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Fig. 1

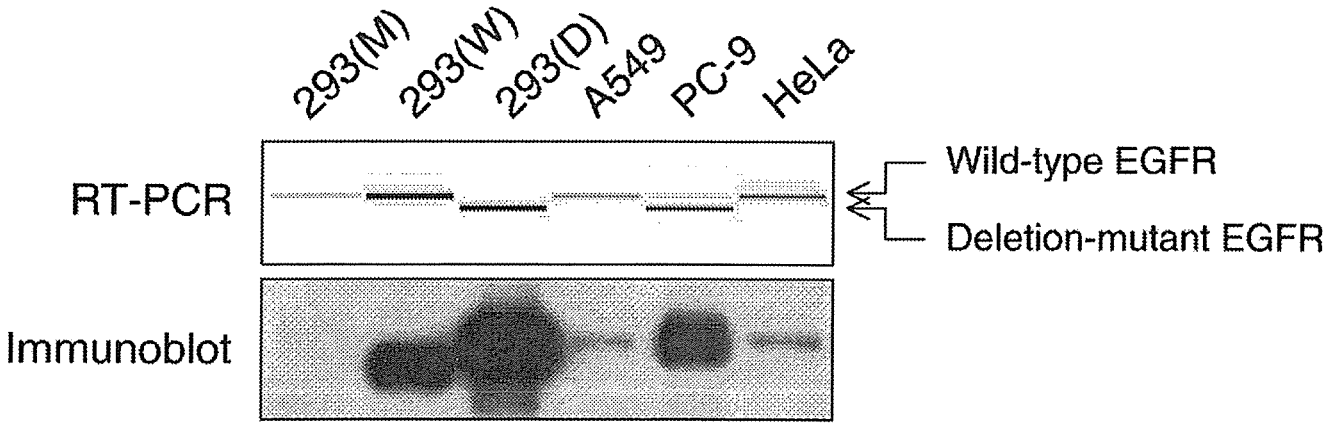

Figure 1. Expression status of epidermal growth factor receptor (EGFR) The EGFR expression level was determined by RT-PCR and immunoblot analysis. The RT-PCR products were analyzed with a 2100 Bioanalyzer. The level of EGFR protein expression was measured by immunoblot with anti-EGFR.

Fig. 2

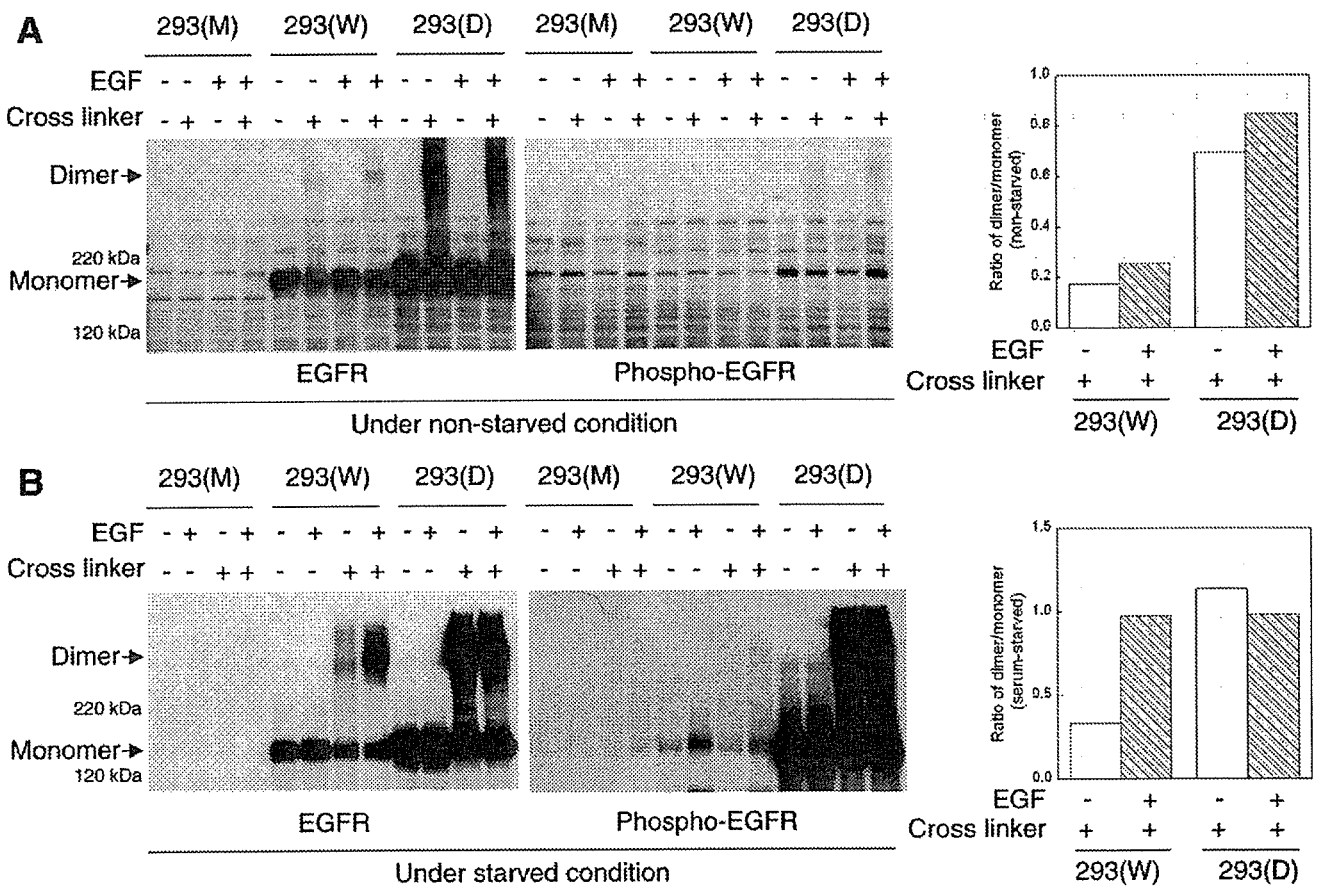

Figure 2. Dimerization and phosphorylation of wild-type EGFR and deletional EGFR. ***A***) The transfected cells were treated with or without epidermal growth factor (EGF) (10 ng/ml) for 10 min under nonstarved conditions. After two washes with ice-cold PBS(+), monolayer cells were incubated with the 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

