5-fluorouracil (5-FU) (B) or interferon-gamma (IFN-γ) (C) at the indicated concentrations. The mean fluorescence intensity (MFI) of human leukocyte antigen (HLA)-ABC, -A2, -A24 and -DR expression was analyzed. The results are expressed as the mean ± SD. *p<0.05, **p<0.01.

Figure 5. *Effects of chemoimmunotherapeutic agents on programmed death receptor ligand-1* (PDL1) *expression by primary tumor cells.* A: Primary intrahepatic cholangiocarcinoma (ICC) cells were treated with gemcitabine (left panel), 5-fluorouracil (middle panel) or interferon-gamma (IFN-γ) (right panel) at the indicated concentrations. The mean fluorescence intensity (MFI) of PDL1 expression was analyzed. The results are expressed as the mean ± SD. *p<0.05, **p<0.01. B: The histogram profile with the dotted line indicates the control (no treatment), and the lined histogram indicates PDL1 expression in the treated ICC cells.

Figure 6. *Effects of chemoimmunotherapeutic agents on calreticulin expression*

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.

References

1. Furuse J and Okusaka T: Targeted therapy for biliary tract cancer. Cancers *3*: 2243-2254, 2011.

2. Ramirez-Merino N, Aix SP and Cortes-Funes H: Chemotherapy for cholangiocarcinoma: An update. World J Gastrointest Oncol *5*: 171-176, 2013.

3. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Metivier D, Larochette N, van Endert P, Ciccosanti F, Piacentini M, Zitvogel L and Kroemer G: Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med *13*: 54-61, 2007.

4. Hodge JW, Garnett CT, Farsaci B, Palena C, Tsang KY, Ferrone S and Gameiro SR: Chemotherapy-induced immunogenic modulation of tumor cells enhances killing by cytotoxic T-lymphocytes and is distinct from immunogenic cell death. Int J Cancer *133*: 624-636, 2013.

5. Takahara A, Koido S, Ito M, Nagasaki E, Sagawa Y, Iwamoto T, Komita H, Ochi T, Fujiwara H, Yasukawa M, Mineno J, Shiku H, Nishida S, Sugiyama H, Tajiri H and Homma S: Gemcitabine enhances Wilms' tumor gene *WT1* expression and sensitizes human pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune response. Cancer Immunol Immunother *60*: 1289-1297, 2011.

6. Koido S, Homma S, Takahara A, Namiki Y, Komita H, Uchiyama K, Ito M, Gong J, Ohkusa T and Tajiri H: Immunotherapy synergizes with chemotherapy targeting pancreatic cancer. Immunotherapy *4*: 5-7, 2012.

7. Obeid M, Panaretakis T, Joza N, Tufi R, Tesniere A, van Endert P, Zitvogel L and Kroemer G: Calreticulin exposure is required for the immunogenicity of gamma-irradiation and UVC light-induced apoptosis. Cell Death Differ *14*: 1848-1850, 2007.

8. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V,

Clavel-Chapelon F, Bourhis J, Andre F, Delaloge S, Tursz T, Kroemer G and Zitvogel L: Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med *13*: 1050-1059, 2007.

9. Koido S, Homma S, Okamoto M, Takakura K, Mori M, Yoshizaki S, Tsukinaga S, Odahara S, Koyama S, Imazu H, Uchiyama K, Kajihara M, Arakawa H, Misawa T, Toyama Y, Yanagisawa S, Ikegami M, Kan S, Hayashi K, Komita H, Kamata Y, Ito M, Ishidao T, Yusa S, Shimodaira S, Gong J, Sugiyama H, Ohkusa T and Tajiri H: Treatment with chemotherapy and dendritic cells pulsed with multiple Wilms' tumor 1 (WT1)-specific MHC class I/II-restricted epitopes for pancreatic cancer. Clin Cancer Res *20*: 2014. in press.

10. National Cancer Institute. Common Terminology Criteria for Adverse Events v.3.0 and v.4.0 (CTCAE). http://ctep.cancer.gov/protocol Development/electronic_applications/ctc.htm, 2011.

