Fig. 2

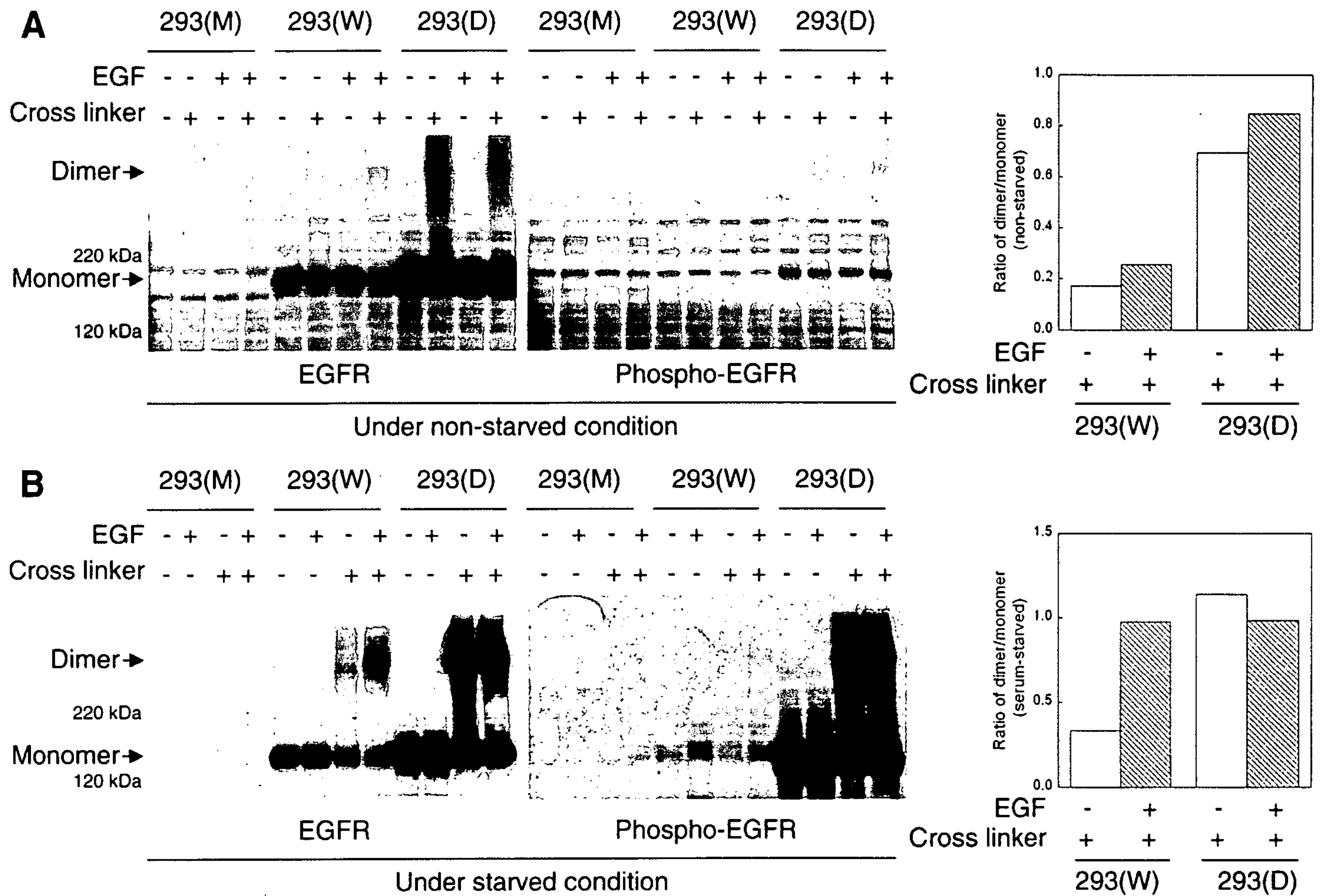

Figure 2. Dimerization and phosphorylation of wild-type EGFR and deletional EGFR. *A*) The transfected cells were treated with or without epidermal growth factor (EGF) (10 ng/ml) for 10 min under nonstarved conditions. After two washes with ice-cold PBS(+), monolayer cells were incubated with the chemical cross-linking reagent BS^3 in PBS(+) as described in the Materials and Methods. Equivalent amounts of protein were separated by 2–15% gradient SDS-PAGE and subjected to immunoblot analysis to detect EGFR and phospho-EGFR. The ratio of dimerized to monomeric EGFR is shown in the ***right panel***. ***B***) The transfected cells were exposed or unexposed to EGF (10 ng/ml) for 10 min after serum starvation. Chemical cross-linking and immunoblotting were performed as described above. The ratio of dimerized to monomeric EGFR is shown in the ***right panel***.

Fig. 3

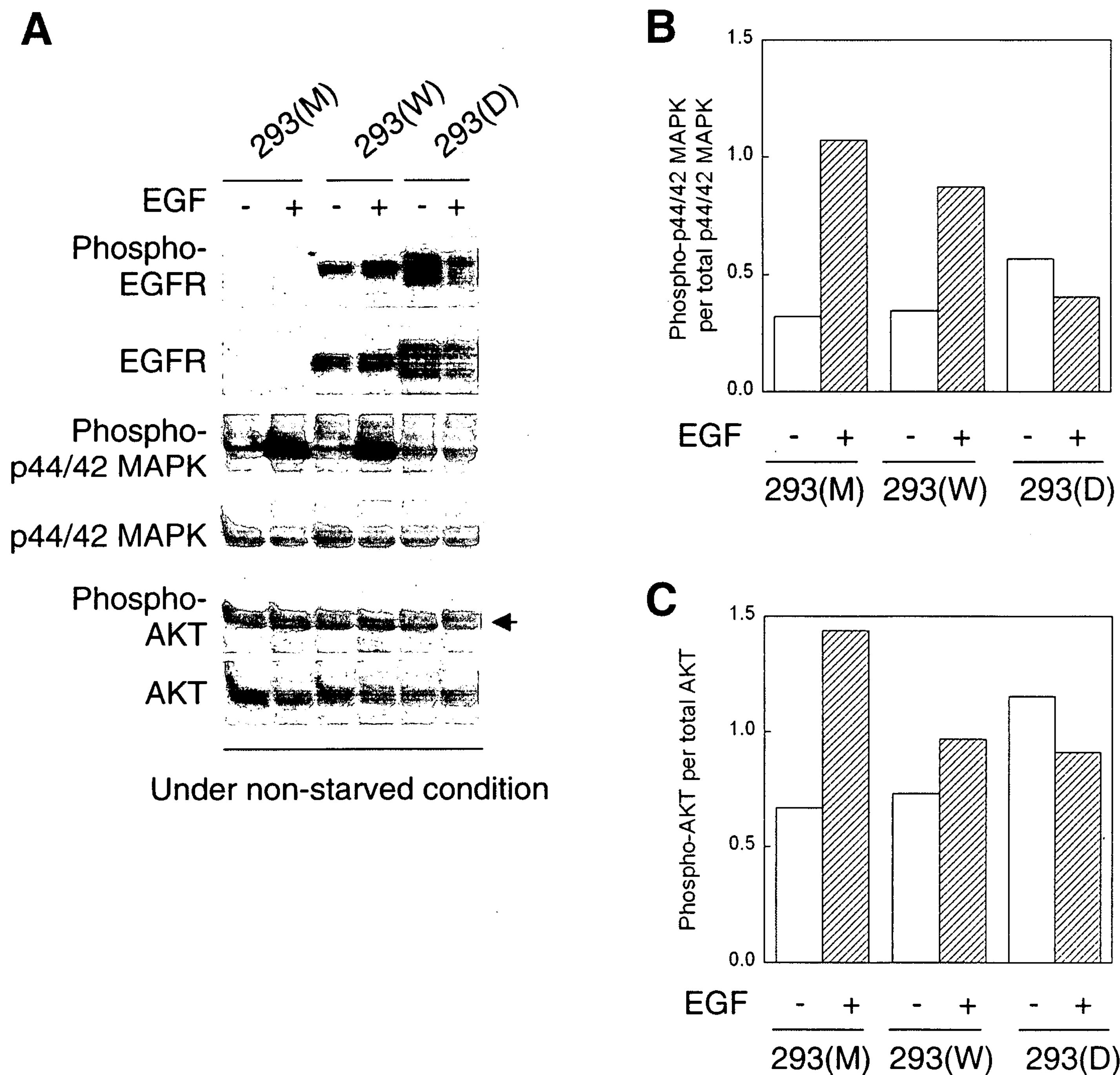

Figure 3. Phosphorylation of EGFR, p44/42 mitogen-activated protein kinase (MAPK), and AKT in the EGFR-transfected 293 cells. ***A***) The 293 (M), 293(W), and 293(D) cells were treated with EGF (10 ng/ml) for 10 min under nonstarved conditions. After two washes with ice-cold PBS(+), monolayer cells were lysed. Equivalent amounts of protein were separated by 2–15% gradient SDS-PAGE for EGFR or 10–20% for p44/42 MAPK, phospho-p44/42 MAPK, AKT, and phospho-AKT, and then subjected to immunoblot analysis. ***B***) Histogram of the degree of p44/42 MAPK activation expressed as phospho-p44/42 MAPK per total p44/42 MAPK. ***C***) Histogram of the degree of AKT activation expressed as phospho-AKT per total AKT.

Fig. 4

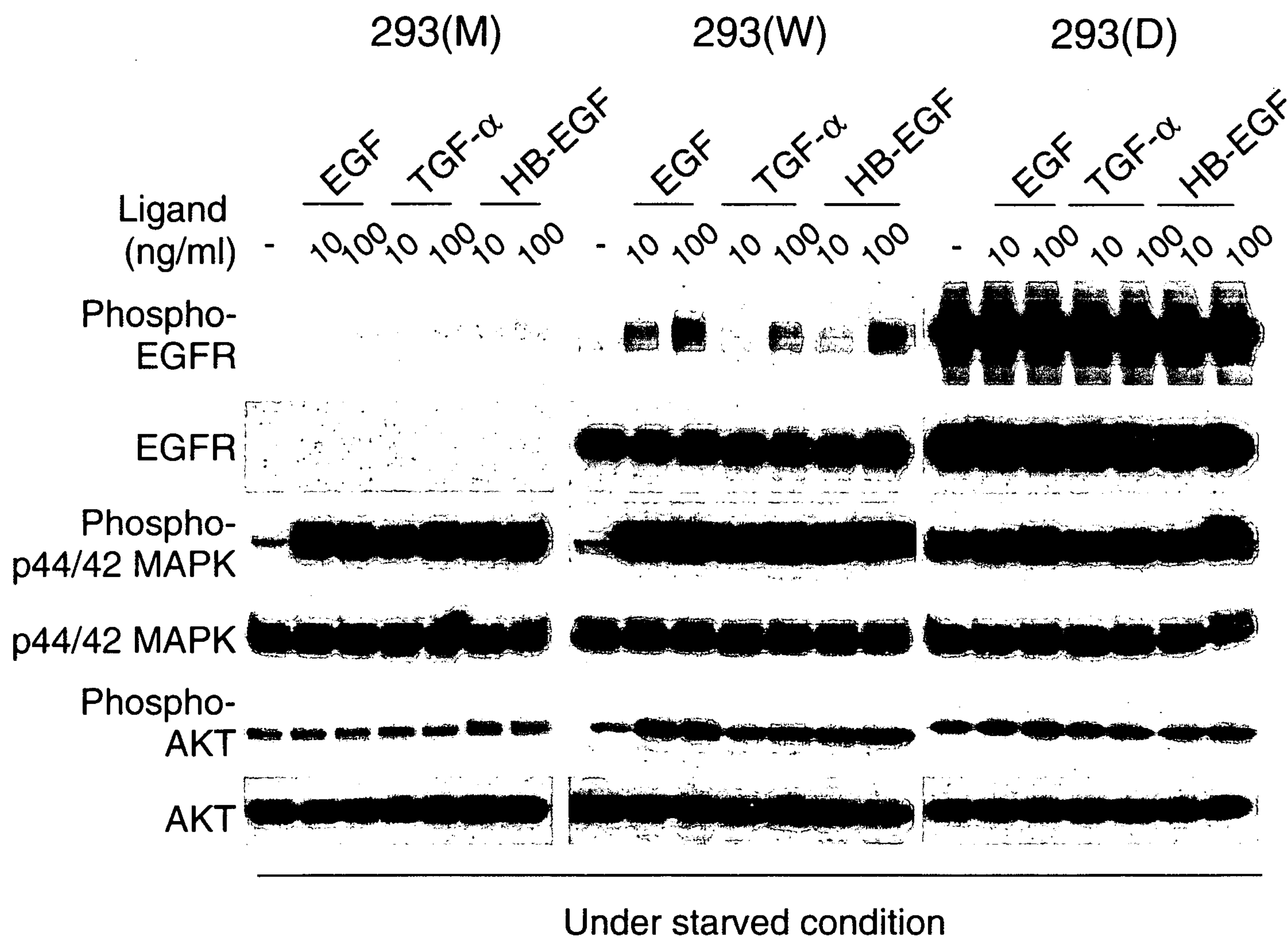

Figure 4. Phosphorylation of EGFR, p44/42 MAPK, and AKT by other ligands. The transfected cells were exposed or not exposed to EGF, TGF-α, and HB-EGF for 10 min under serum-starved conditions. After two washes with ice-cold PBS(+), monolayer cells were lysed. Equivalent amounts of protein were separated by 2–15% gradient SDS-PAGE for EGFR or 10–20% for p44/42 MAPK, phospho-p44/42 MAPK, AKT, and phospho-AKT, and then subjected to immunoblot analysis.

