by tumor cells. Primary intrahepatic cholangiocarcinoma (ICC) cells were treated with gemcitabine (left panel), 5-fluorouracil (5-FU) (middle panel) or interferon-gamma (IFN-γ) (right panel) at the indicated concentrations. The mean fluorescence intensity (MFI) of calreticulin was analyzed. The results are expressed as the mean ± SD. *p<0.05, **p<0.01. B: The histogram profile of the dotted line indicates the control (no treatment), and the lined histogram indicates CRT expression in the treated ICC cells.

Conflicts of interest

The Authors declare that they have no competing interests.

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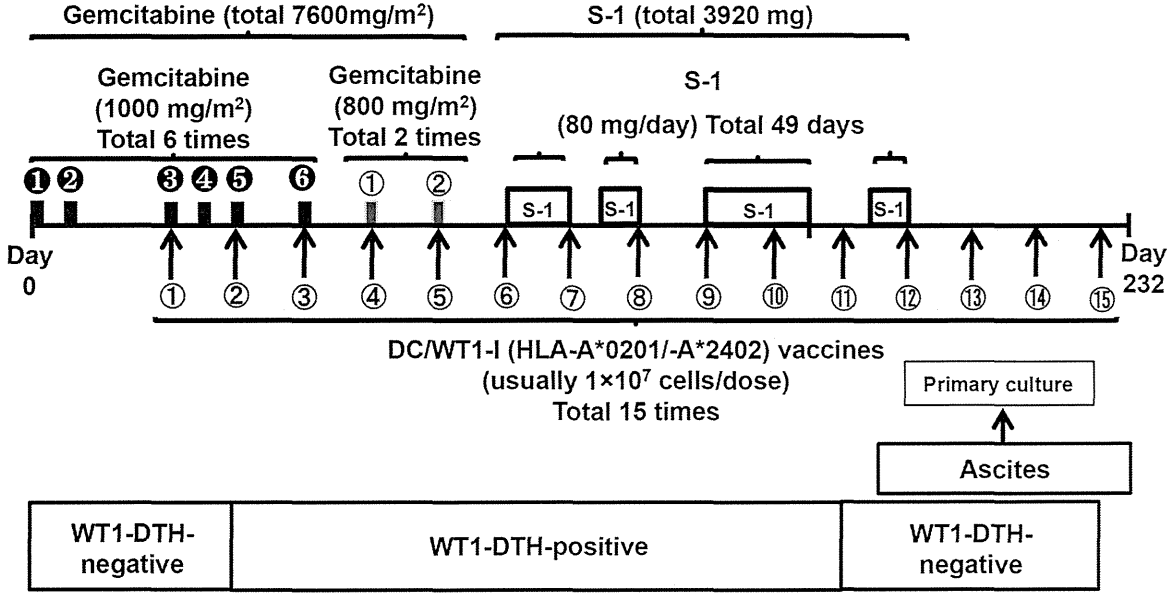
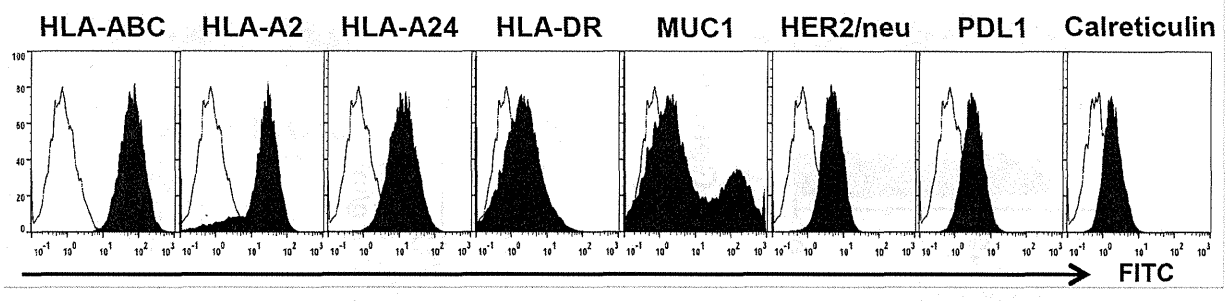
Treatment schedule

