Table 2. Differentially expressed ABC transporters ordered by significance

Gene symbol	Genbank	Parametric p-value*	% CV support	RD[a]	pCR[b]	Fold difference[c]	Description
ABCC5	AF146074	0.000368	100	6009.1	2427.5	2.48	ABC, sub-family C (CFTR/MRP), member 5
ABCC5	BE550362	0.000463	100	3571.5	1234.4	2.89	ABC, sub-family C (CFTR/MRP), member 5
ABCA12	AL080207	0.000795	100	711.7	93.1	7.64	ABC, sub-family A (ABC1), member 12
ABCA1	AL833227	0.000859	100	166.8	50.5	3.3	ABC, sub-family A (ABC1), member 1
CFTR	NM_000492	0.007030	100	27.7	104.4	0.27	cystic fibrosis transmembrane conductance regulator, ABC (sub-family C, member 7)
ABCF2	NM_005692	0.015901	100	49.4	154.1	0.32	ABC, sub-family F (GCN20), member 2
TAP2	M74447	0.019345	89	543.4	1008.5	0.54	Transporter 2, ABC, sub-family B (MDR/TAP)
ABCC13	NM_172025	0.019377	100	157.5	20.9	7.54	ABC, sub-family C (CFTR/MRP), member 13
ABCB6	NM_005689	0.027077	89	1471.9	677.5	2.17	ABC, sub-family B (MDR/TAP), member 6
TAP2	AA573502	0.042069	58	1740.5	2802	0.62	Transporter 2, ABC, sub-family B (MDR/TAP)
ABCC11	AF352582	0.048626	42	160.9	59.4	2.71	ABC, sub-family C (CFTR/MRP), member 11

Table sorted by p-value. * p by random variance t-test.
[a]Geometric mean of intensities in the RD group.
[b]Geometric mean of intensities in the pCR group.
[c]Fold difference of geometric means RD: pCR.

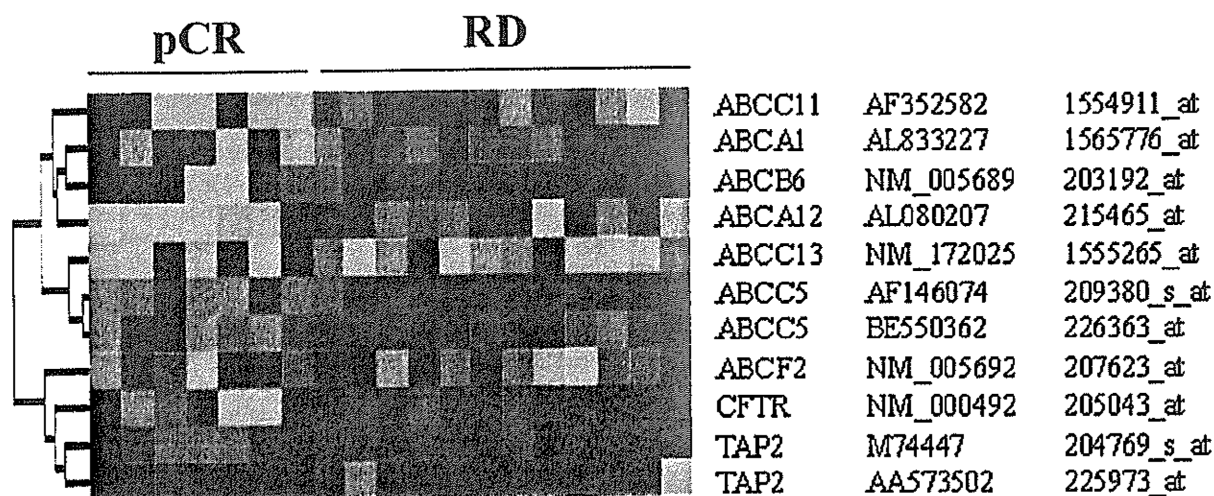

Figure 1. Hierarchical clustering of differentially expressed ABC transporters associated with the response to neoadjuvant chemotherapy in breast cancer patients. The cluster image map shows patterns of differential ABC transporter gene expression in breast cancer patients in respect to the response to neoadjuvant chemotherapy. The hierarchical clustering on each axis was performed using the complete linkage algorithm. Relatively highly expressed genes are shown in red, low expressed genes are shown in green.

an individual basis, there is a real need to develop an appropriate predictor to identify those cancer patients most likely to require or benefit from particular therapies. Resistance to chemotherapy is significant obstacle to appropriate treatment of cancer patients and affects the treatment outcome. Numerous cellular mechanisms exist which are responsible for the treatment failure due to chemoresistance. ABC transporters are the one of the major factors leading to drug resistance. Extensive study has been conducted on the ABC transporters, and ABCB1 (MDR1-P-gp) [1,2], ABCC1-MRP1 [3], and ABCG2-MXR [4] are particularly well known for their role in resistance to several chemotherapeutic agents. Because the members of the ABC transporters are grouped by sequence homology, the remained members may play roles in absorption, distribution, and excretion of chemotherapeutic agent and probably be related to drug resistance although little has been known about most of the functions of these genes. Characterization of the expression of the genes related to chemoresistance is an interesting subject and may lead to clinically useful predictors of response to chemotherapy. The profiling of ABC transporter genes in relation to the clinical response to chemotherapy may also be useful to determine the patient's underlying risk and choose the optimal therapeutic regimen to which the individual cancer patient is most likely to respond.