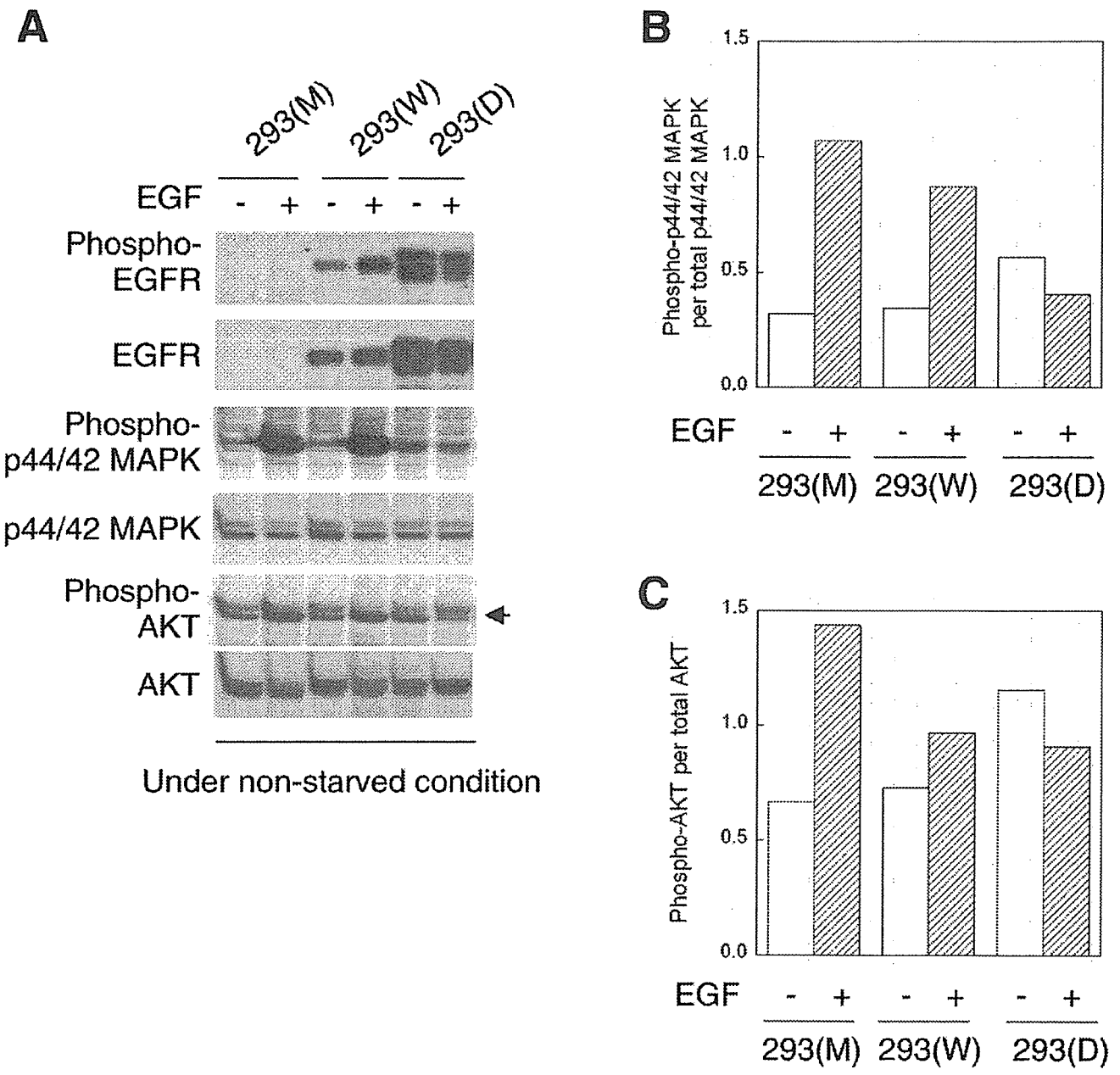

Figure 3. Phosphorylation of EGFR, p44/42 mitogen-activated protein kinase (MAPK), and AKT in the EGFR-transfected 293 cells. ***A***) The 293 (M), 293(W), and 293(D) cells were treated with EGF (10 ng/ml) for 10 min under nonstarved conditions. After two washes with ice-cold PBS(+), monolayer cells 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

