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Estrogen adversely affects the prognosis of patients with lung adenocarcinoma

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Estrogen has been postulated to contribute to the development and progression of lung cancer. We examined the epidemiologic evidence, explored the characteristics of estrogen receptors (ER) in lung adenocarcinoma, and investigated the effect of estrogen on lung cancer cell migration, including the signaling pathway involved. For epidemiologic evidence, a total of 1434 consecutive non-small cell lung cancer patients who underwent standardized staging and homogenous treatment were prospectively enrolled from January 2002 to December 2008, and followed until December 2012. The possible prognostic factors to be analyzed included stage, age, gender, menopausal status, smoking history and histology. For laboratory study, lung cancer cell lines A549 and PE089 and malignant pleural effusions from the patients with lung adenocarcinoma were used. We found that the premenopausal patients had more advanced disease and a shorter survival among the never-smoking female patients with lung adenocarcinoma. ERβ was the predominant ER in the lung cancer cell lines. We proposed a different pathway that estrogen upregulated the expression of osteopontin and then promoted cell migration through αvβ3 integrin binding and activated MEK-ERK signaling pathway, which is a common downstream pathway with epidermal growth factor receptor (EGFR) activation. An additive effect of ER antagonists and EGFR antagonists on the inhibition of cell migration was also noted. Our results suggest that estrogen adversely affects the prognosis of patients with lung adenocarcinoma. Osteopontin contributed to the cross-talk between ER and EGFR signaling pathways. Estrogen, with its receptor, has the potential to be a prognosticator and a therapeutic target in lung cancer.

Estrogen is thought to play an important role in lung cancer carcinogenesis.[1,2] Estrogen has also been reported to adversely affect the prognosis of patients with lung cancer.[3–9] However, there are also some study results that are contradictory to this.[10–14] We previously examined gender-associated differences in non-small cell lung cancer (NSCLC) and noted that elderly women with lung adenocarcinoma had a more significant survival advantage than their male counterparts.[15] In addition to the cumulative adverse effects of smoking in the elderly male patients, the longer survival of the postmenopausal female patients may also have been due to a lower estrogen cancer-promoting effect. With more patients recruited and extended follow up, we re-examined the evidence and a comparison was made between premenopausal and postmenopausal female never-smoking adenocarcinoma patients.

Drugs targeting the estrogen signaling pathway have been shown to suppress the growth of lung cancer cells.[4,16,17] To examine the role of estrogen, the expression of estrogen receptor (ER) in lung cancer cells, the effect of estrogen on cancer cell migration and the relationship between estrogen and osteopontin (OPN) were investigated. The individual and combined effect of ER antagonists and epidermal growth factor receptor (EGFR) antagonists on cancer cell migration was also studied.

Materials and Methods

Epidemiology study design and patients. Consecutive NSCLC patients were prospectively enrolled in an electronic database called the Cancer Information System at the Sun Yat-Sen Cancer Center from January 2002 to December 2008, and followed until December 2012. They were clinically and pathologically staged and underwent homogenous treatment algorithms, as described previously.[15] The possible prognostic factors to be analyzed include stage, age, gender, menopausal status, smoking history and histology. Demographics, histology and disease stage between genders were compared. Survival differences between the men and women in the subgroups of age, smoking history and histology were analyzed.

Laboratory study design and patients. Malignant pleural effusions confirmed by cell block cytology or closed pleural biopsy from 62 female patients with lung adenocarcinoma (age 56.0 ± 12.4 years, included four with smoking history) at its initial presence were collected between July 2009 and

December 2011 at the Sun Yat-Sen Cancer Center. Another 25 female patients (age 66.7 ± 12.6 years) with non-malignant pleural effusion were included as controls. The etiologies include indeterminate lymphocyte predominant exudate (n = 14), transudates secondary to liver cirrhosis or metastasis (n = 4), para-pneumonia (n = 3), heart failure (n = 2), and surgery or radiotherapy (n = 2). All of the pleural effusions were centrifuged at 3000 g for 10 min and fresh frozen at −80°C. The Institutional Review Board of the hospital approved this study as well as the database used to collect the data. All the patients of the cohort for epidemiology study and the subgroup involved in the laboratory study provided written informed consent before study entry. The study was also approved by the local Ethics Committee and was conducted in accordance with the ethical principles stated in the Declaration of Helsinki and the guidelines on good clinical practice.

Chemicals. The drugs and chemicals used in this study were purchased from different companies: β-estradiol (E_2), diarylpropionitrile (DPN, ERβ agonist), ICI 182780 (ER-specific inhibitor), epidermal growth factor (EGF), 4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126; MAP kinase/MEK inhibitor), recombinant human OPN and tamoxifen citrate were purchased from Sigma (St. Louis, MO, USA), Gefitinib from AstraZeneca (Macclesfield, UK), and anti-αvβ3 antibody from Affinity BioReagent (Golden, CO, USA).

Cell cultures. A549 and MCF-7 cell lines were purchased from ATCC (Manassas, VA, USA). The PE089 was characterized as harboring an EGFR exon 19 deletion and derived from a female patient with adenocarcinoma of the lung (courtesy of K. J. Liu from the National Health Research Institute). Both cell lines were maintained in phenol-red free DMEM and nutrient mixture F12 (1:1) (Gibco, Grand Island, NY, USA), supplemented with 5% heat-inactivated and dextran-coated-charcoal-stripped FBS (Life Technologies, Gaithersburg, MD, USA).

