

Hepatocyte growth factor and HER2/neu downregulate expression of apoptosis-inducing factor in non-small cell lung cancer

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Received August 9, 2013; Accepted September 27, 2013

DOI: 10.3892/or.2013.2867

Abstract. Our previous study showed that patients with advanced stages of non-small cell lung cancer (NSCLC) were frequently detected with upregulation of hepatocyte growth factor (HGF). *In vitro*, HGF reduced expression of apoptosis-inducing factor (AIF) and cisplatin sensitivity in NSCLC cells. The effect of HGF was via HGF receptor (c-MET) and the downstream effector, focal adhesion kinase (FAK). In this study, we determined the prognostic value of AIF in NSCLC patients. AIF expression was determined by immunohistochemistry and immunoblotting. Our data show that AIF expression was associated with better prognosis. Expression of AIF inversely correlated with that of positive NSCLC markers, e.g., dihydrodiol dehydrogenase (DDH), c-MET, short oncostatin M receptor (OSMRs), matrix metalloproteinase (MMP)-1, and HER2/neu, which were closely associated with drug resistance, tumor recurrence, metastasis and poor prognosis. Noteworthy, silence of HER2/neu gene expression increases AIF level and drug sensitivity. Addition of HGF inhibits AIF expression in HER2/neu-silenced cells. These results suggested that both HGF and HER2/neu affect drug resistance by regulating AIF expression in NSCLC.

Introduction

Lung cancer is one of the leading causes of cancer death worldwide. In the United States, the annual mortality rate of lung cancer (estimated 180,000 deaths, male: 73.5/10⁵ person-year; female: 41.5/10⁵ person-year) is approximately 30% of total cancer-related deaths (1), and nearly 85-90% of lung cancer deaths are attributed to tobacco smoking (2). In Taiwan, the annual mortality rate of lung cancer is approximately 20% (estimated 6,000 deaths, male: 21/10⁵ person-year; female: 10.3/10⁵ person-year) (3). Lung carcinoma is categorized into small cell lung cancer (SCLC) or non-small cell lung cancer (NSCLC) with neuroendocrine features of the cancer cells. Based on the histopathological characteristics, NSCLC can be subcategorized into adenocarcinoma (ADC), squamous cell carcinoma and large cell carcinoma (4). Of note, among these, ADC, which is associated with a higher frequency of drug resistance and mortality than the other types, is most commonly found in women and smokers (5).

Previous studies have indicated that tobacco smoking is a key risk factor for lung cancer (1,2,5,6). In patients with stage I NSCLC, we demonstrated that tobacco smoking and tumor size, but not visceral pleural invasion, are major factors influencing overall and disease-free survival (6). Moreover, accumulated evidence showed that NSCLC patients who continued to smoke were more resistant to chemotherapy and irradiation, and had poorer prognosis (6-9). Although nicotine per se is not directly associated with tumorigenesis, catalyzed nicotine is carcinogenic (10,11). In addition, nicotine induces NSCLC growth, and increases angiogenesis in tumors probably via activating nicotinic acetylcholine receptor (nAChR), epidermal growth factor receptor (EGFR) and Akt (12-19).

Using differential display alone or in combination with microarray, we previously identified a spectrum of NSCLC-specific tumor markers, such as dihydrodiol dehydrogenase (DDH), c-MET, matrix metalloproteinase (MMP) and short oncostatin M receptor (OSMRs), which were closely associated with resistance to chemotherapy, tumor recurrence, metastasis and poor prognosis (20-24). Although we expected that DDH would correlate with drug resistance (20-22) and MMP with tumor metastasis (23), DDH was not directly involved in

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Abbreviations: AIF, apoptosis inducing factor; ATII, alveolar type II epithelial cells; ATM, ataxia-telangiectasia mutated kinase; ATR, ATM and Rad 3-related kinase; HGF, hepatocyte growth factor; HGFR, HGF receptor/ c-MET; MMP-1, matrix metalloproteinase-1; NSCLC, non-small cell lung cancer; PI3K, phosphoinositide 3-kinase

Key words: apoptosis inducing factor, cigarette smoking, cisplatin resistance, hepatocyte growth factor, non-small cell lung cancer

cisplatin deactivation. However, since DDH overexpression is closely related to tobacco smoking, and tobacco smoking is the key risk factor for carcinogenesis and disease progression of lung cancer, we used the same methods to examine gene expression profiles in biopsy specimens from smokers and non-smokers, and we detected that hepatocyte growth factor (HGF) was frequently overexpressed in smokers with NSCLC. HGF was correlated with tumor stages and poor prognosis (25). Of note, HGF not only increased resistance to cisplatin, but also reduced levels of apoptosis inducing factor (AIF), a vital factor of caspase-independent cell death (CICD), in NSCLC (25,26). Reduced effect of EGFR tyrosine kinase inhibitor (TKI), gefitinib, in patients with smoking habits or amplified c-MET gene suggested that in addition to cisplatin HGF might be involved in resistance to TKI as well (27). However, the prognostic value of HGF downstream effector AIF and its association with metastasis-related genes in NSCLC have not been reported.

In this study, we investigated AIF expression, and evaluated the statistical relationship between AIF expression and the clinicopathological factors as well as the prognostic significance of AIF expression in patients with NSCLC. We also studied the biological correlation between AIF and positive tumor marker genes in NSCLC.

