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Identification of differentially expressed genes according to chemosensitivity in advanced ovarian serous adenocarcinomas: expression of *GRIA2* predicts better survival

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BACKGROUND: The purpose of this study was to identify genes that are differentially expressed in chemosensitive serous papillary ovarian carcinomas relative to those expressed in chemoresistant tumours.

METHODS: To identify novel candidate biomarkers, differences in gene expression were analysed in 26 stage IIIC/IV serous ovarian adenocarcinomas (12 chemosensitive tumours and 14 chemoresistant tumours). We subsequently investigated the immunohistochemical expression of GRIA2 in 48 independent sets of advanced ovarian serous carcinomas.

RESULTS: Microarray analysis revealed a total of 57 genes that were differentially expressed in chemoresistant and chemosensitive tumours. Of the 57 genes, 39 genes were upregulated and 18 genes were downregulated in chemosensitive tumours. Five differentially expressed genes (*CD36*, *LIFR*, *CHL1*, *GRIA2*, and *FCGBP*) were validated by quantitative real-time PCR. The expression of *GRIA2* was validated at the protein level by immunohistochemistry, and patients with GRIA2 expression showed a longer progression-free and overall survival ($P = 0.051$ and $P = 0.031$ respectively).

CONCLUSIONS: We found 57 differentially expressed genes to distinguish between chemosensitive and chemoresistant tumours. We also demonstrated that the expression of GRIA2 among the differentially expressed genes provides better prognosis of patients with advanced serous papillary ovarian adenocarcinoma.

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Ovarian carcinoma remains the most lethal gynaecological tumour, ranking fifth in incidence of cancer deaths in women, and where >70% of those affected are diagnosed with advanced disease (Runnebaum and Stickeler, 2001). Although many patients initially respond to combinations of cytoreductive surgery and platinum/taxane chemotherapy, most patients experience subsequent recurrences (International Collaborative Ovarian Neoplasm Group, 2002; du Bois *et al*, 2003).

It is difficult to predict the clinical outcome of patients with advanced ovarian cancer, and as such, the prognostic factors available today need to be improved. In ovarian cancer, gene expression profiles have so far been used to identify gene expression signatures that correlate with clinical outcome, to determine which genes affect survival and relapse, and to generate biomarkers that could predict patient response to chemotherapy (Collins *et al*, 2004; Lancaster *et al*, 2004; Spentzos *et al*, 2004; Berchuck *et al*, 2005; Hartmann *et al*, 2005). However, none of the genes have been validated adequately or proven to provide superior predictive value in prospective studies (Dupuy and Simon, 2007). Interestingly, there is typically very little overlap between the gene lists reported by various groups. These discrepancies might be related to the use of different microarray platforms with different normalisation methods and differences in the degree of contamination by non-neoplastic cells in specimens, or heterogeneity in the patient population undergoing study (Konstantinopoulos *et al*, 2008).

Although several gene expression profiling studies were performed in homogenous groups of patients (Berchuck *et al*, 2005; Bachvarov *et al*, 2006; Ouellet *et al*, 2006; Trinh *et al*, 2011), many other studies compared tumour groups with different histological subtypes and stages. As some differences in expression could depend on histological tumour type and stage, it is important to study homogeneous tumour groups in survival analyses. Recently, there are publications using microdissected ovarian cancer tissue and a homogenous tumour group to identify prognostic markers for ovarian cancer (Mok *et al*, 2009; Cancer Genome Atlas Research Network, 2011).

In this study, we investigated gene expression differences in a homogeneous group of patients with stage IIIC/IV serous ovarian adenocarcinomas. The gene expression of the 12 tumours from

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chemosensitive patients were compared with 14 tumours from chemoresistant patients. This comparison showed that 57 genes were differentially expressed and the genes were compared in the TCGA dataset. We subsequently investigated the immunohistochemical expression of GRIA2 as a candidate prognostic marker in 48 independent sets of advanced ovarian serous papillary adenocarcinoma specimens.

