B1 and AhR staining (*a*) and (*b*) respectively). The immunostaining (appeared as golden brown stains) were primarily located in the cytoplasm of the tumor cells (scale bar, 50 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

normalized to *GAPDH* mRNA expression. The relative mRNA levels of the target gene $= 2^{-\Delta Ct}$; $\Delta Ct = Ct_{target} - Ct_{18s\ rRNA}$.

Preparation of tissue microarrays

A total of 146 primary NSCLCs were collected from Chung Shan Medical University Hospital between 1998 and 2006 (Institutional Review Board number: CS05091). Lung tissue was fixed with 10% formalin and embedded in paraffin. The pathologist (Dr. Han Chang) used hematoxylin and eosin staining to confirm the tumor area and prepared core samples (2 mm in diameter). These cored tissues were placed in 3 array blocks (approximately 3 cm × 2 cm) and tissue microarrays were constructed by Everlight Biotech Co. Ltd. (Taichung, Taiwan).

Immunohistochemistry

Immunohistochemistry was performed as previously described.[19] The following primary antibodies were used: anti-AhR (1:90 dilution; Biomol, Plymouth Meeting, PA), anti-CYP1B1 (1:1500 dilution; clone WB-1B1, Gentest Corp., Woburn, MA), and anti-FGF-9 (1:50 dilution; R&D Systems, Minneapolis, MN). After incubation with the primary antibodies overnight at 4°C, a streptavidin-biotin peroxidase method was used according to the manufacturer's instructions (Universal LSAB2 kit, Dakocytomation, Glostrup, Denmark). Finally, the sections were counterstained with hematoxylin. Negative controls were performed by phosphate buffer or normal serum instead of primary antibody.

Evaluation of FGF-9, AhR and CYP1B1 protein expression in lung carcinoma tissues

FGF-9, AhR and CYP1B1 immunoreactivities were evaluated by immunostained lung tumors (Fig. 1 and 2). Vascular or bronchiolar walls in lung tissue and hyperplastic prostate sections were used as positive controls for CYP1B1 and AhR immunostaining, respectively.[14] In rats, FGF-9 is detectable in smooth muscle cells of normal carotid arteries.[20] del Moral *et al.*[21] also have observed *Fgf-9* expression in the distal airway epithelium of mouse fetal lung. Consistent with these reports, we used vascular walls of lung tumor tissues and human fetal lungs as FGF-9 positive controls. The immunoreactivity of AhR, CYP1B1 or FGF-9 in lung tumors was graded against positive controls. Immunostaining intensity was scored as a mean value of pixel brightness using the Metamorph 7 image system (Molecular Devices, Downington, PA). Each pixel from RGB images was converted to a grayscale value by the MetaMorph imaging software. Pixel brightness is inversely related to grayscale values, which represents immunostaining intensity. Pixel brightness values of each sample were automatically measured by MetaMorph imaging software. Firstly, the pixel brightness of positive controls was measured in 4 prostatic and 7 lung tissues. Positive control values for CYP1B1, AhR and FGF-9 were 112 ± 24, 127 ± 7 and 117 ± 5 (mean ± standard deviation), respectively. Pixel values of lung tumor samples were measured and compared with of positive controls values. The values of lung tumor samples for CYP1B1, AhR and FGF-9 stains were 136, 134, and 122 (mean plus 1 standard deviation) as cutoff values, respectively. When the tumor values were equal to or lower than the cutoff value, tumor was rated as a high expresser; whereas higher values were graded as low expressers.

Immunoblotting

Cytosolic homogenates were prepared for AhR and FGF-9 immunoblotting as described previously.[19] An aliquot of these extracts were separated by SDS-PAGE (using 10% gels) and transferred to a polyvinylidene difluoride membrane. Membranes were blotted either with anti-AhR (Biomol, Plymouth Meeting, PA), anti-Flag (Sigma-Aldrich, St. Louis, MO) or anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were visualized using an enhanced chemiluminescence kit according to manufacturer's instructions (Amersham, Buckinghamshire, UK).

RNA interference knockdown of AhR expression in H1355 cells

A plasmid containing short hairpin RNA complementary to AhR was generated as previously described.[14] Briefly, 2 oligonucleotides containing partial complementary sequences for a short hairpin RNA template with an overlapping loop, i-AhR-F (5′-GAT CCG AAT ACT TCC ACC TCA GTT GGC TTC AAG AGA-3′) and i-AhR-R (5′-AGC TTC CAA AAA AAA TAC TTC CAC CTC AGT TGG CTCTCT TGA A), were synthesized, ligated with pcDNA3.1/HU6 and *E. coli* XL-10 cells were transformed with it.[22,23] Vector containing short hairpin RNA was transfected into H1355 cells to select stable RNAi clones.