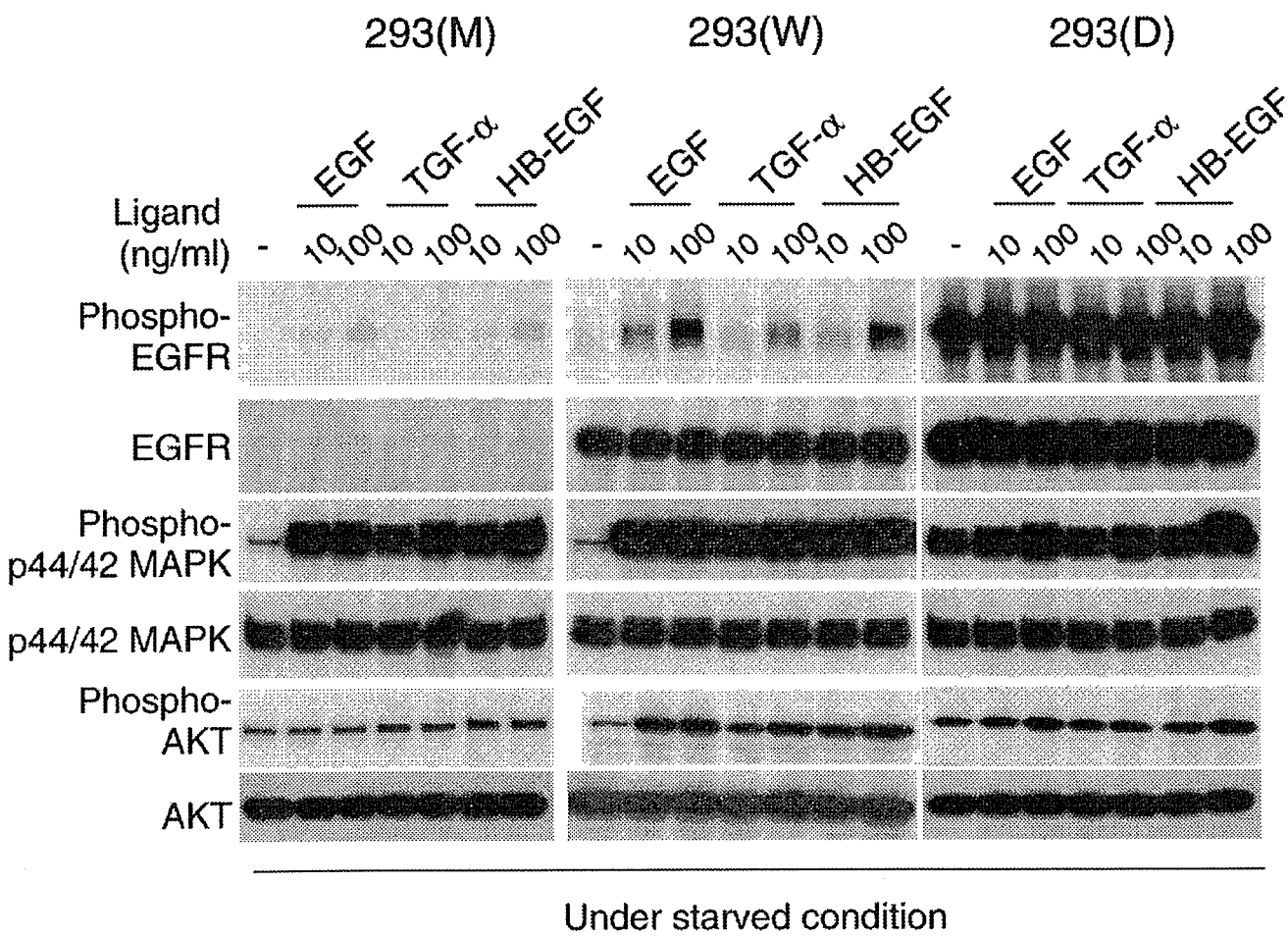

Figure 4. Phosphorylation of EGFR, p44/42 MAPK, and AKT by other ligands. The transfected cells were exposed or not exposed to EGF, TGF-α, and HB-EGF for 10 min under serum-starved conditions. After two washes with ice-cold PBS(+), monolayer cells were lysed. Equivalent amounts of protein were separated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