Materials and methods

Tissue specimens. From August 1986 to November 2003, pathology specimens from 452 patients with NSCLC were reviewed. Pathology samples from all patients, for whom at least one follow-up examination or death was documented, were pathologically confirmed NSCLC. Of the 452 patients, 219 were diagnosed as having lung ADC. The stage of the disease was classified (patients after 1999) or re-classified (patients before 1999) according to the new international staging system for lung cancer (28). The Medical Ethics Committee approved the protocol in 2001, and written informed consent of donating biopsy specimens had been obtained from every patient before surgery since 2001. All patients had undergone surgical resection and radical N2 lymph node dissection. Tumor size, lymph node number, differentiation, vascular invasion and mitotic number were documented. Patients with lymph node involvement or locoregional recurrence received irradiation at the afflicted areas. Those with distant metastasis were treated with chemotherapy. After treatment, patients were routinely followed every 3 to 6 months in the outpatients department. Tumor recurrence and metastasis were diagnosed when biochemical studies, chest radiography, whole body bone scan and computerized tomography scans of chest showed any evidence of the disease. Immunohistochemical staining was carried out using a single-blinded procedure.

Immunoblotting analysis. Total cell lysate was prepared by mixing 5×10^7 cells/100 μ l phosphate-buffered saline with equal volume of 2X loading buffer (50 mM Tris, pH 6.8, 150 mM NaCl, 1 mM disodium EDTA, 1 mM PMSF, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue and 1% SDS). Electrophoresis was carried out in two 10% polyacrylamide gels with 4.5% stacking. One gel was processed for immunoblotting (20-26), and the other gel was stained with Coomassie blue. After electrophoresis, proteins on the first gel

were transferred to a nitrocellulose membrane for immunoblotting. The membrane was probed with specific antibodies. The signal was amplified by biotin-labeled goat anti-mouse IgG, and peroxidase-conjugated streptavidin. The protein was visualized by exposing the membrane to an X-Omat film (Eastman Kodak, Rochester, NY, USA) with enhanced chemiluminescent reagent (NEN, Boston, MA, USA). After getting unsatisfactory results from several batches of commercially obtained antibodies for Her2/neu, we decided to raise our own antibodies.

Preparation of mouse antibodies to HER2/neu. DNA sequence corresponding to C-terminal amino acids 807-1183 of HER2/neu was amplified by primer sequences containing *Sal*I (sense) and *Not*I (antisense) restriction sites respectively. The primer sequences were 5'-TCCGTCGACAAATGGACCATGTCCGGGAAAAC-3' (*Sal*I site is underlined) and 5'-AGCGGCCGCAGTCTTTGACGACCCCATCTT-3' (*Not*I site is underlined).

The 1131-bp cDNA of HER2/neu was cloned into an expression vector pET-32a⁺ (Promega KK, Tokyo, Japan). Bacterial colony containing the pET32⁺-HER2/neu was selected, and induced by isopropyl- β -D-thiogalactopyranoside (IPTG) to mass-produce HER2/neu. The recombinant protein was purified by a nickel-affinity column, and protein identity was determined by MALDI-TOF. Affinity-purified HER2/neu was used to immunize BALB/c mice, and sensitivity of anti-serum (OD₄₀₅ >0.3 at 1:6,000 dilutions) was measured by enzyme-linked immunosorbent assay (ELISA). Specificity of antibodies was determined by showing distinct bands with molecular weight of 185 kDa in the immunoblotting of breast cancer cell extract. Monoclonal antibodies were produced by a hybridoma technique, and HER2/neu-specific antibodies were screened by the above-mentioned methods.

Immunohistochemistry. Immunohistochemical staining was performed according to the immunoperoxidase method previously reported (20-26).

Slide evaluation. In each pathological section, non-tumor lung tissue (NTLT) served as the internal negative control. Slides were evaluated by two independent pathologists blinded to the clinicopathological knowledge. The ImmunoReactive Scoring system was adapted for this study (29). Briefly, a specimen was considered having strong signals when >50% of cancer cells were positively stained; intermediate, if 25-50% of the cells stained positive; weak, if <25% or >10% of the cells were positively stained; and negative, if <10% of the cells were positively stained. Cases with strong and intermediate AIF signals were classified as AIF⁺, and those with weak or negative AIF signals were classified as AIF⁻. Those with AIF detecting in the nuclei were classified as cases with nuclear AIF index (NAI).