Focusing on the ABC transporters, we analyzed the gene expression profile in breast cancer patients using microarray data that contain the transcripts of all the

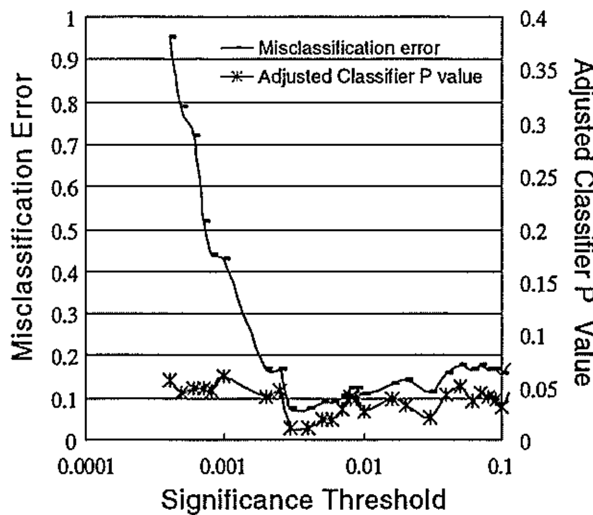

Figure 2. Multivariate predictive classification models in leave-one-out cross-validation and permutation test with an increasing significance threshold at which genes were selected as a classifier. The *x*-axis represents the significance threshold p value used to select the discriminate genes as classifiers. The *y*-axis shows the average of the misclassification error rate determined by leave-one-out cross-validation and the average classifier *p*-value, the probability that a similar low error rate could happen by chance calculated after 2000 permutations. Classifier genes selected as differentials between the 2 classes at a significance threshold $p = 0.003$ level showed the highest discriminate value.

members of ABC transporter family. We compared the expression pattern of the ABC transporters between two classes of pretreatment tumor samples divided by the pathologic response to neoadjuvant chemotherapy (RD versus pCR).

On microarray analysis, several ABC transporters showed differential expression between the two groups of tumors. Of interest, several ABC transporters showed increased expression in the pCR group, including CFTR (NM_000492, ABCC7, fold ratio 0.27, $p = 0.007030$), ABCF2 (NM_005692, fold ratio 0.32, $p = 0.015901$) and ABCB3 (M74447, TAP2, fold ratio 0.54, $p = 0.019345$). ABCB3 is known to be involved in antigen presenting by transporting peptides necessary for the assembly of major histocompatibility complex (MHC) class I molecules from the cytoplasm to the endoplasmic reticulum [18]. It is also known that its reduced expression is associated with HLA class I deficient human tumor cell lines [19] and it has been suggested that it is related to the aggressive features of some kinds of tumors [20–22]. Its increased expression has been found to be associated with pathological complete response in our clinical samples, but any clinical significance in the treatment of in breast cancer remains to be elucidated.

Five ABC transporters ABCC5 (AF146074, fold ratio 2.48, $p = 0.000368$), ABCA12 (AL080207, fold ratio 7.64,

Table 3. Performance of the multivariate classifier; the sensitivity, specificity, PPV and NPV for the pCR group of each predictor model at a significance threshold of $p = 0.003$

	CCV[a]	1NNC[b]	3NNC[c]	NCC[d]	SVM[e]	LDD[f]	Average[g]
Sensitivity	100	85.7	85.7	85.7	71.4	100	88.1
Specificity	100	91.7	91.7	100	100	91.7	95.9
PPV	100	85.7	85.7	100	100	87.5	93.2
NPV	100	91.7	91.7	92.3	85.7	100	93.6
Misclassification error	0	0.05	0.11	0.11	0.05	0.11	0.072
Percent correctly classified	100	95	89	89	95	89	92.8
Classifier P	5.00E-04	0.014	0.025	0.006	0.023	0.005	0.01225

[a]Compound covariate predictor classifier.
[b]1-Nearest neighbor classifier.
[c]3-Nearest neighbor classifier.
[d]Nearest centroid classifier.
[e]Support vector machine classifier.
[f]Linear diagonal discriminant analysis classifier.
[g]Average value of six multivariate classifier models.

Table 4. ABC transporters selected as best classifiers at a significance threshold of 0.003

Gene symbol	Genbank	*t*-Value	Parametric *p*-value*	% CV support	RD[a]	pCR[b]	§Fold difference	Description
ABCC5	AF146074	4.43	0.000368	100	6009.1	2427.5	2.48	ABC, sub-family C (CFTR/MRP), member 5
ABCC5	BE550362	4.32	0.000463	100	3571.5	1234.4	2.89	ABC, sub-family C (CFTR/MRP), member 5
ABCA12	AL080207	4.07	0.000795	100	711.7	93.1	7.64	ABC, sub-family A (ABC1), member 12
ABCA1	AL833227	4.04	0.000859	100	166.8	50.5	3.30	ABC, sub-family A (ABC1), member 1

Table sorted by *p* value.
*Parametric *p*-value by random variance *t*-test.
[a]Geometric mean of intensities in the RD group.
[b]Geometric mean of intensities in the pCR group. §Fold difference of geometric means; RD: pCR.

$p = 0.000795$), ABCA1 (AL833227, fold ratio 3.30, $p = 0.000859$), ABCC13 (NM_172025, fold ratio 7.54, $p = 0.0194$), ABCB6 (NM_005689, fold ratio 2.17, $p = 0.0271$) and ABCC11 (AF352582, fold ratio 2.71, $p = 0.0486$) showed significantly increased expression in the RD group associated with a decreased responsiveness to sequential weekly paclitaxel/FEC (5-fluorouracil, epirubicin and cyclophosphamide) neoadjuvant chemotherapy. Of these, ABCC5 was selected with the highest significance ($p = 0.000368$) and the highest expression level (RD: pCR 6009.1: 2427.5) although correlation between the gene expression level and the functional protein level remains to be seen. The ABCC5 (MRP5) transporter on human chromosome 3q27 has been known to be involved in the transport of nucleoside analogs [23] and has been reported to confer resistance to several drugs including methotrexate, GW1843 and ZD1694 (raltitrexed) [24]. Recently, Pratt et al. demonstrated that ABCC5 confers resistance against 5-fluorouracil [17] that was used in our neoadjuvant chemotherapy regimen. These results suggest that ABCC5 mediates transport of several chemotherapeutic agents and may contribute to resistance against 5-fluorouracil which is presently used in neoadjuvant chemotherapy.