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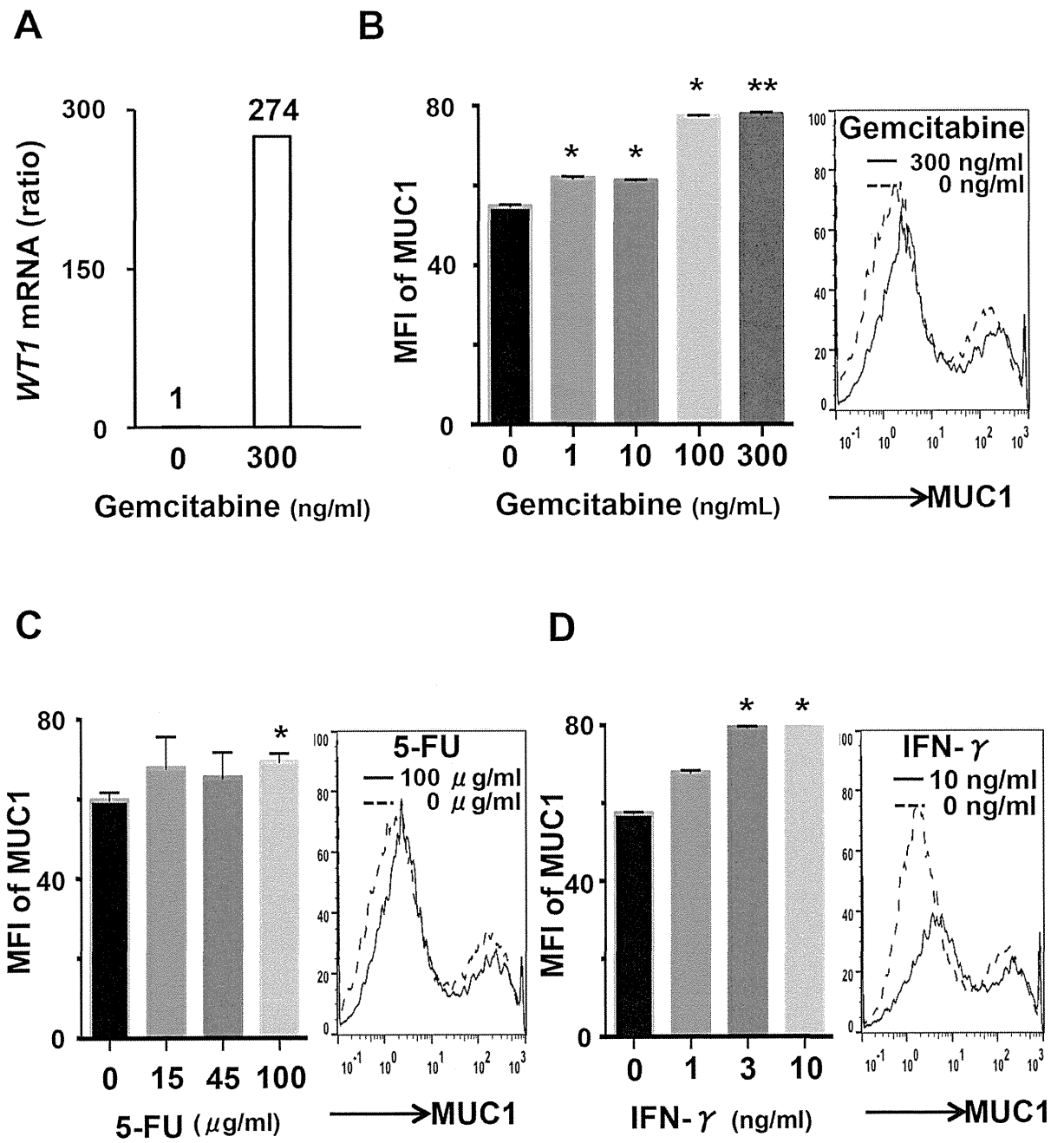
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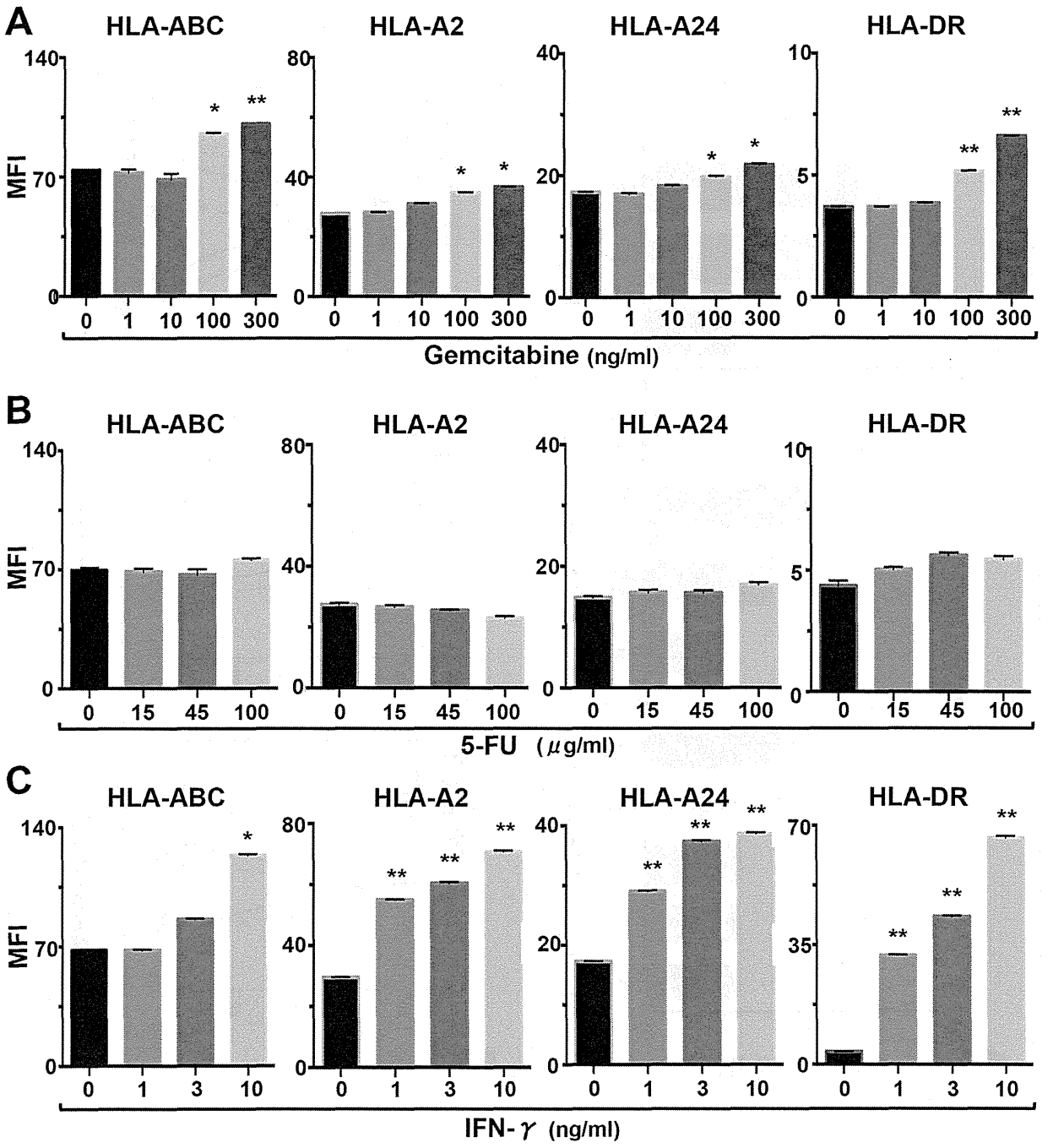
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