Fig. 5

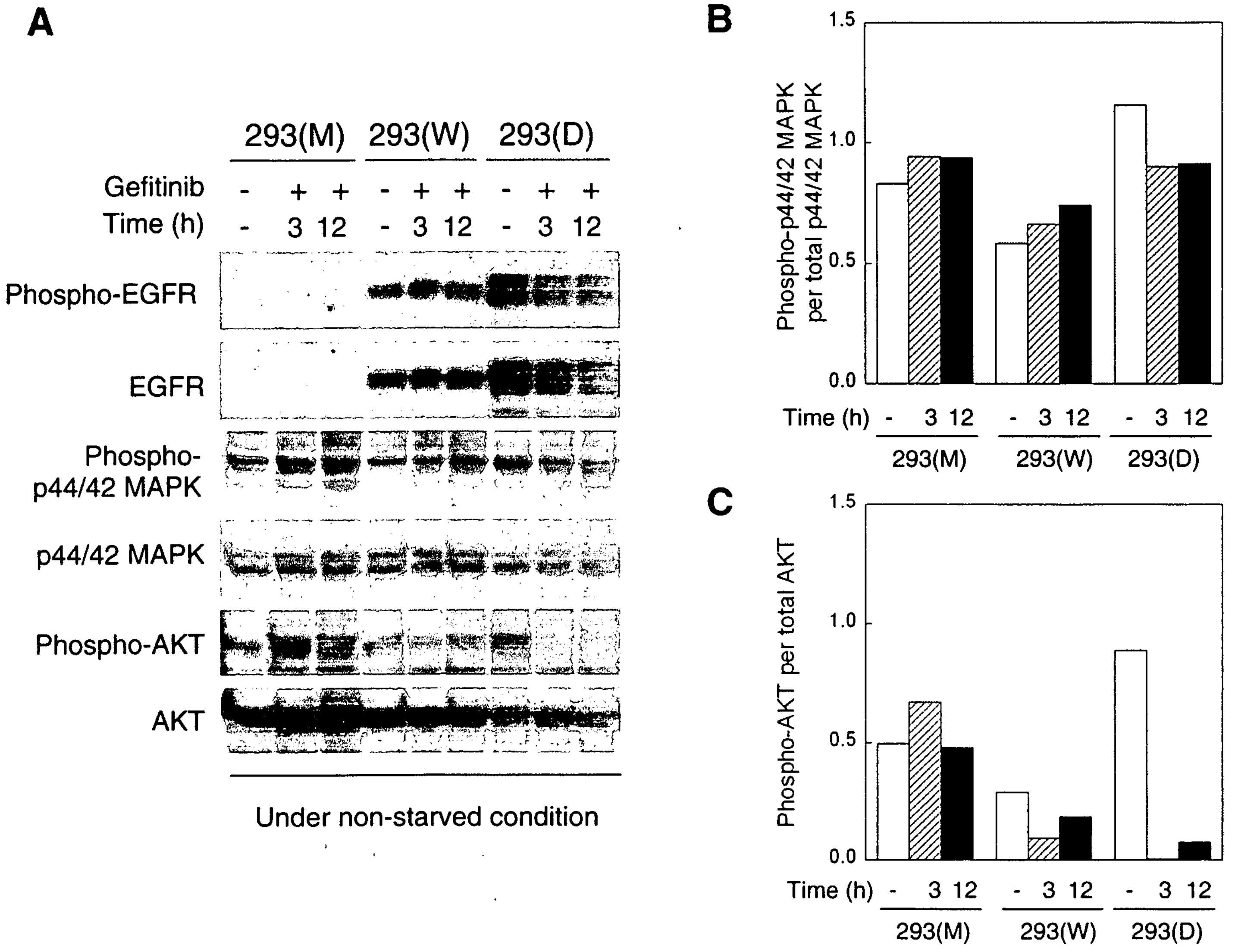

Figure 5. Effect of gefitinib on phosphorylation of EGFR, p44/42 MAPK, and AKT in the EGFR-transfected 293 cells under nonstarved conditions. *A*) The 293(M), 293(W), and 293(D) cells were incubated with gefitinib (0.01 μM) for 3 h or 12 h under nonstarved conditions. After two washes with ice-cold PBS(+), monolayer cells were lysed. Equivalent amounts of protein were separated by 2–15% gradient SDS-PAGE for EGFR or 10–20% for p44/42 MAPK, phospho-p44/42 MAPK, AKT, and phospho-AKT, and then subjected to immunoblot analysis. ***B***) Histogram of the degree of p44/42 MAPK activation expressed as phospho-p44/42 MAPK per total p44/42 MAPK. *C*) Histogram of the degree of AKT activation expressed as phospho-AKT per total AKT.

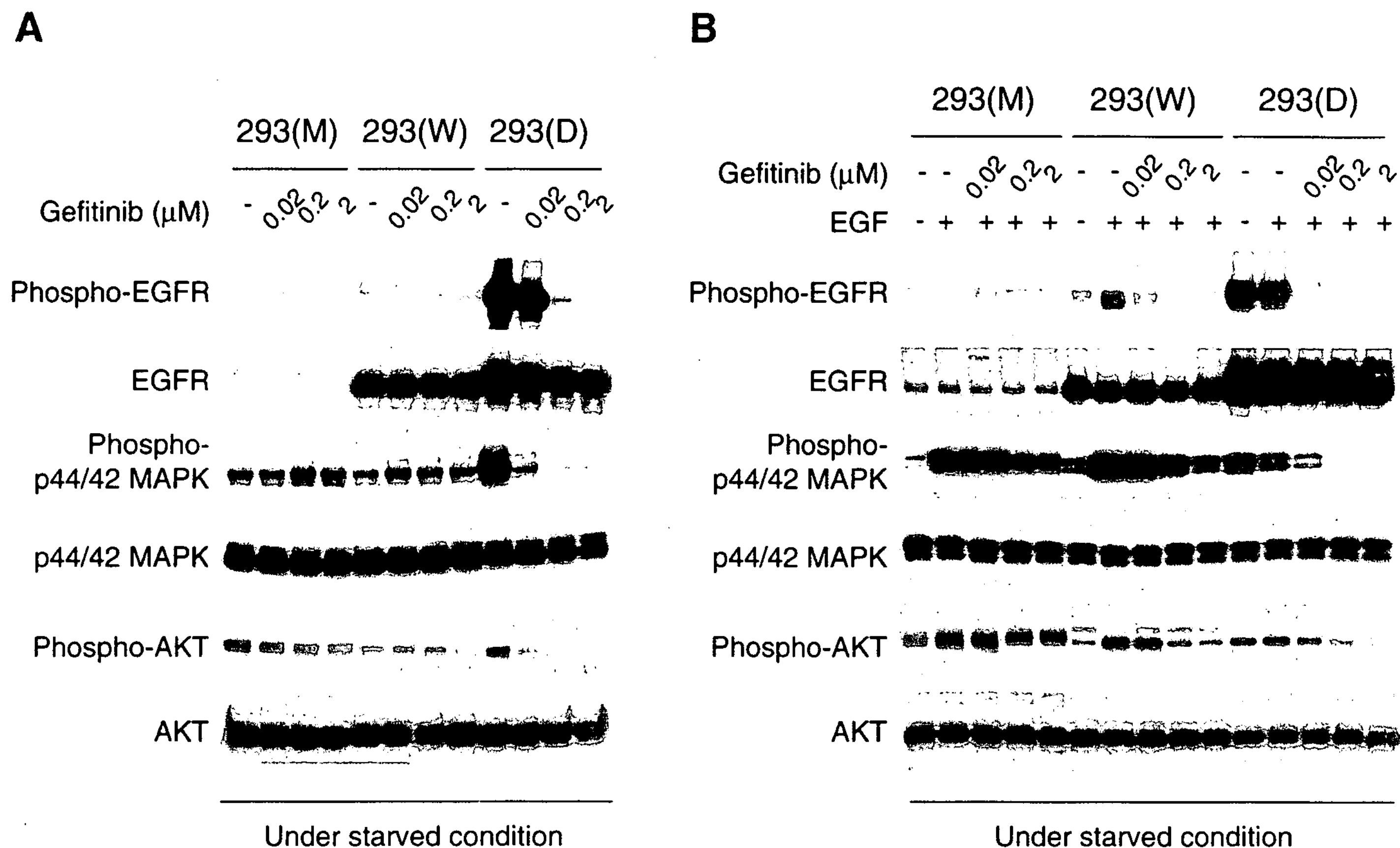

Figure 6. Effect of gefitinib on phosphorylation of EGFR, p44/42 MAPK, and AKT in the EGFR-transfected 293 cells under serum-starved conditions. *A*) The 293(M), 293(W), and 293(D) cells were incubated with gefitinib (0.02, 0.2, 2 μM) for 3 h under serum-starved conditions. After two washes with ice-cold PBS(+), monolayer cells were lysed. Equivalent amounts of protein were separated by 2–15% gradient SDS-PAGE for EGFR or 10–20% for p44/42 MAPK, phospho-p44/42 MAPK, AKT, and phospho-AKT, and then subjected to immunoblot analysis. ***B***) The transfected cells were incubated with gefitinib (0.02, 0.2, 2 μM) for 3 h under serum-starved conditions and stimulated with EGF (100 ng/ml) for 10 min. Immunoblot analysis was performed as described above.