Inducible RNA interference of AhR *in H1355 cells*

To perform AhR RNA interference (RNAi), an expressed short hairpin RNA was driven by an inducible human U6 promoter in the pSingle-tTS-shRNA vector (Clontech, Mountain View, CA). To generate shRNA template, 2 oligonucleotides, i-*AhR*-F (5′-TCG AGG AAT ACT TCC ACC TCA GTT TTC AAG AGA AAC TGA GGT GGA AGT ATT CTT TTT TA-3′) and i-*AhR*-R (5′-AGC TTA AAA AAG AAT ACT TCC ACC TCA GTT TCT

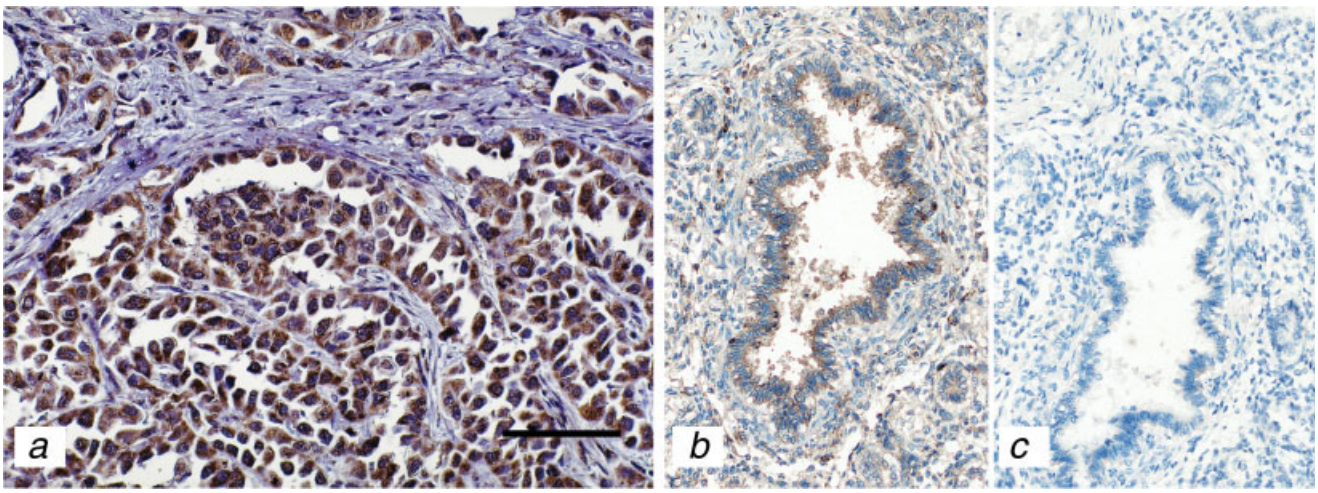

FIGURE 2 – Immunostaining for FGF-9 in human lung adenocarcinoma tissues. (*a*) Strong FGF-9 staining (dark brown color) was demonstrated in the cytoplasm of all the tumor cells. (*b*) Positive control (normal human fetal lung tissue) for FGF-9 staining. (*c*) Negative control for FGF-9 staining was done by performing the staining procedure on normal human fetal lung tissue with normal serum (scale bar, 50 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CTT GAA AAC TGA GGT GGA AGT ATT CC-3′), were synthesized and ligated with pSingle-tTS-shRNA. The ligated product was sent to *E. coli* XL-10 cells. DNA sequences of inserted AhR RNAi were confirmed by sequencing and then transiently transfected into H1355 cells. Transfected cells were treated with 1 μg/ml doxycycline (Clontech, Mountain View, CA) for 48 hr to express the shRNA.

Statistical analysis

Correlation between clinical features and expression of AhR, CYP1B1 and FGF-9 was examined with Pearson χ^2 test and multivariate logistic regression (SPSS 8.0 statistical software package, SPSS, Inc., Chicago, IL). For cell culture experiments, data were compared using the student *t*-test. $p < 0.05$ was considered statistically significant.

Results

Expression of CYP1B1, AhR and FGF-9 in human lung cancer tissues

Immunostainings for CYP1B1 and AhR in human lung cancer tissues as well as in normal bronchiolar epithelia have been demonstrated in our previous report.[14,19] Immunoreactivity for CYP1B1 and AhR was negative or very weak in normal bronchiolar epithelia. Most lung carcinomas are found to be positive in both CYP1B1 (Fig. 1*a*) and AhR (Fig. 1*b*) immunostaining and the immunoreactivity are primarily located in the cytoplasm of the tumor cells (Figs. 1*a* and 1*b*). In our present study, we further demonstrated strong FGF-9 positivity in the cytoplasm of many tumor cells of lung cancer, especially lung adenocarcinoma (Fig. 2*a*).

Noncancerous tissues, such as the adjacent bronchiolar epithelium and the alveolar cells/stroma, are totally negative in FGF-9 staining. To assure specificity of our FGF-9 staining in the tumor specimens, both positive control (distal airway epithelium) and negative control (staining with normal serum) were performed on normal human fetal lung (Figs. 2*b* and 2*c*). Our study provided specific demonstration of the association of elevated expression not only of CYP1B1 and AhR but also of FGF-9 in human lung tumors.