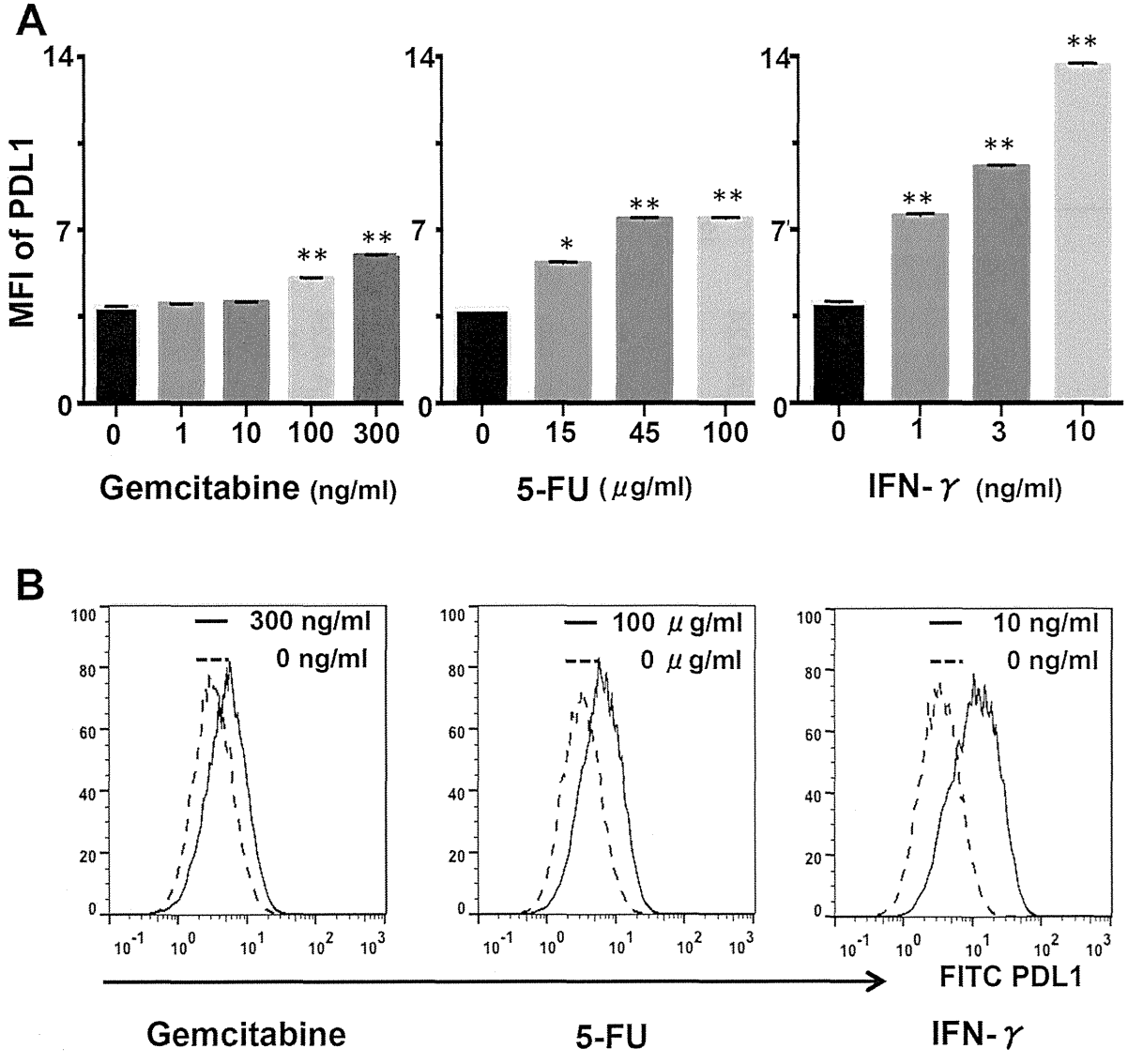
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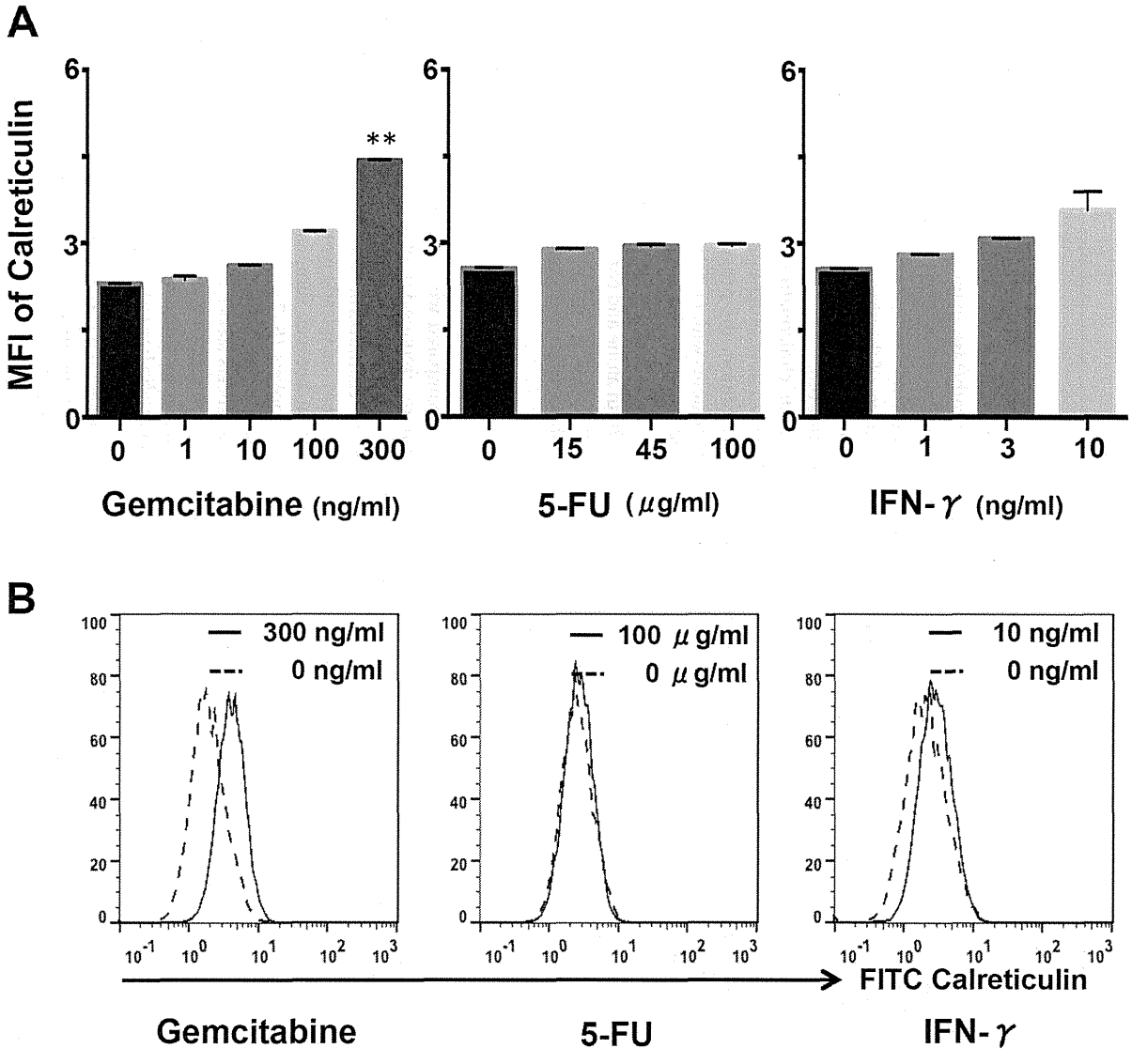
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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