In our clinical trial setting, ABCB1, known to confer resistance to several chemotherapeutic agents including paclitaxel, did not significantly increase in tumors with decreased response to neoadjuvant chemotherapy. Samples used in this study were all from chemotherapy-naïve patients and the time of exposure to the drug may not have been sufficient to induce the gene expression of this transporter. Although several ABC transporters showed high expression levels in the pretreatment samples, ABCB1 did not show significantly high expression. ABCB1 may thus play a greater role in resistance to chemotherapy in a secondary chemotherapy clinical setting than in first line chemotherapy when the exposure time is sufficiently long to induce the gene expression of the transporters known to be inducible by exposure to that chemotherapeutic agent [25,26].

But, some ABC transporters may also play significant role in chemoresistance in early breast cancer. Recently, it was reported that ABCC1 expression predict shorter relapse free survival and overall survival and play important role in resistance to chemotherapy in early breast cancer who underwent CMF (cyclophosphamide, methotrexate, and fluorouracil) adjuvant chemotherapy [27].

A variety of compounds are transported by ABC transporters through the lipid bilayer and still little has been known about the function of individual transporters in transport of chemotherapeutic agents. ABCA1 has been implicated in the control of the extrusion of membrane phospholipids and cholesterol toward specific extracellular acceptors [28] and macrophage interleukin-1 beta secretion and apoptosis [29]. ABCC13, highly expressed in the RD group mapped to chromosome 21q11.2 has been suggested that it might be associated with hematopoiesis. It has also been reported that ABCC13 shows decreased expression during cell differentiates [30]. ABCC11, called MRP8 is known to be a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2′,3′-dideoxycytidine and 9′-(2′-phosphonylmethoxyethyl) adenine [31]. Szakacs et al. [10] suggested ABCC11 mediated resistance may not be confined to nucleoside analog, demonstrating that the ABCC11 transfected cell confers resistance to NSC 671136 by 2–3 fold. ABCB6 is a mitochondrial half transporter that is known to be involved in the transport of a precursor of the Fe/S cluster from mitochondria to the cytosol [32]. A recent report showed that several ABC transporters including ABCB6 amplified drug resistance in a non small cell lung cancer cell line (A549/CPT) in comparison with its parental cell [33].

Although the role in chemoresistant of individual transporters selected in our study to discriminate between the pCR and RD groups remains to be revealed, the transporters may also play roles in response to chemotherapy by influencing absorption, distribution, and excretion of chemotherapeutic agents.

To evaluate the predictive signature of ABC transporters, we examined multigene predictor model of response to neoadjuvant chemotherapy using differentially expressed ABC transporters. Six different multivariate classification models were examined. When the ABC transporters differentially expressed between the two classes at a significance threshold level of 0.003 were used for class prediction, an average 92.8% of predictive accuracy was observed, with a 93.2% positive predictive value for the pCR group, 93.6% negative predictive value, sensitivity for the pCR group of 88.1%, and 95.9% specificity. The classifier *p*-value, the probability that a similar low error rate could happen by chance, was also low ($p = 0.012$). The optimum classifier model included ABCC5, ABCA1, and ABCA12. These genes all showed high expression in tumors in the RD group.

Of interest, although we developed the class prediction model from a small subset of genes, i.e., genes belonging only to the ABC transporter family, the predictive accuracy reached above 90% with quite a low classifier *p*-value although these prediction models based on ABC transporter genes need to be validated in future studies by comparing the classification model with all subsets of genes and with larger numbers of samples.

Our result suggest that several ABC transporters in human breast cancer cells may contribute to the clinical response to neoadjuvant chemotherapy and gene expression profiling of these ABC transporters may be useful in prediction of the pathologic response to sequential weekly paclitaxel/FEC in breast cancer patients.

Acknowledgments

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Address for offprints and correspondence: Kazuto Nishio, Shien Lab, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, 104-0045, Tokyo, Japan; *Tel.:* +81-3-3542-6143; *Fax:* +81-3-3547-5185; *E-mail:* knishio@gan2.res.ncc.go.jp

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

Ikuo Sekine, MD, John D. Minna, MD,† Kazuto Nishio, MD,‡ Tomohide Tamura, MD,* and Nagahiro Saijo, MD**

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.

*Divisions of Thoracic Oncology and Internal Medicine, National Cancer Center Hospital, Tokyo, Japan; †Hamon Center for Therapeutic Oncology Research, The University of Texas Southwestern Medical Center at Dallas, Dallas, TX; ‡Pharmacology Division, National Cancer Center Research Institute, Tokyo, Japan.
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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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ORIGINAL ARTICLE

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

R Yamanaka[1], T Arao[2], N Yajima[1], N Tsuchiya[1], J Homma[1], R Tanaka[1], M Sano[1], A Oide[3], M Sekijima[3] and K Nishio[2]

[1]*Department of Neurosurgery, Brain Research Institute, Niigata University, Niigata City, Japan;* [2]*Pharmacology Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan and* [3]*Mitsubishi Chemical Safety Institute, Ibaraki, Japan*

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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Correspondence: Dr R Yamanaka, Department of Neurosurgery, Brain Research Institute, Niigata University, Asahimachi-dori 1-757, Niigata City 951-8585, Japan.
E-mail: ryaman@bri.niigata-u.ac.jp
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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).

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 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.