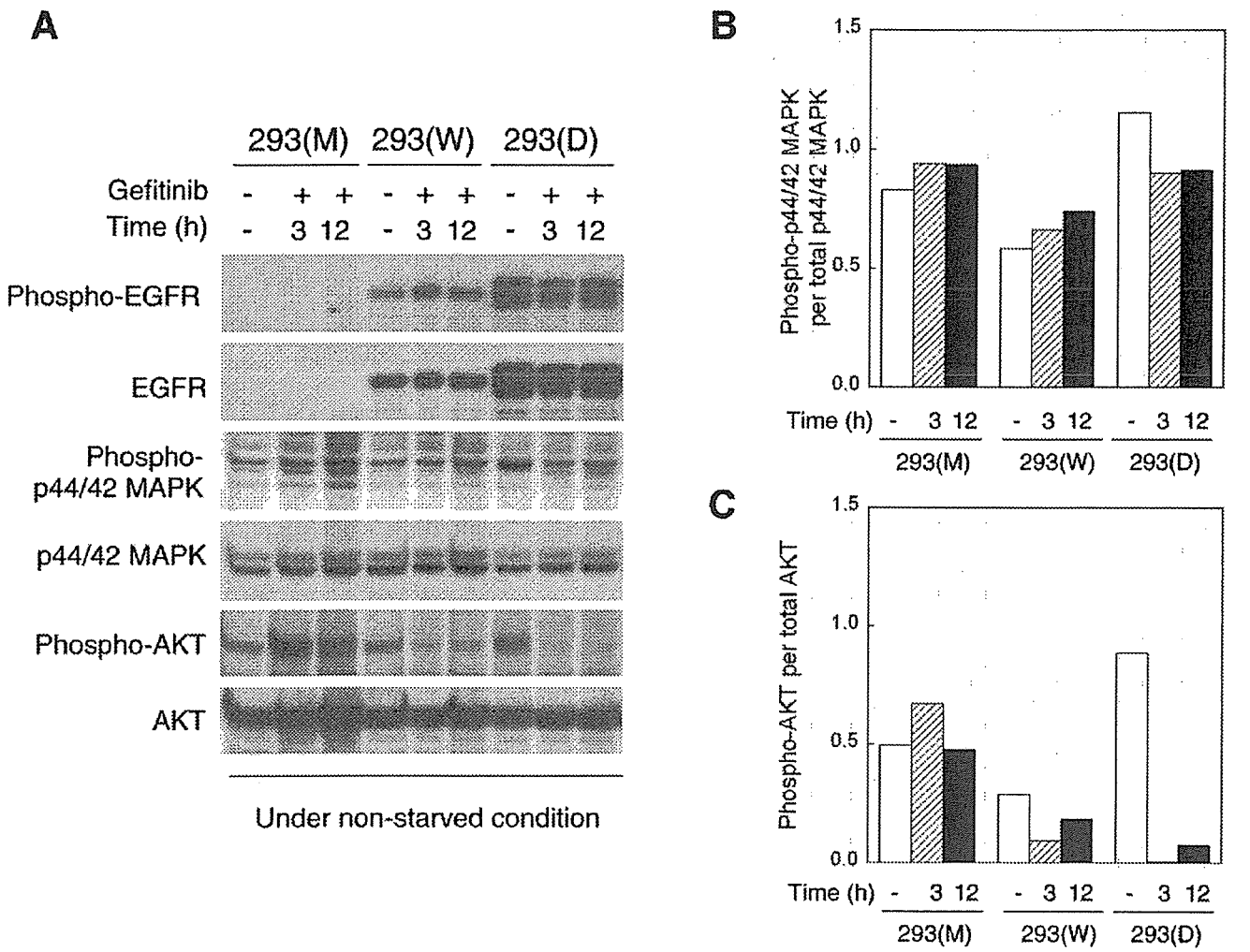

Figure 5. Effect of gefitinib on phosphorylation of EGFR, p44/42 MAPK, and AKT in the EGFR-transfected 293 cells under nonstarved conditions. ***A***) The 293(M), 293(W), and 293(D) cells were incubated with gefitinib (0.01 μM) for 3 h or 12 h under nonstarved conditions. After two washes with ice-cold 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.

Fig. 6

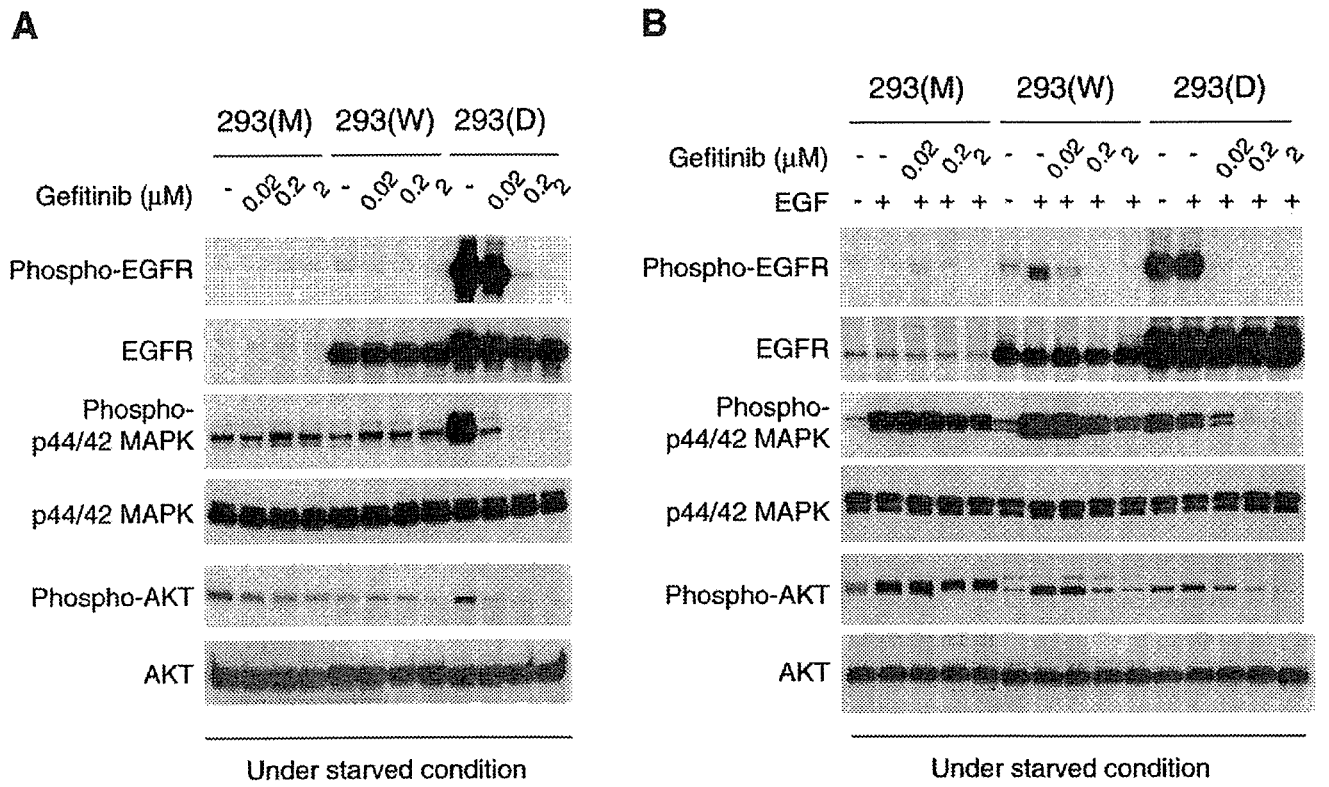

Figure 6. Effect of gefitinib on phosphorylation of EGFR, p44/42 MAPK, and AKT in the EGFR-transfected 293 cells under serum-starved conditions. ***A***) The 293(M), 293(W), and 293(D) cells were incubated with gefitinib (0.02, 0.2, 2 μM) for 3 h under serum-starved conditions. After two washes with 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