Effects of AhR activation on FGF-9 expression in CL5 human lung cancer cells

Previously, it was demonstrated that BaP increased FGF-9 mRNA levels in CL5 human adenocarcinoma cells.[8] Treatment of CL5 cells with 0.1, 1 or 10 μM BaP increased *FGF-9* levels to 2.2, 2.7 or 2.7 fold of control levels, respectively (Fig. 3*a*). PAHs and HAH, such as BaP and TCDD, are AhR agonists[24] that upregulate *CYP1A1* and *CYP1B1* expression.[15,25] All PAHs (BaP, BaA, BkF and DBaA) and HAHs (PCB126, TCDD, PCDD and PCDF) tested in our study significantly increased the expression of CYP1B1 and FGF-9 mRNA in CL5 cells (Fig. 3*b*), except PCB39 which has a weaker TCDD-like activity.[26] Cotreatment of CL5 cells with DMF, an AhR antagonist, significantly reduced *FGF-9* induction by BaP from 3.4- to 2.0-fold of control (Fig. 3*c*). These results suggest that BaP-induced FGF-9 expression was, at least partially, dependent on AhR activation. Furthermore, AhR agonists, TCDD and BaP, significantly increased FGF-9 promoter activity in human lung adenocarcinoma cells H1355 (Fig. 3*d*), indicating that AhR agonists increases FGF-9 transcription. Consistent with the results of Figure 3*b*, treatment with 1 μM BaP or 1 nM TCDD significantly increased FGF-9 protein secreted into the media of CL5 (Fig. 3*e*).

Effects of AhR expression on FGF-9 expression in lung cells

Previously, we established that AhR interfered stable clones in human lung adenocarcinoma cells H1355, and reducing AhR expression in these clones not only prevented CYP1A1/CYP1B1 induction by AhR agonists, but also reduced constitutive CYP1B1 levels.[14] Similarly, reducing AhR expression in the stable clone Si1414-6 significantly reduced constitutive FGF-9 expression and BaP-induced FGF-9 expression (Fig. 4*a*). With transient induction of AhR interference, AhR, FGF-9 and CYP1B1 mRNA levels were simultaneously reduced (Fig. 4*b*). Conversely, transient overexpression of AhR protein in BEAS-2B cells further enhanced both constitutive and BaP-induced FGF-9 and CYP1B1 expression (Fig. 4*c*). AhR and FGF-9 mRNA levels were determined in 6 lung adenocarcinoma and 2 squamous cell carcinoma cells. A positive correlation between AhR and FGF-9 mRNA levels was observed ($R^2 = 0.742$, $p = 0.027$) in 6 adenocarcinoma cell lines but not in the 8 human lung carcinoma cell lines ($R^2 = 0.393$, $p = 0.096$) (Fig. 4*d*). This result suggested that correlation between FGF-9 and AhR expression may specifically occur in human lung adenocarcinomas, but not in squamous cell carcinomas. Previously, we have shown a positive correlation between AhR expression and intracellular oxidative stress in lung adenocarcinoma cells.[14] On the other hand, in order to clarify whether oxidative stress plays a role between AhR overexpression and FGF-9 induction, AhR overexpressed BEAS-2B cells were treated with 1 mM NAC to prevent oxidative stress. However, NAC treatment failed to prevent induction of FGF-9 expression by AhR overexpression (Fig. 4*e*). These results suggest that AhR