Prognostic and Therapeutic Implications of Aromatase Expression in Lung Adenocarcinomas with *EGFR* Mutations

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Abstract

Purpose: Lung adenocarcinomas among never-smokers are more common in females than in males. This implies that gender-dependent hormones promote smoking unrelated lung adenocarcinoma. We therefore investigated mRNA expression of aromatase, an intrinsic estrogen synthetase, in lung adenocarcinoma and assessed its correlation to clinicopathologic factors, including *EGFR* mutations and postsurgical prognosis.

Experimental Design: Aromatase mRNA expression in primary tumor samples from 110 patients with lung adenocarcinoma was evaluated with qRT-PCR. Inhibitory effects of the aromatase inhibitor exemestane were assessed in lung adenocarcinoma cell lines (11-18 and HCC4006), which have *EGFR* mutations, separately and combined with EGFR tyrosine kinase inhibitor erlotinib.

Results: Aromatase gene expression was not correlated with patients' clinicopathologic factors, including *EGFR* mutation status. High aromatase expression was associated with poor prognosis for both recurrence-free survival ($P = 0.004$) and overall survival ($P = 0.003$). In addition, the prognostic significance of aromatase expression was limited to females, never-smokers, and patients with *EGFR* mutations, but not in their counterparts. HCC4006, which has a low aromatase mRNA expression level, was not sensitive to exemestane, either alone or combined with erlotinib. In contrast, growth of 11-18 cells, which have high aromatase expression, was significantly inhibited by exemestane, both alone and combined with erlotinib.