Western blot analysis. Equal amounts of protein were electrophoresed on 8% SDS-PAGE, then transferred to PVDF membranes (GE Healthcare Bioscience, Fribourg, Switzerland) and immunoblotted. The following primary antibodies were used for immunohistochemistry: anti-ERα (HC20), anti-ERβ (H-150), anti-p-ERK (E4), anti-OPN (AKm2A1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ERK1/2 (E31R; GeneTex, Irvine, CA, USA) and anti-GAPDH (#4300; Ambion Silencer, Lakewood, NJ, USA). Secondary antibodies, anti-mouse IgG conjugated HRP (Cell Signaling Technology, Beverly, MA, USA) were applied followed by enhanced chemiluminescence detection using an ECL system (GE Healthcare Bioscience).

RNA extraction, reverse-transcription and real-time quantitative PCR. Total RNA was extracted with a RNeasy Mini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA synthesis was performed with 5 U MMLV reverse transcriptase (Epicentre, Madison, WI, USA) with 1 μg RNA. The *OPN* (*spp1*) mRNA level was determined by real-time quantitative PCR (RT-qPCR) using a Roche LightCycler FastStart DNA master SYBR Green kit (Roche Diagnostics GmbH, Germany). The forward and reverse primers used for *OPN* were 5′-CACCTGTGCCATACCAGTTAA-3′ and 5′-GGTGATGTCCTCGTCTGTAGCATC-3′, respectively, and for β-*globin* 5′-ACCTGACTCCTGAGGAGAAG-3′ and 5′-GATCCTGAGACTTCCACACT-3′, respectively.

Wound healing assay. The cells were treated with 10 μg/mL of mitomycin-c (Sigma) to inhibit proliferation, and allowed to migrate. A culture-insert was used to create a discrete zone to form a cell-free zone into which cells at the edges of the wound could migrate. Molecules of interest, including 10 nM E_2, 10 nM DPN, 10 μM ICI 182780, 10 μM tamoxifen, 100 ng/mL EGF, 10 μM gefitinib, 10 μM U0126 or 1.25 μM OPN, were added to the wells and images of cell movement were captured.

Plasmid transfection. Serum-starved cells were transfected with pRST-*ESR2* (*ER*β) (Addgene, Cambridge, MA, USA), shRNA targeting *ER*β (with an oligonucleotide sequence of 5′-CCTTAATTCTCCTTCCTCCTA-3′; Academia Sinica, Taipei, Taiwan), pcDNA3.1 (scramble shRNA) or siOPN (Ambion Silencer) using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA). Cells transfected with the plasmids were selected by 400 μg/mL G418 for 4 weeks.

Estradiol measurements. The E_2 levels in pleural effusions were measured with an ARCHITECT E_2 assay (Estradiol Reagent Kit 7K72; Abbott Labs, Abbott Park, IL, USA). The results were expressed as picograms per milliliter for E_2.

Enzyme-linked immunosorbent assay for osteopontin. A Human Osteopontin TiterZyme Enzyme Immunometric Assay (EIA) kit (Gentaur Europe, Brussels, Belgium) was used. The enzyme reaction was stopped and read at 405 nm. The standard curve was established at 32, 16, 8, 4 and 2 ng/mL OPN. The OPN levels were expressed as ng/mL.

Statistical analysis. For epidemiological results, the Pearson χ^2-test was used to evaluate the associations between stage, smoking history, gender and histology. The Wilcoxon rank-sum test was used to test differences in median age. Kaplan–Meier plots and the log-rank test were used to assess the association of groups with survival. Multivariate analysis of overall survival was performed according to the Cox proportional hazards model using the clinical prognostic factors. Laboratory data were presented as mean ± standard deviation. The results of different experimental treatments were analyzed using Student's t-tests. A two-sided P-value of <0.05 was considered to be significant. Analysis was performed using the statistical software package SAS, version 9.1.3 (SAS Institute, Cary, NC, USA).

Results

Epidemiology study. *Patient characteristics.* A total of 1434 NSCLC patients (668 women) were enrolled in the study. The

Table 1. Patient characteristics of epidemiology study, stratified by gender, smoking history, histology and stage

Variable	Women (n = 668)	Men (n = 766)	P-value
Median age, years	59	64	<0.001
History of smoking (%)	42 (7)	574 (75)	<0.001
Histology			<0.001
Smoker (%)			
Adenocarcinoma	31 (91)	307 (68)	0.005
Squamous cell carcinoma	3 (9)	145 (32)	
Never-smoker (%)			
Adenocarcinoma	511 (95)	149 (94)	0.80
Squamous cell carcinoma	28 (5)	9 (6)	
Stage of NSCLC (%)			
I	86 (13)	86 (11)	0.18
II	25 (4)	37 (5)	
III	186 (28)	246 (32)	
IV	371 (56)	397 (52)	

NSCLC, non-small cell lung cancer.

patient characteristics are shown in Table 1. The female patients were younger and a higher percentage of female patients had adenocarcinomas. Seventy-five percent of the male patients and 7% of female patients had smoking history.

Survival analysis. In the patients with lung adenocarcinoma, multivariate analysis indicated that stage (hazard ratio [HR], 2.13; $P < 0.01$), age (HR, 1.36; $P < 0.01$) and smoking history (HR, 1.08; $P < 0.01$) were independent prognostic factors. Gender did not reach statistical significance (HR, 1.38; $P = 0.41$). The never-smokers had a longer survival than the smokers (median survival: 695 *vs* 493 days; $P < 0.01$) (Fig. 1a). The survival was similar between never-smoking women and men (median survival: 715 *vs* 677 days; $P = 0.39$) (Fig. 1b).