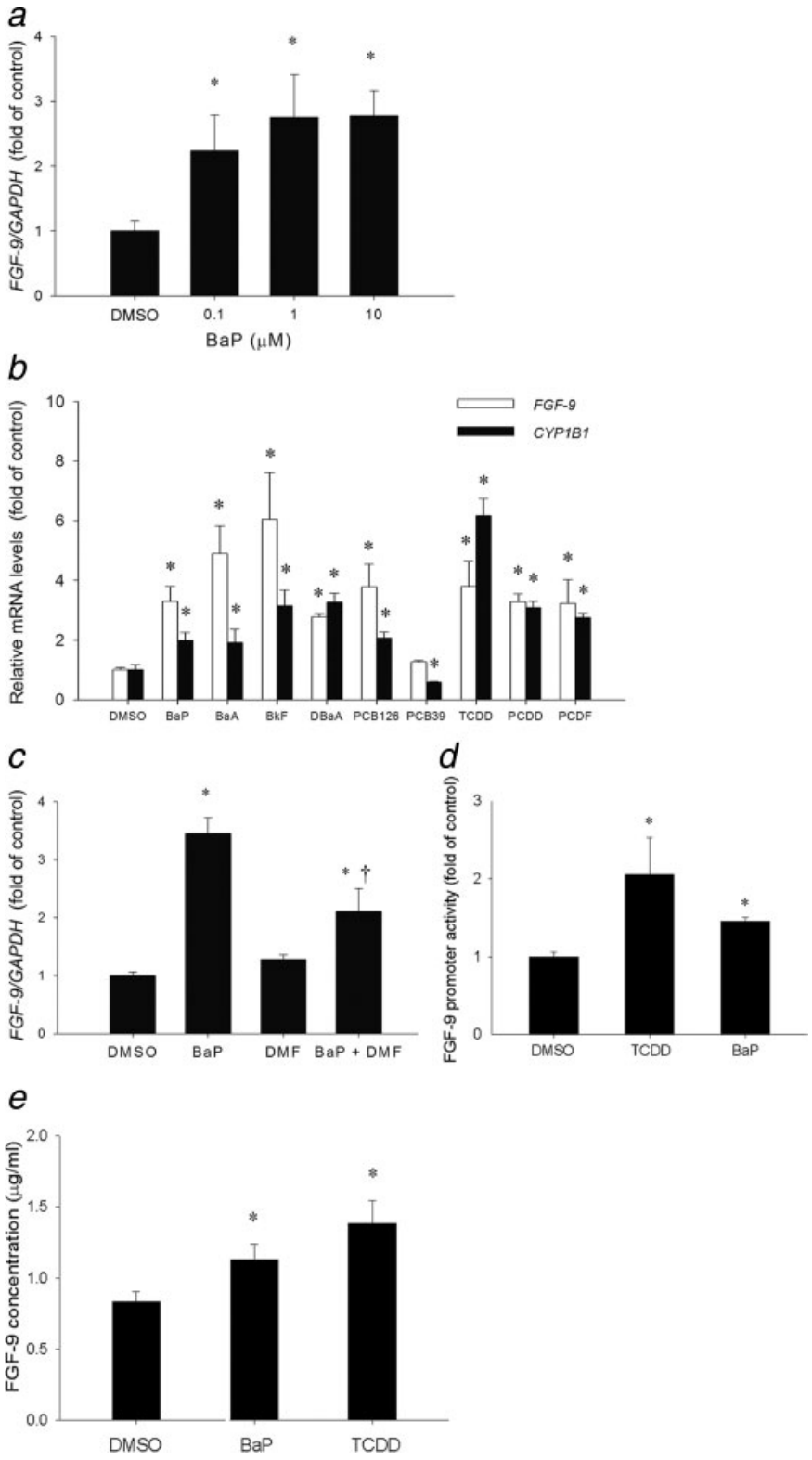

FIGURE 3 – AhR activation increased FGF-9 expression in lung adenocarcinoma cells. (*a*) CL5 cells were treated with 0.1% DMSO or BaP (0.1, 1 or 10 μM) for 6 hr. (*b*) CL5 cells were treated with 0.1% DMSO, PAH (10 μM BaP, BaA, BkF, or DBaA), PCB (10 μM PCB126 or PCB39) or dioxin (10 nM TCDD, PCDD or PCDF) for 6 hr. (*c*) CL5 cells were treated with 0.1% DMSO, 1 μM BaP and/or 20 μM 3′4′-DMF for 6 hr. Relative CYP1B1 or FGF-9 mRNA levels were measured using the real-time RT-PCR. Data are presented as the mean ± SD ($n = 3$). *$p < 0.05$, compared with DMSO-treated cells; †$p < 0.05$, compared with 1 μM BaP-treated cells. (*d*) H1355 cells were transiently transfected with pGL3-FGF-9 and then following by treatment with 0.1% DMSO, 1 nM TCDD or 1 μM BaP for 48 hr. FGF-9 promoter activity was presented as folds of control and normalized by the FGF-9 promoter activity in 0.1% DMSO treated cells. Data are presented as the mean ± SD ($n = 4$). *$p < 0.05$, compared with 0.1% DMSO-treated cells. (*e*) CL5 cells were treated with 0.1% DMSO, 1 μM BaP or 1nM TCDD for 1 day. Concentration of secreted FGF-9 was determined with ELISA in CL5 media. Data are presented as the mean ± SD ($n = 5$). *$p < 0.05$, compared with 0.1% DMSO-treated cells.

expression is correlated with FGF-9 expression in human lung cells and is independent of oxidative stress.

Effects of FGF-9 on cell growth and communication between fibroblasts and CL5 cells

Several studies suggest that FGF-9 increases growth of cancer cells, and plays an important role in regulating communication between fibroblasts and cancer cells.[11,12] Treatment with FGF-9 (10 ng/ml) significantly increased growth of human lung WI-38 fibroblasts, but not CL5 cells (Fig. 5*a*). However, when CL5 cells were cultured with conditioned media from FGF-9-treated WI-38 cells, growth of CL5 cells increased 1.6 folds, compared with cells cultivated with conditioned media from untreated WI-38 cells (Fig. 5*b*). Thus, it appears that FGF-9 may stimulate secretion of