Conclusions: Aromatase is a candidate prognostic factor in patients with lung adenocarcinoma, especially in those with *EGFR* mutations, and may also be a beneficial therapeutic target in those patients. *Clin Cancer Res; 20(13); 3613–22. ©2014 AACR.*

Introduction

Worldwide, lung cancer is the leading cause of cancer death in males, and the second leading cause of cancer death in females (1). Although tobacco smoking is the predominant risk factor for lung cancer, approximately 25% of lung cancer cases are not attributable to tobacco use (2). The proportion of never-smokers among patients with non–small cell lung cancer (NSCLC) has significantly increased for decades. NSCLC in never-smokers is more frequent in females and the adenocarcinoma cell type, and has a better prognosis compared with NSCLC in ever smokers (3, 4). Furthermore, frequencies of oncogenic drivers, such as mutations in *KRAS* or epidermal growth factor receptor (*EGFR*), or echinoderm microtubule–associated protein-like 4 (*EML4*)–anaplastic lymphoma kinase (*ALK*) fusion are different between lung cancers in never-smokers and those in smokers (5–7). These striking differences in epidemiologic, clinical, and molecular characteristics suggest that lung cancers associated with smoking and those unassociated with smoking are separate entities (2, 3).

The higher proportion of females among patients with lung cancer who have never smoked suggests a possible role for gender-dependent hormones in lung cancer development. Estrogen reportedly affects differentiation and maturation of the normal lungs (8) and stimulates lung tumor growth in both laboratory-based (9–12) and clinical studies (13–16). Epidemiologic studies also have suggested that endogenous and exogenous estrogen affect development of lung cancer (17). A *post hoc* analysis of a randomized controlled trial in postmenopausal women showed that hormone replacement therapy (HRT) may increase the risk of death from lung cancer (18). A prospective cohort study confirmed dose-dependent increase in lung cancer risk among women who received HRT (19). A decreased incidence of lung cancer was observed in patients with breast

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

The proportion of females among patients with lung cancer with no smoking history is reportedly increasing, which implies that female hormones may affect the development of lung cancer. We examined mRNA expression of aromatase (*CYP19A1*), a possible intrinsic estrogen-synthetase, in patients with primary lung adenocarcinoma, and assessed its correlation with clinicopathologic factors including *EGFR* mutation status and prognosis. High aromatase gene expression was associated with poor outcomes. The prognostic significance of aromatase expression was also demonstrated in females, never-smokers, and patients with *EGFR* mutations, whereas such significance was not observed in their counterparts. *In vitro* analysis showed an antitumor effect of aromatase inhibitor in a lung adenocarcinoma cell line with an *EGFR* mutation and high aromatase expression. Our findings suggest aromatase is a possible therapeutic target in lung adenocarcinomas with *EGFR* mutations.

cancer treated with an aromatase inhibitor, exemestane, after tamoxifen therapy compared with patients who continued tamoxifen therapy (20). These data strongly support an important effect of female hormones in lung cancer development.

Aromatase (CYP19A1) is a cytochrome P-450 enzyme that converts androstenedione and testosterone to estrone and estradiol, respectively, and supports breast and endometrial cancer growth via autocrine and paracrine stimulation (21, 22). In not only gonadal tissue, but also in lung tissue, estrogen is synthesized mainly by aromatase (12, 23). Treatment with aromatase inhibitor has been found to suppress growth in lung cancer cell lines and mice tumor xenografts (12). In early-stage lung cancer, high aromatase expression reportedly correlates with poor prognosis in women ages $\geq$65 years (13). These studies suggest that aromatase affects lung cancer development, although the precise pathway is unclear.

After somatic *EGFR* mutations were discovered in NSCLC, many studies found higher mutation frequencies in East Asians, women, never-smokers, and adenocarcinomas (5, 6). The prevalence of *EGFR* mutation among female patients implies interactions between female hormones and *EGFR* mutations. Interactions between estrogen receptor (ER) and EGFR pathways have been extensively investigated *in vitro* (10, 11, 24, 25) and in tumor specimens (14, 15, 26, 27), but the influence of aromatase on EGFR-dependent growth is not clear.

We studied expression and prognostic significance of aromatase, with regard to *EGFR* mutation status, in patients with primary lung adenocarcinoma. We also examined growth inhibition by aromatase inhibitor combined with EGFR tyrosine kinase inhibitor (TKI) on lung cancer cell lines with *EGFR* mutations.

Materials and Methods

Human tissue samples

Primary tumor and corresponding nonneoplastic lung specimens were collected from 110 consecutive patients who underwent complete resections (R0) for primary lung adenocarcinoma from April 2007 to March 2011 at the Department of Surgery and Science, Kyushu University Hospital (Fukuoka, Japan), for whom surgical specimens were available and *EGFR* mutation status were determined (Table 1). This study included 44 men and 66 women, with a mean age of 67.7 years (range: 37–85 years) at surgical resection. Almost all of the women were postmenopausal. Histologic tumor diagnoses were based on hematoxylin and eosin-stained preparations, using the WHO 2004 classification (28). Pathologic staging was performed according to the 7th edition of the TNM Classification of Malignant Tumors (29). *EGFR* mutation tests used the peptide nucleic acid–locked nucleic acid (PNA-LNA; Mitsubishi Chemical Medience, Tokyo, Japan) polymerase chain reaction (PCR) clamp method (30) with formalin-fixed paraffin-embedded sections of surgical specimens. No patient was treated with chemotherapy or radiotherapy before surgery. Thirty-nine (35.5%) patients received postoperative chemotherapy: 21 received oral tegafur and uracil, 17 were enrolled into a clinical trial for the postoperative adjuvant chemotherapy (S-1 or cisplatin-S-1), and 1 received paclitaxel. A routine check-up with a physical examination, blood cell counts, serum chemistry, serum tumor markers including carcinoembryonic antigen and cytokeratin fragment 19, and chest X-rays were performed on an outpatient basis 4 times a year for the first 3 years, and thereafter twice annually. Computed tomography was performed twice a year for the first 3 years, and thereafter at least annually. Brain magnetic resonance imaging, and bone scintigram or fluorodeoxyglucose positron-emission tomography were performed annually. This study was approved by the Kyushu University Institutional Review Board for Clinical Research (no. 24–173).