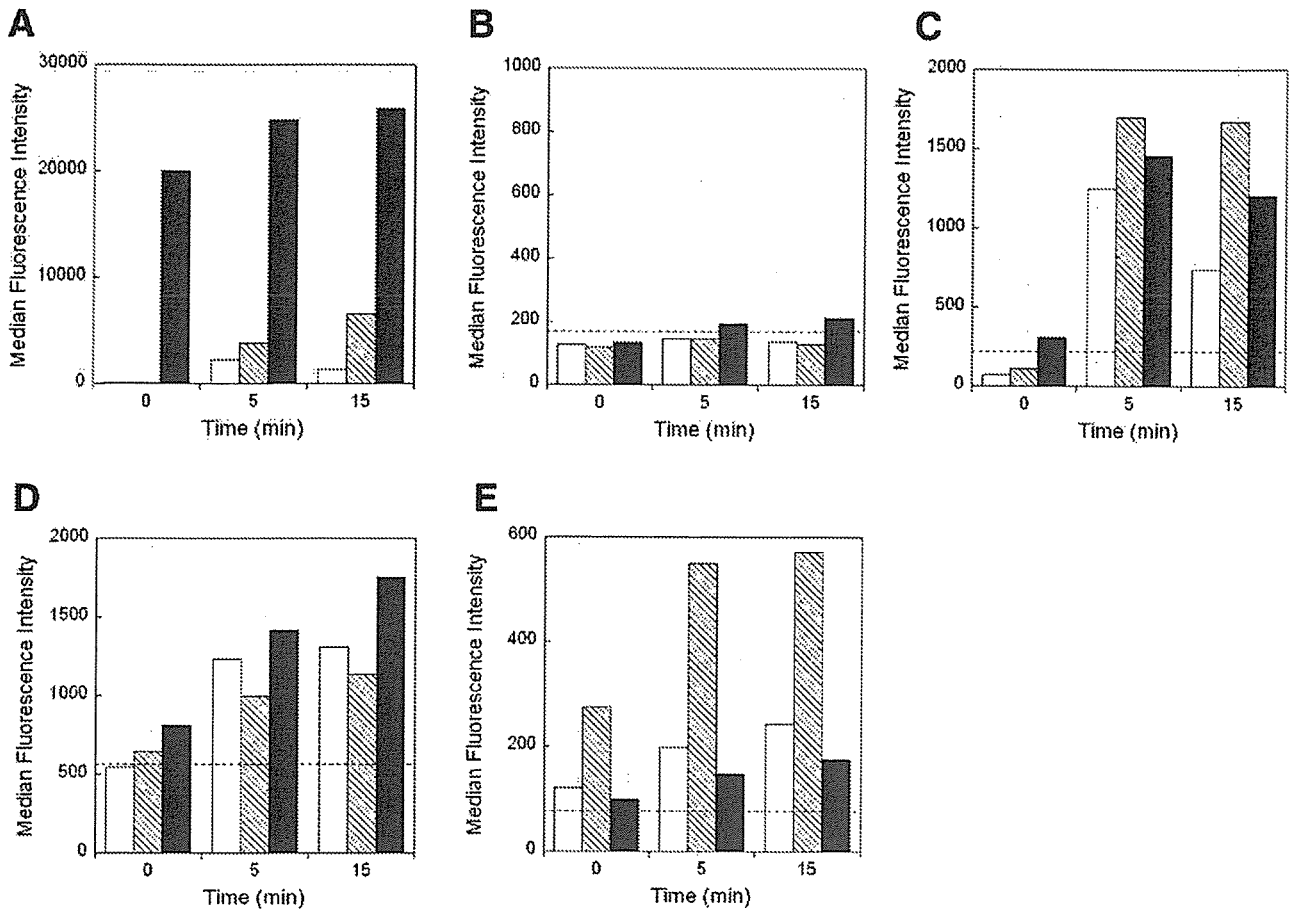

Figure 7. Monitoring of phosphoproteins after EGF stimulation in the EGFR-transfected 293 cells. The phosphoproteins in the 293(M) (open bars), 293 (W) (hatched bars), and 293(D) (solid bars) cells were analyzed by bead-based multiplex assay at the indicated intervals after addition of EGF (100 ng/ml) 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