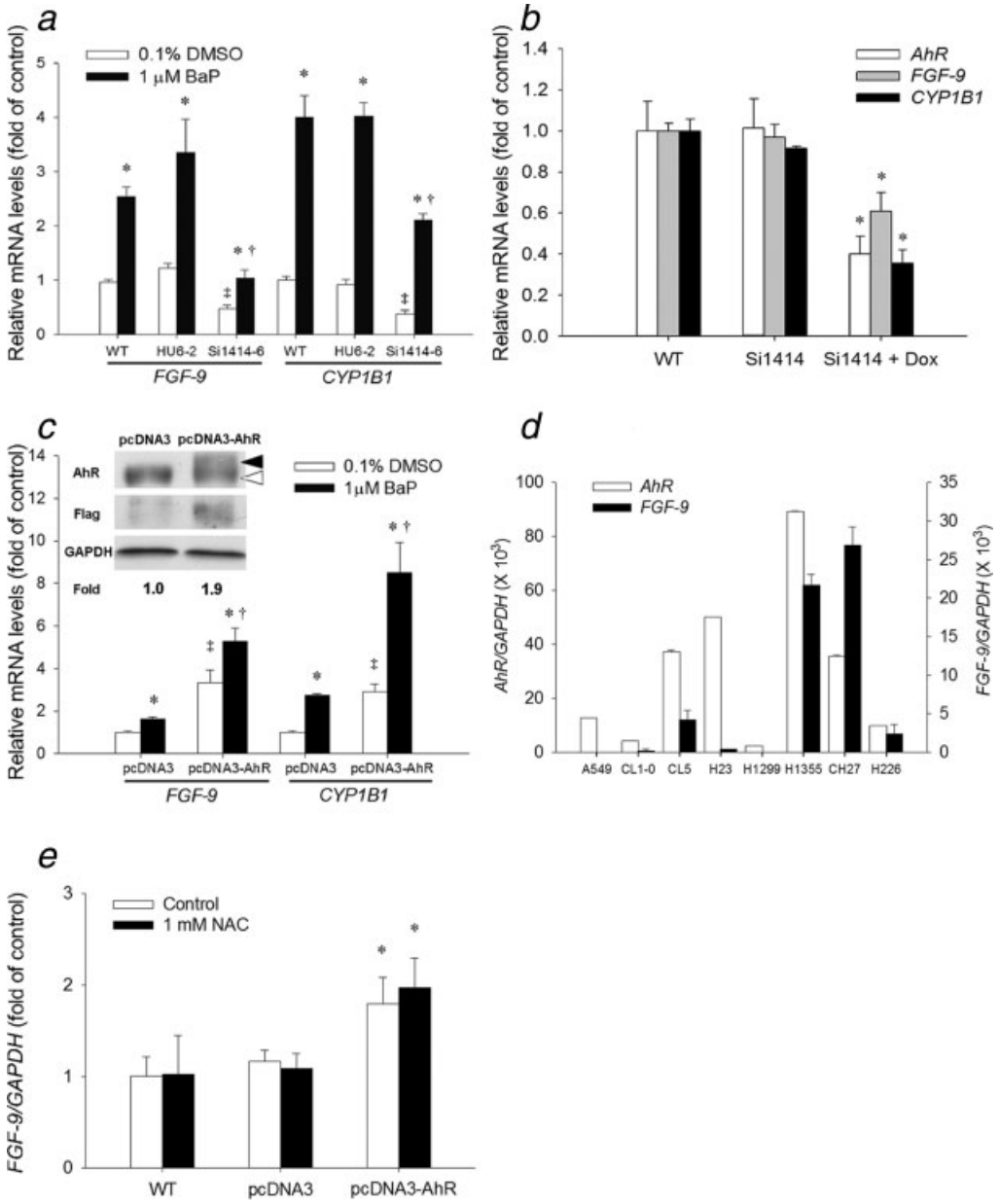

Figure 4 – Effect of AhR RNAi or overexpression on AhR, FGF-9 and CYP1B1 expression in lung cells. (*a*) The AhR interfered stable clone Si1414-6 was treated with 0.1% DMSO or 1 μM BaP for 24 hr. WT, wild type; HU6-2, vector control; Si1414-6, AhR interfered clones; *$p < 0.05$, compared with DMSO controls; † or ‡ $p < 0.05$, compared with BaP- or DMSO-treated WT cells, respectively. (*b*) AhR interference was transiently induced by doxycycline in H1355 cells for 48 hr. AhR, WT, wild type; Si1414, AhR interfered cells; Si1414 + Dox, AhR interfered cells treated with 1 μg/ml doxycycline; *$p < 0.05$, compared with wild type. (*c*) FLAG-AhR was transiently overexpressed in BEAS-2B cells. FLAG-AhR and endogenous AhR protein levels were determined by immunoblotting and normalized to GAPDH. Total AhR was increased to 1.8-fold of vector control (pcDNA3). *$p < 0.05$, compared with vector control. Insert: Western blots of AhR, FLAG-AhR and GAPDH. The black arrowhead indicates the FLAG-AhR protein. The white arrowhead indicates endogenous AhR protein. (*d*) AhR and FGF-9 mRNA levels in 6 human adenocarcinoma cell lines and 2 squamous cell carcinoma cell lines. (*d*) FLAG-AhR was transiently overexpressed in BEAS-2B cells and treated with 1 mM NAC for 48 hr. WT, wild type; *$p < 0.05$, compared with WT BEAS-2B cells. All mRNA levels were determined using real-time RT-PCR.