METHODS

Ovarian cancer samples

Twenty-six snap-frozen stage IIIC/IV serous papillary adenocarcinomas of the ovary were collected prospectively from patients diagnosed between 2003 and 2007 at the Samsung Medical Center with IRB approval. Each tumour specimen was $\sim 1\,cm^3$ in size and were maintained a temperature of less than $-80^{\circ}C$. Each sample was analysed histologically by a staff pathologist, and only tumour samples containing at least 70% tumour epithelial cells were included. The clinicopathological features are summarised in Table 1. All of the patients were treated with maximal debulking surgery, which was followed by a combination of paclitaxel/carboplatin. Cases were staged according to the 1988 FIGO staging system. Debulking status was defined according to the size of the nodules left after surgery (<1 cm, optimal; $\geqslant 1$ cm, suboptimal). Fourteen patients had recurrences within 6 months following treatment and were considered chemoresistant. The remaining 12 patients had no recurrences or recurrences beyond 24 months. The median follow-up time was 41 months (range, 11–86 months). For the present study, FIGO stages I–IIIB tumours or patients with follow-up time <24 months were excluded.

For validation of PCR, another 40 snap-frozen stage III–IV serous adenocarcinomas were selected. Patients were divided into two groups according to the sensitivity for taxane/platinum combination chemotherapy: platinum resistant was defined as platinum-free interval <6 months; platinum-sensitive, and platinum-free interval $\geqslant 6$ months. The clinical features are summarised in Supplementary Table 1.

Forty-eight paraffin-embedded serous ovarian adenocarcinoma specimens were selected for immunohistochemical staining, and grade 1 or stage I tumours were excluded in the cohort.

RNA isolation and gene expression profiling

In the present study, we performed global gene expression analyses using AffymetrixGeneChip Human Gene 1.0 ST oligonucleotide arrays (Affymetrix, http://www.affymetrix.com). The sample preparation was performed according to the instructions and recommendations provided by the manufacturer. Total RNA was isolated using RNeasy Mini Kit columns as described by the manufacturer (Qiagen, Hilden, Germany). The quality of RNA was assessed by an Agilent 2100 bioanalyser using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands), and the quantity was determined by a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) (Supplementary Figure 1). For each RNA sample, 300 ng was used as input into the Affymetrix procedure, as recommended by protocol. Briefly, 300 ng of total RNA from each sample was converted to double-strand cDNA. Using a random hexamer incorporating a T7 promoter, amplified RNA (cRNA) was generated from the double-stranded cDNA template though an *in vitro* transcription reaction and purified with the Affymetrix sample cleanup module. cDNA was regenerated through a random-primed reverse transcription using a dNTP mix containing dUTP. The cDNA was then fragmented by UDG and APE 1 restriction endonucleases and end-labelled by a terminal transferase reaction incorporating a biotinylated dideoxynucleotide. Fragmented end-labelled cDNA was hybridised to the GeneChip Human Gene 1.0 ST arrays for 16 h at 45°C and 60 r.p.m., as described in the Gene Chip Whole Transcript Sense Target Labelling Assay Manual (Affymetrix). With ~ 4 probes per exon and roughly 26 probes per gene, the GeneChip Human Gene 1.0 ST arrays enables two complementary levels of analysis-gene expression and alternative splicing. After hybridisation, the chips were stained and washed in a Genechip Fluidics Station 450 and scanned using a Genechip Array scanner 3000 7G. The assays were performed blinded to the study end point.

The expression intensity data were extracted from the scanned images using Affymetrix Command Console Software (version 1.1) and stored as .cel files. To remove bias between the samples, the intensity values were normalised using the robust multi-average (RMA) algorithm implemented in the Affymetrix Expression Console Software (version 1.1). To determine whether or not the genes were differentially expressed between the two groups, we performed an unpaired Student's *t*-test on the RMA expression values, and genes with *P*-values <0.05 were extracted (Irizarry *et al*, 2003). Highly expressed genes that showed greater than a two-fold difference between the average signal values of the control and test groups were selected for further study. To classify the coexpression gene groups which have similar expression patterns, hierarchical clustering analysis was performed with the Multi Experiment Viewer software (version 4.4; http://www.tm4.org) (Saeed *et al*, 2003, 2006). The web-based tool Database for Annotation, Visualisation, and Integrated Discovery was used to perform the biological interpretation of the differentially expressed genes (Huang da *et al*, 2009a, b). The genes were classified based on the information of gene function in Gene ontology and KEGG pathway databases (http://david.abcc.ncifcrf.gov/home.jsp).