11. Sugiyama H: Cancer immunotherapy targeting Wilms' tumor gene *WT1* product. Expert Rev Vaccines *4*: 503-512, 2005.

12. Kaida M, Morita-Hoshi Y, Soeda A, Wakeda T, Yamaki Y, Kojima Y, Ueno H, Kondo S, Morizane C, Ikeda M, Okusaka T, Takaue Y and Heike Y: Phase 1 trial of Wilms tumor 1 (WT1) peptide vaccine and gemcitabine combination therapy in patients with advanced pancreatic or biliary tract cancer. J Immunother *34*: 92-99, 2011.

13. Kimura Y, Imai K, Shimamura K, Tsukada J, Tomoda T, Sunamura M, Shimodaira S, Yokokawa K, Koido S, Homma S and Okamoto M: Clinical and immunologic evaluation of dendritic cell-based immunotherapy in combination with gemcitabine and/or S-1 in patients with advanced pancreatic carcinoma. Pancreas *41*:195-205, 2012.

14. Nishida S, Koido S, Takeda Y, Homma S, Komita H, Takahara A, Morita S, Ito T, Morimoto S, Hara K, Tsuboi A, Oka Y, Yanagisawa S, Toyama Y, Ikegami M, Kitagawa T, Eguchi H, Wada H, Nagano H, Nakata J, Nakae Y, Hosen N, Oji Y, Tanaka T, Kawase I, Kumanogoh A, Sakamoto J, Doki Y,

Mori M, Ohkusa T, Tajiri H and Sugiyama H: Wilms tumor gene 1 (*WT1*) peptide–based cancer vaccine combined with gemcitabine for patients with advanced pancreatic cancer. J Immunother *37*:105-114, 2014.

15. Algarra I, Garcia-Lora A, Cabrera T, Ruiz-Cabello F and Garrido F: The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother *53*: 904-910, 2004.

16. Obeid M, Panaretakis T, Tesniere A, Joza N, Tufi R, Apetoh L, Ghiringhelli F, Zitvogel L and Kroemer G: Leveraging the immune system during chemotherapy: moving calreticulin to the cell surface converts apoptotic death from 'silent' to immunogenic. Cancer Res *67*: 7941-7944, 2007.

17. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, Shen S, Kepp O, Scoazec M, Mignot G, Rello-Varona S, Tailler M, Menger L, Vacchelli E, Galluzzi L, Ghiringhelli F, di Virgilio F, Zitvogel L and Kroemer G: Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. Science *334*: 1573-1577, 2011.

18. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, Chen S, Klein AP, Pardoll DM, Topalian SL and Chen L: Colocalization of inflammatory response with B7H1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med *4*: 127-137, 2012.

19. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A and Wigginton JM: Safety and activity of anti-PDL1 antibody in patients with advanced cancer. N Engl J Med *366*: 2455-2465, 2012.