Fig. 7

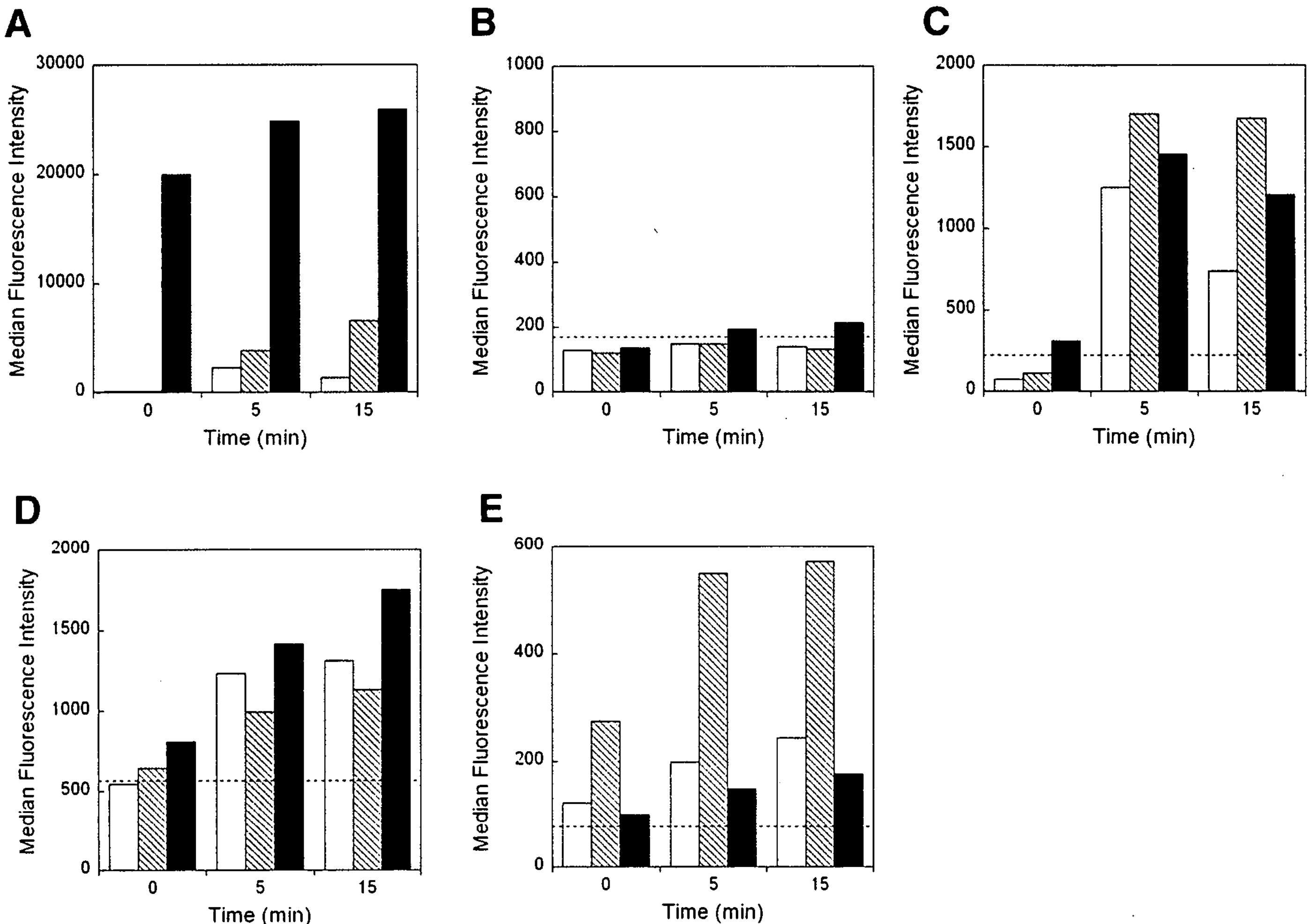

Figure 7. Monitoring of phosphoproteins after EGF stimulation in the EGFR-transfected 293 cells. The phosphoproteins in the 293(M) (open bars), 293 (W) (hatched bars), and 293(D) (solid bars) cells were analyzed by bead-based multiplex assay at the indicated intervals after addition of EGF (100 ng/ml) under serum-starved conditions. After two washes with ice-cold PBS(+), monolayer cells were lysed. The fluorescence intensity of phosphoproteins in the transfected cells was counted by bead-based multiplex assay. ***A***) phospho-EGFR; ***B***) phospho-IκB-α; ***C***) phospho-p44/42 MAPK; ***D***) phospho-ATF-2; ***E***) phospho-JNK. The dotted line shows the signal intensity of nontreated HeLa cells as a background control.

Fig. 8

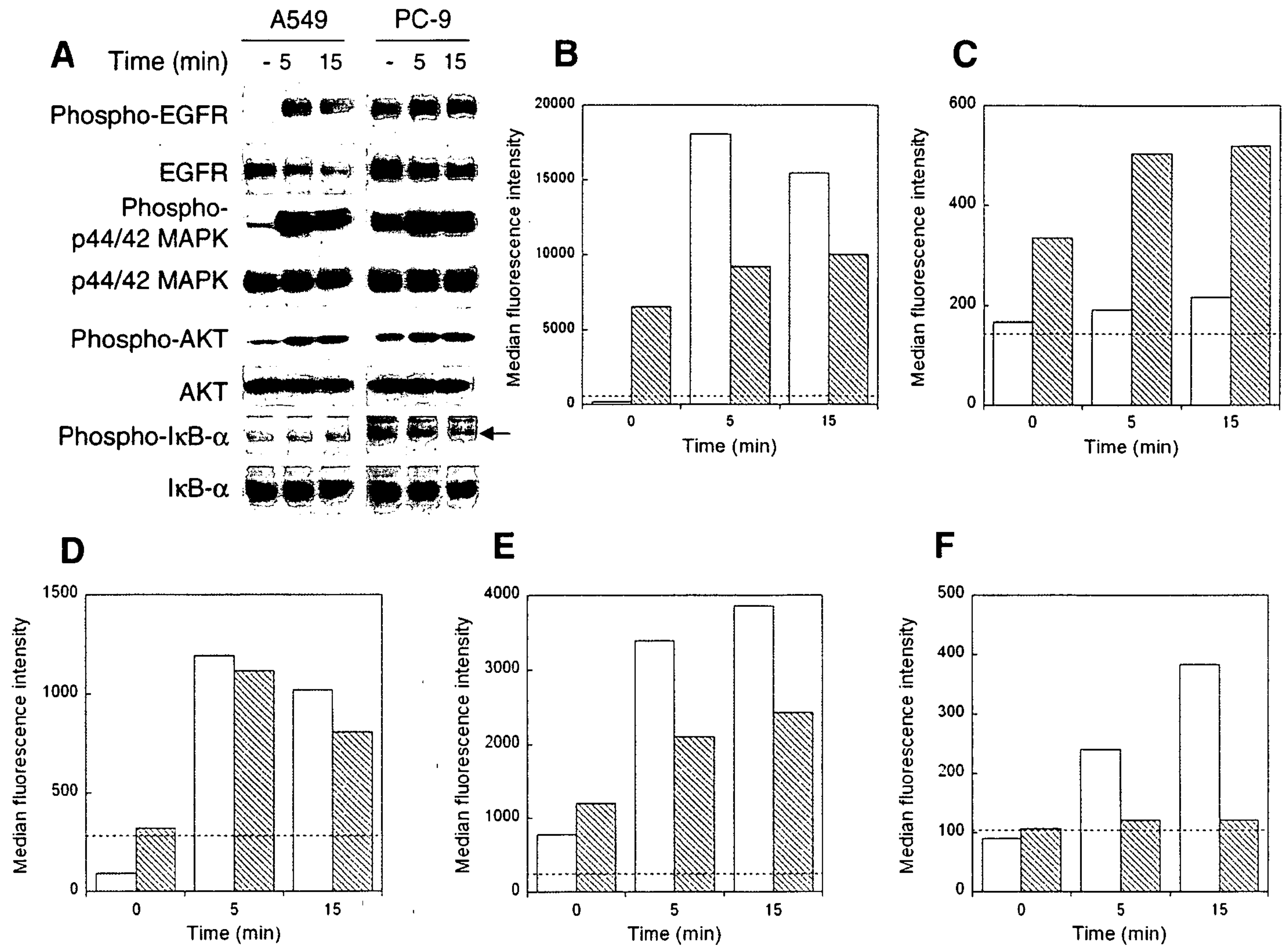

Figure 8. Monitoring of phosphoproteins after EGF stimulation in NSCLC cells. The phosphoproteins in NSCLC cells were analyzed for the indicated time intervals after addition of EGF (100 ng/ml) under serum-starved conditions. ***A***) After two washes with ice-cold PBS(+), monolayer cells were lysed. Equivalent amounts of protein were separated by 2–15% gradient SDS-PAGE for EGFR and phospho-EGFR or 10–20% for p44/42 MAPK, phospho-p44/42 MAPK, AKT, phospho-AKT, IκB-α, and phospho-IκB-α, and then subjected to immunoblot analysis. The fluorescence intensity of phosphoproteins in the A549 (open bars) and PC-9 (hatched bars) cells was counted by bead-based multiplex assay. ***B***) phospho-EGFR. ***C***) phospho-IκB-α, ***D***) phospho-p44/42 MAPK, ***E***) phospho-ATF-2, ***F***) phospho-JNK. The dotted line shows the signal intensity of nontreated HeLa cells as a background control.

A Literature Review of Molecular Markers Predictive of Clinical Response to Cytotoxic Chemotherapy in Patients with Lung Cancer

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Background: To find candidate genes for a predictive chemosensitivity test in patients with lung cancer by using a literature review.
Methods: Using MEDLINE searches, "in vitro chemosensitivity associated genes" and articles on association of the gene alteration with clinical chemosensitivity in lung cancer patients were selected. We calculated odds ratios (ORs) and their 95% confidence intervals (95% CIs) of response rates for patients who had tumors with or without gene alteration. Combined ORs and 95% CIs were estimated using the DerSimonian-Laird method.
Results: Of the 80 in vitro chemosensitivity-associated genes identified, 13 genes were evaluated for association with clinical chemosensitivity in 27 studies. The median (range) number of patients in each study was 50 (range, 28-108). The response rates of lung cancer with high and low P-glycoprotein expression were 0% and 73% to 85%, respectively ($p < 0.001$). Glutathione S-transferase pi expression (OR 0.22, 95% CI 0.06-0.79), excision repair cross-complementing 1 alterations (combined OR 0.53, 95% CI 0.28-1.01; $p = 0.055$), and tumor suppressor p53 mutation (combined OR 0.25, 95% CI 0.12-0.52) were associated with clinical chemosensitivity.
Conclusion: In total, 80 in vitro chemosensitivity-associated genes were identified in the literature, and high and low P-glycoprotein, glutathione S-transferase pi expression, excision repair cross-complementing 1 alterations, and tumor suppressor p53 mutation were candidates for future clinical trials of chemosensitivity tests in lung cancer patients.

Key Words: chemotherapy, drug response, molecular markers, prediction, lung cancer

(*J Thorac Oncol.* 2006;1: 31–37)

Lung cancer is the leading cause of death in many countries despite extensive basic research and clinical trials. Approximately 80% of patients with lung cancer have developed distant metastases either by the time of initial diagnosis or during recurrence after surgery for local disease. Systemic chemotherapy against lung cancer, however, has limitations in efficacy such that patients with distant metastases rarely live long.[1]

Tumor response to chemotherapy varies among patients, and objective tumor response rates to standard chemotherapy regimens are approximately 20 to 40% in patients with non—small-cell lung cancer and 60 to 90% in patients with small-cell lung cancer. Thus, it would be extremely useful to know in advance whether patients have tumors that respond to chemotherapy agents and whether the tumors would be resistant to such therapy. For this purpose, cell culture-based chemosensitivity tests have been investigated for more than 20 years, but they are not widely accepted because of technical problems such as the large amount of material required, a low success rate for the primary culture, length of time required, and poor correlation with the clinical response.[2–5]

To overcome these obstacles, DNA-, RNA-, and protein-based chemosensitivity tests have been created, but gene alterations that are predictive of the clinical drug response are not established. Recently, as many as 400 genes whose expression was associated with drug response were identified by cDNA microarray studies, but their functions do not seem to be related to drug sensitivity or resistance.[6–10] In addition, the genes identified by microarray studies were highly unstable and depended on the selection of patients used for gene identification.[11,12] The purpose of this study was to provide an overview of gene alterations in lung cancer that are associated with chemotherapy drug response to identify candidate genes for predictive chemosensitivity tests in patients with lung cancer.

MATERIALS AND METHODS

Because one set of genes associated with chemosensitivity is those directly involved in drug resistance mechanisms, we conducted a MEDLINE search for articles on tumor drug resistance published in the years 2001–2003. This search yielded 112 studies, including several review articles. By searching manually through these articles, we identified 134 genes or gene families that may be involved in drug resistance based on their function. We conducted the second MEDLINE searches for papers of in vitro studies on the 134 genes or gene families by using their names as a keyword.