Table 1 Clinical and pathological characteristics of the patients with serous ovarian carcinoma

	Chemoresistant	Chemosensitive	*P*-value
No.	14	12	
Age, years			0.70
Median (range)	50 (33–68)	49 (40–75)	
Histological grade			0.26
1,2	4 (28.6%)	2 (16.7%)	
3	10 (71.4%)	10 (83.3%)	
Stage			0.24
IIIC	12 (85.7%)	8 (66.7%)	
IV	2 (14.3%)	4 (33.3%)	
Optimal debulking	5 (35.7%)	7 (58.3%)	0.43
Percentage of tumour cells			
Median (range)	83% (70–90)	85% (70–95)	0.52

Validation of gene expression by quantitative real-time PCR

To validate the results of the oligonucleotide microarray analysis, five genes (*CD36*, *LIFR*, *CHL1*, *GRIA2*, and *FCGBP*) that were shown to be differentially expressed were analysed with a real-time (RT)–PCR. The cDNA synthesis was performed with a High Capacity cDNA Archive kit (4368813; Applied Biosystems, Foster City, CA, USA) following the protocol supplied. TaqMan PCR was done with an ABI PRISM 7900HT Fast-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR primers and probes for *CD36* (Hs01567186), *LIFR* (Hs01123581), *CHL1* (Hs00544091), *GRIA2* (forward 5′-GTTTTCC TTGGGTGCCTTTATGC-3′, reverse 5′-CCAACAATGCGCCCAGAGA-3′, probe 5′-TTCGCCAAGATCCC-3′), *FCGBP* (Hs01553051), and

GAPDH (4310884E) were purchased from Applied Biosystems (Supplementary Figure 2). The relative expressions of these mRNAs were normalised to the amount of GAPDH in the same cDNA by using the $\Delta\Delta C_t$ method described by the manufacturer (Lee *et al*, 2008).

Immunohistochemistry on formalin-fixed tissues

We also performed immunohistochemical staining for GRIA2 to determine whether or not there was a correlation between the gene expression and clinical outcomes. *GRIA2* was found to be upregulated in chemosensitive tumours. Interestingly, there have been conflicting reports on the role of GRIA2 with regard to cancer biology. It has been reported not only to be upregulated in the neuroendocrine carcinoma cells (Tsibris *et al*, 2003; Leja *et al*, 2009), but also has been reported to be associated with a low degree of malignancy compared with high-grade glioblastoma multiforme (GBM; Beretta *et al*, 2009). In addition, there has been no report on *GRIA2* in ovarian cancer. Thus, we selected the GRIA2 expression as a candidate prognostic marker in this study.

Immunohistochemical staining was performed with the standard peroxidase/DAB method (DakoCytomation, Inc., Carpinteria, CA, USA) on formalin-fixed, paraffin-embedded 4-μm thick tissue sections. To increase specificity and sensitivity, samples were pretreated with target retrieval solution (pH 9, S2367; DakoCytomation) at 97°C for 20 min. GRIA2 expression was detected using primary rabbit polyclonal, mono-specific antibody (ANTI-GRIA2, HPA008441; Atlas Antibodies, Stockholm, Sweden) at a 1 : 20 dilution for 24 h at room temperature in a humidified chamber. Immunohistochemical procedures were performed as described previously (Kim *et al*, 2010). Antigen antibody complexes were detected with the Dako REAL DAB/Chromogen (K5007; DakoCytomation) according to the manufacturer's instructions. Tissue sections were lightly counterstained with haematoxylin, and then examined by light microscopy. To verify antibody specificity, anti-mouse IgG (AI-2000; Vector Laboratories, Burlingame, CA, USA) was used in place of the primary antibody as a negative control, and brain (cerebellum) was used as positive control.