Compared with the male patients, the survival superiority of the female patients with lung adenocarcinoma appeared to be more significant in the elderly group (Fig. 1c–e). Among the never-smoking female patients with lung adenocarcinoma, the premenopausal women had a shorter survival than the postmenopausal women (median survival: 643 *vs* 735 days; $P = 0.01$) (Fig. 1f). The multivariate analysis including stage, age and menopausal status showed that stage was the only independent prognostic factor (HR, 2.15; $P < 0.01$). Premenopausal women had more advanced disease (stage I 8%, stage

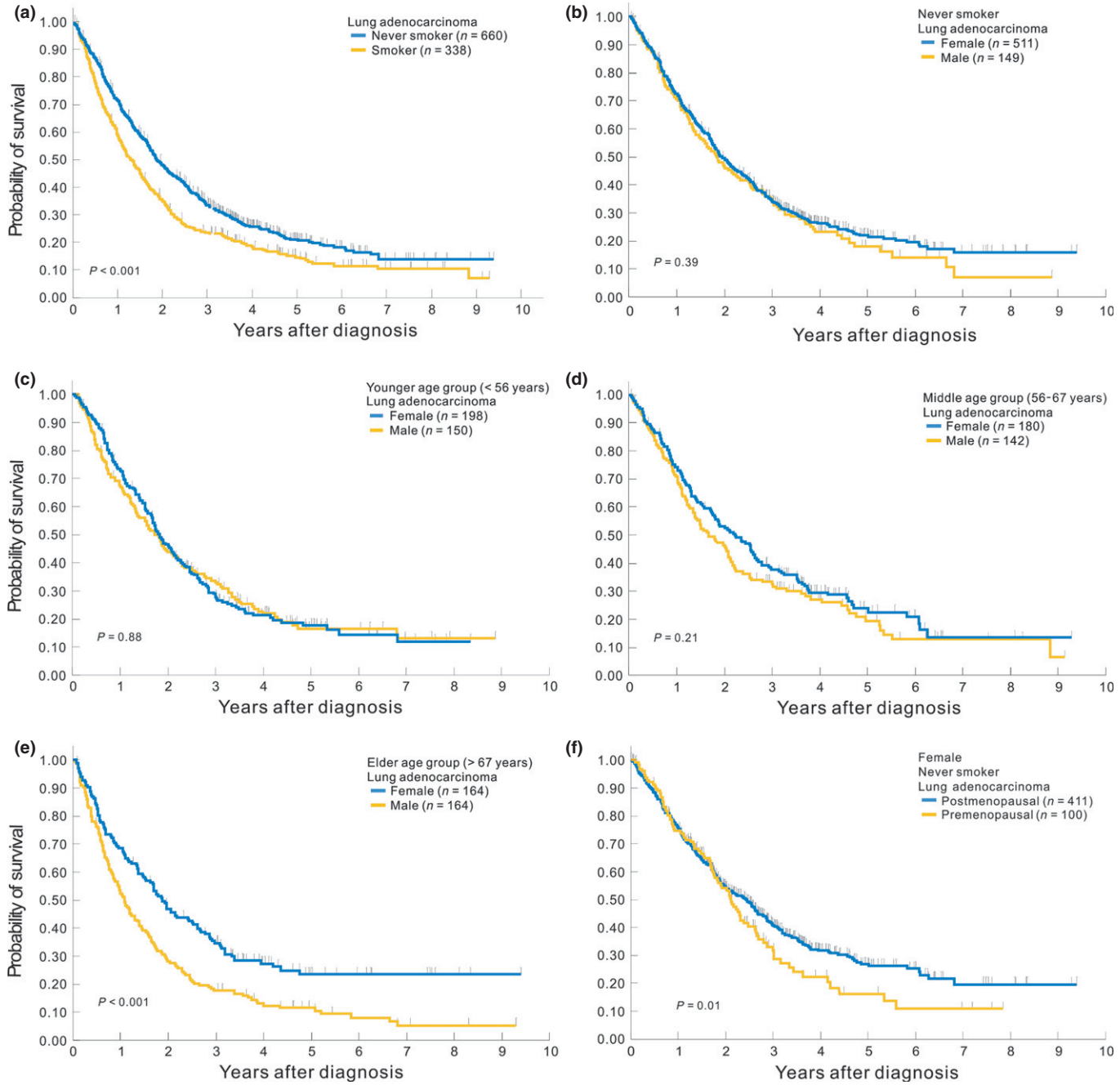

Fig. 1. Among the never-smoking female patients with lung adenocarcinoma, the premenopausal had a shorter survival. Kaplan–Meier survival estimates of: (a) lung adenocarcinoma patients divided by smoking history; (b) never-smoking lung adenocarcinoma patients divided by gender; (c–e) lung adenocarcinoma patients divided by gender in different age groups; and (f) never-smoking female lung adenocarcinoma patients divided by the menopausal status.

II 5%, stage III 21% and stage IV 66%) than the postmenopausal women (stage I 16%, stage II 4%, stage III 27% and stage IV 53%; $P = 0.04$).

Laboratory study. *Estrogen promoted cell migration via ERβ activation of the MEK/ERK signaling pathway.* For quantification of the wound healing, the wound area of nontreated cells for 24 h is defined as 100%. Wound healing assays of A549 or PE089 cells incubated with E_2 showed a significant decrease in wound area. The PE089 cells showed a higher migratory ability than the A549 cells in the presence of E_2. Tamoxifen significantly reduced the E_2-induced cell migration in A549 and PE089 cells (Fig. 2a,b).