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From the 134 genes, we selected genes that met the following definition of "in vitro chemosensitivity associated genes": 1) alteration of the gene was identified in a human drug-induced resistant, solid tumor cell line; 2) transfection of the gene induced drug resistance; or 3) down-regulation of the gene or its encode protein increased drug sensitivity. In this latter category, we included studies in which the gene expression or function was suppressed by antisense RNA, hammerhead ribozyme, or an antibody against the gene product. We excluded studies in which drugs were used to inhibit function because the specificity of the drug against the target may not have been complete. We performed a third MEDLINE search for articles on the association between the gene alteration and chemosensitivity of lung cancer cell lines by using the name of the gene as a keyword. Articles in which the association was evaluated in 20 or more cell lines were included in this study. Finally, we searched MEDLINE for studies on the association between the gene alteration and clinical drug response in patients with lung cancer by using the name of the gene as a keyword. Articles in which the association was evaluated in 25 or more patients with advanced lung cancer were included in this study. Studies in which gene expression was evaluated with microarray were excluded because result analysis and interpretation of this technique have not been established, as indicated by the fact that the list of genes identified by microarray studies was highly variable without overlap between these gene sets.[11,12] Clinical studies on concurrent chemoradiotherapy were excluded. We constructed 2 × 2 tables from the response data and calculated odds ratios (ORs), their variances, and 95% confidence intervals (95% CIs) for the patients who had tumors with gene alteration relative to those who had tumors without gene alteration. Combined ORs and 95% CIs were estimated using the DerSimonian-Laird method.[13] When a response rate was 0, association with gene alteration was evaluated using the χ^2 test because 95% CIs for ORs cannot be calculated. The name of each gene was standardized according to Human Gene Nomenclature Database of National Center for Biotechnology Information.

RESULTS

Of the 134 genes or gene families found, a gene alteration in drug-induced resistant cells, an increased or decreased resistance in transfected cells, and an altered sensitivity in gene down-regulated cells were reported for 45, 57, and 32 genes, respectively. In total, 80 genes met the definition of "in vitro chemosensitivity associated gene" (Table 1).

Gene alteration was associated with in vitro chemosensitivity in 15 (50%) of 30 studies on 15 (56%) of 27 gene alterations (Table 2). Clinical drug response was evaluated in 27 studies on 13 gene alterations. The methods used to identify gene alteration included immunohistochemical protein expression analysis (n = 18), polymerase chain reaction (PCR)-based mRNA expression analysis (n = 3), and PCR-based mutation analysis (n = 6). All but one clinical study was retrospective, and the median (range) number of patients in each study was 50 (28-108). Gene alteration was associated with clinical response in 8 of the 27 (30%) studies (Table 2).

TABLE 1. In Vitro Chemosensitivity-Associated Genes

Transporters: ABCA2, ABCB1, ABCB11, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCG2, MVP, ATP7A, ATP7B, SLC29A1, SLC28A1, SLC19A1

Drug targets: TUBB, TUBB4, TUBA, TYMS, TOP1, TOP2A, TOP2B, DHFR,

Target-associated proteins : MAP4, MAP7, STMN1, KIF5B, HSPA5, PSMD14, FPGS

Intracellular detoxifiers: GSTP1, GPX, GCLC, GGT2, MT, RRM2, AKR1B1

DNA damage recognition and repair proteins: HMGB1, HMGB2, ERCC1, XPA, XPD, MSH2, MLH1, PMS2, APEX1, MGMT, BRCA1, GLO1

Cell cycle regulators: RB1, GML, CDKN1A, CCND1, CDKN2A, CDKN1B

Mitogenic signal regulators: ERBB2, EGFR, KRAS2, HRAS, RAF1

Survival signal regulators: AKT1, AKT2

Integrin: ITGB1

Transcription factors: JUN, FOS, MYC, NFKB1

Apoptosis regulators: TP53, MDM2, TP73, BCL2, BCL2L1, MCL1, BAX, BIRC4, BIRC5, TNFRSF6, CASP3, CASP8, HSPB1

We evaluated the association between transporter P-glycoprotein/multidrug resistance 1 (ABCB1) expression and clinical chemosensitivity in four studies. The response rate of lung cancer with high ABCB1 expression was consistently 0%, whereas that for lung cancer with low ABCB1 expression was 73 to 85% (Table 3). Among drug targets, only topoisomerase II-beta (TOP2B) expression was associated with clinical drug response in patients with small-cell lung cancer (OR 0.29, 95% CI 0.09-0.95). The intracellular detoxifier glutathione s-transferase pi (GSTP1) was associated with both in vitro and clinical drug response (OR 0.22, 95% CI 0.06-0.79) (Table 4). DNA repair gene excision repair cross-complementing 1 (ERCC1) alterations were associated with drug response among patients with non—small-cell lung cancer with marginal statistical significance; the combined OR (95% CI) for ERCC1 alteration was 0.53 (0.28-1.01; p = 0.055) (Table 5). Tumor suppressor p53 (TP53) mutation was the only alteration associated with drug response among patients with non—small-cell lung cancer among genes involved in cell cycle and apoptosis. A combined OR (95% CI) for TP53 among patients with non—small-cell lung cancer was 0.25 (0.12-0.52) (Table 6). B-cell CLL/lymphoma 2 (BCL2) and its family protein expression was not associated with clinical drug response (Table 7).

DISCUSSION

We identified 80 in vitro chemosensitivity-associated genes in our literature search. Of these, 13 were evaluated clinically in 27 studies; ABCB1, TOP2B, GSTP1, and ERCC1 expression and TP53 mutation were associated with changes to drug responses among patients with lung cancer.

Classical drug resistance is believed to be the result of molecular changes inhibiting the drug-target interaction. ABCB1, an ATP-binding cassette protein, acts as an energy-dependent transmembrane efflux pump and decreases the intracellular accumulation of anticancer drugs, including anthracyclines, vinca alkaloids, taxanes, and epipodophyllotox-

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TABLE 2. Chemosensitivity-Associated Genes and Association with Chemosensitivity

		Association with chemosensitivity					
		In vitro studies (n)			Clinical studies (n)		
Category	No of Genes	Total	Yes	%	Total	Yes	%
Transporter	15	9	5	55	4	4	100
Drug target	8	2	1	50	5	1	20
Target-associated protein	7	0	0		0	0	
Intracellular detoxifier	7	3	3	100	1	1	100
DNA repair	10	1	1	100	6	0	0
Damage recognition protein	2	0	0		0	0	
Cell cycle	6	4	2	50	2	0	0
Mitogenic signal	5	3	1	33	1	0	0
Survival signal	2	0	0		0	0	
Transcription factor	4	3	0	0	0	0	
Cell adhesion-mediated drug-resistance protein	1	0	0		0	0	
Apoptosis	13	5	2	40	8	2	25
Total	80	30	15	50	27	8	30

TABLE 3. ABCB1 (P-Glycoprotein) and Clinical Response to Chemotherapy

Author	Histology	Drugs	Method	Expression	Patients (n)	RR (%)	Odds ratio
Yeh et al.[30]	Non-small cell	Paclitaxel	IHC	Low	35	80	0
				High	15	0	$p < 0.001$*
Kawasaki et al.[31]	Small cell	CAV or EP	IHC	Low	26	85	0
				High	4	0	$p < 0.001$*
Hsia et al.[32]	Small cell	EP	IHC	Low	37	73	0
				High	13	0	$p < 0.001$*
Savaraj et al.[33]	Small cell	CAV, CEV, or EP	RT-PCR	Low	24	75	0
				High	7	0	$p < 0.001$*

Combined odds ratio for ABCB expression in patients with SCLC: 0

IHC, Immunohistochemical analysis; RR, response rate; RT-PCR, reverse transcriptase-polymerase chain reaction.
*Calculated using the χ^2 test because the confidence interval cannot be calculated.

ins. Overexpression of this protein gives tumor cells a multidrug resistance phenotype in vitro, which is thought to be associated with clinical chemoresistance.[14] Our review showed that the response rate of tumors with ABCB1 overexpression was 0 in all studies of lung cancer, whereas that for lung cancer tumors with low ABCB1 expression was 73 to 85% (Table 3).

There is a close relationship between drug sensitivity and quantitative and qualitative alterations of the drug's target, including tamoxifen sensitivity and estrogen receptor expression and trastuzumab response and Her-2/neu overexpression in breast cancer,[15] imatinib resistance and BCR-ABL gene amplification and mutations in Philadelphia chromosome-positive leukemias,[16] and imatinib response and KIT gene mutations in gastrointestinal stromal tumors.[17] In all of these cases, the target molecule is a receptor or a mutated tyrosine kinase located at the entry of growth-stimulating signal transduction pathways. Recently, gefitinib, a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR), has been developed, and two large phase II trials showed a response rate of 18% and 12% in patients with non—small-cell lung cancer who were previously treated with conventional chemotherapy.[18,19] Responses to the drug have been unpredictable, but mutations of the EGFR gene were identified in patients with gefitinib-responsive lung cancer.[20,21] Furthermore, all mutations in these tumors were restricted to the activation loop of the kinase domain of EGFR, which are in distinct contrast to mutations in extracelluar and regulatory domains of EGFR in glioblastoma, which are unresponsive to gefitinib.[22] Thus, molecular developments of structure and function of the targets hold the promise of targeted cancer therapy. The target molecules of many anticancer cytotoxic agents have not been clearly defined; therefore, the relationship between the target molecule status and sensitivity to the agent has not been established. TOP2B expression was associated with drug response in patients with small-cell lung cancer, with a response rate of 71% for high TOP2B expression tumors versus 90% for low TOP2B expression tumors (OR 0.29, 95% CI 0.09-0.95).[23] This result, however, is in contrast with the idea that a higher

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TABLE 4. Drug Targets, Intracellular Detoxifier, and Clinical Response to Chemotherapy

Author	Histology	Drugs	Method	Expression	Patients (n)	RR (%)	Odds ratio (95% CI)
Beta-tubulin class III							
Rosell et al.[34]	Non-small cell	Paclitaxel,	Real-time	Low	13	46	0.39
		Vinorelbine	PCR	High	24	25	(0.09-1.62)
Topoisomerase II-alpha							
Dingemans et al.[23]	Small cell	CEV or EP	IHC	Low	65	85	0.65
				High	23	80	(0.20-2.17)
Dingemans et al.[35]	Non-small cell	Platinum-based	IHC	Low	30	47	0.67
				High	8	38	(0.14-3.40)
Topoisomerase II-beta							
Dingemans et al.[23]	Small cell	CEV or EP	IHC	Low	48	90	0.29
				High	35	71	(0.09-0.95)
Dingemans et al.[35]	Non-small cell	Platinum-based	IHC	Low	18	50	0.86
				High	13	46	(0.21-3.58)
Glutathione s-transferase pi							
Nakanishi et al.[36]	Non-small cell	Cisplatin-based	IHC	Low	17	47	0.22
				High	37	16	(0.06-0.79)

CI, confidence interval; IHC, immunohistochemical analysis; PCR, polymerase chain reaction; RR, response rate; CEV, cyclophosphamide, etoposide, and vincristine; EP, etoposide and cisplatin.