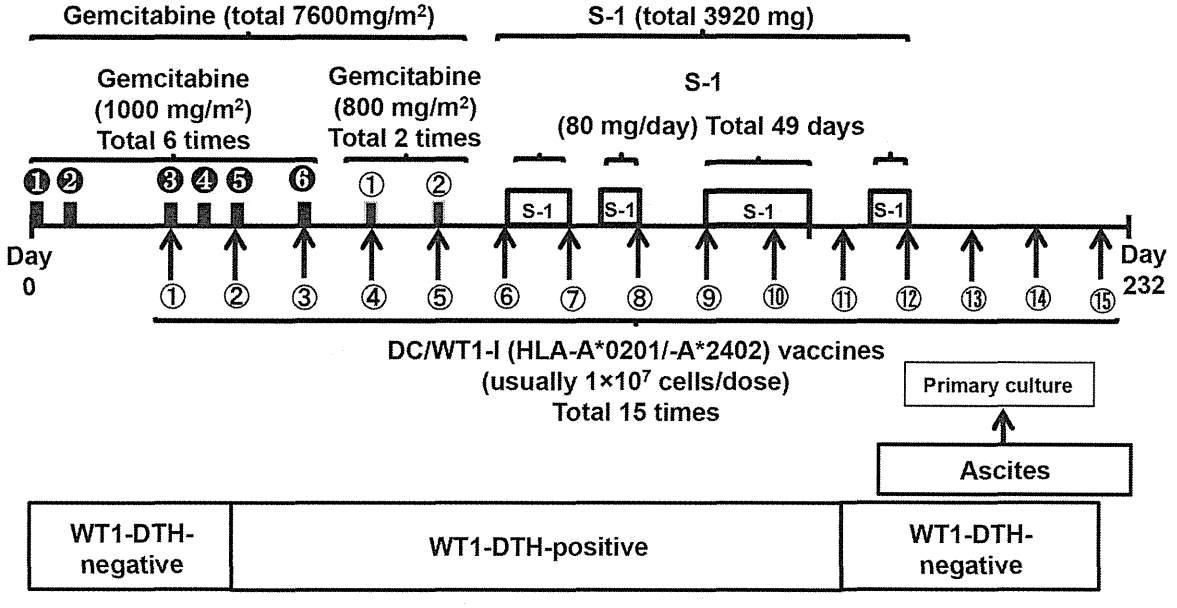
20. Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, Nakamura S, Enomoto K, Yagita H, Azuma M and Nakajima Y: Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. Clin Cancer Res *13*: 2151-2157, 2007.

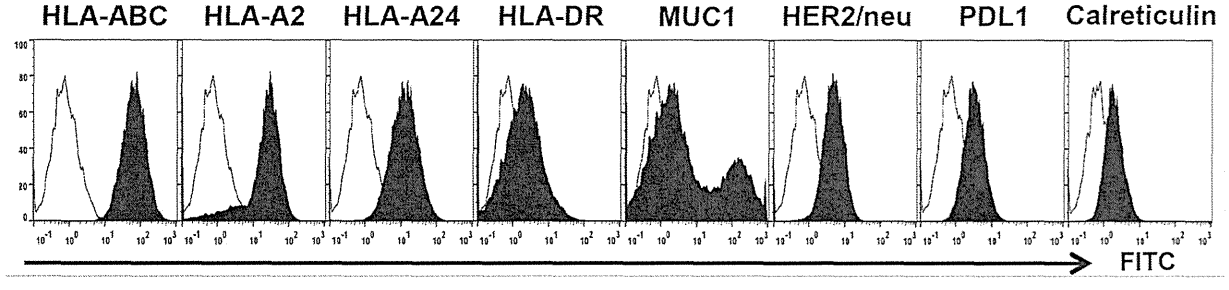
21. Blank C, Brown I, Peterson AC, Spiotto M, Iwai Y, Honjo T and Gajewski TF: PDL1/B7H1 inhibits the effector phase of tumor rejection by T-cell receptor (TCR) transgenic $CD8^+$ T-cells. Cancer Res *64*: 1140-1145, 2004.