Statistical analysis. The relationship between AIF expression and clinicopathological parameters was analyzed by Chi-square test. Survival curves were plotted using the Kaplan-Meier estimator (30). Statistical difference in survival among different groups was compared by the log-rank test (between AIF⁺ and AIF⁻ groups) and log-rank test for trend [among NAI,

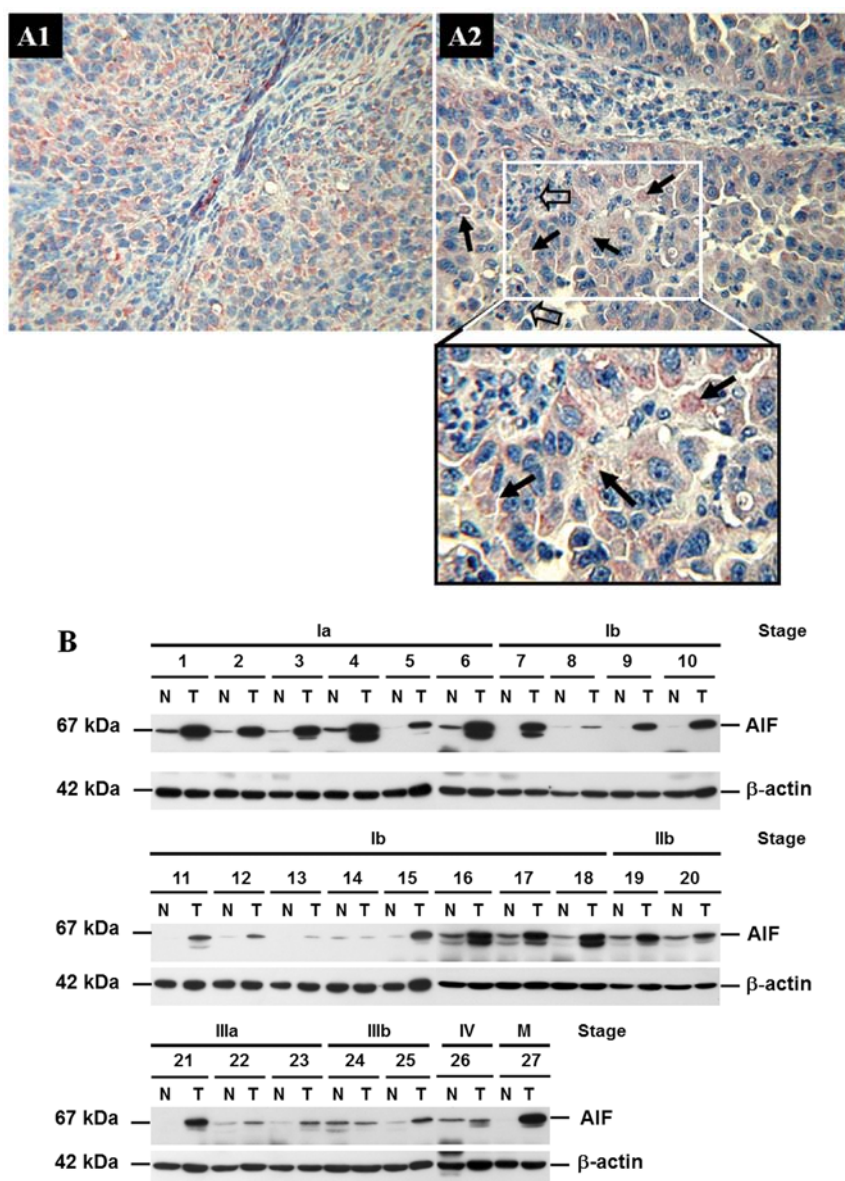


Figure 1. Expression of AIF in pathological specimens of NSCLC. (A) Representative examples of AIF expression in NSCLC. (A1) Expression of AIF was detected by immunohistochemistry (as crimson color precipitates in cytoplasm). (A2) Some AIF signals were detected in nuclei of tumor cells (indicated by arrows), when infiltrates of white blood cells were identified around tumor nests (indicated by white arrows). (B) Expression of AIF was confirmed by immunoblotting. N, non-tumor lung tissue; T, tumor fraction of surgical resections.

cAIF⁺ (cytoplasmic AIF, AIF⁺ patients minus NAI patients) and AIF⁻ groups] (31). Statistical analysis was performed using GraphPad Prism5 statistical software (San Diego, CA, USA). Statistical significance was set at p-value <0.05.

Results

Expression of AIF in NSCLC and correlation with patient survival. Using AIF-specific monoclonal antibodies, we detected AIF expression (Fig. 1A1 and A2) in tumor cells in 109 NSCLC patients (24.1%). In 14 (12.84%) of the 109 patients, some AIF signals were identified in the nuclei of tumor cells (Fig. 1A2). Nuclear AIF indicated that these tumor cells are under apoptosis. Judging from vicinity of the tumor, apoptotic cancer cells could be associated with the infiltrating immune cells. AIF was also detected in 40.45% (36/89) of metastatic

lymph nodes. However, no nuclear AIF was identified in the metastatic lymph nodes. AIF expression in was verified by immunoblotting (Fig. 1B). Expression of AIF decreased following advances of tumor stage. From stage 1b, AIF level reduced markedly.