TABLE 5. DNA Repair Genes and Clinical Response to Chemotherapy

Author	Histology	Drugs	Method	Alteration	Patients (n)	RR (%)	Odd ratio (95% CI)
Excision repair cross-complementing 1 expression							
Lord et al.[37]	Non-small cell	Cisplatin,	Real-time	Low	23	52	0.38
		gemcitabine	PCR	High	24	36	(0.11-1.26)
Excision repair cross-complementing 1 (ERCC1) polymorphism at codon 118							
Ryu et al.[38]	Non-small cell	Cisplatin-based	PCR	C/C	54	54	0.61
			Hybridization	C/T or T/T	53	42	(0.28-1.31)
Combined odds ratio (95% C.I.) for ERCC1 alteration in patients with NSCLC0.53 (0.28-1.01, p = 0.055)							
Xeroderma pigmentosum group D polymorphism							
At codon 231							
Ryu et al.[38]	Non-small cell	Cisplatin-based	PCR	G/G	100	48	1.08
			Hybridization	G/A or A/A	8	50	(0.26-4.57)
At codon 312							
Camps et al.[39]	Non-small cell	Cisplatin,	PCR	G/G	18	17	3.33
		gemcitabine	Sequencing	G/A or A/A	15	40	(0.66-16.7)
At codon 751							
Camps et al.[39]	Non-small cell	Cisplatin,	PCR	A/A	22	23	2.04
		gemcitabine	Sequencing	A/C or C/C	16	38	(0.49-8.45)
Ryu et al.[38]	Non-small cell	Cisplatin-based	PCR	A/A	96	49	0.74
			Hybridization	A/C	12	42	(0.22-2.51)
Combined odds ratio (95% CI) for XPD polymorphism in patients with NSCLC: 1.38 (0.68-2.78).							

CI, confidence interval; PCR, polymerase chain reaction; RR, response rate; NSCLC, non—small-cell lung cancer; XPD, xeroderma pigmentosum group D.

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TABLE 6. Cell Cycle Regulators, Mitogenic Signals, Tumor Protein p53, and Clinical Response to Chemotherapy

Author	Histology	Drugs	Method	Alteration	Patients (n)	RR (%)	Odds ratio (95% CI)
Retinoblastoma 1 expression							
Gregorc et al.[40]	Non-small cell	Cisplatin-based	IHC	Low	61	51	0.45
				High	41	32	(0.20-1.03)
Cyclin-dependent kinase inhibitor 1A, p21 expression							
Dingemans et al.[23]	Small cell	CEV, EP	IHC	Low	63	90	0.57
				High	22	71	(0.17-1.92)
Kirsten rat sarcoma 2 viral oncogene homolog mutation							
Rodenhuis et al. [41, a]	Aenocarcinoma	Ifosfamide, carboplatin	PCR-MSH	Normal	46	26	0.65
				Mutated	16	19	(0.16-2.70)
Tumor protein p53 (P53) mutation							
Nakanishi et al.[36]	Non-small cell	Cisplatin-based	IHC	Normal	11	45	0.19
				Mutated	29	15	(0.04-0.94)
Gregorc et al.[40]	Non-small cell	Cisplatin-based	IHC	Normal	56	57	0.26
				Mutated	46	26	(0.11-0.62)
Combined odds ratio (95% CI) for P53 mutation in patients with NSCLC: 0.25 (0.12-0.52)							
Kawasaki et al.[31]	Small cell	CAV or EP	IHC	Normal	10	70	1.3
				Mutated	20	75	(0.24-6.96)
Dingemans et al.[23]	Small cell	CEV or EP	IHC	Normal	47	85	0.81
				Mutated	45	82	(0.27-2.45)
Combined odds ratio (95% C.I.) for P53 mutation in patients with SCLC: 0.93 (0.37-2.35).							

CI, confidence interval; IHC, immunohistochemical analysis; PCR-MSH, polymerase chain reaction-mutation specific hybridization; RR, response rate; CEV, cyclophosphamide, etoposide, and vincristine; EP, etoposide and cisplatin.
[a]Prospective study.

TABLE 7. B-Cell CLL/Lymphoma 2 (BCL2) Family Expression and Clinical Response to Chemotherapy

Author	Histology	Drugs	Method	Expression	Patients (n)	RR (%)	Odds ratio (95% CI)
BCL2							
Krug et al.[42]	Non-small cell	Docetaxel, vinorelbine	IHC	Low	26	46	1.75
				High	5	60	(0.25-12.3)
Dingemans et al.[23]	Small cell	CEV or EP	IHC	Low	20	79	1.36
				High	71	85	(0.38-4.86)
Takayama et al.[43]	Small cell	CAV or EP	IHC	Low	17	76	0.50
				High	21	62	(0.12-2.08)
Combined odds ratio (95% CI) for BCL2 expression in patients with SCLC: 0.87 (0.33-2.32)							
BAX (BCL2-associated X protein)							
Krug et al.[42]	Non-small cell	Docetaxel, vinorelbine	IHC	Low	9	56	0.72
				High	19	47	(0.15-3.54)

CI, confidence interval; IHC, immunohistochemical analysis; RR, response rate; CEV, cyclophosphamide, etoposide, and vincristine; EP, etoposide and cisplatin.

expression of topoisomerase II enzymes correlates with greater chemosensitivity in patients with breast cancer.[24]

In addition to genes involved in classical drug resistance, genes that act downstream of the initial damage induced by a drug-target complex are thought to play an important role in chemosensitivity.[25] ERCC1 is a key enzyme in nucleotide excision repair, one of the key pathways by which cells repair platinum-induced DNA damage. High levels of ERCC1 mRNA have been associated with platinum resistance in the treatment of ovarian and gastric cancer.[26,27] The codon 118 in exon 4 of ERCC1 gene is polymorphic with the nucleotide alteration AAC to AAT. Although this base change results in coding for the same amino acid, it may affect gene expression based on the usage frequency of synonymous codons.[28] The associations between drug response and both ERCC1 gene expression and polymorphism at codon 118 in patients with non—small-cell lung cancer have been reported in the literature. A combined OR (95%

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CI) for these ERCC1 alterations was 0.53 (0.28-1.01, p = 0.055), although each study failed to show statistical significant association. Thus, ERCC1 may be a candidate for evaluation of the predictability of drug response in future clinical trials.

TP53, which is mutated or deleted in more than half of lung cancer cells, has a remarkable number of biological activities, including cell-cycle checkpoints, DNA repair, apoptosis, senescence, and maintenance of genomic integrity. Because most anticancer cytotoxic agents induce apoptosis through either DNA damage or microtubule disruption, mutated TP53 may decrease chemosensitivity by inhibiting apoptosis or, in contrast, may increase chemosensitivity by impairing DNA repair after drug-induced DNA damage.[29] This review showed that mutated TP53 was associated with poor drug response in patients with non—small-cell lung cancer (Table 6).

No other genes located downstream (including xeroderma pigmentosum group D, retinoblastoma 1, cyclin-dependent kinase inhibitor 1A, Kirsten rat sarcoma 2 viral oncogene homolog, B-cell CLL/lymphoma 2, and B-cell CLL/lymphoma 2-associated X protein) were associated with clinical drug response (Tables 5-7). The association was evaluated for only 8 of 43 *in vitro* chemosensitivity-associated downstream genes; therefore, key genes may be among the remaining 35 genes. Most clinical studies included a limited number of patients with various background characteristics such as tumor stage and chemotherapy regimen administered, which resulted in low statistical power to identify the association. Finally, because all but one study was retrospective, the quality of tumor samples may vary, and it is therefore unclear whether the gene alteration was detected in all samples. Thus, in future prospective clinical studies, the method of tumor sample collection and preservation, as well as immunohistochemistry and polymerase chain reaction-based methods, should be standardized, and the sample size of patients should be determined with statistical consideration.

The recently developed microarray technique enables investigators analyze mRNA expression of more than 20,000 genes at once, and as many as 100 to 400 genes were selected statistically as chemosensitivity-related genes.[6-8,10] Among them, however, only a limited number of genes were functionally related to chemosensitivity, and only ABCB1 and BAX corresponded with the 80 chemosensitivity-associated genes identified in this literature review, which were picked because of their known function and contribution to in vitro chemosensitivity. Thus, it will be interesting to evaluate the role of expression profile of these genes using microarray analysis.

The association between the expression and alterations of genes and clinical drug responses should be studied further in prospective trials. ABCB1, GSTP1, ERCC1, and TP53, and other genes identified by exploratory microarray analyses should be evaluated in those trials. Simple methods to identify gene alterations, such as immunohistochemistry and polymerase chain reaction-based techniques, will be feasible in future clinical trials because of their simplicity, cost, and time. The median number of patients in retrospective studies analyzed in this review was 50 (range, 28-108). In future prospective trials, sample size consideration for statistical power will also be important.

In conclusion, we identified 80 in vitro chemosensitivity-associated genes in a review of the literature; ABCB1, GSTP1, and ERCC1 expression and TP53 mutation were associated with drug responses among patients with lung cancer.

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ZD6474 inhibits tumor growth and intraperitoneal dissemination in a highly metastatic orthotopic gastric cancer model

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Angiogenesis inhibitors have been used to treat some cancers, but the therapeutic potential of these agents for gastric cancer has remained unclear. To investigate their therapeutic potential, we examined the effect of ZD6474, an agent that selectively targets vascular endothelial growth factor receptor-2 (VEGFR-2; KDR) tyrosine kinase and epidermal growth factor receptor (EGFR) tyrosine kinase, in a highly metastatic orthotopic model using an undifferentiated gastric cancer cell line, 58As1. ZD6474 (100 mg/kg/day, p.o., 2 weeks) significantly inhibited tumor growth ($p < 0.05$ *vs.* control) and reduced tumor dissemination into the peritoneal cavity ($p < 0.05$ *vs.* control). In addition, to identify putative tumor biomarkers that would reflect the effects of ZD6474 treatment in clinical settings, we examined the gene expression profiles of implanted gastric tumors treated with ZD6474 *in vivo*. Twenty-eight candidate genes were identified, including *IGFBP-3, ADM, ANGPTL4, PLOD2, DSIPI, NDRG1, ENO2, HIG2* and *BNIP3L*, which are known to be hypoxia-inducible genes. These genes and gene products may be useful biomarkers for monitoring the effects of ZD6474 treatment. ZD6474 also improved the survival of mice with implanted another undifferentiated gastric cancer cell line, 44As3. In conclusion, our results suggest that ZD6474 may have clinical activity against gastric cancer, particularly undifferentiated gastric cancer with peritoneal dissemination. We also identified putative biomarkers for monitoring the pharmacodynamic effects of ZD6474 by gene expression profiling.
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Key words: ZD6474; gastric cancer; intraperitoneal dissemination; VEGF; oligonucleotide microarray

Various anti-cancer agents have been examined for efficacy against gastric cancer over the past two decades, but the median survival time of patients remains around 7 months,[1,2] and the prognosis of gastric cancer patients remains poor. Peritoneal dissemination is common in patients with unresectable gastrointestinal cancer, and many suffer from peritoneal carcinomatosis in the terminal stage. Because undifferentiated gastric cancer is particularly invasive and often accompanied by peritoneal dissemination,[3] a new treatment strategy is needed.

Vascular endothelial growth factor (VEGF) is a key mediator of tumor growth and is known to have multiple functions in angiogenesis, vascular permeability, and the regulation of endothelial cell proliferation and migration.[4–6] VEGF receptors (VEGFR) are activated by ligand stimulation with VEGF and commonly expressed in vascular endothelial cells. VEGFR-2 (KDR/Flk-1) is thought to be important for angiogenesis.[7] Because the VEGF-VEGFR system plays a key role in angiogenesis and tumor growth *in vivo*, the therapeutic potential of many agents targeting this system is being investigated.[8] A recent study has shown that a combination of monoclonal antibody against VEGF and chemotherapy produces a clinically meaningful survival benefit for patients with metastatic colorectal cancer,[5] and these results may lead to changes in the standard treatment for colorectal cancer.[9]

ZD6474 is a novel orally available VEGFR-2 (KDR) tyrosine kinase inhibitor that is also known to selectively target epidermal growth factor receptor (EGFR) tyrosine kinase, both of which are parts of key pathways in tumor growth.[10–13] We demonstrated previously the evidence suggesting that ZD6474 inhibits angiogenesis and tumor growth by targeting EGFR.[14,15]

In our present study, we tested ZD6474 for an inhibitory effect on tumor growth and intraperitoneal dissemination, and for improvement of survival in a newly established, highly metastatic orthotopic gastric tumor model *in vivo*. In addition, we also identified putative biomarkers to monitor the effects of ZD6474 treatment using gene expression profiling.