To identify whether the molecular signals controlled E_2-mediated cell migration through the MEK/ERK pathway, A549 cells and PE089 cells were subjected to wound healing assays in the presence of E_2 and/or U0126 for 24 h. U0126 significantly decreased the E_2-induced cell migration (Fig. 2c), and reduced the E_2-induced phosphorylation of ERK (p-ERK, Fig. 2d). These results indicated that the estrogen-induced cell migration was via activation of the MEK/ERK signaling pathway, which is also a downstream pathway of EGFR activation.

Western blot analysis revealed that ERβ was the predominant receptor expressed in the lung cancer cell lines (Fig. 2e). To further investigate the effects of ERβ, we established two cell clones: one with pRST-*ESR2* overexpressing ERβ (ERβ O/E), and the other transfected with ERβ shRNA (ERβ knockdown) (Fig. 2f). A 1.5-fold increase in growth rate was found in the ERβ O/E cell clone with E_2 stimulation for 24 h (Fig. 2g). DPN (ERβ agonist) treatment stimulated cell migration in a similar fashion to E_2. ERβ knockdown with shRNA, tamoxifen and ICI 182780 (ICI) resulted in a significant reduction of cell migration (Fig. 2h).

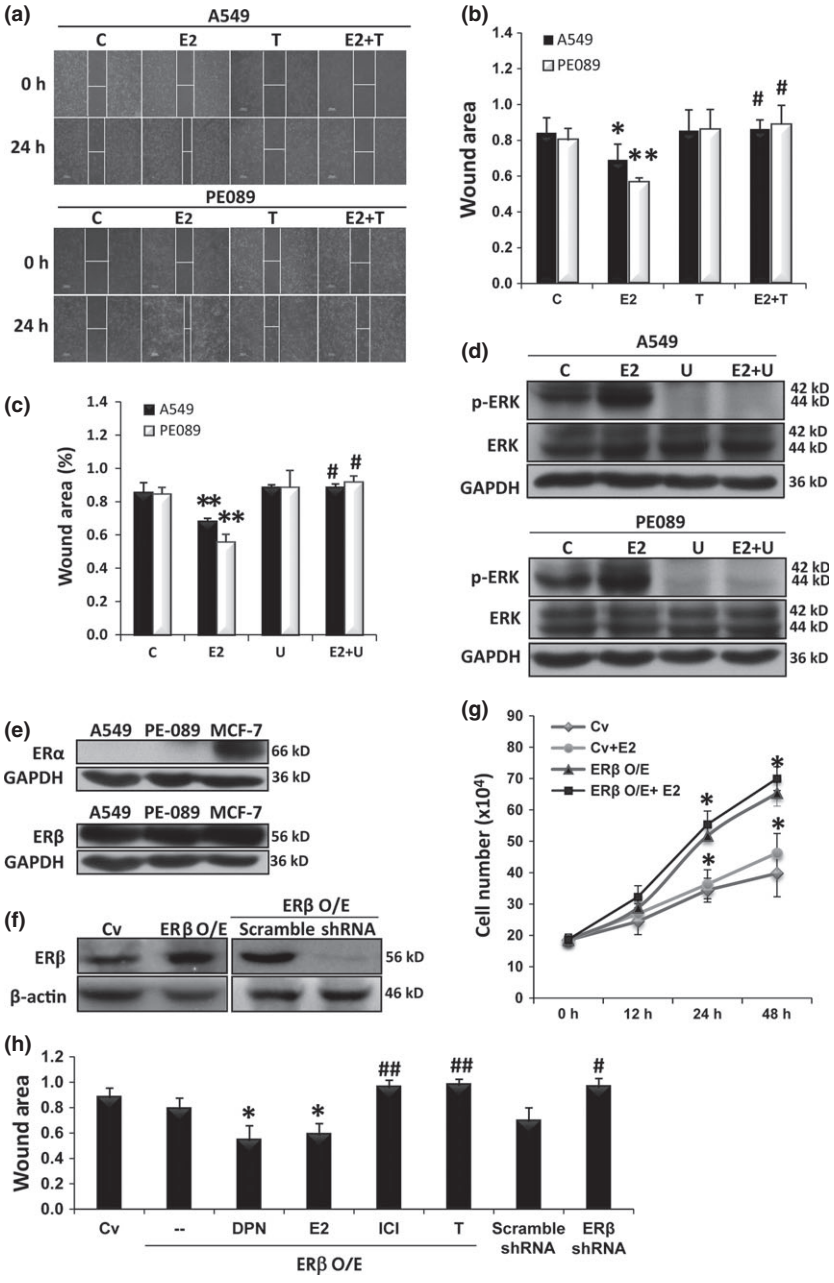

Fig. 2. Estrogen promoted cell migration via estrogen receptor β (ERβ) activation of the MEK/ERK signaling pathway. (a) A549 cells and PE089 were subjected to wound healing assays for 24 h in the presence of 100 nM estradiol (E_2), 10 μM tamoxifen citrate (T, ER antagonist), co-treatment with E_2 and tamoxifen citrate (E_2 + T), or ethanol equivalent as the control. (b) The wound area of A549 or PE089 cell migration was analyzed. The lines indicate the boundary of the edges of the wound after 24 h. The bars represent mean ± standard deviation relative to the cells treated with ethanol. (c) A549 and PE089 cells were cultured in the presence of E_2 and/or U0126 (U, MEK inhibitor) for 24 h. U0126 reduced E_2-induced cell migration. (d) Western blotting of the ERK and phosphorylated ERK of the cells treated with E_2 and/or U0126 showed estrogen-induced ERK phosphorylation was inhibited by U0126. (e) Western blotting showed that ERβ was the predominant receptor type in the lung cancer cell lines. (f) Transfection of the A549 cells with the human ERβ gene or ERβ shRNA was performed to establish an ERβ overexpression cell clone (ERβ O/E) and ERβ depletion cell clone (shRNA), respectively. (g) ERβ O/E with E_2 stimulation resulted in a maximal increase in cell growth rate. (h) The migratory ability of the various indicated cells was analyzed. DPN (ERβ agonist) and E_2 induced migration of ERβ O/E cell. ER inhibitor (ICI 182780, ICI), tamoxifen and ERβ knockdown with shRNA resulted in a reduction of cell migration. $*P < 0.05$; $**P < 0.01$ compared with the control; $\#P < 0.05$; $\#\#P < 0.01$ compared with the E_2 group. Cv, control vector; O/E, overexpression.