Among the 109 patients whose tissue samples had high AIF expression, 31 (28.44%) patients had tumor recurrence. Among the 343 patients whose tissue samples had low AIF expression, 168 (48.98%) patients had tumor recurrence during follow-up examination. All 199 patients who had recurrence developed new tumors within 18 months after operation and cisplatin-based chemotherapy. Recurrence rate of patients with low AIF expression was 1.72-fold higher than that of patients with high AIF expression. The difference was significant (p<0.01). Moreover, survival of patients with high AIF levels was significantly better than that of patients with low AIF

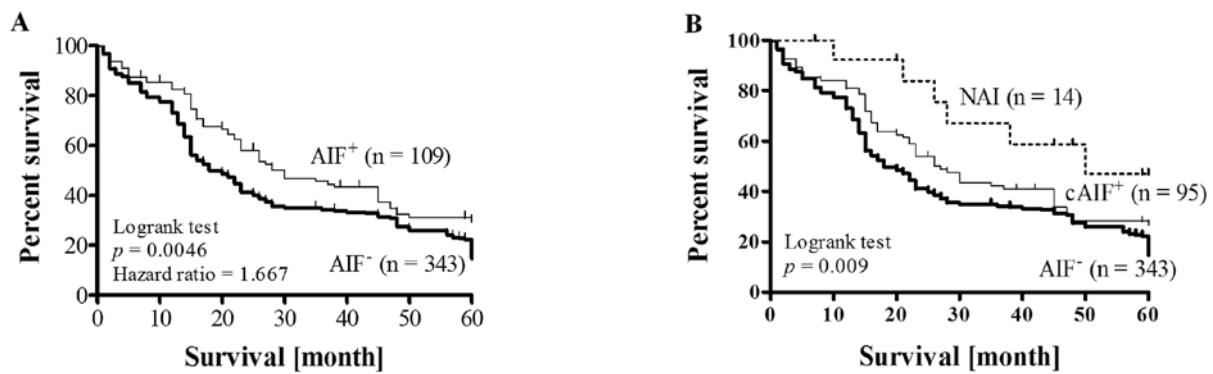


Figure 2. Comparison of Kaplan-Meier product limit estimates of survival analysis in patients with NSCLC. (A) Patients were divided into two groups based on AIF expression in pathological specimens of NSCLC. AIF⁻, NSCLC with low AIF expression; AIF⁺, NSCLC with high AIF expression. Survival difference between the two groups was compared by a log-rank test, $p=0.0046$; (B) Patients were divided into three groups based on AIF expression and detection of AIF in the nuclei of NSCLC. Survival rate in patients with nuclear AIF index (NAI) was the best among three patient groups. The difference in cumulative survival among the three groups was significant ($p=0.009$).

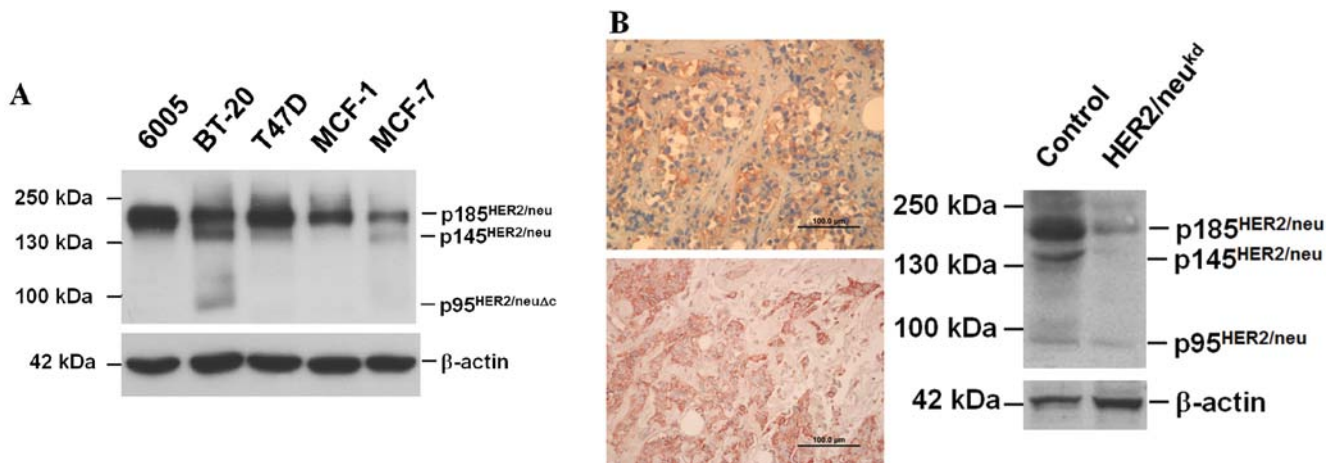


Figure 3. Characterization of monoclonal antibodies to HER2/neu. (A) A unique 185-kDa protein (p185^{HER2/neu}) was detected by immunoblotting in breast cancer cells. In some cancer cell lines, p145^{HER2/neu} and p95^{HER2/neuΔc} (HER2/neu with deletion of extracellular domain) fragments were detected. (B) Immunohistochemical staining showed that HER2/neu was abundantly present on the plasma membranes of breast cancer cells (left panels). Infection with lentivirus expressing siRNA to knockdown HER2/neu (HER2/neu^{kd}) expression validated that our monoclonal antibodies uniquely recognized HER2/neu (right panel). Reduction of HER2/neu expression is ~87% as determined by band intensity scan (EDAS 290, Kodak, Rochester, NY, USA).

levels (Fig. 2A). Survival rate of patients with high nuclear AIF index (NAI) was the best among patient groups (Fig. 2B). The differences in cumulative survival between groups were significant ($p=0.0046$ and $p=0.009$).