Material and methods

Reagents

ZD6474 and gefitinib (Iressa®) were provided by AstraZeneca (Cheshire, UK).

Cell cultures

The newly established highly metastatic human signet-ring cell gastric cancer cell lines 58As1 and 44As3 produce large volumes of ascitic fluid and spontaneously metastasize to the peritoneal cavity after orthotopic (gastric wall) implantation.[16,17] 58As1 and 44As3 and human non-small cell lung cancer cell line PC-9 were maintained in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FBS (Gibco BRL, Grand Island, NY). The PC-9 cells were a gift of Tokyo Medical University. Human embryonic kidney cell line 293 (HEK293) was obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVEC) were maintained in EBM-2 medium (Clonetics, Walkersville, MD) supplemented with EGM-2 kit (Clonetics), according to the manufacturer's instructions.

In vitro *growth-inhibition assay*

The cell-growth inhibitory effects of ZD6474 and gefitinib were assessed by the thiazole blue tetrazolium bromide (MTT) assay (Sigma). Briefly, 180 µl/well of cell suspension was seeded on to Sumilon® 96-well microculture plates (Sumitomo Bakelite, Akita, Japan) and incubated in 10% FBS-containing medium for 24 hr. The cells were then treated with ZD6474 at various concentrations (4 nM–80 µM) and cultured at 37°C in a humidified atmosphere for 72 hr. After the culture period, 20 µl volume of MTT reagent was added, and the plates were further incubated for 4 hr. After centrifuging the plates, the culture medium was discarded and the wells were filled with dimethyl-sulfoxide. The optical density of the cultures was measured at 562 nm with Delta-soft software on a

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Macintosh computer interfaced to a Bio-Tek Microplate Reader EL-340 (BioMetallics, Princeton, NJ). The experiment was conducted in triplicate.

Immunoblotting

EGFR, phospho-EGFR (specific for Tyr 1068), and anti-rabbit horseradish peroxidase (HRP)-conjugated antibody were purchased from Cell Signalling (Beverly, MA). Cell pellets were lysed in RIPA buffer (Tris-HCl, 50 mM; pH 7.4; NP-40, 1%; Na-deoxycholate, 0.25%; NaCl, 150 mM; EDTA, 1 mM; phenylmethyl-sulfonyl fluoride, 1 mM; aprotinin, leupeptin, pepstatin, 1 mg/ml each; Na_3VO_4, 1 mM; NaF, 1 mM). Cell extracts were electrophoresed on 7.5% (w/v) polyacrylamide gels and transferred to a polyvinylidene di-fluoride membrane (Nihon Millipore, Tokyo, Japan). The membrane was incubated in Tris-buffered saline containing 0.5% Tween 20 with 3% BSA and then reacted with the primary antibodies and the HRP-conjugated secondary antibody for 90 min each. Visualization was achieved with an enhanced chemiluminescent detection reagent (Amersham Bioscience, Buckinghamshire, UK).

RT-PCR

A 5 μg of total RNA from each cultured cell line was converted to cDNA with a GeneAmp® RNA-PCR kit (Applied Biosystems, Foster City, CA). The primers used for the PCR were: human-specific beta-actin, forward: 5′-GGAAATCGTGCGTGACATT-3′ and reverse: 5′-CATCTGCTGGAAGGTGGACAG-3′; mouse-specific beta-actin, forward: 5′-GAAATCGTGCGTGACATCAAA-3′ and reverse: 5′-TCATGGTGCTAGGAGCCA-3′; VEGF-A, forward: 5′-GCCTTG-CCTTGCTGCTCTAC-3′ and reverse: 5′-CA-GGGATTTTCTTGTC-TTGC-3′; VEGF-C, forward: 5′-AAACAAGGAGCTGGATGAA-GAG-3′ and reverse: 5′-CAATATGAAGGGACACAACGAC-3′; VEGFR-1, forward: 5′-TAGCGTCACCAGCAGCGAAAGC-3′ and reverse: 5′-CCTTTCTTTTGGGTCTCTGTGC-3′; VEGFR-2, forward: 5′-CAGACGGAC-AGTGGTATGGTTC-3′ and reverse: 5′-ACCTGCTGGTGGAAAGAACAAC-3′; VEGFR-3, forward: 5′-AGCCATTCATCAACAAGCCT-3′ and reverse: 5′-GGCAACAG-CTGGATGTCATA-3′; IGFBP3, forward: 5′- AATGCTAGTGA-GTCGGAGGAAGAC-3′ and reverse: 5′- GGCGACACTGCTTTT-TCTTATAAA-3′; ADM, forward: 5′- CCTGGGTTCGCTCGCCTT-CCTA-3′ and reverse: 5′- GGCTGGAGCCCCGTGTG- CTTGT-3′.

PCR amplification was carried out for 35 cycles (95°C for 45 sec, 56–62°C for 45 sec, and 72°C for 60 sec) with a final extension step at 72°C for 7 min. The bands were visualized by ethidium bromide staining.

Sequencing

Exons 18–21 of the EGFR cDNA from the tumor cell lines were sequenced, and the cDNAs were amplified using the following primers: forward, 5′-TCCAAACTGCACCTACGGATGC-3′, and reverse, 5′-CATCAACTCCCAAACGGTCACC-3′. The PCR amplification consisted of 25 cycles (95°C for 45 sec, 55°C for 30 sec and 72°C for 60 sec). The sequences of the PCR products were determined using ABI prism 310 (Applied Biosystems). Amplification and sequencing were carried out in duplicate for each tumor cell line. The sequences were compared to the GenBank-archived human sequence of EGFR (accession number: NM_005228.3).

Orthotopic model in vivo

ZD6474 was dissolved in sterile water containing 1% TWEEN 80 (Sigma). Six-week old female BALB/c nude mice were purchased from CLEA Japan Inc. (Tokyo) and maintained under specific pathogen-free conditions. A total of 1×10^6 58As1 cells was inoculated into the gastric wall of each mouse after laparotomy. Three days after the inoculation, the mice were given ZD6474 50 mg/kg/day ($n = 6$) or 100 mg/kg/day ($n = 6$) or a vehicle control ($n = 6$) p.o. for 14 days. After euthanizing the mice on Day 19, tumor volume was measured and tumor samples and intraperitoneal lavage fluid were collected. The tumor samples were formalin fixed ($n = 3$) or stored in Isogen ($n = 3$) (Nippon Gene, Tokyo, Japan). The intraperitoneally disseminated cells were collected from 2 ml of PBS that had been used to wash the peritoneal cavity.

In the survival study, mice were inoculated with 1×10^6 58As1 or 44As3 cells into the gastric wall after laparotomy. Three days after inoculation, the mice were given ZD6474 50 mg/kg/day of ZD6474 p.o. ($n = 7$) or i.p. ($n = 7$) or the vehicle control p.o. ($n = 7$) for 14 days. The "visible ascites," which was evident a few days before death in this model, was used as a surrogate for survival time to consider for animal welfare. Mice were euthanized when ascites became visible, implantation of the gastric cancer cells was confirmed in all of the euthanized mice. No cancer cell was found in one mouse (ZD6474 100 mg/kg/day, 44As3 implanted), and it was excluded from the analysis. The experimental animal protocols were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the Guidelines for Animal Experiments of the National Cancer Center.

Oligonucleotide microarray study

A DNA microarray procedure was used to prepare the *in vitro* transcription products, and oligonucleotide array hybridization and scanning were carried out according to the Affymetrix protocols (Santa Clara, CA). In brief, total RNA extracted from the tumor samples was analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and cRNA was synthesized with the GeneChip® 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). The cRNA were then labeled and purified for use as probes. Hybridization was carried out to the Affymetrix GeneChip HG-U133 Plus2.0 array for 16 hr at 45°C. After washing the glass slides, spot intensity was measured by scanning with a GeneChip® Scanner3000 (Affymetrix) and converted to numerical data with GeneChip Operating Software, Ver.1 (Affymetrix).

Six GeneChips were used to primary implanted 58as1 tumor samples from the vehicle control group ($n = 2$), and the ZD6474-treated group ($n = 2$, 50 mg/kg group; $n = 2$, the 100 mg/kg group).

Statistical analysis

All statistical calculations, except the analysis of the microarray data, were carried out using StatView version 5 software (SAS Institute Inc., Cary, NC). A p-value of <0.05 was considered significant. The microarray data were analyzed with GeneSpring software (Silicon Genetics, Redwood City, CA). The expression data were normalized across the sample set by the 50th percentile of each chip's intensity range. Relative expression data for each probe set were generated by median normalization. The fold change (mean value of the ZD6474-treatment group/mean value of the vehicle control group) was calculated, and genes with >2-fold change or <0.5-fold change were selected.

Results

Cell sensitivity to ZD6474 in vitro *and expression of VEGFR and EGFR*

Cell sensitivity to ZD6474 and the expression levels of EGFR, VEGFR and VEGF were examined in the 58As1 cells. The growth-inhibitory effect of ZD6474 and gefitinib was assessed by an MTT assay. The IC_{50} values of ZD6474 and gefitinib for 58As1 cells were 5.8 ± 1.8 and 11.0 ± 3.0 μM, respectively, suggesting that 58As1 cells are not sensitive to ZD6474 or gefitinib *in vitro*, compared to the "hypersensitive" PC-9 cells (IC_{50} values = 0.09 and 0.03 μM, respectively).[15] The 58As1 cells expressed a relatively high level of EGFR compared to the cells expressing high (PC-9) and low (HEK293) levels of EGFR, but the phosphorylation status was low (Fig. 1*a*). The expression levels of VEGFR and VEGF-A,C were measured by RT-PCR. A low

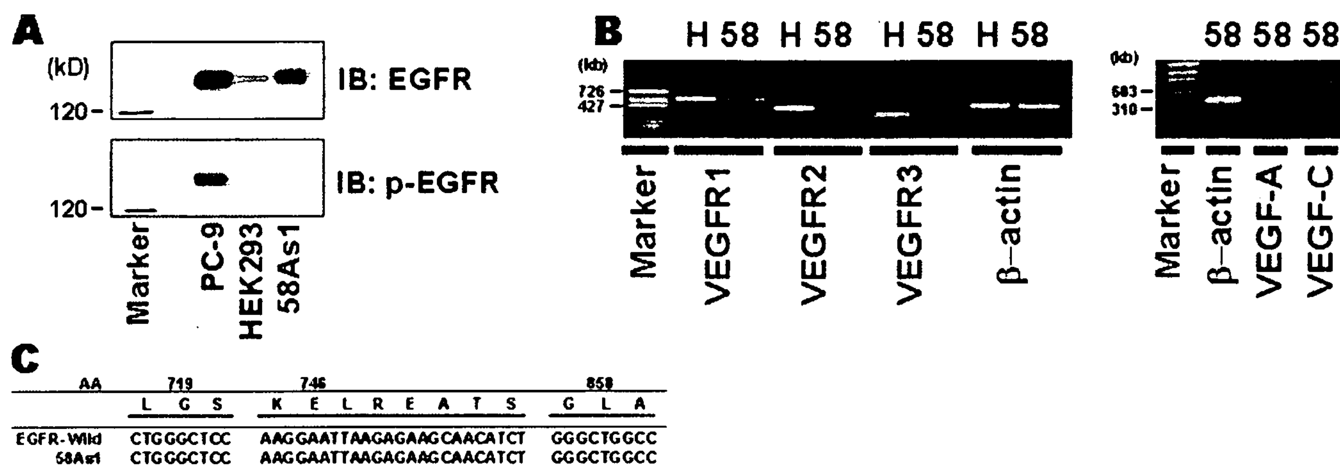


FIGURE 1 – Cellular characteristics of 58As1 cells. (*a*) EGFR expression and phosphorylation levels determined by Western blotting. A moderately high level of EGFR expression was observed in 58As1 cells, compared to cells expressing high (PC-9) and low (HEK293) levels of EGFR. The phosphorylation of EGFR status in 58As1 cells was low under normal culture conditions. IB, immunoblotting. Molecular marker: 120 kD. (*b*) Expression levels of VEGFR and VEGF-A and VEGF-C were measured by RT-PCR. A low level of VEGFR1 expression was detected in 58As1 cells, but no expression of VEGFR-2 or 3 was detected. 58As1 cells expressed VEGF-A but not VEGF-C. H, human umbilical vein endothelial cells. 58: 58As1. (*c*) EGFR sequence in 58As1 cells. No mutations were detected near the ATP-binding domains in 58As1 cells. AA, amino acid.

level of VEGFR1 expression was found in the 58As1 cells, but no VEGFR2 or VEGFR3 expression was detected. The 58As1 cells expressed VEGF-A, but not VEGF-C (Fig. 1*b*). Our results suggest that the lymphatic-metastasis-related VEGF-C and VEGFR3 are not involved in the inhibitory effect of ZD6474 on tumor dissemination observed in our present study *in vivo*.