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
M1 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

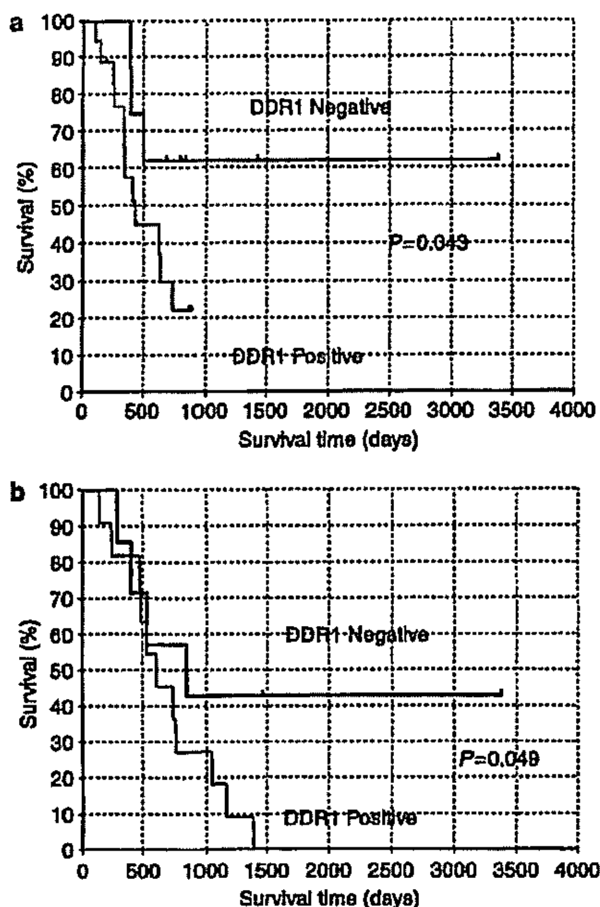

Figure 1 DDR1 protein expressions and patient survivals. Kaplan–Meier survival curves for patients, stratified according to levels of DDR1 expressions in tumors (low DDR1 staining: 0–1 score; high DDR1 staining: 2–3 score; log-rank test). (**a**) A significant trend for worse outcome was observed in the DDR1-positive group ($P = 0.043$). (**b**) DDR1 protein expressions and patient survivals in independent groups of gliomas. Kaplan–Meier survival curves for patients, stratified according to levels of DDR1 expressions in tumors (low DDR1 staining: 0–1 score; high DDR1 staining: 2–3 score; log-rank test). A significant trend for worse outcome was observed in the DDR1-positive group ($P = 0.049$).

groups: positive and negative groups according to immunostaining score. Positive staining for DDR1 was confirmed to be associated with unfavorable overall survival time ($P = 0.043$; Figure 1a). Next, in new independent 19 glioma samples, similar results were obtained ($P = 0.049$; Figure 1b). Although our results were based on relatively small sample size, the correlation between DDR expression and survival was confirmed by real-time quantitative PCR and also confirmed immunohistochemical analysis in independent samples.

Glioma cell proliferation and invasion are inhibited by DDR1 siRNA

DDR1 overexpression was linked to aggressiveness of glioma in our analysis. In order to determine whether downregulation of endogenous DDR1 suppresses proliferation and invasive behavior of gliomas, we synthesized short interfering RNA (siRNAs) against DDR1 mRNA to reduce expression of DDR1 protein. We analysed efficacy of siRNA-mediated inhibition of DDR1 mRNA synthesis in U251, GI-1, and T98G cells by real-time PCR. As shown in Figure 2a, when U251 cells were transfected with siRNAs against DDR-1 (DDR1-#1 and DDR1-#2), DDR1 mRNA was downregulated 48 h later ($P < 0.01$), whereas transfection with a related control siRNA failed to modify DDR1 mRNA expression. When GI-1 and T98G cells were transfected with siRNAs against DDR-1 (DDR1-#1 and DDR1-#2), DDR1 mRNA was downregulated by 10–15% of control siRNA ($P < 0.01$).

After transfection with siRNAs against DDR-1, U251 cell counts within 48 h were approximately 40–60% of untreated or control-siRNA-treated cells during this same period of time ($P < 0.01$; Figure 2b). GI-1 and T98G cell counts within 48 h were approximately 35–50% of untreated or control-siRNA-treated cells during this same period of time ($P < 0.01$). Cell proliferation was significantly suppressed by siRNA against DDR1, as reflected in reduction of mRNA expression.

For invasion assays, transfectants were seeded onto Matrigel-coated invasion chambers, incubated for 24 h and total numbers of cells on the underside of each filter were determined. As shown in Figure 2c, transfections of U251 cells with anti-DDR1 siRNA inhibited cell invasion through the Matrigel by more than 80%, whereas the use of control siRNA had no effect ($P < 0.01$). Transfections of GI-1 and T98G cells with anti-DDR1 siRNA inhibited cell invasion through the Matrigel by more than 70–80%, whereas the use of control siRNA had no effect ($P < 0.01$). Therefore, invasion by cells was significantly suppressed by siRNA against DDR1, as reflected by reduced mRNA expression.