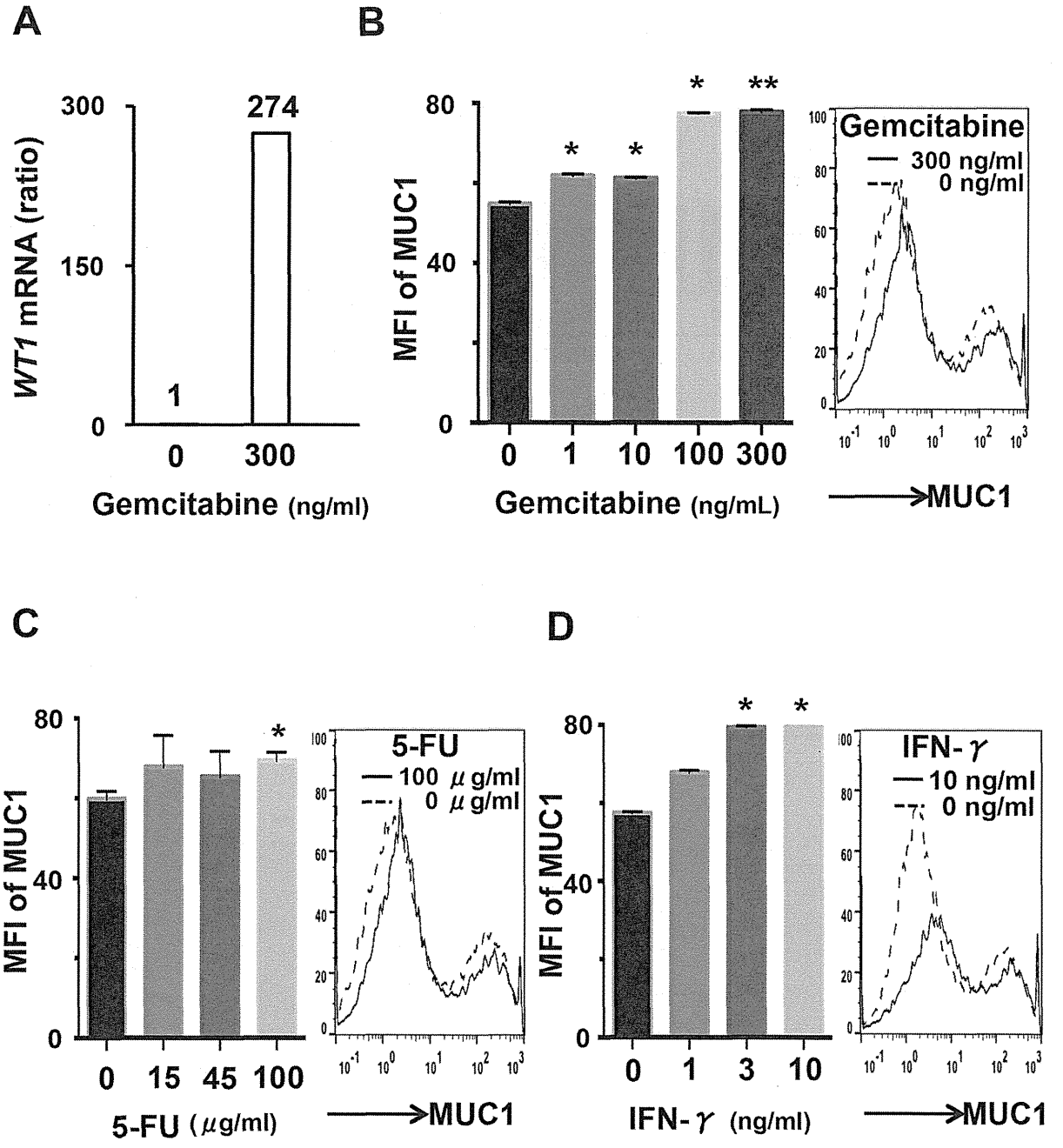
22. Butte MJ, Keir ME, Phamduy TB, Sharpe AH and Freeman GJ: Programmed death-1 ligand 1 interacts specifically with the B7-1 co-stimulatory molecule to inhibit T cell responses. Immunity *27*: 111-122, 2007.