Because EGFR mutations may be a determinant of tumor cell sensitivity to ZD6474,[15] exons 18–21 of EGFR mRNA from 58As1 cells were sequenced. No mutations near the ATP-binding domains[18,19] were detected, the 58As1 cells were concluded to express the wild-type EGFR.

Growth-inhibitory effect of ZD6474 in the orthotopic model in vivo

To examine the antitumor effect of ZD6474 on gastric cancer, we assessed the growth-inhibitory effect of ZD6474 by measuring implanted tumor volume after 14 days of p.o. treatment *in vivo*. A significant growth-inhibitory effect was observed in the ZD6474 (100 mg/kg/day) group in comparison with the vehicle control group ($p = 0.027$) in athymic mice implanted with 58As1 cells (Fig. 2*a*). Average tumor volume in the vehicle control group, 50 mg/kg/day ZD6474 group and 100 mg/kg/day ZD6474 groups was 106.3 ± 81.8 mm^3, 79.9 ± 70.0 mm^3, and 42.3 ± 24.8 mm^3, respectively.

Histological examination of H&E stained specimens showed a marked reduction in the number of cancer cells in the sub-mucosal lesions and the presence of necrotic tissue in the ZD6474 groups (Fig. 2*b*), suggesting that ZD6474 inhibits the growth of primary gastric tumor *in vivo*.

Inhibitory-effect of ZD6474 on peritoneal dissemination

To monitor the inhibitory effect of ZD6474 on peritoneally disseminated human cancer cells, the mRNA expression ratio of human β-actin/murine β-actin was measured with appropriate specific primers in cells collected from intraperitoneal lavage fluid. A significantly lower level of human-derived β-actin was observed in the 100 mg/kg/day ZD6474 group than in the vehicle control group ($p = 0.049$) (Fig. 2*c*,*d*), indicating that ZD6474 inhibits the intraperitoneal dissemination of gastric cancer in a dose-dependent manner.

Effect of ZD6474 on survival

In the survival experiment, we examined the effect of ZD6474 (p.o. or i.p.) on the survival of mice implanted with 58As1 or 44As3 cells. Both p.o. and i.p. administration of ZD6474 50 mg/kg/day significantly improved the survival of 44As3-implanted mice ($p = 0.0009$, $p = 0004$ *vs.* control, Fig. 3*b*), but did not significantly improve the survival of 58As1-implanted mice ($p = 0.09$, $p = 0.4$ *vs.* control, Fig. 3*a*). The median survival time of the 58As1-implanted mice was 33 days in the control group, 40 days in the i.p. group, and 46 days in the *p.o.* group, whereas in the 44As3-implanted mice, it was 34 days, 43 days and 53 days, respectively. Oral administration of ZD6474 was more effective than i.p. injection ($p = 0.049$) in the 44As3-implanted mice (Fig. 3*b*). These results suggest that ZD6474 is an active against gastric cancer.

Regulation of the gene expression by ZD6474 in vivo

To identify putative tumor biomarkers that reflect the efficacy of ZD6474 in vivo, we analyzed the gene expression profiles of implanted-tumor samples with oligonucleotide microarray. Expression of 26 genes was upregulated by 2-fold or more in the ZD6474 treatment group compared to the control group, whereas 2 genes were downregulated (Fig. 4*a*). Interestingly, of 26 upregulated genes, 9 of these genes were reported previously to be hypoxia-inducible: *IGFBP3* (*insulin-like growth factor binding protein 3*), *ADM* (*adrenomedullin*), *ANGPTL4* (*angiopoietin-like 4*), *PLOD2* (*procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2*), *DSIPI* (*delta sleep inducing peptide, immunoreactor*), *ENO2* (*enolase 2*), *NDRG1* (*N-myc downstream regulated gene 1*) *HIG2* (*hypoxia-inducible protein 2*) and *BNIP3L* (*BCL2/adenovirus E1B 19 kDa interacting protein 3-like*). To confirm upregulation of the genes, we measured the expression levels of representative genes, *IGFBP3* and *ADM*, by RT-PCR in murine tumor samples (Fig. 4*b*).

Discussion

A correlation between somatic EGFR mutations in non-small cell lung cancer cells and sensitivity to EGFR-specific tyrosine kinase inhibitors, including gefitinib and erlotinib, has been demonstrated recently,[18–20] and a similar observation was made in regard to ZD6474 *in vitro*.[15] We demonstrated previously that cells transfected with mutated EGFR were ~60-fold more sensitive to ZD6474 *in vitro*. EGFR tyrosine kinase inhibitors may pro-

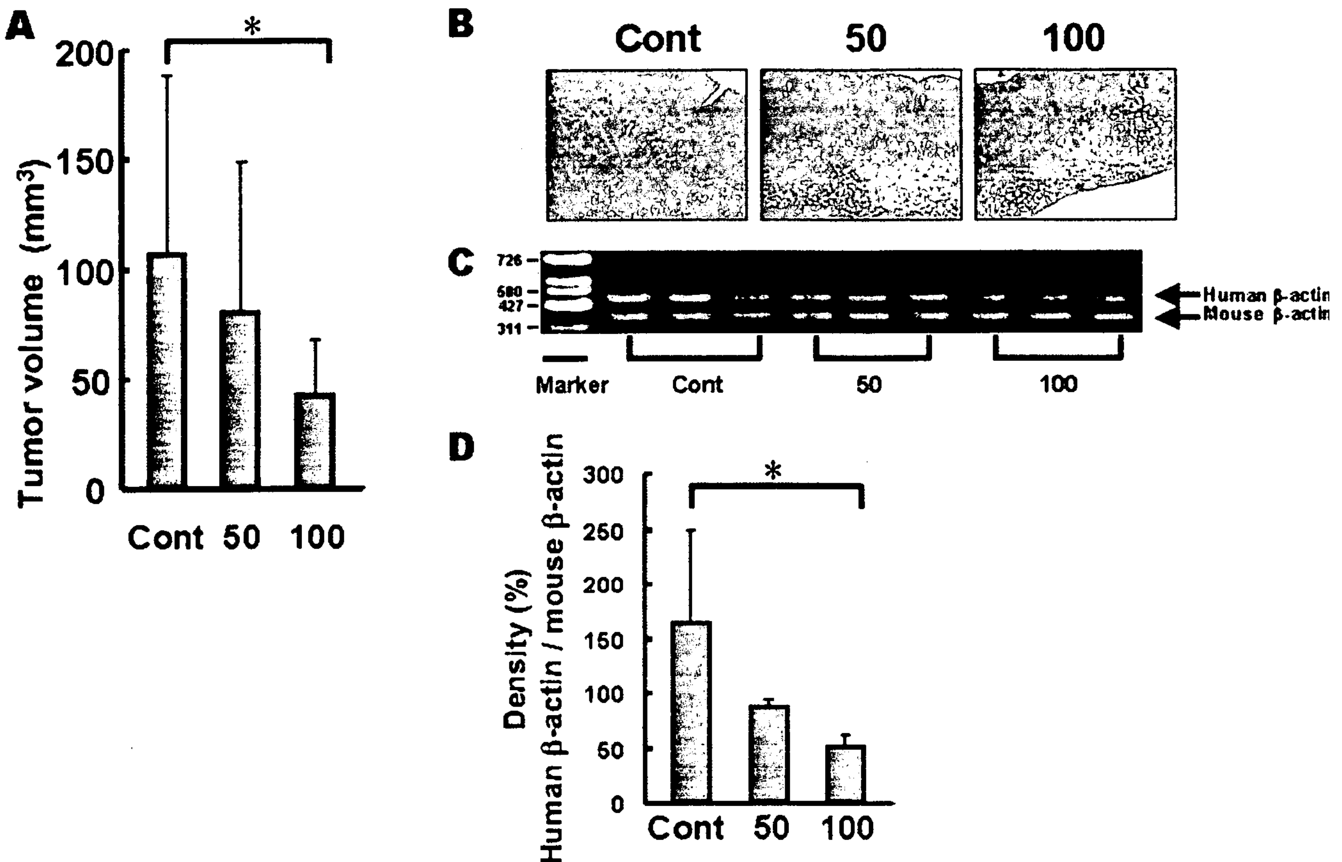
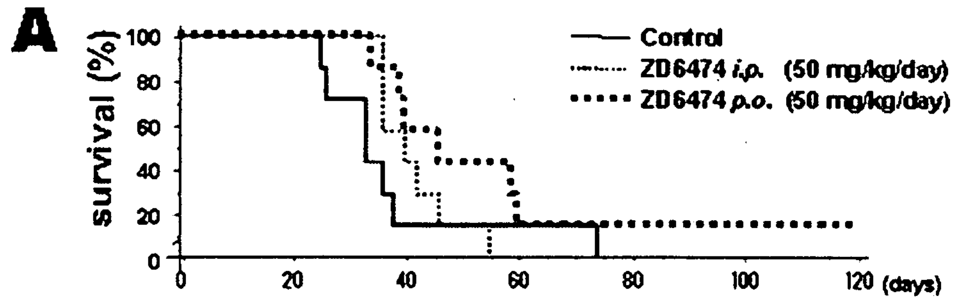


FIGURE 2 – Antitumor effect of ZD6474 in an orthotopic dissemination model *in vivo*. (*a*) *In vivo* growth-inhibitory effect of ZD6474. The implanted tumor volume was calculated after 14 days of treatment (p.o.). *Athymic mice (n = 7) per group were implanted with 58As1 cells, and a significant growth inhibitory effect was observed in the 100 mg/kg/day group, compared to the vehicle control group (p = 0.027). (*b*) Representative HE staining of tumor tissue in mice treated with ZD6474. The number of cancer cells in the sub-mucosal lesions was clearly lower and necrotic tissue was visible in the ZD6474 group, compared to the control group. (*c*) Disseminated cancer cells were collected from intraperitoneal lavage fluid to measure the ratio of tumor-derived human β-actin to murine β-actin using RT-PCR and specific primers (3 mice/group). Total RNA was equalized to 5 μg for each sample. (*d*) Densitometric analysis. Ratio of β-actin levels. *Significantly lower level of human-derived β-actin was detected in the 100 mg/kg/day ZD6474 group than in the control group (p = 0.049). The data shown are means ± SD. Cont, vehicle control; 50, ZD6474 50 mg/kg/day group; 100, ZD6474 100 mg/kg/day group. Significance was analyzed by Student's t-test.

vide particularly effective therapy for the subset of lung cancer patients whose tumor cell growth is highly dependent on EGFR signaling, including patients with tumor cells harboring activated, mutated EGFR. The proportion of patients with tumors highly dependent on EGFR signaling may be relatively small among various cancer patient populations. Therefore, additional treatment options for patients with cancers less dependent on EGFR signaling are also needed. It was concluded that 58As1 cells expressing wild-type EGFR are not highly dependent on EGFR signaling *in vitro* because the IC_{50} for growth inhibition by ZD6474 (5.8 μM) fell within the range of sensitivity reported by others for tumor cells *in vitro* (2.7–13.5 μM)[10] and because the IC_{50} for growth inhibition by gefitinib, a highly selective EGFR tyrosine kinase inhibitor, was >10μM. As a result, the concentration of ZD6474 required to inhibit 58As1 cell growth *in vitro* was 97-fold greater than required to inhibit VEGF-dependent HUVEC proliferation.[10] Nonetheless, ZD6474 significantly inhibited 58As1 tumor growth *in vivo* (Fig. 2*a*), suggesting that ZD6474 is active against gastric cancers expressing wild-type EGFR *in vivo* and that ZD6474 inhibition of tumor angiogenesis is likely to contribute significantly to this antitumor effect.