Tumor samples and corresponding nonneoplastic lung tissues (most distant from tumor) were obtained immediately after resection, frozen in liquid nitrogen, and stored at −80°C.

Cells and reagents

We obtained 21 lung adenocarcinoma cell lines and the breast cancer line MCF-7. A549, LK87, PC-9, and 11-18 cell lines were the kind gift of Dr. M. Takeshita. HCC4006 cell line was the kind gift of Dr. A.F. Gazdar, and was confirmed by identification of the rare *EGFR* deletion mutation (del L747_E749, A750P) in this cell line (31). The ACC-LC-319 cell line was a kind gift from Dr. T. Hida. Total RNAs from other cell lines were extracted in previous analyses (32, 33) or were the kind gifts of Dr. K. Tomizawa and Dr. T. Mitsudomi.

Driver mutations of the cell lines were *KRAS* mutations: A549, ACC-LC-94, H23, H358, H2009, LK87, and SK-LU1; *EGFR* mutations: H3255, HCC827, HCC4006, PC-9, and 11–18; *MET* mutation: H596; *MET* amplifications: ACC-

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Table 1. Clinicopathologic characteristics by aromatase expression ($n = 110$)

Characteristic		Number ($n = 110$)	Aromatase expression		P
			Low ($n = 83$)	High ($n = 27$)	
Age (y)	< 70	58	44	14	0.92
	≥ 70	52	39	13	
Sex	Male	44	33	11	0.93
	Female	66	50	16	
Smoking history	Never	60	46	14	0.75
	Current or former	50	37	13	
EGFR mutation	Negative	56	44	12	0.44
	Positive	54	39	15	
SUV_{max}[a]			6.1 ± 4.7	6.4 ± 4.5	0.76
Tumor size (cm)			2.9 ± 1.7	3.0 ± 1.5	0.47
Histologic grade	G1	53	44	9	0.21
	G2	41	28	13	
	G3	16	11	5	
	G4	0	0	0	
Pleural invasion[b]	Negative	89	68	21	0.55
	Positive	20	14	6	
Lymphatic invasion	Negative	97	73	24	0.90
	Positive	13	10	3	
Vascular invasion	Negative	78	61	17	0.30
	Positive	32	22	10	
Pathologic stage	I	81	64	17	0.15
	II, IIIA	29	19	10	

[a]Data not available for 19 of the aromatase-low patients and 5 of the aromatase-high patients.
[b]Data not available for one of the aromatase-low patients.

LC-319 and H1993; *Ros* fusion: HCC78; *HER2* mutation: H1781; *EML4/ALK* fusion, H2228; unknown: HCC193, SK-LC-3, and VMRC-LCD.

Cells were maintained in RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum (Life Technologies), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C.

EGFR-TKI erlotinib and aromatase inhibitor exemestane were purchased from Selleck Chemicals and LKT Laboratories, respectively.

RNA extraction and quantitative RT-PCR

The aromatase mRNA expression levels were evaluated by quantitative RT-PCR. Total RNA was extracted from resected lung tissues and cell lines using ISOGEN (Nippon Gene) according to the manufacturer's protocol. cDNA was synthesized using a SuperScript III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's protocol. Quantitative PCR amplification was performed using Applied Biosystems StepOnePlus real-time PCR system (Life Technologies). TaqMan gene expression assay (Applied Biosystems) for *CYP19A1* (Hs00903413_m1) was used and *β-actin* (Hs99999903_m1) was used as an internal control. The BD qPCR total RNA human reference (Clontech Laboratories, Inc.), corresponding to a standardized mixture of total RNAs from a collection of adult human tissues, was used as a standard for quantitation. Relative aromatase mRNA expression levels of each sample (tissue and cell line) were standardized to those of *β-actin* and calculated relative to that of the total RNA human reference. Each sample was tested with triplicate measurements, and the mean value of the triplicate measurements was defined as a final value. We divided patients with adenocarcinoma into 2 groups based on the expression level of aromatase compared with human reference; high aromatase expression was defined as being above the human reference expression, whereas low expression was defined as being below it.

Cell-proliferation assay

HCC4006, 11-18, H358, H2228, and ACC-LC-319 cells (5×10^3) were plated into each well of 96-well flat-bottomed plates and grown in phenol red-free RPMI 1640 (Life Technologies) containing 10% dextran-coated charcoal-stripped fetal bovine serum (Biological Industries). Twenty-four hours later, dimethyl sulfoxide (DMSO), erlotinib, exemestane, or a combination of these drugs was added to achieve the indicated drug concentration, and cells were incubated for an additional 72 hours. The viability of drug-treated cells was determined by a WST-8 method using Cell Count Reagent SF (Nacalai Tesque) according to the

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manufacturer's instructions. Percent growth was determined relative to DMSO-treated controls.