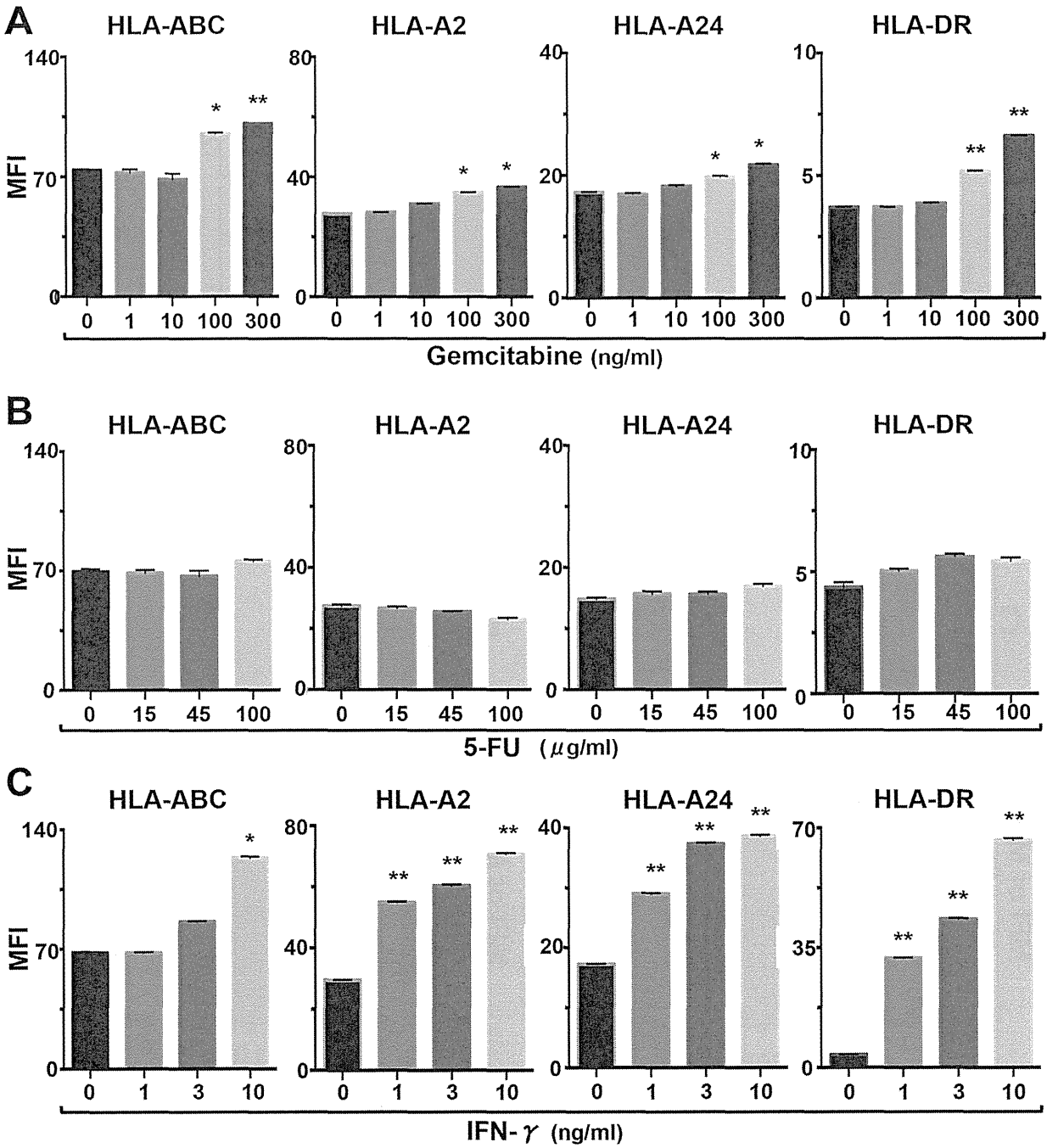
23. Zitvogel L and Kroemer G: Targeting PD1/PDL1 interactions for cancer immunotherapy. Oncoimmunology *1:* 1223-1225, 2012.