unknown factor(s) from lung fibroblasts that promote growth of lung cancer cells. Typically, FGFs bind to FGFR2b, 2c, 3b and 3c for activating downstream signaling pathways. Therefore, we further examined these FGFRs expression in WI-38 and CL5 cells by using RT-PCR method. As shown in Figure 5*c*, WI-38 cells only expressed FGF3b and 3c, whereas CL5 cells expressed all FGFR2b, 2c, 3b, and 3c. Since FGFR2 is critical for proliferation of lung epithelial cells, blocking FGFR2 function might prevent growth of CL5 cells induced by conditioned media of FGF-9-treated WI-38 cells. As shown in Figure 5*d*, cotreatment with 1 μg/ml anti-FGFR2 prevented growth of CL5 cells induced by conditioned media. These results suggested that FGFR2 might play an essential role for the communication between lung tumor cells and stromal fibroblasts.

Association between FGF-9 and AhR or CYP1B1 expression in NSCLC cases

Eighty-six of the 146 NSCLC samples expressed high levels of FGF-9 protein and it was prevalent in adenocarcinomas (76.7%)

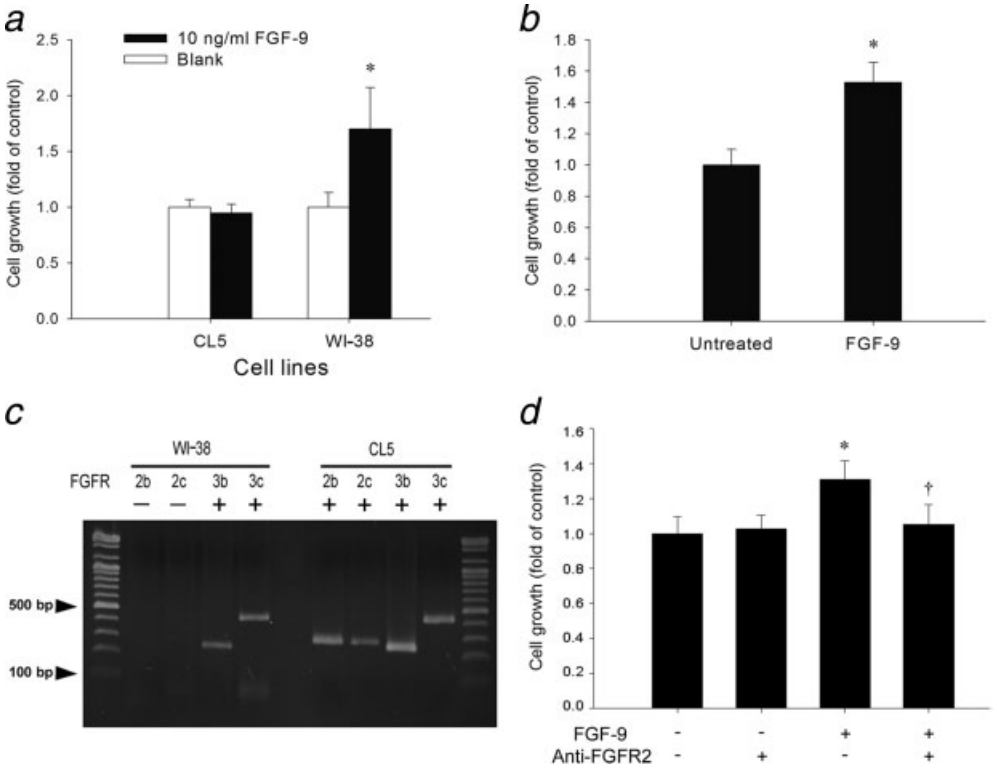

FIGURE 5 – Effects of FGF-9 and conditioned media of WI-38 cells on growth of CL5 cells. (*a*) CL5 and WI-38 cells were treated with FGF-9 (10 ng/ml) for 3 days. *$p < 0.05$, compared to blank WI-38 cells. (*b*) WI-38 cells were incubated with or without FGF-9 (10 ng/ml) for 1 day. CL5 cells were then cultivated in conditioned media of WI-38 cells for 3 days. (*c*) Expression of *FGFR2b, 2c, 3b,* and *3c* in CL5 and WI-38 cells. Expression of *FGFR2b*, *2c*, *3b* and *3c* were detected using RT-PCR. CL5 cells expressed FGFR2b, 2c, 3b and 3c, whereas WI-38 cells expressed FGFR3b and 3c only. (*d*) WI-38 cells were incubated with or without FGF-9 (10 ng/ml) for 1 day. CL5 cells were then cultivated in conditioned media of WI-38 cells with or without anti-FGFR2 (1 μg/ml) for 3 days. Cell growth was determined with the MTT assay. All data of MTT assay are the mean of at least twice from 8 replicates each time. *$p < 0.05$, compared to conditioned media of untreated WI-38 cells. †$p < 0.05$, compared to conditioned media of FGF-9-treated WI-38 cells.