Immunoreactivity of GRIA2 in cancer cells was evaluated according to intensity and area (Michalski *et al*, 2008). The intensity of cancer cells was recorded as 'no staining (0)', 'weak to moderate staining (1)' or 'strong staining (2)'. The area of stained cancer cells was recorded as $<33\%$ (1), 33–66% (2) or $>66\%$ (3) of all cancer cells. These numbers were then multiplied resulting in a score of 0–6. We arbitrarily classified a score >2 as GRIA2 positive. The cutoff points were based on the distribution of the staining results and statistical significance. We judged the expression as either positive or negative according to the membranous staining.

Data analysis

After confirming whether the data were normally distributed using the Shapiro–Wilk test, we used the Wilcoxon rank sum test to compare the PCR data. Fisher's exact probability test or the chi-square test was used to test possible associations between the expression of GRIA2 and various clinicopathological factors. Overall survival was defined as the interval from the date of initial surgical resection to the date of last known contact or death. Progression-free survival was defined as the interval from the date of initial surgical resection to the date of progression, date of recurrence, or date of last known contact if the patient was alive and has not recurred. Kaplan–Meier curves were plotted to assess the effects of GRIA2 expression on survival. These survival curves were compared using the log-rank test. The Cox proportional hazard models were used to assess multiple factors. *P*-values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS (version 15.0; SPSS, Inc., Chicago, IL, USA).

RESULTS

Gene expression analysis and clustering of chemosensitive and chemoresistant tumours

Comprehensive gene expression profiles of 14 chemoresistant and 12 chemosensitive samples were generated with high-density oligonucleotide arrays. After the most 'absent' genes were filtered out, a significance analysis of the microarrays test revealed a total of 57 genes that had a *P*-value <0.05 and at least a two-fold change. Of the 57 genes, 39 genes were found to be upregulated (Table 2), and 18 genes were downregulated (Table 3) in chemosensitive tumours. To further visualise differences in gene expression between the chemosensitive and chemoresistant tumours, a hierarchical clustering of the 57 genes was performed (Figure 1).

Validation of gene expression by quantitative RT–PCR

Five differentially expressed genes (*CD36*, *LIFR*, *CHL1*, *GRIA2*, and *FCGBP*) were selected for verification of the microarray data with quantitative (Q)PCR analysis. The quantitative RT–PCR data were correlated with the microarray data, confirming the reliability of our expression data (Figure 2, Supplementary Figures 3 and 4). In four of five genes, we found a statistically significant difference in the gene expressions between the chemosensitive and the chemoresistant tissues (Figure 2A, Supplementary Figure 1). The differences were also validated in an additional 40 patients cohort (Figure 2B).

Expression of GRIA2 correlates with survival

As shown in Figure 3, a strong expression for GRIA2 protein was found on the membranes of the malignant cells. GRIA2 expression was detected in 6 of 48 samples (12.5%), and was not correlated with age, stage, and residual tumour after surgery (Table 4). And interestingly, GRIA2 expression was correlated with a longer progression-free and overall survival (Figure 4). On the basis of multivariate analysis, GRIA2 expression remains a significant predictor of better progression-free survival ($P=0.026$; Table 5).

Downregulation of GRIA2 increases cell survival of ovarian cancer cells *in vitro*

Next, we evaluated the effect of GRIA2 on the survival of epithelial ovarian cancer cells (Figure 5). We tried to examine whether regulation of expression level of GRIA2 could change cell survivability *in vitro* using siRNA. First, we estimated the protein expression level of GRIA2 in the epithelial ovarian cancer cell lines and selected SKOV3 and HeyA8 cells for *in vitro* test. After checking the effect of GRIA2 siRNA, we estimated GRIA2 siRNA induced modification of cell survival of two cells by MTT assay. Interestingly, GRIA2 siRNA increased 0.1- and 0.13-fold relative cell survival of SKOV3 and HeyA8 cells, respectively (Figure 5D and E). These results might imply the negative association of GRIA2 expression and cell survivability of epithelial ovarian cancer cells.