Additive effect of estrogen receptor antagonist (tamoxifen) and epidermal growth factor receptor antagonist (gefitinib) on the inhibition of lung cancer cell migration. The effects of E_2 and EGF on cancer cell migration were then compared, and the highest stimulation of cell migration was observed when both E_2 and EGF were present in the culture (EE group in Fig. 3a). A significant difference in the wound healing assay was found between EE + TG and EE + G ($P < 0.05$, Fig. 3a). Up to a 3.3- and 2.6-fold increase of p-ERK were found in the EE group of PE089 cell line and A549 cell line, respectively (Fig. 3b). The E_2 and EGF induced ERK phosphorylation was downregulated in the presence of tamoxifen and/or gefitinib. Tamoxifen and gefitinib together acted in an additive manner to inhibit E_2 and EGF induced cell migration and ERK phosphorylation (Fig. 3a,c).

Estrogen induced osteopontin expression. To test whether OPN was a downstream molecule of the estrogen signaling pathway and whether it contributed to cell migration, the expression levels of the OPN gene were quantified. A 2.2-fold increase in OPN mRNA level was detected in the E_2-treated A549 cells (Fig. 4a,b). The protein levels of OPN also increased in the medium cultured with E_2 (Fig. 4c). Both the E_2-induced OPN expressions were reduced in the presence of tamoxifen (Fig. 4a–c). OPN-induced cell migration and ERK phosphorylation were significantly inhibited by U0126 (MAP kinase/MEK inhibitor) (Fig. 4d,e). The integrin receptor αvβ3 interacts with OPN via the arginine-glycine-aspartate (RGD) motif. The anti-αvβ3 Ab significantly inhibited OPN-induced cell migration and E_2-stimulated or EGF-stimulated cell migration (Fig. 4f). These results suggested that estrogen induced OPN expression and promoted cancer cell migration through αvβ3 integrin binding and MEK/ERK signaling, and implied that OPN-αvβ3 integrin-induced cell migration was involved in the activation of ER and EGFR signaling pathways.

Estradiol and osteopontin both elevated and positively correlated in the malignant pleural effusions from patients with lung adenocarcinoma. To investigate whether the cell-free supernatants of malignant pleural effusions provide a migratory niche for A549 or PE089 cells, the cells were incubated with the malignant pleural effusions from patients with lung adenocarcinoma ($n = 24$). In Figure 5(a), the wound healing assays were treated with the malignant pleural effusion from a female never-smoker patient with lung adenocarcinoma harboring an EGFR exon 21 L858R mutation. We found that the malignant pleural effusions more intensely promoted cell migration than E_2 or EGF. Additive inhibition of the malignant pleural effusion-induced cell migration was found with tamoxifen and gefitinib supplementations (Fig. 5a,b). Patients with malignant pleural effusion had higher pleural fluid E_2 (median, 13.0 *vs* 10.0 pg/mL; $P = 0.085$) and OPN (median, 318.77 *vs* 256.28 ng/mL; $P = 0.289$) concentrations than those with non-malignant pleural effusion, although statistical significance