TABLE II – FGF-9 EXPRESSION AND CLINICAL FEATURES OF PATIENTS WITH NSCLCs

	All cases (%)	Numbers of high FGF-9 expression (%)	*p* value[1]	Odds ratio (95% CI)[2]
Total	146 (100)	86 (100)		
Age (year)			0.542	
<55	18 (12.3)	11 (12.8)		1.00
55–64	40 (27.3)	22 (25.5)		0.79 (0.09–7.03)
65–74	58 (39.7)	32 (37.2)		0.61 (0.08–4.23)
≥75	30 (20.5)	21 (24.4)		0.66 (0.10–4.31)
Gender			0.165	
Male	90 (61.6)	49 (56.9)		1.00
Female	56 (38.3)	37 (43.0)		0.80 (0.25–2.48)
Tumor type			0.002	
AD	97 (66.4)	66 (76.7)		3.81 (1.27–1.50)*
SQ	49 (33.5)	20 (23.2)		1.00
Cigarette smoking			0.052	
Yes	76 (52.0)	39 (45.3)		1.00
No	70 (47.9)	47 (54.6)		1.17 (0.33–4.10)

CI, confidence interval; AD, adenocarcinoma; SQ, squamous cell carcinoma.
[1]Pearson χ^2 test was used to compare categorical variables *versus* frequencies of high FGF-9 expressers; differences in data were considered statistically significant at *p* value < 0.05.–[2]Multivariate logistic regression modeling was used to adjust age, gender and cigarette smoking. **p* value < 0.05.

and associated with tumor type ($p = 0.002$) (Table II). FGF-9 expression in NSCLC was not associated with age, gender or smoking status, and by multivariate logistic regression only tumor type was independently associated with expression of FGF-9 (Table II). Accordingly, adenocarcinomas cases were more likely to show high FGF-9 expression compared to squamous cell carcinomas (odds ratio, 3.81; 95% confidence interval, 1.275–1.509). Previously we reported that AhR and CYP1B1 expression were more prevalent in adenocarcinomas than in squamous cell carcinomas.[14] FGF-9 expression was well correlated with AhR and CYP1B1 ($P < 0.001$) in NSCLC cases, especially adenocarcinomas (Table III).

TABLE III – CORRELATION OF FGF-9, AHR AND CYP1B1 EXPRESSION IN HUMAN NSCLCs

	Numbers of high FGF-9 expression (%)		
	Total	AD	SQ
AhR expression			
Low	30 (34.9)	19 (28.8)	11 (55.0)
High	56 (65.1)	47 (71.2)	9 (45.0)
p value*	<0.001	0.001	0.458
CYP1B1 expression			
Low	19 (22.1)	8 (12.1)	11 (55.0)
High	67 (77.9)	58 (87.9)	9 (45.0)
p value*	<0.001	0.001	0.126

AD, adenocarcinoma; SQ, squamous cell carcinoma.
*Pearson χ^2 test, significant at the level of $p < 0.05$.

Discussion

Aberrant FGF-9 expression has recently been reported in human cancers, including ovarian endometrioid adenocarcinoma and prostate cancer.[11,12] FGF-9 is a critical regulator of proliferation of airway epithelium and mesenchymal growth during lung embryogenesis.[10] In the present study, we show that FGF-9 expression was more common in lung adenocarcinomas than in lung squamous cell carcinomas, and positively correlated with AhR expression in both lung adenocarcinoma cell lines and clinical cases. FGF-9 expression was also upregulated *via* AhR signaling and AhR overexpression in lung adenocarcinoma cells. Treatment of lung fibroblasts with FGF-9 may induce secretion of other growth factors that increase proliferation of lung adenocarcinoma cells. These results suggest that AhR overexpression may alter communication between fibroblasts and tumor cells *via* FGF-9, thereby promoting growth of lung adenocarcinomas.

Several lines of evidence indicates that FGF-9 mRNA is elevated in lung adenocarcinoma cells by AhR activation. In CL5 cells, AhR agonists but not non-AhR agonist (PCB39) increased FGF-9 mRNA levels. FGF-9 induction was significantly reduced by cotreatment with an AhR antagonist. In H1355 cells, *AhR* expression positively modulated the BaP-induced increase in FGF-9 mRNA. It is well known that activated AhR heterodimerizes with AhR nuclear translocator (Arnt) are present in the nucleus where they interact with DRE in the promoter region of some genes (*e.g. CYP1B1*).[27] Three DREs were located at −556, −1186 and −1413 bp from the transcription start site of the FGF-9 promoter region.[17] Indeed, AhR agonists enhanced FGF-9 promoter activity. Thus, activated AhR might increase transcription of *FGF-9*.