Discussion

Several works (Sallinen *et al.*, 2000; Khan *et al.*, 2001; Ramaswamy *et al.*, 2001; Rickman *et al.*, 2001; Agrawal *et al.*, 2002; Kim *et al.*, 2002; Veer *et al.*, 2002; Vijver *et al.*, 2002; Boom *et al.*, 2003; Godard *et al.*, 2003; Hunter *et al.*, 2003; Mischel *et al.*, 2003; Nutt *et al.*, 2003; Shai *et al.*, 2003; Sorlie *et al.*, 2003; Freije *et al.*, 2004; Mischel *et al.*, 2004; Hoelzinger *et al.*, 2005; Liang *et al.*, 2005; Nigro *et al.*, 2005; Rich *et al.*, 2005; Somasundaram *et al*, 2005; Wong *et al.*, 2005) showed the usefulness of utilizing methods of analysis of multiple forms of data including both clinical and multiple genes, to achieve a more precise discrimination of outcomes for individual patients. The same logical use of multiple forms of data and methods of analysis has been applied in the present study to accurately achieve better classification and prediction of glioma patients. In the present study, we used expression arrays to identify genes that reflect patient's survival. The groups of patients used represented the two extremes of

glioma with respect to outcomes. Nutt *et al.* (2003) and Freije *et al.* (2004) reported the use of microarrays to predict outcomes for glioma patient. Nutt *et al.* involved a group of 50 glioma patients who were not selected based on survival duration. The investigators used Affymetrix U 95 GeneChips to develop a model to classify cases into unfavorable and favorable groups that exhibited significantly different survivals. They picked up 20 genes different from our study that highly correlated with class distinction. On the other hand, Freije *et al.* (2004) also reported the use of microarrays to predict outcomes for all histological types of 85 gliomas. The investigators used Affymetrix HG 133 GeneChips to develop a 44-gene model to classify cases into unfavorable and favorable groups that exhibited significantly different survivals. From these two studies, there were no attempt to predict survivals of individual patients, but results were consistent with ours, and together suggested that clinical differences in outcomes were reflected in global patterns of gene expression that could be appreciated using microarrays.

Some of the genes that were critical components of patterns that were used to discriminate between long-term and short-term survivors are known to affect virulence of the malignant phenotype. Several groups have confirmed prognostic markers of glioma such as Insulin-like growth factor-binding protein 2 (IGFBP2) (Kim *et al.*, 2002; Godard *et al.*, 2003), vascular endothelial growrh factor (VEGF) (Godard *et al.*, 2003), Osteonectin, Doublecortex, Semaphorin 3B (Rich *et al.*, 2005) and brain-type fatty acid-binding protein (FABP7) (Liang *et al.*, 2005).

We have selected *DDR1*, *KSP37* and *DYRK3* from a 21-gene model (21 genes derived from multivariate analysis) to classify cases into unfavorable and favorable groups that exhibited significantly different survivals. We observed that glioma cell proliferation and invasion were significantly suppressed by siRNA against DDR1. The DDR1 is a tyrosine receptor kinase activated by various types of collagen, and is involved in cell–matrix communication (Vogel, 1999). DDR1 is activated independently of $\beta 1$ integrin (Vogel *et al.*, 2000). DDR1–collagen interaction facilitates the adhesion, migration, differentiation/maturation and cytokine/chemokine production of leukocytes (Yoshimura *et al.*, 2005). DDR1 is overexpressed in several tumors including high-grade brain, esophageal and breast cancers (Weiner and Zagzag, 2000). Based on our data and Ram *et al.* (2005), DDR1 may play a potential role in proliferation and invasion of gliomas. Invasive phenotype is caused by activation of matrix metalloproteinase-2 in DDR1-overexpressing cells (Ram *et al.*, 2006). Glioma cell adhesion, including intercellular and