Statistical analysis

Statistical analysis was performed using JMP statistical software version 9.0.2 (SAS Institute Inc.). All variables are expressed as the mean ± standard deviation (SD). Qualitative variables were compared using χ^2 tests, and quantitative variables were compared using Wilcoxon tests. Multivariate models were constructed using logistic regression, including sex, smoking history, and *EGFR* mutation status, with aromatase expression (high/low) as the outcome of interest. Survival curves were drawn using the Kaplan–Meier method. Significant differences among subgroups were compared using the log-rank test. The Cox proportional hazard regression model was used to explore the effects of the clinicopathologic variables and aromatase expression on survival. Factors showing prognostic significance in the univariate analyses were adopted as variables in multivariate analysis. $P < 0.05$ was considered statistically significant.

Results

Expression of aromatase mRNA in lung adenocarcinoma tissues and corresponding nonneoplastic lung tissues

We first examined the mRNA expression level of aromatase in lung adenocarcinoma and corresponding nonneoplastic lung tissues, using quantitative RT-PCR. Relative aromatase mRNA expression in carcinoma tissues (0.83 ± 1.06) was significantly higher than in corresponding nonneoplastic lung tissues (0.55 ± 0.46; $P = 0.025$; Fig. 1). Aromatase mRNA expression in nonneoplastic lung tissues did not significantly differ among subgroups divided by age (<70 vs. ≥70), sex, smoking history, or *EGFR* mutation status ($P = 0.07$, 0.58, 0.46, and 0.61, respectively).

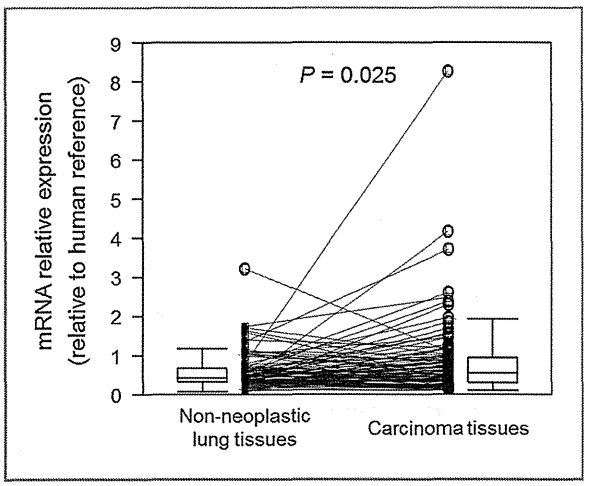

Figure 1. Differences in aromatase mRNA expression levels between carcinoma tissues and corresponding nonneoplastic lung tissues ($n = 94$). Each value is shown in an open circle; paired values of the same patient are connected by a line. Two data groups are shown as box-and-whisker plots, with the bottom and top of the box at the first and third quartiles, and the band inside the box at the median. Upper and lower whiskers indicate 90th and 10th percentiles, respectively. Aromatase mRNA level for the human reference RNA is set as 1. Statistical difference was determined by a Wilcoxon matched-pair signed-rank test.

Relationship between aromatase expression and clinicopathologic factors

In univariate analysis, no significant correlation was identified between tumor aromatase expression level and clinicopathologic factors that are associated with smoking-unrelated lung cancer, such as sex, smoking history, and *EGFR* mutation status (Table 1). The same results were observed for other clinicopathologic characteristics; age, maximum standardized uptake value (SUV_{max}), tumor size, histologic grade, pleural invasion, lymphatic invasion, vascular invasion, and pathologic stage. In addition, in multivariate analysis, no significant association between high aromatase expression and sex, smoking history, or *EGFR* mutation status was identified (Supplementary Table S1). We also examined tumor aromatase expression as a continuous variable. However, any statistically significant correlation was still not found between aromatase expression level and clinicopathologic factors, although tendencies were seen in vascular invasion ($P = 0.06$) and pathologic stage (I vs. ≥II, $P = 0.051$).

Influence of aromatase gene expression level on survival

A survival analysis was performed in 110 patients who underwent curative resections. Median follow-up time was 35 months (range: 9–66 months). High expression of aromatase was associated with poor prognosis in terms of both recurrence-free survival (RFS; $P = 0.004$; Fig. 2A) and overall survival (OS; $P = 0.003$; Fig. 2B).

Cox regression analyses of clinical variables for RFS and OS are shown in Table 2. Among the variables, sex, aromatase expression, and pathologic stage were identified as potential predictors of RFS. A multivariate analysis that included the above variables also showed aromatase expression to be a significant prognostic factor, with a relative risk of 2.37 [95% confidence interval (CI), 1.05–5.31; $P = 0.039$] for RFS. Multivariate analysis for OS was not performed because of the small number of events (deaths).