DISCUSSION

In this study we used oligonucleotide microarrays to identify differences in expression levels in 26 stage IIIC/IV ovarian serous adenocarcinomas (12 chemosensitive tumours *vs* 14 chemoresistant tumours). We detected 57 differentially expressed genes, 5 of which were validated with QPCR analysis. In the present study we focused on *GRIA2* because this gene had low *P*-values and displayed a large fold change. We showed that *GRIA2* was downregulated in chemoresistant tumours, which strengthen

Table 2 Upregulated genes expressed at least two-fold higher in chemosensitive tumours compared with chemoresistant tumours

Gene symbol	Gene name	P-value	FDR	Log2 ratio
IMPG2	Interphotoreceptor matrix proteoglycan 2	0.0008	0.0003	1.96
NTS	Neurotensin	0.0107	0.2488	1.83
AREG	Amphiregulin	0.0120	0.0147	1.65
SEMA3A	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted (semaphorin) 3A	0.0248	0.0053	1.49
CHL1	Cell adhesion molecule with homology to L1CAM (close homologue of L1)	0.0053	0.0312	1.47
GRIA2	Glutamate receptor, ionotropic, AMPA 2	0.0460	0.0200	1.41
MPPED2	Metallophosphoesterase domain containing 2	0.0037	0.0279	1.39
CD36	CD36 molecule (thrombospondin receptor)	0.0312	0.0421	1.37
EDNRB	Endothelin receptor type B	0.0237	0.0399	1.30
LIFR	Leukaemia inhibitory factor receptor alpha	0.0002	0.0033	1.28
AREG	Amphiregulin	0.0248	0.0309	1.24
CT45A6	Cancer/testis antigen family 45, member A6	0.0447	0.2977	1.21
RERG	RAS-like, oestrogen-regulated, growth inhibitor	0.0145	0.0899	1.19
CRISP3	Cysteine-rich secretory protein 3	0.0444	0.2970	1.19
CCL18	Chemokine (C–C motif) ligand 18 (pulmonary and activation-regulated)	0.0429	0.2942	1.17
IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3	0.0195	0.2551	1.17
EIF4A2	Eukaryotic translation initiation factor 4A, isoform 2	0.0264	0.0002	1.16
SULT1C2	Sulfotransferase family, cytosolic, 1C, member 2	0.0234	0.2596	1.15
ANK2	Ankyrin 2, neuronal	0.0155	0.0844	1.15
TKTL1	Transketolase-like 1	0.0136	0.2494	1.14
CT45A4	Cancer/testis antigen family 45, member A4	0.0395	0.2886	1.14
CT45A2	Cancer/testis antigen family 45, member A2	0.0423	0.2935	1.12
—	8084878	0.0161	0.0006	1.12
ARMC3	Armadillo repeat containing 3	0.0292	0.0142	1.12
SNORA4	Small nucleolar RNA, H/ACA box 4	0.0119	0.0002	1.11
CT45A5	Cancer/testis antigen family 45, member A5	0.0411	0.2919	1.08
CDC2	Cell division cycle 2, G1 to S and G2 to M	0.0130	0.0032	1.07
HSDL2	Hydroxysteroid dehydrogenase like 2	0.0052	0.0062	1.06
MLLT3	Myeloid/lymphoid or mixed-lineage leukaemia (trithorax homologue, *Drosophila*); translocated to, 3	0.0008	0.0084	1.05
NRG4	Neuregulin 4	0.0331	0.0845	1.05
SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1	0.0228	0.0977	1.04
—	8124846	0.0153	0.0006	1.03
AP3M2	Adaptor-related protein complex 3, mu 2 subunit	0.0013	0.0001	1.03
C5orf54	Chromosome 5 open reading frame 54	0.0082	0.0020	1.02
GPM6B	Glycoprotein M6B	0.0213	0.0247	1.02
PMCHL2	Pro-melanin-concentrating hormone-like 2	0.0214	0.0101	1.02
PMCHL2	Pro-melanin-concentrating hormone-like 2	0.0207	0.0117	1.01
—	7946567	0.0113	0.0008	1.00
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6 (nonspecific cross reacting antigen)	0.0245	0.2601	1.00