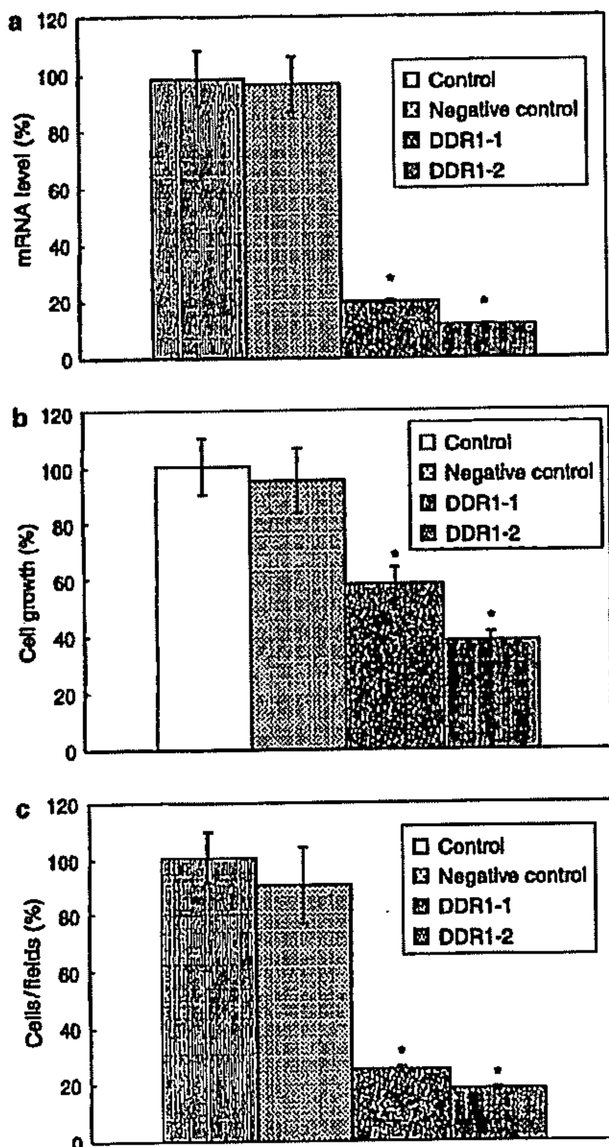

Figure 2 Effects of DDR1 knockdown by RNA interference on proliferation and invasiveness of human glioma cell lines. U251 cells were transiently transfected with short interfering RNAs (siRNA) and subjected to semiquantitative PCR analysis, proliferation assay or Matrigel invasion assays. (**a**) Reduction of DDR1 mRNA expression by siRNAs against DDR1 was determined by semiquantitative PCR analysis. Transfection with DDR1 siRNAs significantly reduced DDR1, whereas transfection with siRNAs targeted to an unrelated mRNA had no effect on DDR1 expression. *$P<0.01$ compared with both control groups. (**b**) Cell proliferation assay. Cells were cultured in 96-well plates in 100 μl of serum-enriched medium. When 80% confluence was reached, 25 μl of 100 nM siRNA in cytofectin was added drop wise. Numbers of viable cells were evaluated after 48 h culture by incubation with Tetra color one, and numbers obtained were compared with those of controls. After transfection with DDR1 siRNAs, U251 cell counts within 48 h were approximately 40–60% of untreated or control-siRNA-treated cells during this same period of time. *$P<0.01$ compared with both control groups. (**c**) For the invasion assays, transfectants were seeded onto Matrigel-coated invasion chambers and incubated for 24 h. Total numbers of cells on the underside of each filter were determined. Invading cells were significantly suppressed by siRNAs against DDR1, as reflected by reduction of mRNA expression. Control, no siRNA treatment; negative control, control siRNA treated. DDR1-#1, DDR1-#2; DDR1 siRNA treated. **$P<0.01$ compared with both control groups.

cell–matrix adhesions, is critical to the maintenance of structural integrity, polarity and cell–cell communication, and their expression is frequently observed in tumor cells concordant with a breakdown of cellular organization, causing an uncontrolled leakage of nutrients and other factors necessary for the survival and growth of tumor cells, and loss of cell–cell contact inhibition leading to increased cell motility. Thus, DDR1 may be a novel molecular target for therapy, and provide an important predictive marker for survival in patients with glioma. KSP37 protein is constitutively secreted by Th1-type CD4-positive lymphocytes and lymphocytes with cytotoxic potential, and may be involved in an essential process of cytotoxic lymphocyte-mediated immunity (Ogawa *et al.*, 2001). Down-regulation of KSP37 protein may correlate with poor prognosis of glioma patients with immunosuppressive state. DYRK3 is a member of dual-specificity tyrosine-regulated kinases with roles in cell growth and development. DYRK3 was reported to be expressed in erythroid progenitor cells, and to play roles in kinase activation (Li *et al.*, 2002). Although KSP37 and DYRK3 are unique molecules, their roles in glioma progression are unclear, and should be further investigated in the future.

Regardless of their roles in tumorigenesis, all these markers offer potential clinical applications for the treatment and detection of malignant gliomas. To our knowledge, this study is the first to address these molecules as molecular targets for therapeutics. Values of gene-expression-based predictors for prognosis of malignant glioma patients will not be fully realized until additional therapies are available for patients destined to have poor survival, following conventional chemotherapy. In this regard, expression profiles may not only predict the likelihood of long-term survival following nitrosourea chemotherapy, but may also yield clues on individual genes involved in tumor development, progression and response to therapy. It is likely that some of the most differentially expressed genes such as those discussed above will represent therapeutic molecular targets. Moreover, the ability to histologically distinguish ambiguous gliomas will enable appropriate therapies to be tailored to specific tumor subtypes. Class prediction models based on defined molecular profiles allow classification of malignant gliomas in a manner that will better correlate with clinical outcomes than with standard pathology.

Materials and methods

Patients

Mean age of patients was 53.2 years old (range, 18–80). Twenty-two patients were men and seven were women. Tissues were snap-frozen in liquid nitrogen within 5 min of harvesting, and stored thereafter at −80°C. Clinical stage was estimated from accompanying surgical pathology and clinical reports. Samples were specifically re-reviewed by a board-certified pathologist in our institution, using observation of sections of paraffin-embedded tissues that were adjacent or in close proximity to the frozen sample from which the RNA was extracted. Histopathology of each collected specimen was reviewed to confirm adequacy of the sample (i.e., minimal contamination with non-neoplastic elements), and to assess the extent of tumoral necrosis and cellularity. Histological characteristics of tumor samples and clinical disease stage were included as supplements in Table 1.

After surgical resection of tumor, patients had a course of external beam radiation therapy (standard dose of 40 Gy to the tumor with a 3-cm margin, and 20 Gy boost to the whole brain) and nitrosourea-based chemotherapy. Patients were monitored for recurrences of tumor during the initial and maintenance therapy by magnetic resonance imaging or computed tomography. Treatments were carried out at the Department of Neurosurgery, Niigata University Hospital. Informed consent was obtained from all patients for the use of samples in accordance with the guidelines of the Ethical Committee on Human Research, Niigata University Medical School. Overall survival was measured from the date of diagnosis. Survival end points corresponded to dates of death or last follow-up.

RNA extraction

Total RNA was extracted with 1 ml Isogen (Nippongene, Toyama, Japan) per 100 mg frozen glioma tissues, following the manufacturer's instructions. Each tissue type was homogenized with a Polytron (Fisher Scientific) for 30 s and cleared by a 10-min centrifugation at 10 000 *g*. For each ml Isogen, 0.2 ml chloroform was added and samples were vigorously shaken for 20 s and then incubated on ice for 10 min. The aqueous phase was separated by centrifugation at 10 000 *g* for 10 min, decanted and an equal volume of isopropanol was added. The mixture was allowed to precipitate for 10 min and the precipitate was collected by centrifugation at 12 000 *g* for 10 min. The pellet was washed with 70% ethanol, collected by brief centrifugation, air dried and re-suspended in H_2O. RNA was further purified using an RNeasy column (Qiagen, Valencia, CA, USA). The purified RNA was quantified using a UV spectrophotometer, and RNA quality was evaluated by capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with 28S/18S ratios >0.7 and with no evidence of ribosomal peak degradation were included in the study.