Functional characterization of monoclonal antibodies to HER2/neu. Specificity of the monoclonal antibodies was determined by an immunoblotting analysis of the whole cell lysate. The unique 185-kDa protein was detected by monoclonal antibodies (Fig. 3A). Immunohistochemical staining showed that HER2/neu was present on plasma membranes of breast cancer cells (Fig. 3B, left panels). Infection with lentivirus expressing siRNA to HER2/neu reduced HER2/neu expression validated that our monoclonal antibodies uniquely recognized HER2/neu (Fig. 3B, right panel).

Levels of AIF inversely correlates with expression of positive tumor markers in NSCLC. Our previous studies showed that overexpression of DDH, c-MET, MMP1 and OSMRs in NSCLC was associated with drug resistance, tumor recur-

rence, metastasis and poor prognosis (20-26). Moreover, our recent data suggested that expression of HGF, which was frequently detected in smokers with NSCLC, reduced levels of AIF (25). In this study, we investigated the correlation between levels of AIF and expression of positive tumor markers, e.g., DDH, c-MET, MMP1, HGF, OSMRs and HER2/neu (Fig. 4A), in NSCLC. HER2/neu in patients with the early stages was mainly p95^{HER2/neuΔc} (HER2/neu with deletion of extracellular domain) (32); in advanced stages it was the p185^{HER2/neu}. As shown in Fig. 4B, higher AIF expression ratio was detected in 37 (43.5%) of 85 tumor specimens. When gene expression, tumor staging and smoking habit were used to categorize patient groups, our data showed that AIF expression, as determined by immunoblotting, was inversely correlated with positive tumor markers, tumor staging and cigarette smoking. Noteworthy, cancer samples that had higher level of HER2/neu expressed less AIF. Levels of checkpoint kinase 1 (CHK1) and Nijmegen breakage syndrome 1 (NBS1, nibrin) protein, which are essential for mediating cell cycle arrest and maintaining genome stability during DNA replication, on the other hand,

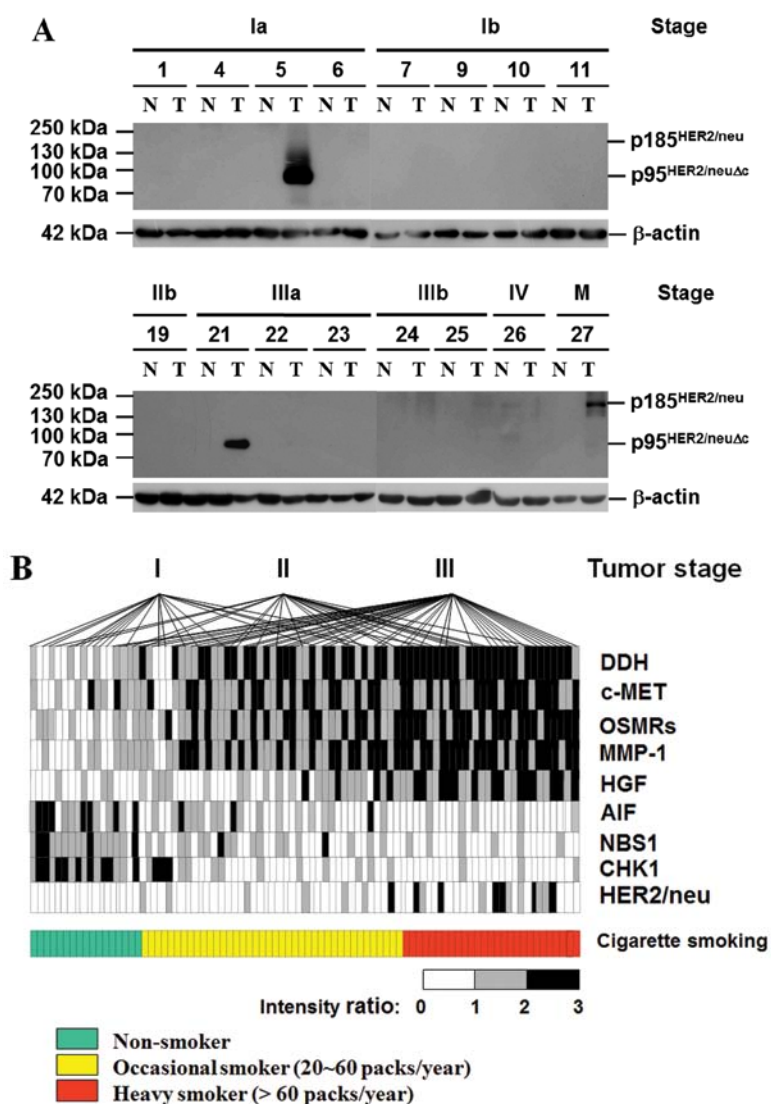


Figure 4. Expression of AIF and some positive tumor markers in NSCLC. (A) Expression of HER2/neu was detected by immunoblotting. Expression of β -actin was used as a monitoring standard. N, non-tumor lung tissue; T, tumor fraction of surgical resections. (B) Gene cluster analysis of 85 human NSCLC samples by AIF expression in patients with NSCLC. Level of gene expression was determined by measuring the intensity ratio between the specific protein and β -actin of immunoblots.

were proportional to those of AIF. The results are consistent with our *in vitro* data that AIF level is comparative to that of NSB1 (22) and HGF inhibits AIF expression (26).