Another major finding of this study is that FGF-9 is primarily expressed in lung adenocarcinomas and is well correlated with AhR and CYP1B1 expression in NSCLCs. In the absence of AhR agonists, basal levels of FGF-9 and CYP1B1 mRNA in H1355 lung adenocarcinoma cells were modulated by AhR expression. Although cigarette smoke is an AhR agonist[28,29] and habitual cigarette smoking is associated with elevated CYP1A1 expression in lung adenocarcinomas,[14] neither FGF-9 nor CYP1B1 expression were associated with cigarette smoking in the NSCLCs. Because FGF-9 and CYP1B1 expression were correlated in lung adenocarcinomas, it is likely that exogenous ligand-free AhR protein modulates CYP1B1 and FGF-9 mRNA levels *via* a common pathway. Cyclic AMP (cAMP) also regulates CYP1B1 expression.[30] There are 2 CREs in the CYP1B1 promoter region.[31] The FGF-9 promoter region also contains 1 CRE-like sequence at the −256 bp position. In the absence of AhR agonists, cAMP can trigger nuclear translocation of AhR.[32] Therefore, AhR protein may interact with a common cofactor or messenger, such as cAMP, to upregulate CYP1B1 and FGF-9 expression.

NAC is an antioxidant that prevents BaP-induced elevation of FGF-9 mRNA in CL5 cells.[8] Moreover, intracellular oxidative stress and FGF-9 mRNA is reduced in Si1414-6 cells when expression of AhR is downregulated.[14] Thus, it appears that AhR activation or overexpression increases oxidative stress and elevates FGF-9 mRNA in lung cancer cells. However, our results demonstrated that NAC treatment in AhR-overexpressed BEAS-2B cells failed to inhibit *FGF-9* induction. It suggested that increased oxidative stress by AhR overexpression might not be involved in FGF-9 induction.

At least 22 types of FGF have been identified in a variety of organisms.[33] FGFs activates 4 different types of FGF receptors (FGFR); alternative splicing generates b and c isoforms.[34] FGF-9 binds to and activates FGFR2 and FGFR3, with greater affinity for the 'c' splice form.[11,35] FGF-9 specifically binds to stromal FGFR3 in a prostate tumor model.[11] During lung embryogenesis, the airway epithelium and pleura secrete FGF-9 to stimulate mesenchymal proliferation and migration.[10,21] FGF-9 also promotes neoplastic transformation of immortalized rat kidney epithelial (RK3E) cells and invasion of human endothelial and RK3E cells.[12] In the present study, FGF-9 induced growth of WI-38 lung fibroblasts, implying that WI-38 cells express FGFR2c and/or FGFR3c. Our data confirmed that WI-38 cells expressed FGFR3c, which may respond to FGF-9 stimulation. Although CL5 lung adenocarcinoma cells expressed both FGFR2c and 3c, they failed to respond to FGF-9 treatment. It is likely that either the binding affinity of FGF-9 to FGFR2/3c is abnormally low in CL5 cells, or signaling pathways downstream of FGFR2/3c are not activated following FGF-9 binding in CL5 cells. On the other hand, FGFR2b is required for FGF-9-induced epithelial proliferation in embryonic lung.[21] FGFR2b, is mainly found in epithelia and binds FGFs secreted from mesenchymal cells (*i.e.* FGF-1, FGF-3, FGF-7 and FGF-10).[10,36] Our results showed that CL5 cells expressed FGF2b and responded to other growth factors secreted from FGF-9-treated WI-38 cells. Furthermore, blocking of FGF2b by antibody reduced CL5 cell growth in conditioned medium from FGF-9-treated WI-38 cells. It is conceivable that expression of FGF2b in CL5 may render cells response to other growth factor secreted from FGF-9 treated WI-38 cells. These results suggest that FGF-9 might promote the development of lung adenocarcinomas by altering the paracrine loop of growth factors secreted from the surrounding stromal cells.

In the past, AhR was thought to regulate the expression of drug metabolizing enzymes involved in metabolic activation of some carcinogens.[15,37,38] More recently, several studies also demonstrated that AhR is a potential drug target for cancer therapy.[14,39] In prostate, ovarian, endometrial and breast cancers, AhR agonists inhibited the growth of tumor cells *via* mediating estrogen[40–43] or androgen receptors.[44–46] In pancreatic cancer, TCDD activated AhR and suppressed cell growth of pancreatic cancer cells by inducing cyclin-dependent kinase inhibitor 21.[39] Therefore, recent studies suggested that relative nontoxic AhR agonists could serve as a potential clinical treatment for breast, ovarian, pancreatic and endometrial cancers.[39,41,42,47] However, similar effects were not reported in liver cancer. These findings also suggested that the role of AhR and its agonist on cancer is highly tumor-specific. In the present study, we identified a developmentally regulated cancer-related gene, FGF-9, which is upregulated by AhR overexpression and activation in lung adenocarcinomas. We demonstrated that FGF-9 could change the tumor microenvironment and promoted growth of tumor cells. Thus, disruption or prevention of AhR overexpression or activation could modulate the tumor microenvironment and prevent development of lung adenocarcinomas.

Target therapy with inhibitors for EGF receptor has been effective in lung adenocarcinoma patients.[48,49] Similar to EGF, FGFs interact with FGF receptors to activate distinct signaling pathways.[34] Inhibitors for tyrosine kinase activity of FGF receptors were suggested to have anti-neoplastic activity in pituitary tumor and thyroid carcinoma.[50,51] Thus, our present results suggest that interruption of FGF9-stimulated signaling pathways may be a potential target for therapy for lung adenocarcinomas.

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