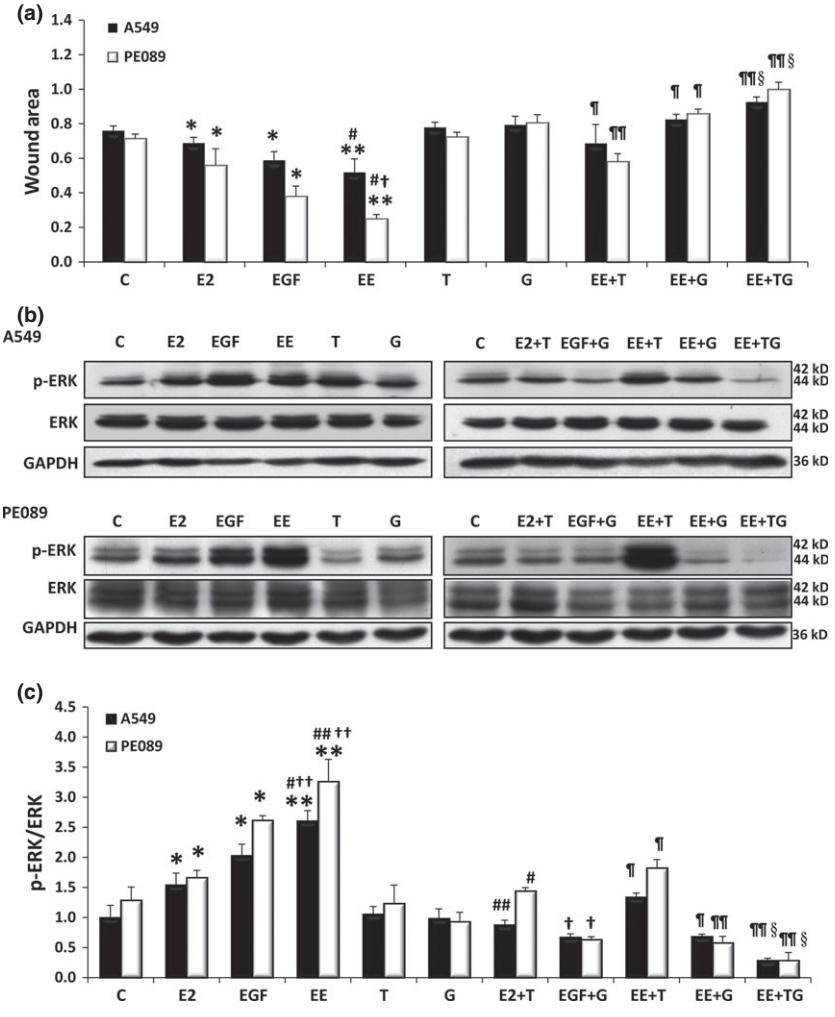

Fig. 3. Additive effect of estrogen receptor (ER) antagonist and epidermal growth factor receptor (EGFR) antagonist on the inhibition of lung cancer cell migration. (a) In wound healing assay, both E_2 and EGF promoted cell migration. ER antagonist (tamoxifen, T) and EGFR antagonist (gefitinib, G) inhibited cell migration. (b) Estradiol and EGF (EE group) activated ERK phosphorylation. Tamoxifen and gefitinib inhibited ERK phosphorylation. (c) The levels of p-ERK and total ERK were quantified and expressed as a p-ERK/ERK ratio for each group. EE, E_2 and EGF; $^*P < 0.05$; $^{**}P < 0.01$ compared with the control; $\#P < 0.05$; $\#\#P < 0.01$ compared with the E_2 group; $\dagger P < 0.05$; $\dagger\dagger P < 0.01$ compared with the EGF group; $\P P < 0.05$; $\P\P P < 0.01$ compared with the EE group; $\S P < 0.05$ compared with the G + EE group.