Table 3 Downregulated genes expressed at least two-fold higher in chemosensitive tumours compared with chemoresistant tumours

Gene symbol	Gene name	P-value	FDR	Log2 ratio
FBXO32	F-box protein 32	0.0152	0.0360	−1.00
ITGB4	Integrin, beta 4	0.0375	0.0104	−1.01
KRT19	Keratin 19	0.0078	0.0084	−1.01
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	0.0420	0.0513	−1.01
PDZK1IP1	PDZK1 interacting protein 1	0.0273	0.2615	−1.03
CLDN1	Claudin 1	0.0143	0.2494	−1.03
LRRN4	Leucine rich repeat neuronal 4	0.0183	0.2549	−1.05
SNORA65	Small nucleolar RNA, H/ACA box 65	0.0203	0.0015	−1.06
PLCD3	Phospholipase C, delta 3	0.0014	0.0049	−1.06
L1CAM	L1 cell adhesion molecule	0.0366	0.0379	−1.09
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	0.0041	0.0314	−1.11
GPX3	Glutathione peroxidase 3 (plasma)	0.0387	0.0348	−1.11
C3	Complement component 3	0.0043	0.0144	−1.18
WNT7A	Wingless-type MMTV integration site family, member 7A	0.0389	0.0210	−1.21
C1orf186	Chromosome 1 open reading frame 186	0.0359	0.2808	−1.26
LOC613266	Hypothetical LOC613266	0.0247	0.0706	−1.29
FCGBP	Fc fragment of IgG-binding protein	0.0007	0.0331	−1.76
CLDN16	Claudin 16	0.0066	0.0733	−2.22

GRIA2 as a target for further evaluation as a prognostic marker for ovarian cancer. This study has some limitations. First, the tumour tissue was not microdissected, and therefore it may comprise up to 30% non-tumour tissue. Second, our data were based on a small sample size. Nonetheless, to our knowledge, this is the first report demonstrating the correlation between *GRIA2* expression and prognosis in patients with ovarian cancer.

The 56 genes found in this study were tested in a set consisting of 489 samples available through TCGA. Using univariate Cox model, expression of the four genes (*CCL18*, *GPM6B*, *MMP9*, and