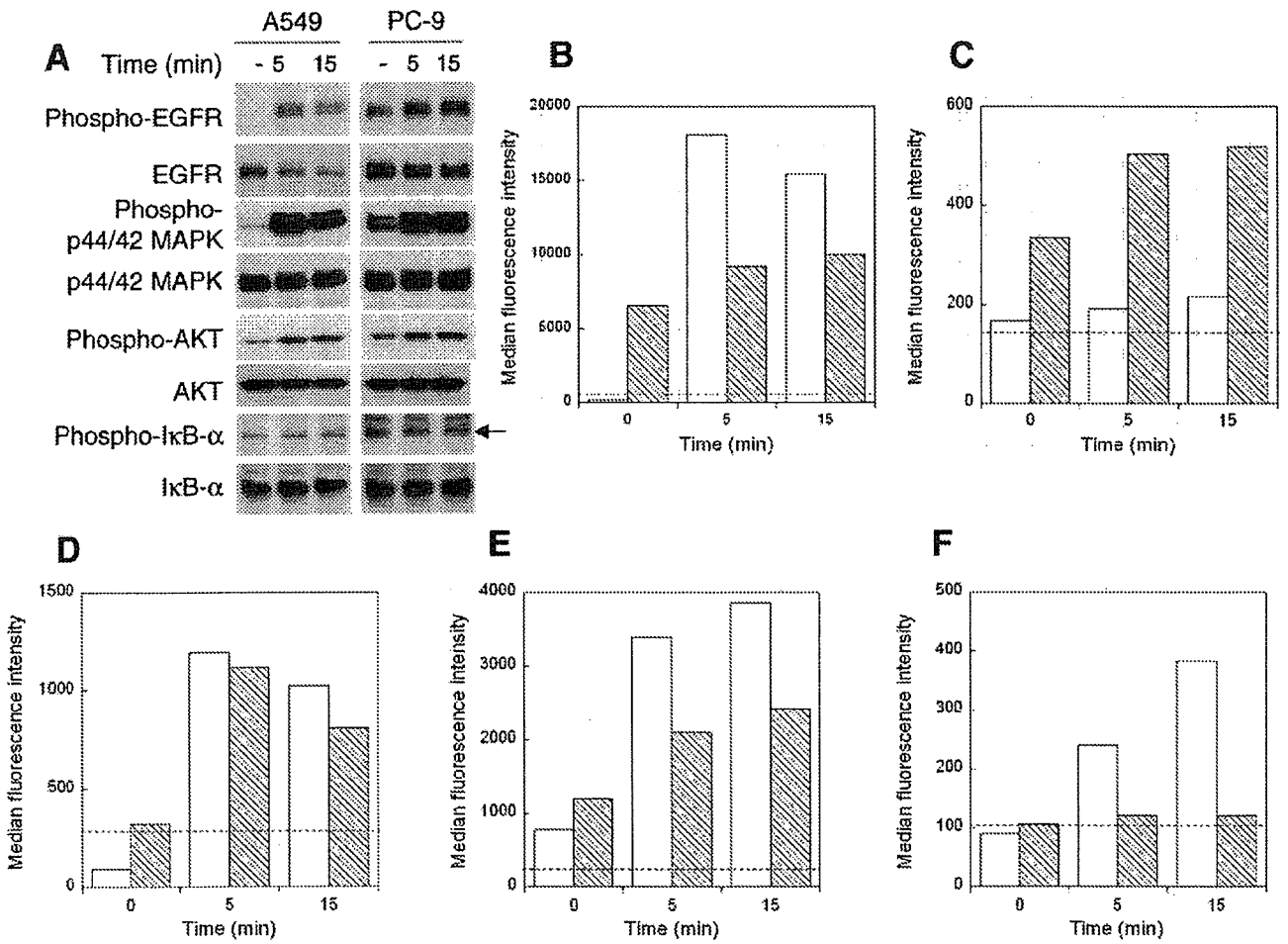

Figure 8. Monitoring of phosphoproteins after EGF stimulation in NSCLC cells. The phosphoproteins in NSCLC cells were analyzed for the indicated time intervals after addition of EGF (100 ng/ml) under serum-starved conditions. ***A***) After two washes with ice-cold PBS(+), monolayer cells were lysed. 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

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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-

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

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.

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%

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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ORIGINAL ARTICLE

Identification of expressed genes characterizing long-term survival in malignant glioma patients

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Better understanding of the underlying biology of malignant gliomas is critical for the development of early detection strategies and new therapeutics. This study aimed to define genes associated with survival. We investigated whether genes coupled with a class prediction model could be used to define subgroups of high-grade gliomas in a more objective manner than standard pathology. RNAs from 29 malignant gliomas were analysed using Agilent microarrays. We identified 21 genes whose expression was most strongly and consistently related to patient survival based on univariate proportional hazards models. In six out of 10 genes, changes in gene expression were validated by quantitative real-time PCR. After adjusting for clinical covariates based on a multivariate analysis, we finally obtained a statistical significance level for *DDR1* (discoidin domain receptor family, member 1), *DYRK3* (dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 3) and *KSP37* (Ksp37 protein). In independent samples, it was confirmed that DDR1 protein expression was also correlated to the prognosis of glioma patients detected by immunohistochemical staining. Furthermore, we analysed the efficacy of the short interfering RNA (siRNA)-mediated inhibition of DDR1 mRNA synthesis in glioma cell lines. Cell proliferation and invasion were significantly suppressed by siRNA against DDR1. Thus, DDR1 can be a novel molecular target of therapy as well as an important predictive marker for survival in patients with glioma. Our method was effective at classifying high-grade gliomas objectively, and provided a more accurate predictor of prognosis than histological grading.
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Introduction

Glioblastoma, which is pathologically the most aggressive form, has a median survival range of only 9–15 months (Karpeh *et al.*, 2001; Stewart, 2002; Stupp *et al.*, 2005). Advances in the basic knowledge of cancer biology and surgical techniques, chemotherapy and radiotherapy have led to little improvement in the survival rates of patients suffering from glioblastoma (Stewart, 2002). Poor prognosis is attributable to difficulties of early detection, and to a high recurrence rate during post-initial treatment observation periods. Therefore, it is important to devise more effective therapeutic approaches, to reveal more clearly the biological features of glioblastoma, and identify novel target molecules for diagnosis and therapy of the disease. Several histological grading schemes exist, but the two-tiered World Health Organization (WHO) system is currently the most widely used (Kleihues and Cavenee, 2000). A high WHO grade correlates with clinical progression and decreased survival. However, there are still many individual variabilities within diagnostic categories, leading to the need for developing additional prognostic markers. As prognostic markers are based on morphology, identification of new treatment strategies is limited. Identification of distinct molecular pathways has become critical for developing molecular targeted therapies.