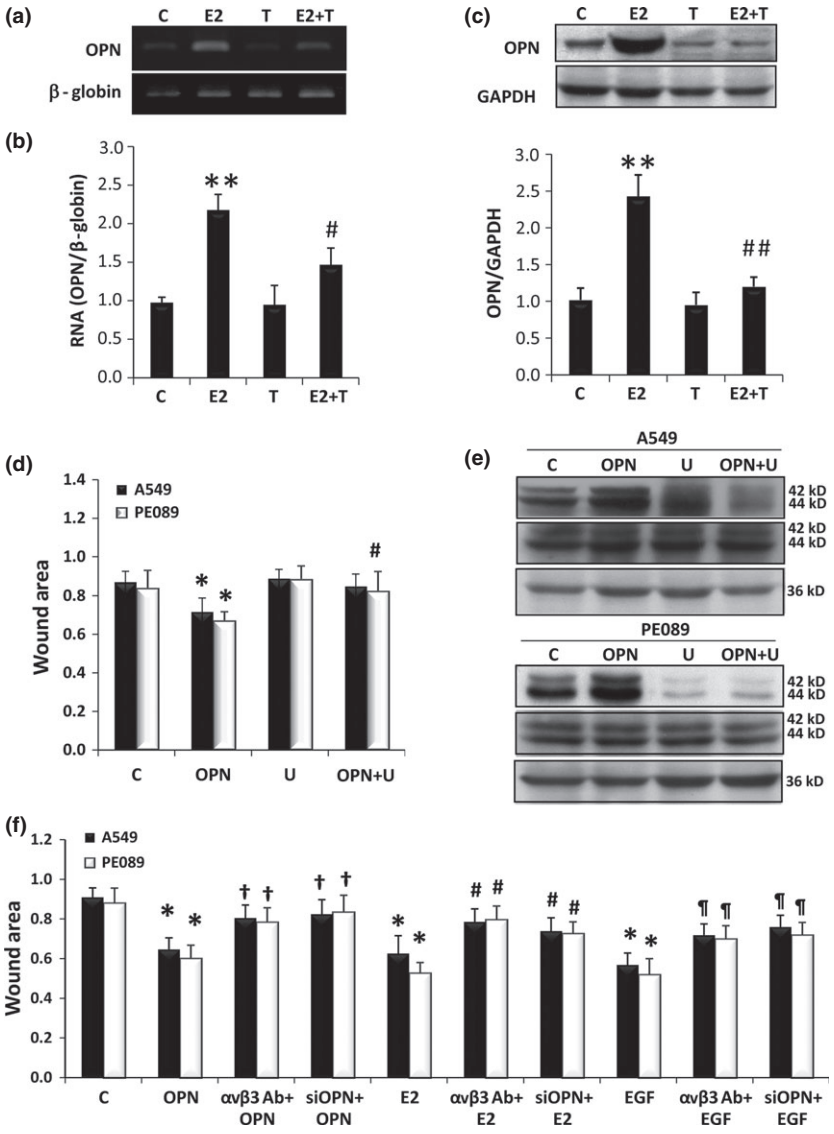

Fig. 4. Estrogen induced osteopontin (OPN) expression and promoted cell migration through αvβ3 integrin binding and MEK/ERK signaling. (a) Gel electrophoretogram of OPN (*spp1*) gene expression of A549 cells in the presence of E_2 and/or tamoxifen. (b) The expression levels of OPN mRNA were quantified by quantitative real-time PCR. $^{**}P < 0.01$ compared with the control; $^{\#}P < 0.05$ compared with the E_2 group. (c) Western blotting of OPN in the culture media from the cells treated with E_2 and/or tamoxifen was analyzed. Both the E_2-induced OPN mRNA and protein expression were reduced in the presence of tamoxifen. $^{**}P < 0.01$ compared with the control; $^{\#\#}P < 0.01$ compared with the E_2 group. (d) Wound healing assay of A549 or PE089 cells incubated for 24 h with OPN and/or U0126 (U). U0126 inhibited OPN-promoted cell migration. $^{*}P < 0.05$ compared with the control; $^{\#}P < 0.05$ compared with the OPN group. (e) Western blotting of ERK and phosphorylated ERK of the cells treated with OPN and/or U0126. OPN-induced ERK phosphorylation was inhibited by U0126. (f) Wound healing assay of A549 or PE089 cells incubated with OPN, E_2, EGF with/without anti-αvβ3 antibody (αvβ3 Ab) or siOPN for 24 h. αvβ3 Ab and siOPN attenuated the E_2 or EGF-induced cell migration. $^{*}P < 0.05$ compared with the control; $^{\dagger}P < 0.05$ compared with the OPN group; $^{\#}P < 0.05$ compared with the E_2 group; $^{\P}P < 0.05$ compared with the EGF group.

was not reached. The premenopausal women had higher pleural fluid E_2 (median, 21.5 *vs* 13.0 pg/mL; $P = 0.044$) concentrations than the postmenopausal women. Pleural fluid E_2 and OPN concentrations were positively correlated ($n = 62$, $r = 0.74$, $P = 0.002$; Fig. 5c).

Discussion

There is a lung adenocarcinoma epidemic in Taiwanese women who have never smoked.[18] In addition to the role of cooking fumes and passive smoking frequently discussed, estrogen is speculated to play an important role in lung cancer carcinogenesis and to adversely affect patients' prognosis. In the study, smoking history remained a poor prognostic factor for patients with lung adenocarcinoma. Never-smoking women and men with lung adenocarcinoma had similar survival rates. An estrogen cancer-promoting effect appears to be responsible for the more advanced disease in the premenopausal female never-smoking adenocarcinoma patients and contributes to a shorter survival, in spite of more comorbidities existing in the postmenopausal women.

Estrogen receptors are consistently found in lung cancer tissues and lung cancer cell lines (especially adenocarcinoma), mostly in the form of ERβ.[19–22] In addition to cell proliferation, cell migration and degradation of the extracellular matrix are crucial steps in tumor progression. In the study, we demonstrated that cancer cell migration could be stimulated by E_2 and that an ER antagonist inhibited this effect. Through establishing cell clones with ERβ overexpression or knockdown and the application of an ERβ-specific agonist or antagonist, we demonstrated that estrogen promotes cell migration via ERβ.

Recent research has elucidated the pivotal role of OPN, a small integrin-binding ligand N-linked glycoprotein in regulating the cell signaling that controls tumor progression and metastasis.[23,24] Enhanced OPN expression has been noted in the plasma of advanced lung cancer patients, and OPN has also been speculated to be involved in the formation of malignant pleural effusions.[25–29] Estrogen-related receptors (ERR) have been known to regulate the synthesis of osteopontin and ERRalpha response elements have been identified in the OPN promoter.[30,31] Estrogen was demonstrated to regulate OPN expression and lead to angiogenesis and metastasis in breast cancer.[32] Our results demonstrated that E_2 can augment OPN expression and secretion and induce cell migration through αvβ3 integrin binding in lung adenocarcinoma. The elevated and positively correlated pleural fluid E_2 and OPN