In vivo, expression level of AIF is comparatively low in the center of cigarette smoking (CS)-induced lung adenocarcinoma in BALB/c3 mice. To study the *in vivo* effect of cigarette smoking on AIF expression, male BALB/c mice (National Animal Center, National Science Council, Taipei, Taiwan) at six weeks of age were treated with passive cigarette smoking daily (5 min a day in a 40 cm x 40 cm x 60 cm chamber, and each cigarette contained 0.9 mg of nicotine, Marlboro, USA) for 25 weeks before histopathological and biochemical examinations (Animal exposure protocols were approved by the Institutional Animal Care and Use Committee of National Chung-Hsing University). Among 100 male mice, lung cancer was detected in seven mice. Compared to alveolar type II (ATII) pneumocytes and cancer cells in the periphery (Fig. 5A), which were highly expressing AIF, AIF level decreased toward the center of the

tumor (Fig. 5B). The results are consistent with our *in vitro* data that nicotine reduced AIF expression (26,33).

Effect of HER2/neu on AIF expression and cell survival following cisplatin challenge in NSCLC cells. Eight NSCLC cells, H23, H225, H226, H838, H1437, H2009, H2087 and A549, examined by immunoblotting, expressed various levels of HER2/neu: high in H2009 and H2087; and low in H838 and A549 (Fig. 6A). HER2/neu was not detected in H23, H225, H226 and H1437. Of note, silence of HER2/neu expression by siRNA increased AIF expression; however, it did not affect the HGF effect on downregulation of AIF (Fig. 6B). The data indicated that HER2/neu also influences AIF expression in NSCLC cells. Since c-MET and HER2/neu share FAK as a common signal transducer, we examined the effect of p60^{src} and p23^{ras} on FAK expression. As shown in Fig. 6C, overexpression of p60^{src} protected FAK protein; however, p23^{ras} had no effect. The presence of HGF saved FAK protein from proteolytic degradation and facilitated cell moving out of the agarose trap (Fig. 6D).

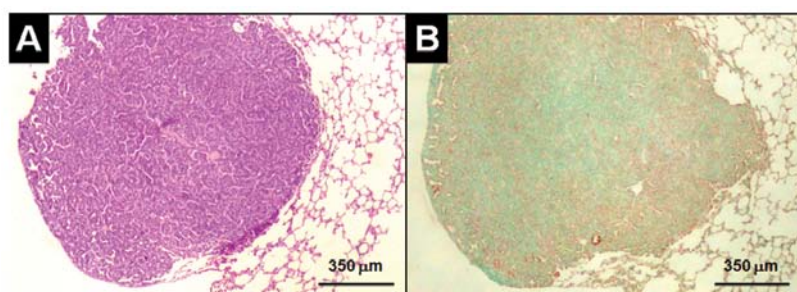


Figure 5. Expression level of AIF is comparatively low toward the center of cigarette smoking-induced lung adenocarcinoma in BALB/c mice. Male BALB/c mice (National Animal Center, National Science Council, Taipei, Taiwan) at six weeks of age were treated with passive cigarette smoking daily (5 min per day in a 40 cm x 40 cm x 60 cm chamber, and each cigarette contained 0.9 mg of nicotine, Marlboro, USA) for 25 weeks before histopathological and biochemical examinations. Among 100 male mice, lung cancer was detected in seven mice. (A) Representative example of cigarette smoking-induced lung adenocarcinoma in BALB/c mice (hematoxylin-eosin-stained); (B) Compared to ATII pneumocytes and cancer cells in the periphery, which highly expressed AIF, AIF level decreased toward the center of tumor. Expression of AIF was detected by immunohistochemistry (as crimson color precipitates in cytoplasm). The slide was counterstained with methyl green.