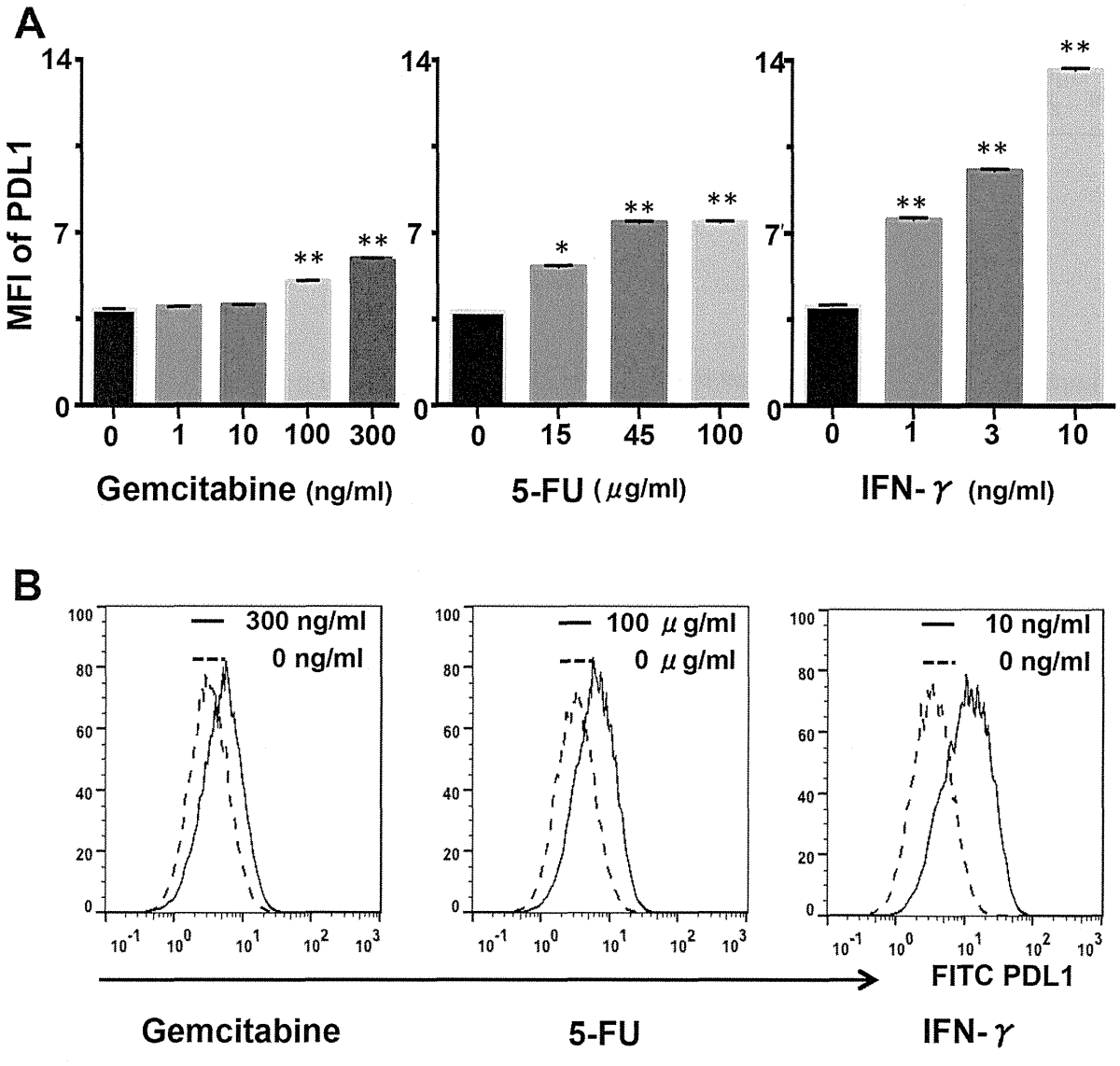
Treatment schedule

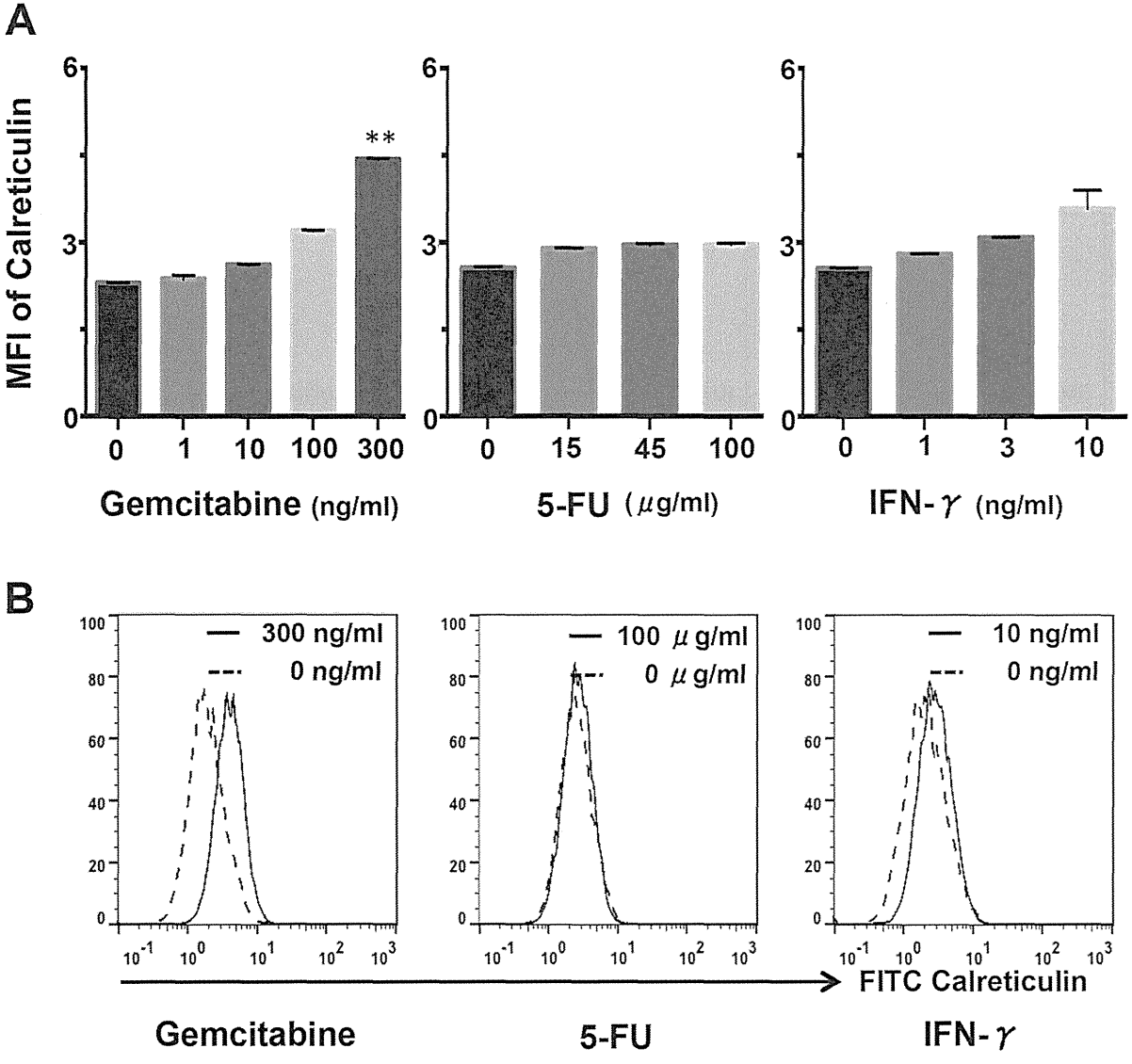



HLA-ABC
HLA-A2
HLA-A24
HLA-DR
MUC1
HER2/neu
PDL1
Calreticulin
FITC


A
300
150
0
274
1
0
300
WT1 mRNA (ratio)
Gemcitabine (ng/ml)
B
80
40
0
*
*
*
**
0
1
10
100
300
MFI of MUC1
Gemcitabine (ng/mL)
Gemcitabine
300 ng/ml
0 ng/ml
MUC1
C
80
40
0
*
0
15
45
100
MFI of MUC1
5-FU (μg/ml)
5-FU
100 μg/ml
0 μg/ml
MUC1
D
80
40
0
*
*
0
1
3
10
MFI of MUC1
IFN-γ (ng/ml)
IFN-γ
10 ng/ml
0 ng/ml
MUC1