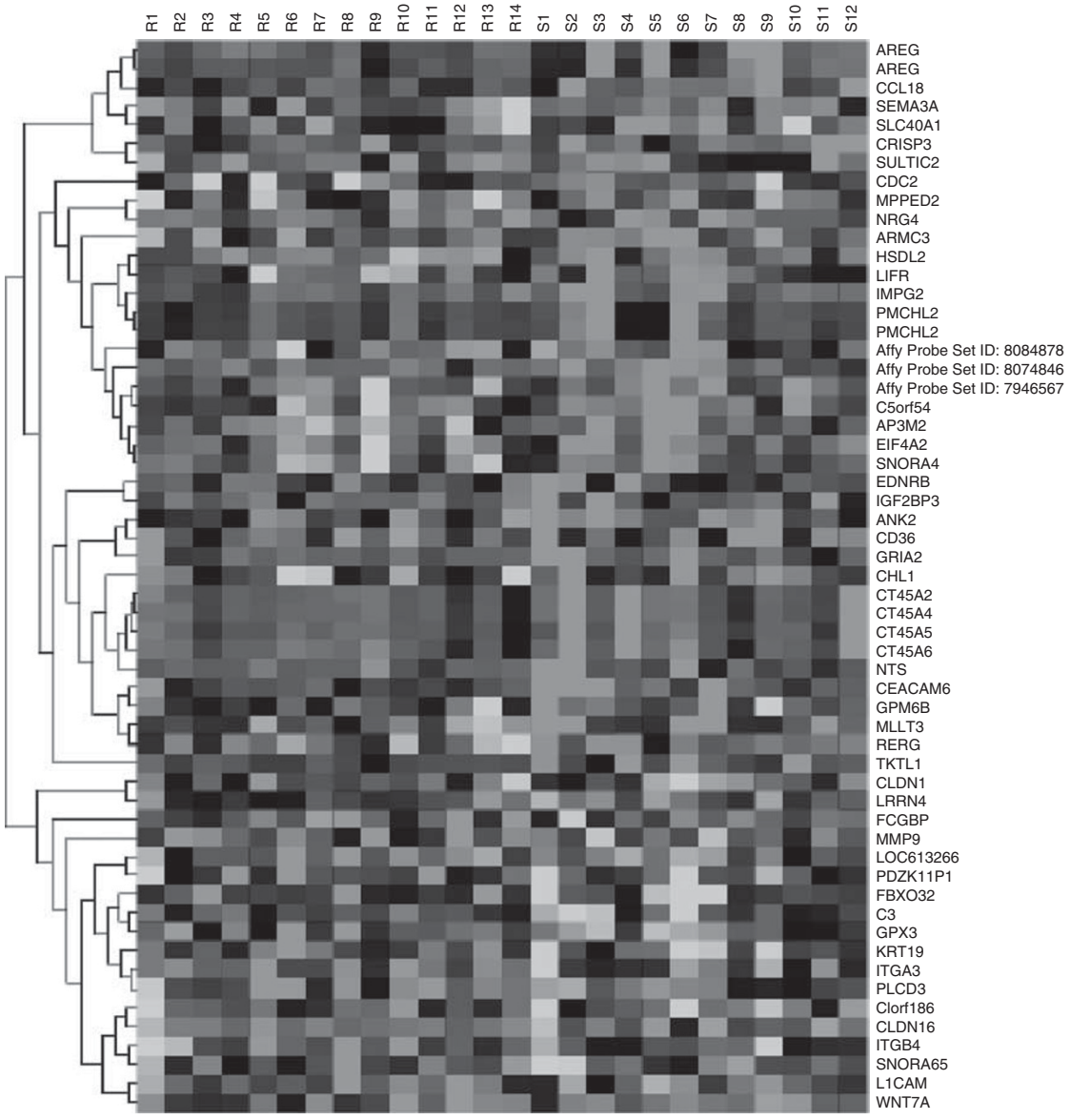

Figure 1 Unsupervised hierarchical clustering analysis of 26 serous papillary ovarian carcinomas (14 chemoresistant tumours and 12 chemosensitive tumours). We identified 39 upregulated (more than two-fold change) and 18 downregulated (more than two-fold change; $P < 0.05$) genes in chemosensitive tumours. Abbreviations: R, chemoresistant tumours; S, chemosensitive tumours.

FCGBP) was associated with overall survival in the validation set (Supplementary Table 2). Kaplan–Meier survival curve showed that six of the nine genes selected (*CHL1*, *GRIA2*, *CD36*, *LIFR*, *FCGBP*, *CCL18*, *GPM6B*, and *MMP9*) were correlated with overall survival (Supplementary Figures 5 and 6).

GRIA2 encodes an alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor, which acts as an excitatory neurotransmitter at many synapses in the central nervous system. AMPA receptors are composed of four types of subunits (GluR1, GluR2, GluR3, and GluR4), which combine to form tetramers (Hansen *et al*, 2007).