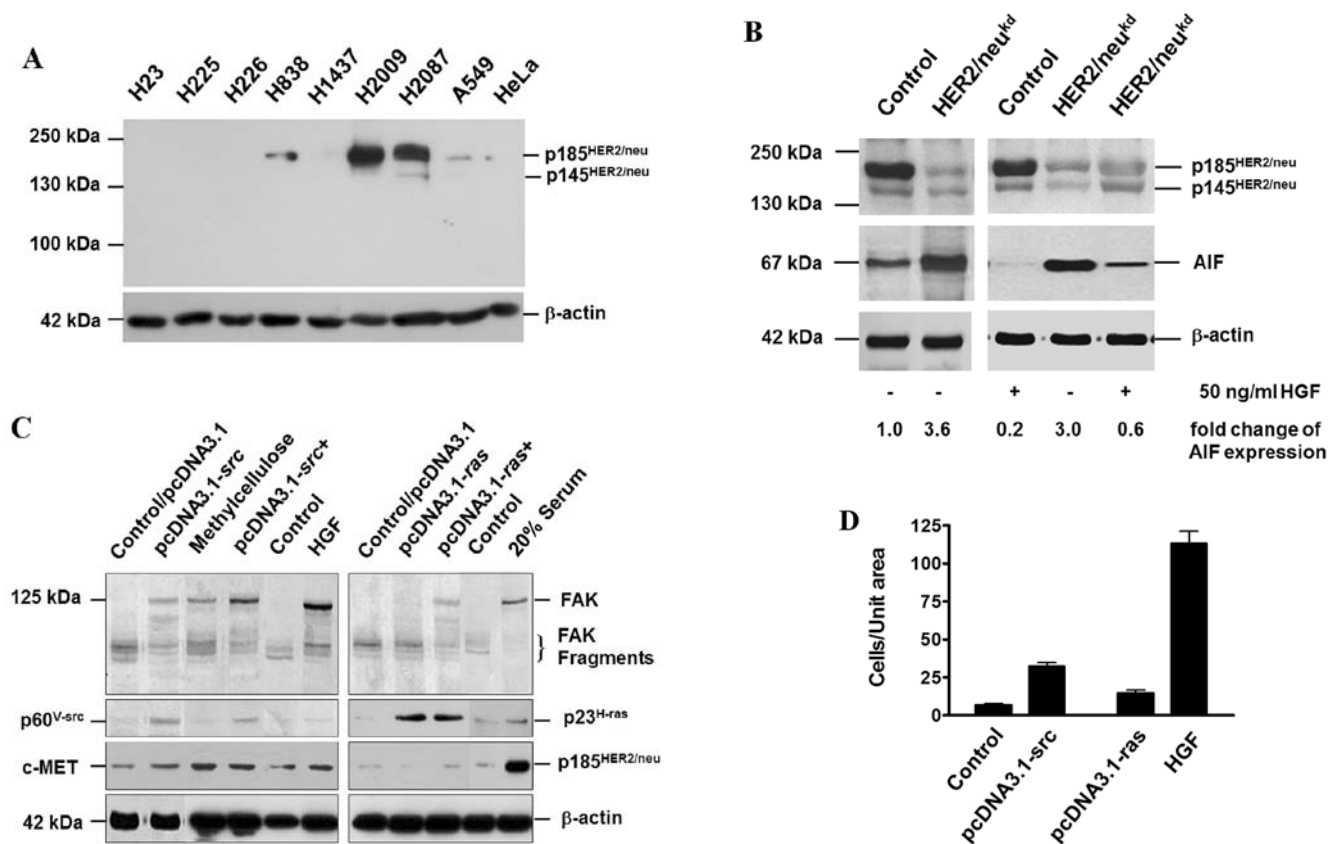


Figure 6. Expression of HER2/neu and the influence of HER2/neu on AIF expression in cultured ADC cells. (A) Expression of HER2/neu was detected in four human lung ADC cell types by immunoblotting. Expression of HER2/neu was high in H2009 and H2087, and low in H838 and A549. HER2/neu was not detected in H23, H225, H226 and H1437. (B) Infection with lentivirus expressing siRNA to knock down HER2/neu (HER2/neu^{kd}) expression increases AIF levels. Addition of HGF enhances the inhibitory effect of HER2/neu on AIF expression. (C) Transfection with pcDNA3.1-src carrying the full length src, or growing H2126 cells in 1% methylcellulose or addition of HGF increased expression levels of p125FAK. Expression of p125FAK was additionally increased in pcDNA3.1-src-transfected H2126 cells which were grown in 1% methylcellulose. Although transfection with pcDNA3.1-H-ras did not affect p125FAK expression, growing pcDNA3.1-H-ras-transfected H2026 cells in 1% methylcellulose increased expression levels of p125FAK. Addition of 20% fetal calf serum for 24 h increased both levels of HER2/neu and p125FAK. (D) The respective effect of p60^{V-src} and p23^{H-ras} on cell migration activity of H838 cells (10⁵ cells seeded in 0.3% agarose for 24 h). Cells treated with 50 ng/ml HGF were used as positive control.

Discussion

The results show that AIF expression in NSCLC is inversely correlated with tumor stage and patient cigarette smoking history. By demonstrating that in patients with more advanced

lung cancer, especially, in those who smoked more than 20 pack-years, AIF expression was low in tumor cells (~67.6% of specimens), our data suggest that, besides being involved in lung carcinogenesis, cigarette smoking increases drug resistance by downregulating expression of AIF in lung tumor

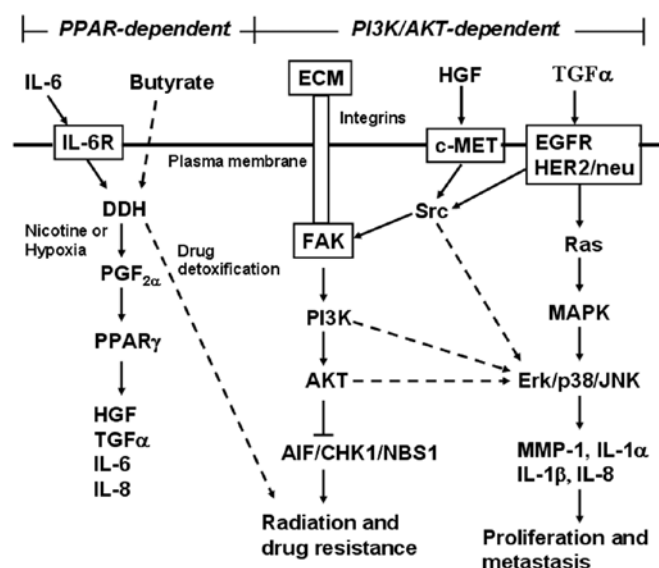


Figure 7. The putative network among integrins and receptor kinase-associated gene activation on expression of AIF, NBS1 and CHK1 as well as their consequences of drug- and radio-resistance in NSCLC.

cells. Reduced AIF expression correlated with poor prognosis and was inversely associated with expressions of positive tumor marker genes.