Our present study is unique because our aggressive and spontaneous intraperitoneal-dissemination model is considered a very good model of tumor progression in gastric cancer patients clinically, especially of the undifferentiated-type. Indeed, Paclitaxel and CPT-11 have been demonstrated to exhibit a growth-inhibitory effect and survival benefit in this model,[17] but gefitinib did not in preliminary result (data not shown). The positive results with ZD6474 in our present study suggest that this agent may be clinically useful in gastric cancer. We had hypothesized that direct intraperitoneal injection of ZD6474 is more effective than oral administration to inhibit intraperitoneal dissemination and result in better survival, however, the result showed that oral administration led to better survival in 44As3-inplanted mice (Fig. 3*b*).

ZD6474 inhibited the intraperitoneal dissemination of gastric cancer cells in our dissemination model. Although the mechanisms underlying this effect remain unclear, a few possibilities can be speculated: based on clinical evidence, the depth of tumor invasion and clinical staging is thought to be closely related to peritoneal dissemination.[21] Thus, one possible mechanism of the reduction of intraperitoneal dissemination may have resulted from a reduction in the serosal penetration of the cancer cells by "antitumor effect of ZD6474" on the implanted tumors. Although it is unclear whether ZD6474 has an inhibitory effect against distal metastasis to the liver and lymph nodes, for examples, it is not surprising that ZD6474 inhibits "intraperitoneal dissemination." Evaluation of its effect on distal metastasis will be the subject of a future investigation in our laboratory. Small tumor lesions (up to 2 mm) may obtain the oxygen and nutrients



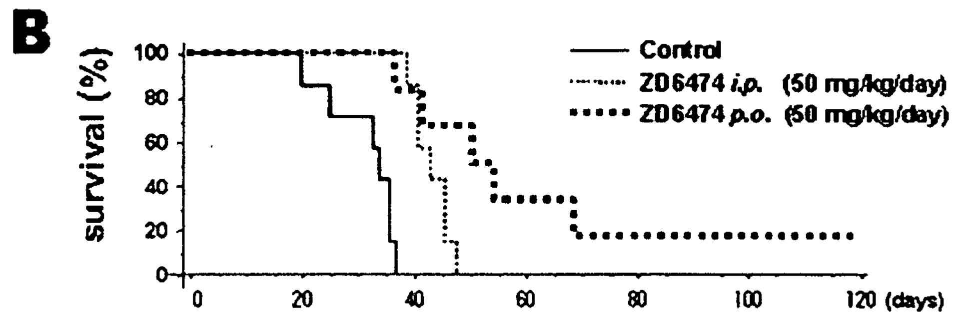
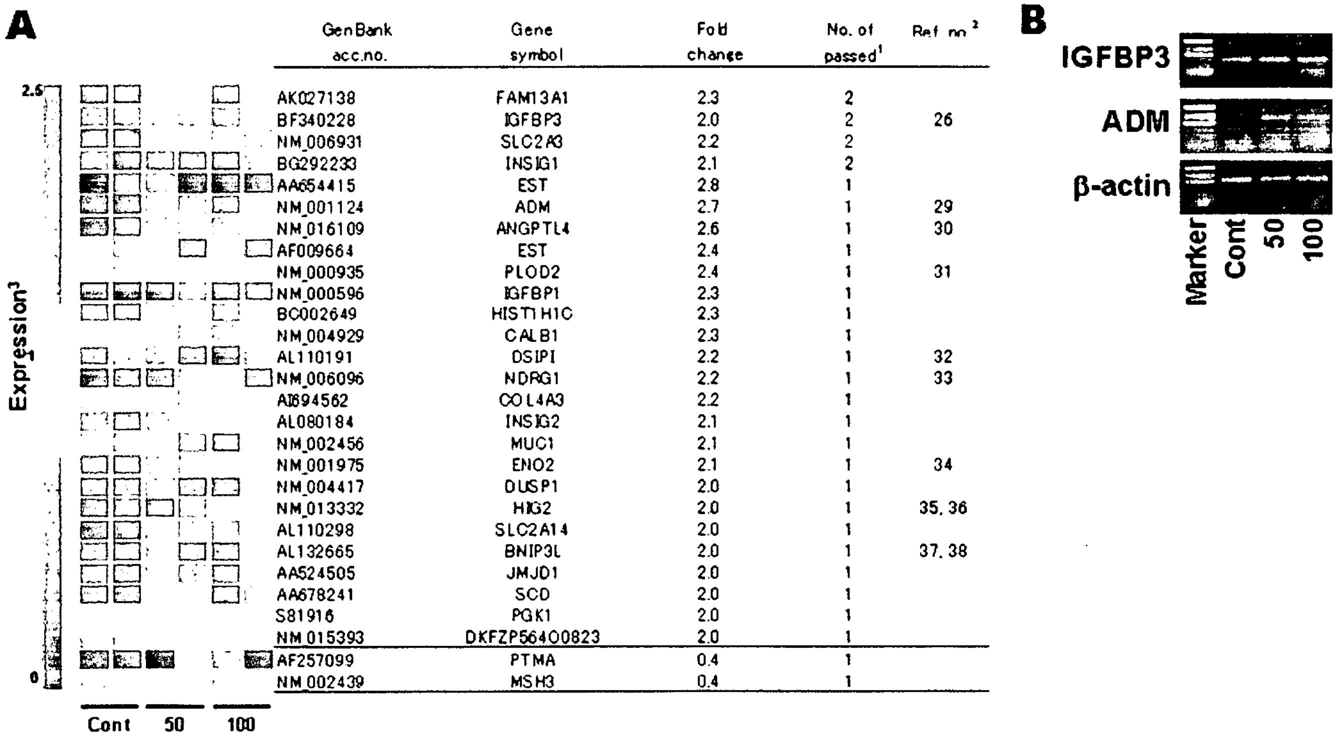


FIGURE 3 – Survival curve of 58As1 cells- (*a*) and 44As3 cells- (*b*) implanted mice treated with ZD6474. Both p.o. and i.p. administration of ZD6474 50 mg/kg/day significantly improved the survival of 44As3-implanted mice ($p = 0.0009$, $p = 0004$ *vs.* control), but did not significantly improve the survival of mice implanted with 58As1 cells ($p = 0.09$, $p = 0.4$ *vs.* control). The p values were calculated by the log-rank test.



GenBank acc.no.	Gene symbol	Fold change	No. of passed[1]	Ref no.[2]
AK027138	FAM13A1	2.3	2	
BF340228	IGFBP3	2.0	2	26
NM_006931	SLC2A3	2.2	2	
BG292233	INSIG1	2.1	2	
AA654415	EST	2.8	1	
NM_001124	ADM	2.7	1	29
NM_016109	ANGPTL4	2.6	1	30
AF009664	EST	2.4	1	
NM_000935	PLOD2	2.4	1	31
NM_000596	IGFBP1	2.3	1	
BC002649	HIST1H1C	2.3	1	
NM_004929	CALB1	2.3	1	
AL110191	DSIPI	2.2	1	32
NM_006096	NDRG1	2.2	1	33
AI694562	COL4A3	2.2	1	
AL080184	INSIG2	2.1	1	
NM_002456	MUC1	2.1	1	
NM_001975	ENO2	2.1	1	34
NM_004417	DUSP1	2.0	1	
NM_013332	HIG2	2.0	1	35, 36
AL110298	SLC2A14	2.0	1	
AL132665	BNIP3L	2.0	1	37, 38
AA524505	JMJD1	2.0	1	
AA678241	SCD	2.0	1	
S81916	PGK1	2.0	1	
NM_015393	DKFZP564O0823	2.0	1	
AF257099	PTMA	0.4	1	
NM_002439	MSH3	0.4	1	

FIGURE 4 – Candidate genes for biomarkers regulated by ZD6474 treatment. Each colored block represents the expression level of a given gene in an individual sample. (*a*) Upregulated genes with a >2-fold change or <0.5-fold change are shown (mean value in the ZD6474 group/vehicle control group). Cont, vehicle control group, $n = 2$; 50, ZD6474 50 mg/kg/day group, $n = 2$; 100, ZD6474 100 mg/kg/day group, $n = 2$. [1]Number of different probes that passed fold-change criteria above. [2]Reference number for genes whose expression has been reported to be related to hypoxia. [3]Red represents increased expression and blue represents decreased expression relative to the normalized expression of the gene across all samples. (*b*) mRNA expression levels of 2 representative genes, *IGFBP-3* and *ADM*, detected by RT-PCR in tumors treated with ZD6474. *IGFBP-3* and *ADM* mRNA expression was induced in response to ZD6474.

they need by passive diffusion, but angiogenesis is required for the growth of tumors larger than 2 mm.[22] A second possible mechanism is that ZD6474 may inhibit the growth or migration of tumor vascular endothelial cells in "small tumor lesions" by inhibiting VEGFR2-dependent intracellular signaling. This effect would be expected to limit metastatic tumor growth due to lack of oxygen and nutrients, and reduce the dissemination of cancer cells.

To identify putative biomarkers of the pharmacodynamic effects of ZD6474 *in vivo*, we identified 28 candidate genes from implanted 58As1 tumor samples by oligonucleotide microarray analysis (Fig. 4*a*). IGFBP-3 has multiple functions, including in the induction of apoptosis,[23] the inhibition of cancer cell proliferation,[24] and carcinogenesis[25] and IGFBP-3 expression is transcriptionally upregulated under hypoxic conditions.[26] A recent study has also shown that EGFR regulates IGFBP-3 expression and secretion.[27] The inhibitory effect of ZD6474 on EGFR kinase may be associated with the upregulation of IGFBP-3. ADM, which was first identified in a human pheochromocytoma, is known to regulate circulation by acting as a hormone.[28] Adrenomedullin is also induced by hypoxia and may have a role in protecting against hypoxic cellular damage in human retinal pigment epithelial cells.[29] Expressions of nine of the upregulated genes, *IGFBP-3*, *ADM*, *ANGPTL4*, *PLOD2*, *DSIPI*, *NDRG1*, *ENO2*, *HIG2* and *BNIP3L*, has been reported previously to be induced by hypoxia.[26,29–38] We hypothesize that ZD6474 inhibits neovascularization in tumors, thereby limiting the oxygen and nutrient supply and resulting in tumor hypoxia and upregulation of hypoxia-inducible genes. If this hypothesis is correct, hypoxia-regulated genes and gene products might be useful biomarkers for the pharmacodynamic effects of ZD6474 and other anti-angiogenic agents in preclinical and clinical settings. We are now investigating whether these genes and gene products can be used as biomarkers for the efficacy of ZD6474 in a correlative study.

Future directions of our study include: (*i*) to compare the antitumor effect of other "anti-vascular agents" with ZD6474 in this model; (*ii*) to evaluate combination therapy with ZD6474 plus anticancer agents; (*iii*) to evaluate the antitumor effect of ZD6474 against micro-metastasis *in vivo*; and (*iv*) to confirm the usefulness of the 9 candidate genes as biomarkers in clinically.

In conclusion, we demonstrated that ZD6474 inhibited tumor growth, suppressed intraperitoneal dissemination, and prolonged survival in a highly metastatic orthotopic gastric cancer model. We carried out a microarray analysis of tumor samples and we identified 9 hypoxia-inducible genes as candidate biomarkers for monitoring the effects of ZD6474 therapy. These findings provide a strong preclinical rationale for investigating ZD6474 for the treatment of gastric cancer in the clinic.

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