Agilent cDNA microarrays

Agilent human 1 cDNA microarrays (Agilent Technologies) contained 13 156 clones from Incyte's human cDNA library. Test and normal brain RNAs were labeled with both Cy3-dCTP and Cy5-dCTP nucleotides (Amersham Biosciences, Tokyo, Japan) and hybridized on two slides (dye-swap hybridizations) according to the direct-labeling method provided by the manufacturer. Following hybridization, slides were scanned and analysed using the Feature Extraction software (version A.4.0.45, Agilent Technologies), as recommended by the manufacturer. Spots that did not pass quality control procedures in the Feature Extraction software were flagged and removed from further analysis. Clones with the same GenBank accession number were averaged.

Expression profiling on Agilent cDNA microarrays

Total RNA (20 μg) was reverse transcribed using the Agilent direct-label cDNA synthesis kit (Agilent Technologies), following the manufacturer's directions. Labeled cDNA was purified using QIAquick PCR Purification columns (Qiagen, Valencia, CA, USA), followed by concentration by vacuum centrifugation. cDNA was suspended in hybridization buffer and hybridized to Agilent human 1 cDNA microarrays (Agilent Technologies) for 17 h at 65°C, according to the

Agilent protocol. To avoid generation of false between-group differences by randomly pairing glioma samples on the two-channel cDNA arrays, each sample was individually labeled and co-hybridized with a normal brain sample labeled with a complementary dye. Normal brain samples were generated by pooling equal amounts of RNA from each control sample and labeling as for individual samples. In addition, Cy dye switch hybridizations were performed for each sample. Normal brain samples were purchased from Clontech (Tokyo, Japan). All microarray data and clinical features have been submitted to Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo; accession no. GSE4381).

Statistical analysis
Univariate analysis for clinical features was performed by log-rank test using SAS software ver. 9.1.3 (SAS Institute Inc., Cary, NC, USA). In microarray analysis, normalization and survival analysis were performed using the BRB Array Tools software ver. 3.3.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) developed by Dr Richard Simon and Amy Peng. In brief, a log base 2 transformation was applied to the microarray raw data, and global normalization was used to median the center of log ratios on each array in order to adjust for differences in labeling intensities of the Cy3 and Cy5 dyes. Genes showing minimal variation across the set of arrays were excluded from the analysis. Genes whose expression differed by at least 1.5-fold from the median in at least 20% of the arrays were retained. Genes were also excluded if percent of data missing or filtered out exceeds 50%. Then, genes that passed filtering criteria were considered for further analysis.

We computed a statistical significance level for each gene based on univariate proportional hazards models ($P<0.005$) and identified genes whose expression was significantly related to survival of the patient. These P-values were then used in a multivariate permutation test in which survival times and censoring indicators were randomly permuted among arrays. To adjust the expression of six candidate genes (DDR1, DYRK3, KSP37, ITGA5, SLN and ALCAM) for clinical features (WHO grade, age, gender, PS), clinical data and normalized microarray expression data of six genes were imported into SAS software ver. 9.1.3 (SAS Institute Inc.) and Cox regression model was performed for multivariate analysis against each variable (WHO grade, age, gender, PS, expression levels of six genes). Three samples were excluded for multivariate analysis because there were a few defected expression data. A P-value <0.05 was considered significant. The differences between subgroups of DDR1 siRNA and control groups were tested for statistical significance using the analysis of variance test and statistical significance was determined at the $P<0.01$ level.

Validation of differential expression by real-time quantitative PCR
Total RNA (2 μg) was subjected to DNase treatment in a 10 μl reaction containing 1 μl 10 × DNase I reaction buffer (Invitrogen, Tokyo, Japan) and 1 μg DNase I at room temperature for 10 min. Ethylenediamine tetraacetic acid (1 μl, 25 mM) and 1 μl oligo dT (0.5 μg/μl; Invitrogen) were added to the DNase reaction, and heated to 70°C for 15 min to inactivate DNase I activity and eliminate RNA secondary structure. Samples were placed on ice for 2 min and collected by brief centrifugation. RNA was then reverse-transcribed into cDNA by adding 8 μl master mix containing 4 μl of 5 × first strand buffer, 2 μl dithiothreitol (0.1 M), 1 μl dNTPs (10 mM each) and 1 μl SuperScript II (200 U/μl) (Invitrogen), followed by incubation at 42°C for 45 min. The reaction was diluted 10-fold with dH_2O and stored at 4°C.

Each sample was subjected to 40 cycles of real-time PCR with a LightCycler (Idaho Technology, Salt Lake City, UT, USA). PCR reagents contained 1 × LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany), 0.5 μM of each primer, 3 mM $MgCl_2$ and 2 μl cDNA template. PCR conditions were as follows: one cycle of denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 5 s and 72°C for 10 s. A melting curve was obtained at the end of amplification cycles to verify specificity of the PCR products. Points at which signal fluorescence exceeded background, for each sample and for each gene, were compared to a standard curve generated by four, 10-fold serial dilutions of concentrated cDNA control of each sample subjected to real-time analysis to determine an expression value. All determinations were performed in duplicate. A Student's t-test was conducted to analyse expression values for long- and short-term survivors to determine statistical significance. For amplification of target genes, the following primers were used (Takara, Yotsukaichi, Japan):