Prognostic significance of aromatase expression in lung adenocarcinomas with EGFR mutations

Next, we compared survival between subgroups divided by clinicopathologic factors that are related to smoking-unrelated lung cancer such as sex, smoking history, and *EGFR* mutation status. High aromatase expression was associated with a poor prognosis in females ($P = 0.008$ for RFS and $P < 0.001$ for OS; Fig. 2C and D), in never-smokers ($P = 0.009$ for RFS and $P < 0.001$ for OS; Fig. 2E and F), and in patients with *EGFR* mutations ($P = 0.005$ for RFS and $P = 0.003$ for OS; Fig. 2G and H), but not in males ($P = 0.14$ for RFS and $P = 0.65$ for OS; Supplementary Fig. S1A and S1B), not in current or former smokers ($P = 0.16$ for RFS and $P = 0.58$ for OS; Supplementary Fig. S1C and S1D) and not in patients without *EGFR* mutations ($P = 0.19$ for RFS

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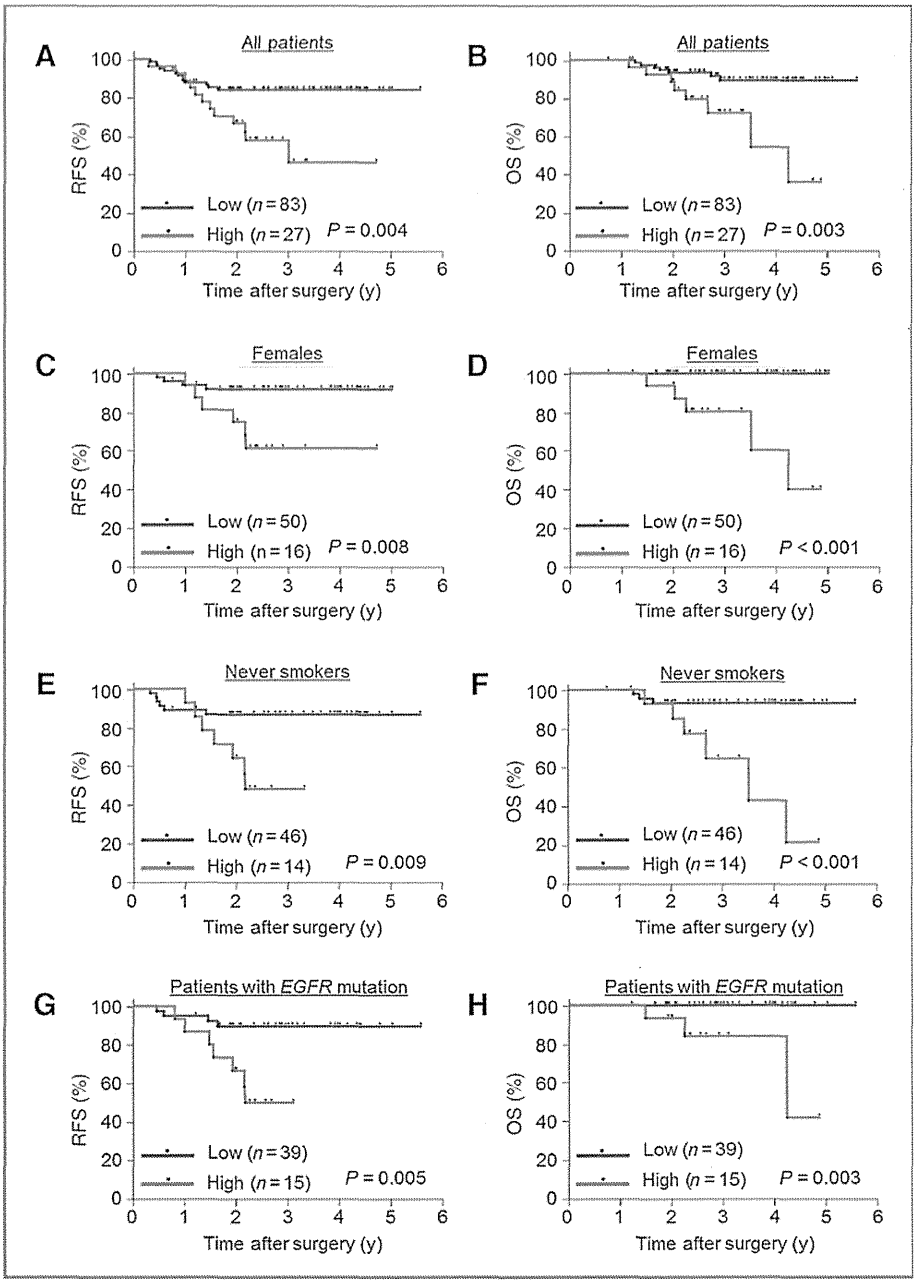

Figure 2. Kaplan–Meier postoperative RFS and OS curves according to aromatase expression level. *N*, number of patients in each category. RFS (A) and OS (B) curves for all cohort patients. RFS (C) and OS (D) curves for females. RFS (E) and OS (F) curves for never-smokers. RFS (G) and OS (H) curves for patients with *EGFR* mutations.

and $P = 0.07$ for OS; Supplementary Fig. S1E and S1F). Eleven patients with *EGFR* mutations had recurrent disease, and among them 8 patients received EGFR-TKI gefitinib after recurrence. There was no patient who received EGFR-TKI before recurrence.

Cox regression analyses for potential predictors of survival in patients with *EGFR* mutations are shown in Table 3. Among the parameters, aromatase expression and pathologic stage were identified as potential predictors of RFS. Multivariate analysis was not performed because of the small number of recurrences.

Growth inhibition of lung adenocarcinoma cell line by aromatase inhibitor

We examined aromatase mRNA expression in 21 human lung adenocarcinoma cell lines (Fig. 3A). No correlation was demonstrated between aromatase expression level and driver mutation type. Because patients with high aromatase expression had worse prognoses than those with low aromatase expression among those with *EGFR* mutations (Fig. 2G and H), we next investigated whether aromatase had therapeutic potential in lung adenocarcinomas with *EGFR* mutations. We chose 11-18 as high-aromatase mRNA-