The results corresponded well with our previous study that HGF was upregulated by tobacco smoke in AII and NSCLC cells (25). Increase of HGF in turn reduces AIF expression and cisplatin sensitivity (26), which could be mediated via c-MET, FAK, phosphoinositide kinase 3 (PI3K) and protein kinase B (PKB, also called AKT) pathway in NSCLC cells (26,34). This study showed that HER2/neu was also involved in down-regulation of AIF expression. Since signals from HER2/neu and c-MET converge onto a common transducer FAK protein, our results suggested that these two receptors might in part play a role in activation of PI3K and AKT (26), and activated AKT decreased expression levels of AIF, a vital factor of caspase-independent cell death, to induce drug resistance in NSCLC (25,26). Expression level of FAK alone, however, was not associated with that of AIF or drug resistance (data not shown).

In addition, AIF level correlated with expression of critical sensor proteins of DNA damage and replication stress, NBS1 and CHK1, which are essential for mediating cell-cycle arrest and DNA-repair mechanisms (35). Noteworthy, FERM (band four point one, Ezrin/Radixin/Moesin) region of FAK protein interacts with the N-terminal transactivation domain of p53, which binds mouse double minute 2 (MDM-2), a ubiquitin ligase (36). Selective binding of p53 to MDM-2 or FAK facilitates cell transformation and epithelial-mesenchymal transition (37). By showing that overexpression of v-src protein and addition of HGF or fetal calf serum (FCS) protected FAK from integrin-related protein degradation, our data suggested that integrin, v-src and H-ras might have different effects on FAK function. ATR, a cell stress-responsive kinase that is essential for sensing DNA replication error, contains a stretch of focal adhesion targeting (FAT) and PI3K motifs (38). Myers *et al* demonstrated that ATR and Chk1 alleviate replication-related stress by suppressing a caspase-3-dependent

apoptotic response (39). Our data supported their findings and showed that in combination with reduced AIF expression, downregulation of NBS1 and CHK1, two mediators of ATR, might elude the inhibitory effect of p53 on cell cycle progression, and increase genomic heterogeneity as well as resistance to doxorubicin, etoposide, cisplatin and gemcitabine in NSCLC cells, which overexpressed HER2/neu (40). By showing that levels of HER2/neu increased following addition of FCS, our data suggested that HER2/neu expression might not be constitutive (41), but could be upregulated by yet to be determined serum factors.

Moreover, our previous studies showed that drug resistance, tumor recurrence, metastasis and poor prognosis of NSCLC correlated with overexpression of DDH, c-MET, MMP1, HGF and OSMRs (20-25). Recently, by confocal immunofluorescence microscopy we found that HGF, which was frequently detected in smoker patients, was expressed on surface of cancer cells (33). Therefore, the overexpressed HGF could only interact with nearby cells. Of note, HGF overexpression was induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which was synthesized by DDH when cells were under hypoxic condition (33). As shown in cigarette smoking-induced murine lung ADC, AIF expression was much lower in the center of tumor mass when tumor size was larger than one millimeter. However, we are less certain whether this phenomenon was caused by tumor size-related hypoxia or the effect of tobacco smoke could not reach the interior of tumor mass. In addition to reducing levels of AIF, HGF upregulates expression of interleukin (IL)-1 α , -1 β , -6, -8 and -24 (a member of IL-10 family), as well as that of tumor necrosis factor (TNF) superfamily member 10 (TNFSF10), MMP1 and transforming growth factor α (TGF- α), an alternative ligand of EGFR (33). TGF- α activates EGFR and IL-6 induces overexpression of DDH (22), which constitute a vicious cycle to maintain cancer cell survival (Fig. 7); in particular, activated EGFR is vital for cell proliferation and DDH is essential for detoxification of anticancer drugs, including cisplatin (21), doxorubicin, etoposide, mitoxantrone, gefitinib and erlotinib, of which chemical structures are highly similar to polycyclic aromatic hydrocarbons (PAH) (20).

These results considered together with the current data provided *in vitro* explanations to support our previous findings that tobacco smoking and tumor size are the two major factors influencing overall and disease-free survival in patients with stage I NSCLC (6); in particular in patients with stage Ib disease AIF levels were markedly reduced. Patients who continued to smoke after proper resections were more resistant to chemotherapy and irradiation, and had poorer prognosis (6-8,20-25). Cigarette smoking and hypoxia respectively induce expression of HGF, which decreases AIF levels and drug sensitivity in NSCLC cells (25,26). In conclusion, our data show that AIF expression was frequently downregulated in patients with NSCLC, especially in those with a previous or current smoking history. Statistical analysis showed that decreased AIF level in NSCLC was closely associated with patient survival. These results suggest that cigarette smoking plays an important role in drug resistance and cancer cell survival, which were probably mediated via HGF, HER2/neu and FAK activation-induced downregulation of AIF, CHK1 and NBS1 expression in patients with NSCLC (42).

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