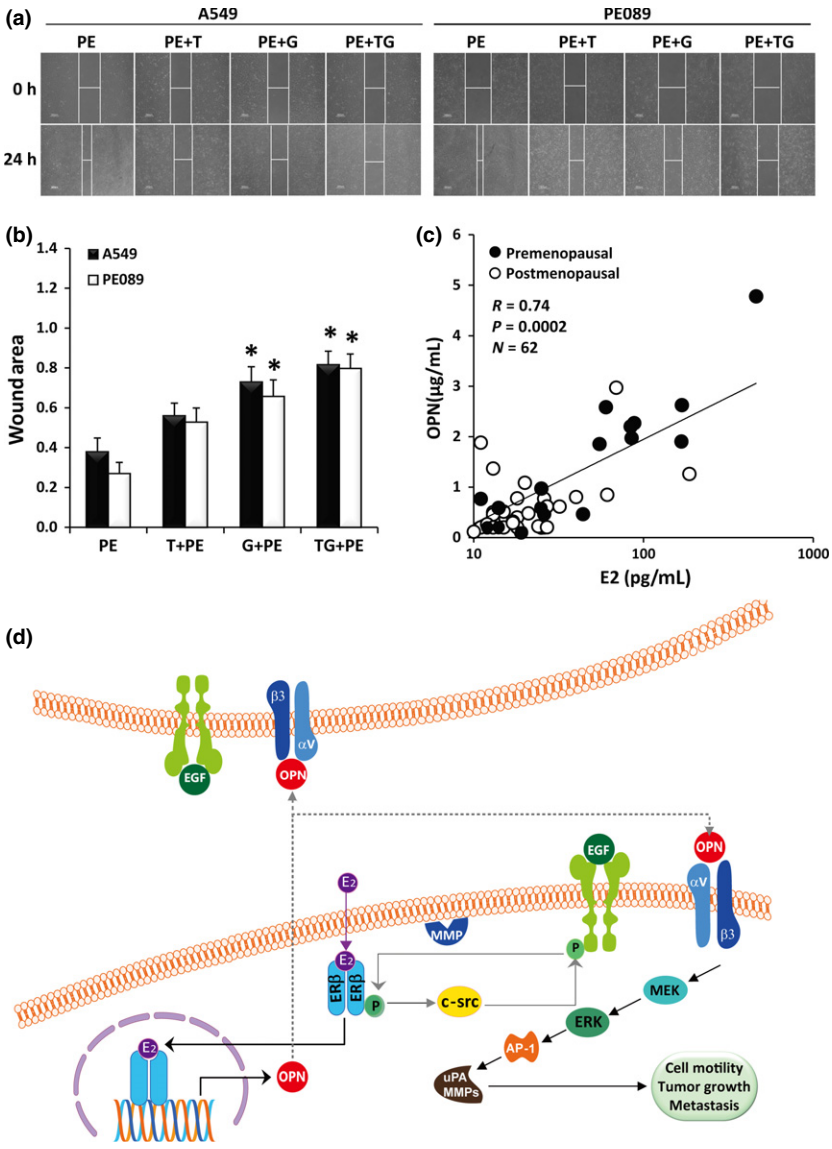

Fig. 5. Both estrogen and osteopontin (OPN) were elevated and positively correlated in the malignant pleural effusions from the patients with lung adenocarcinoma. (a,b) Wound healing assay of A549 or PE089 cells incubated for 24 h with the cell-free supernatants of malignant pleural effusions (PE) from a patient with lung adenocarcinoma. Tamoxifen and/or gefitinib inhibited the cell migration induced by the malignant pleural effusion. $*P < 0.05$ compared with the control. (c) Estradiol and OPN were both elevated and positively correlated in the malignant pleural effusions from the 62 female patients with lung adenocarcinoma. (d) Schematic diagram illustrating the proposed mechanism of how estrogen affects lung cancer cell migration. Estrogen upregulates OPN expression and promotes lung cancer cell migration via the MEK/ERK signaling pathway. OPN contributes to the cross-talk between ER and EGFR signaling pathways.

concentrations in patients with lung adenocarcinoma supported the *in vitro* findings.

For the elevated pleural fluid E_2 concentrations in malignant pleural effusion, we measured the pleural fluid vascular endothelial growth factor (VEGF) and urokinase-type plasminogen activator concentrations in a subsequent study of the same population. Although patients with malignant pleural effusion had higher VEGF than those with non-malignant pleural effusion (median, 1028.82 *vs* 462.85 pg/mL; $P = 0.022$), there was no significant correlation between pleural fluid E_2 and VEGF concentrations, which is a marker of pleural vascular hyperpermeability (unpublished data by Hsu LH, Hsu PC, Liao TL, Feng AC, Chu NM and Kao SH). Increased pleural permeability is the predominant mechanism of malignant pleural fluid formation, but we could not attribute the elevated pleural fluid E_2 concentration to the increased E_2 permeation into pleural cavity. However, we also observed that some postmenopausal women had extraordinary high pleural fluid E_2 concentrations. Studies with bigger case numbers are needed to determine whether increased production of E_2 or increased E_2 permeation into pleural cavity contributed to the higher pleural fluid E_2 concentration in malignant pleural effusion.

Patients with lung adenocarcinoma have specific mutations in the EGFR gene which lead to the activation of growth factor signaling and conferring susceptibility to tyrosine kinase inhibitors.[32–35] Treatment of tumor cells *in vitro* or in nude mice with lung tumor xenografts *in vivo* with inhibitors of aromatase, an estrogen synthase, has been shown to lead to significant suppression of tumor growth.[4,16,17] In addition, a combination of ER antagonists and EGFR-tyrosine kinase inhibitors has been shown to decrease cell proliferation and tumor growth more than one individual treatment in both *in vitro* and *in vivo* studies.[17,36–38] Stabile *et al.*[36] found that EGFR protein expression was downregulated in response to estrogen and upregulated in response to anti-estrogens *in vitro*. Conversely, ERβ expression was found to be decreased in response to EGF and increased in response to gefitinib. A strong association has been reported between the expression of ERβ and EGFR mutations in adenocarcinoma of the lung.[39,40] These studies provide evidence of a functional interaction between the ER and EGFR pathways in lung cancer and support a rationale to use combined therapy.[41,42] In the study, treatment with U0126, an MEK inhibitor, attenuated the cancer cell migration stimulated by E_2 and OPN, implicating their

actions through the MEK-ERK signaling pathway, which is also a downstream signaling pathway after activation of EGFR and possibly contributes to the crosstalk among E_2, OPN and EGF (Fig. 5d). The combination of estrogen and EGF resulted in maximal stimulation of cell migration and ERK phosphorylation. Conversely, the combination of tamoxifen and gefitinib resulted in maximal inhibition of these effects.

We used a large, prospective cohort epidemiologic study and controlled for the confounding effect of smoking to evaluate the role of estrogen. However, there are potential limitations. We were not able to include hormone replacement therapy in the analysis. However, the biological plausibility was still generalized when compared to the relatively short-term effect of hormone replacement therapy with the survival trend over a long period. Analysis of its use was also subject to recall bias. For laboratory evidence, whether or not differences exist in the data endpoints in relation to EGFR mutation status should be confirmed in more than one cell line of each type to make any conclusions.

In summary, we proposed a different pathway of ER activation and interaction with EGFR (Fig. 5d). Estrogen, with its receptor, has the potential to be a prognosticator and a therapeutic target in lung cancer. ER antagonist may become a new effective treatment modality for patients with lung adenocarcinoma and an alternative treatment for patients with acquired resistance to EGFR antagonists.[43,44] Considering the conflicting results in the literature,[10–14,45] further clinical research and laboratory experiments should be undertaken to clarify the interaction of estrogen with various mediators and to elucidate its role in carcinogenesis, cell proliferation, migration and invasion.

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Disclosure Statement

The authors have no conflict of interest to declare.

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