Recently, developed microarray technology has permitted development of multi-organ cancer classification including gliomas (Ramaswamy *et al.*, 2001; Rickman *et al.*, 2001; Kim *et al.*, 2002; Hunter *et al.*, 2003; Mischel *et al.*, 2004), identification of tumor subclasses (Khan *et al.*, 2001; Mischel *et al.*, 2003; Shai *et al.*, 2003; Sorlie *et al.*, 2003; Liang *et al.*, 2005; Nigro *et al.*, 2005; Wong *et al.*, 2005), discovery of progression markers (Sallinen *et al.*, 2000; Agrawal *et al.*, 2002; van de Boom *et al.*, 2003; Godard *et al.*, 2003; Hoelzinger *et al.*, 2005; Rich *et al.*, 2005; Somasundaram *et al.*, 2005) and prediction of disease outcomes (van't Veer *et al.*, 2002; van de Vijver *et al.*, 2002; Nutt *et al.*, 2003; Freije *et al.*, 2004). Unlike clinicopathological staging, molecular staging can predict long-term outcomes of any individual based on gene expression profile of the tumor at diagnosis. Analysis of expression profiles of genes in

clinical materials is an essential step toward clarifying the detailed mechanisms of oncogenesis and discovering target molecules for the development of novel therapeutic drugs.

The human 1 cDNA microarray (Agilent Technologies, Palo Alto, CA, USA) contains 12811 clones from more than 7000 UniGene clusters. Each clone is represented by a PCR-amplified, double-stranded complementary DNA (cDNA) product, immobilized on the slide. mRNAs obtained from two biological samples were separately converted to cDNA labeled with distinct fluorescent dyes, usually cyanines 3 (Cy3) and 5 (Cy5), mixed together and hybridized to a single array. Hybridization intensities from the two dyes were measured, and compared for each gene within the array, to identify gene expression differences between the two samples. Utilization of a common reference sample for each array allowed objective comparisons between samples on separate arrays. In the present study, we used agilent cDNA microarrays to define expression patterns to distinguish between short-term and long-term survival of malignant gliomas.

Results

High-grade gliomas in this study

Patients initially showed histologically proven glioblastoma (grade IV), anaplastic astrocytoma or other malignant gliomas (grade III) corresponding to the WHO criteria. Seven patients with grade III and 22 patients with grade IV were included in this study (Table 1). Univariate analysis of clinical features was performed against pathological diagnoses, age, gender and performance status (PS) with respect to survival. Pathological diagnoses, age and gender were not independent predictors of survival (Table 2). Once all gliomas were sorted according to PS, significant difference was found between survival of patients with PS 0–60 and patients with PS 70–100 in our cases (Table 2).

Table 1 Patient characteristics

No.	*Histological diagnosis*	*Age, gender*	*WHO grade*	*PS*	*Survival time*
1	Anaplastic oligoastrocytoma	59, M	III	80	263
2	Anaplastic oligodendroglioma	60, M	III	90	294
3	Anaplastic oligodendroglioma	72, M	III	90	305
4	Anaplastic astrocytoma	32, M	III	100	545
5	Anaplastic astrocytoma	73, M	III	70	617
6	Anaplastic astrocytoma	45, M	III	60	698
7	Anaplastic astrocytoma	65, M	III	90	762
8	Glioblastoma	18, F	IV	60	111
9	Glioblastoma	64, F	IV	50	154
10	Glioblastoma	28, M	IV	70	202
11	Glioblastoma	45, M	IV	60	261
12	Glioblastoma	54, M	IV	40	268
13	Glioblastoma	68, M	IV	80	286
14	Glioblastoma	62, M	IV	70	347
15	Glioblastoma	80, M	IV	80	349
16	Glioblastoma	78, F	IV	60	350
17	Glioblastoma	69, M	IV	90	352
18	Glioblastoma	67, M	IV	50	396
19	Glioblastoma	63, M	IV	60	405
20	Glioblastoma	20, F	IV	90	417
21	Glioblastoma	71, M	IV	80	436
22	Glioblastoma	31, M	IV	90	453
23	Glioblastoma	56, M	IV	80	506
24	Glioblastoma	55, M	IV	80	630
25	Glioblastoma	52, F	IV	90	641
26	Glioblastoma	27, F	IV	90	757
27	Glioblastoma	42, F	IV	70	880
28	Glioblastoma	47, M	IV	90	908
29	Glioblastoma	42, M	IV	90	1189

Abbreviation: PS, performance status; WHO, World Health Organization.

Table 2 Univariate analysis of clinical features

Variable	*No. of patients*	*Median survival time (days)*	P *(log-rank test)*
WHO grade			
Grade III	7	617	0.56
Grade IV	22	417	
Age (years)			
<60	16	641	0.069
⩾60	13	352	
Gender			
Male	22	436	0.979
Female	7	417	
PS			
70–100	21	617	0.0033
0–60	8	309	

Abbreviation: PS, performance status; WHO, World Health Organization.

Identification of prognosis-related genes

We performed the univariate proportional hazard model to identify a set of genes that better correlated with censored survival time. Genes were selected if their *P*-value was less than 0.005 and the *P*-value was then used in a multivariate permutation test. We identified 21 genes whose expression was most strongly and consistently related to survival. These genes are listed in Table 3, and include several genes that we believe to be biologically active such as DDR1 (discoidin domain receptor family, member 1) and KSP37 (Ksp37 protein) (see Discussion).