ALCAM-FW:	5'-CCAGATGGCAATATCACATGGTACA-3',
ALCAM-RW:	5'TCCAGGGTGGAAGTCATGGTATAGA-3',
DDR1–FW:	5'ACTTTGGCATGAGCCGGAAC-3',
DDR1–RW:	5'ACGTCACTCGCAGTCGTGAAC-3',
DYRK3-FW:	5'AGCTGCCTCCAGTTGTTGGGAATAG-3',
DYRK3-RW:	5'TGCATCTCTGGGCATATCTCTGTC-3',
ITGA5-FW:	5'TCCCAGTAAGCGACTGGCATC-3',
ITGA5-RW:	5'GTTCCAGCACACCCTGGCTAA-3',
ITGB2-FW:	5'ATCGTGCTGATCGGCATTCTC-3',
ITGB2-RW:	5'GGTTCATGACCGTCGTGGTG-3',
KSP37-FW:	5'CTTCCGAGGGTGACAGGTGA-3',
KSP37-RW:	5'TCCAGTGTGAGAACGTTGGATTG-3',
LDHC-FW:	5'TCATCTGTACTGATTGCGCCAAG-3',
LDHC-RW:	5'ACGGCACCAGTTCCAACAATAGTAA-3',
LOC340371-FW:	5'GGAACATGCCAGGGCTTCA-3',
LOC340371-RW:	5'CTGCTCAACACGGTCTGGA-3',
SLN-FW:	5'GGAGTTGGAGCTCAAGTTGGAGAC-3',
SLN-RW:	5'GAACTGCAGGCAGATTTCTGAGG-3',
SLC2A3-FW:	5'GCCTTTGGCACTCTCAACCAG-3',
SLC2A3-RW:	5'GCTGCACTTTGTAGGATAGCAGGAA-3'.

Immunohistochemistry
Sections (5 μm) from formalin-fixed, paraffin-embedded tissue specimens were deparaffinized in xylene and dehydrated in a graded series of ethanol, followed by a phosphate-buffered saline (PBS) wash. Antigen retrieval was carried out by incubation at 121°C for 10 min in 10 mM sodium citrate (pH 6.0), followed by incubation with 0.3% H_2O_2 to quench endogenous peroxidase activity. Slides were blocked in 10% normal serum and incubated with rabbit polyclonal anti-DDR1 antibody (dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 16 h at 4°C. After washing, the slides were incubated with an avidin–biotin–peroxidase system (Vectastain elite ABC kit, Vector Labs, Burlingame, CA,

USA). Finally, sections were exposed for 10–20 min to 0.01% 3,3-diaminobenzidine (Sigma, Tokyo, Japan) and PBS containing 0.01% hydrogen peroxide. Immunohistochemistry scoring was performed as follows. Staining intensity was classified as none (0 point), weak (1 point), moderate (2 point) or strong (3 point). Intensity of signal of stained areas was estimated by light microscopy, based on 25 percentiles in a representative field. Scores were calculated as weighted averages (sum of points × area%). Averages of three independent measurements were calculated to the first decimal place and used for statistical analysis. Observers were not aware of case numbers.

siRNA treatment and cell proliferation assay

Specific siRNA oligonucleotides directed against human DDR1 were purchased from Invitrogen. The Validated Stealth sequence information is DDR1-#1: 5′-GCUAUGUGGAGAU GGAGUUUGAGUU-3′ and DDR1-#2: 5′-GGCCCUGG UUACUCUUCAGCGAAAU-3′. siRNAs were introduced into glioma cell lines by cytofectin-mediated transfection according to the manufacturer's instructions (Qiagen, Tokyo, Japan). Cells were cultured in 96-well plates in 100 μl of serum-enriched medium. When 80% confluence was reached, 25 μl 100 nM siRNA in cytofectin was added drop wise to the cell culture. Numbers of viable cells were evaluated 48 h after culture, by incubating with Tetra color one (Seikagaku CO., Tokyo, Japan), and numbers obtained were compared with those of controls. Control experiments were performed using Cy3-labeled siRNA (Qiagen) directed against an unrelated mRNA (Luciferase; $siRNA_{LUC}$: Qiagen). Transfection efficiency was confirmed with Cy3-labeled $siRNA_{LUC}$ in each assay. All proliferation experiments were repeated as independent experiments at least twice. Results were reported as means ± s.d. of two independent experiments.

Cell invasion of Matrigel

A Transwell containing an 8-μm diameter pore membrane (Becton-Dickinson, Tokyo, Japan) was coated with 500 μl Matrigel (Becton-Dickinson) at 100 μg/ml. Cells were either left untreated, treated with control or DDR1-#1, #2 siRNAs and transfected as described above. After 24-h incubation, cells were detached with cell dissociation solution (Sigma), washed twice with PBS and resuspended in minimum essential medium (MEM) (Nissui Pharmaceutical Inc., Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Gibco, Tokyo, Japan). When siRNAs were used, a second transfection 24 h after the first was performed. In all cases, 2×10^3 cells were seeded into the upper, Matrigel-coated chamber of the Transwell. The lower chamber was filled with MEM supplemented with 10% FBS. After 24-h incubation at 37°C, the non-migrating cells in the upper chamber were gently detached by scraping and adherent cells present on the lower surface of each insert were stained with Giemsa. Ten fields were counted by light microscopy at ×200 magnification. Results were calculated with reference to control values observed after incubation of untreated control, for control and DDR1 siRNA.

Cell lines and culture

All glioma cell lines were cultured in MEM supplemented with 10% FBS. The T98G, GI-1 and U251 cell lines were purchased from Cell Bank, RIKEN BioResource Center (Tsukuba, Japan).

Abbreviations

ALCAM, activated leukocyte cell adhesion molecule; cDNA, complementary DNA; Cy, cyanine; DDR1, discoidin domain receptor family, member 1; DYRK3, dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 3; FBS, fetal bovine serum; ITGA5, integrin alpha 5; ITGB2, integrin beta 2; KSP37, Ksp37 protein; LDHC, lactate dehydrogenase C; LOC340371, hypothetical protein LOC340371; MEM, minimum essential medium; PBS, phosphate-buffered saline; SLC2A3, solute carrier family 2 member 3; SLN, sarcolipin; s.d., standard deviation; siRNA, short interfering RNA.

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