A
HLA-ABC
HLA-A2
HLA-A24
HLA-DR
MFI
140
70
0
80
40
0
40
20
0
10
5
0
0 1 10 100 300
Gemcitabine (ng/ml)
B
HLA-ABC
HLA-A2
HLA-A24
HLA-DR
MFI
140
70
0
80
40
0
40
20
0
10
5
0
0 15 45 100
5-FU (μg/ml)
C
HLA-ABC
HLA-A2
HLA-A24
HLA-DR
MFI
140
70
0
80
40
0
40
20
0
70
35
0
0 1 3 10
IFN-γ (ng/ml)


A
MFI of PDL1
14
7
0
0 1 10 100 300
Gemcitabine (ng/ml)
0 15 45 100
5-FU (μg/ml)
0 1 3 10
IFN-γ (ng/ml)
B
300 ng/ml
0 ng/ml
100 μg/ml
0 μg/ml
10 ng/ml
0 ng/ml
FITC PDL1
Gemcitabine
5-FU
IFN-γ


A
MFI of Calreticulin
6
3
0
**
0 1 10 100 300
Gemcitabine (ng/ml)
0 15 45 100
5-FU (μg/ml)
0 1 3 10
IFN-γ (ng/ml)
B
300 ng/ml
0 ng/ml
100 μg/ml
0 μg/ml
10 ng/ml
0 ng/ml
100
80
60
40
20
0
10^-1 10^0 10^1 10^2 10^3
FITC Calreticulin
Gemcitabine
5-FU
IFN-γ

Published OnlineFirst May 6, 2014; DOI: 10.1158/1078-0432.CCR-13-2683

Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

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

Mikihiro Kohno[1], Tatsuro Okamoto[1], Kenichi Suda[1], Mototsugu Shimokawa[2], Hirokazu Kitahara[1], Shinichiro Shimamatsu[1], Hideyuki Konishi[1,4], Tsukihisa Yoshida[1], Mitsuhiro Takenoyama[3], Tokujiro Yano[5], and Yoshihiko Maehara[1]

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

Authors' Affiliations: [1]Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University; [2]Clinical Research Institute, National Kyushu Cancer Center; [3]Department of Thoracic Oncology, National Kyushu Cancer Center, Fukuoka; [4]Gotemba Research Laboratories, Chugai Pharmaceutical Co. Ltd., Gotemba; and [5]Clinical Research Institute, National Hospital Organization Beppu Medical Center, Beppu, Japan

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

Corresponding Author: Tatsuro Okamoto, Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-5466; Fax: 81-92-642-5482; E-mail: tatsuro@surg2.med.kyushu-u.ac.jp

doi: 10.1158/1078-0432.CCR-13-2683

Published OnlineFirst May 6, 2014; DOI: 10.1158/1078-0432.CCR-13-2683

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-

Published OnlineFirst May 6, 2014; DOI: 10.1158/1078-0432.CCR-13-2683

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

Published OnlineFirst May 6, 2014; DOI: 10.1158/1078-0432.CCR-13-2683

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).

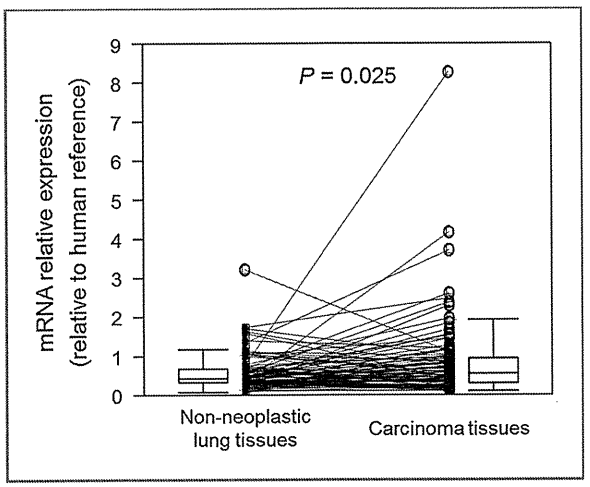


Figure 1. Differences in aromatase mRNA expression levels between carcinoma tissues and corresponding nonneoplastic lung tissues ($n = 94$). Each value is shown in an open circle; paired values of the same patient are connected by a line. Two data groups are shown as box-and-whisker plots, with the bottom 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