Relationships between results obtained by microarray analysis and by real-time PCR

We chose 10 genes that were not previously associated with gliomas, to measure their mRNA levels by real-time quantitative reverse transcription–PCR. From 29 microarray-measured tumor samples, total RNAs from 27 tumor samples (14 long-term survivors and 13 short-term survivors) were analysed for expressions of ALCAM (activated leukocyte cell adhesion molecule), DDR1, DYRK3 (dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 3), ITGA5 (integrin alpha 5), ITGB2 (integrin beta 2), KSP37, LDHC (lactate dehydrogenase C), LOC (hypothetical protein

Table 3 Identification of prognosis-related genes

GenBank	*Symbol*	*Description*	*Hazard ratio*	P-*value*
BC005261	SLN	Sarcolipin	0.41	0.000263
U13680	LDHC	Lactate dehydrogenase-C	0.24	0.000851
AL1 37662	NRBP2	Nuclear receptor binding protein 2	5.5	0.00101
AB021123	KSP37	Ksp37 protein	0.12	0.00102
M20681	GLUT3	Glucose transporter-like protein-III	0.37	0.00107
BC007952	PKM2	Pyruvate kinase, muscle	0.15	0.0013
N92498	PDCD4	Programmed cell death 4	3.1	0.00205
M10036	TPI1	Triosephosphate isomerase 1	0.16	0.00222
BC015061	RAB32	RAB32, member RAS oncogene family	0.51	0.00260
U20362	TTC10	Intraflagellar transport 88 homolog	4.5	0.00290
BE045190	DDR1	Discoidin domain receptor family, member 1	4.2	0.00308
AF327561	DYRK3	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 5	0.17	0.00312
BC005861	ITGB2	Integrin, beta 2	4.1	0.00352
BAB22510		Putative	8.0	0.00365
BC007835	STK40	Serine/threonine kinase 40	0.40	0.00369
AB026706	EMILIN2	Elastin microfibril interfacer 2	0.27	0.00389
AF231512	RBM8B	RNA binding motif protein 8B	4.3	0.00403
BC008786	ITGA5	Integrin, alpha 5	0.36	0.00419
AA404652	ISGF3G	Interferon-stimulated transcription factor 3, gamma (48 kD)	2.8	0.00431
Y10183	ALCAM	Activated leukocyte cell adhesion molecule	2.8	0.00440
MI 9482	ATP synthase	Human ATP synthase beta subunit gene, exons 1–7	0.28	0.00445

A subset of the 21 genes expressed differentially in good and poor prognosis group, listed by category. Included with name of each gene is the GeneBank accession number, a brief description of the gene and the *P*-value that was computed.

Table 4 mRNA levels by real-time quantitative RT–PCR

	Short-term survivor (n = *13*)	*Long-term survivor* (n = *14*)	P
ALCAM (ng/ml)	6.6 ± 14.5	0.06 ± 0.1	<0.05
DDR1 (pg/ml)	416.8 ± 56.5	40.6 ± 11.1	<0.01
DYRK3 (ng/ml)	116.1 ± 96.2	449.3 ± 108.7	<0.05
ITGA5 (pg/ml)	38.7 ± 47.1	707.6 ± 85.6	<0.01
ITGB2 (pg/ml)	0.02 ± 0.01	0.03 ± 0.05	NS
KSP37 (pg/ml)	18.9 ± 24.6	8402.9 ± 855.6	<0.01
LDHC (pg/ml)	1.4 ± 1.0	7.5 ± 12.5	NS
LOC (pg/ml)	1.2 ± 1.1	1.7 ± 2.1	NS
SLN (pg/ml)	8.9 ± 1.9	15.5 ± 4.5	<0.05
SLC2A3 (ng/ml)	7.5 ± 8.3	19.1 ± 23.9	NS

Abbreviations: NS, not significant; RT-PCR, reverse transcription–PCR. For other abbreviations, see Table 3.

Table 5 Multivariate analysis

Variable	*Hazard ratio*	*95% CI*	P
WHO grade	9.55	1.24–73.8	0.0305
Age (⩾60)	5.88	1.1–31.4	0.038
Gender (male)	8.16	0.748–88.9	0.0851
PS (70–100)	18.2	2.47–134	0.0044
DDR1	21.5	2.12–217	0.0094
DYRK3	0.067	0.006–0.798	0.0325
KSP37	0.008	0.000–0.235	0.0053
ITGA5	0.698	0.146–3.34	0.6525
SLN	2.85	0.658–12.4	0.1615
ALCAM	1.67	0.446–6.274	0.4453

Abbreviations: CI, confidence interval; PS, performance status; WHO, World Health Organization. For other abbreviations see Table 3.

LOC340371), SLN (sarcolipin) and SLC2A3 (solute carrier family 2 member 3). Results are shown in Table 4, and are expressed as means ± standard deviation (s.d.). Patterns of gene expression between long- and short-term survivors analysed by microarray paralleled patterns observed using real-time PCR for ALCAM, DDR1, DYRK3, ITGA5, KSP37 and SLN (Table 3).

DDR1, DYRK3 and KSP37 were selected based on a multivariate analysis

To adjust for relevant clinical covariates against six PCR-confirmed genes, we performed a multivariate analysis (Table 5). In incorporating multivariate analysis, high DDR1 expression was negatively correlated with survival ($P = 0.0094$; hazard ratio = 21.5; 95% confidence interval (CI), 2.12–217), high DYRK3 expression was positively correlated with survival ($P = 0.0325$; hazard ratio = 0.067; 95% CI, 0.006–0.798) and high KSP37 expression was positively correlated with survival ($P = 0.0053$; hazard ratio = 0.008; 95% CI, 0.000–0.235). The expression of DDR1 and KSP37 were more closely correlated with survival compared to histological grade (Table 5). Thus, in gliomas, these results suggested that expression of *DDR1*, *DYRK3* and *KSP37* might be a strong predictive factor for patient's survival better than WHO grading.

Immunohistochemical analysis of potential candidate genes

To confirm our results from microarray analysis, we chose to investigate DDR1 expression as a prognostic marker for glioma and performed the immunohistochemical analysis. Firstly, we analysed the protein expression of DDR1 against 29 microarray-measured specimens, and investigated the correlations with patient survivals. DDR1 was expressed in the cytoplasm of neoplastic cells and patients were divided into two