The presence of the GluR2 subunit determines AMPA receptor impermeability to Ca^{2+} (Seeburg, 1993; Hollmann and Heinemann, 1994; Rao and Finkbeiner, 2007). As AMPA receptor activation regulates the differentiation, proliferation, and migration of embryonic stem cells (Behar *et al*, 1999; Ikonomidou *et al*, 1999; Joo *et al*, 2007), it has been hypothesised that modulation of AMPA receptor-mediated signals might be involved in carcinogenesis. This hypothesis has subsequently been proven for some tumour entities, such as astrocytomas, glioblastomas, breast carcinomas, lung carcinomas, colon adenocarcinomas, and prostate carcinomas (Yoshioka *et al*, 1996; Takeda *et al*, 2000; Rzeski *et al*, 2001; Abdul and Hoosein, 2005; Ishiuchi *et al*, 2007). Alexander *et al* have shown that glutamate-mediated AMPA receptor activation increases invasion and migration of pancreatic cancer cells via activation of the classical MAPK pathway (Herner *et al*, 2011).

In contrast, there are reports that have shown endogenous GluR2 expression is associated with a low degree of malignancy. Beretta *et al* (2009) have shown that endogenous GluR2 is expressed in slow-growing GBM-derived tumour stem cells (GBM TSCs) and low-grade tumour specimens, but not in fast-growing gliomas or high-grade tumour specimens. More remarkably, GluR2 overexpression in U-87MG cells inhibits proliferation by inactivating extracellular signal-regulated kinase1/2-Src phosphorylation and induces apoptosis. Moreover, RNAi experiments in a low-grade cell line have shown that downregulation of GluR2 causes a significant acceleration of cell proliferation. With regard to the study

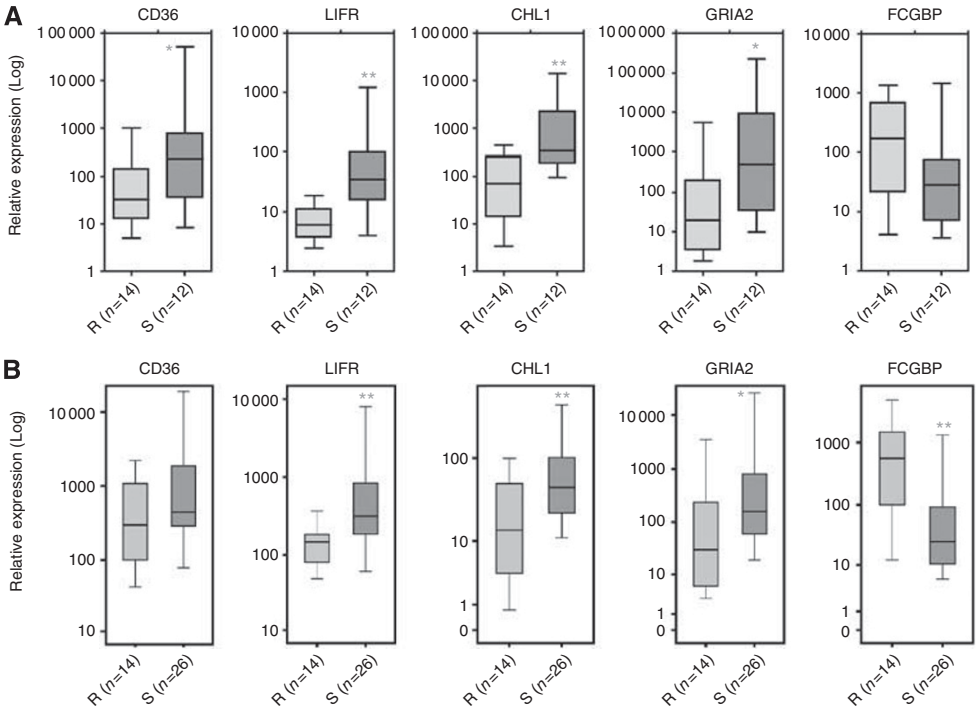

Figure 2 Real-time quantitative PCR analysis of the five selected genes (*CD36*, *LIFR*, *CHL1*, *GRIA2*, and *FCGBP*) differentially expressed between chemosensitive (pink colour) and chemoresistant (blue colour) tumours in the same cohort (**A**) and in another 40 patients cohort (**B**). Statistically significant difference between the chemosensitive and the chemoresistant tissues were found in four of five genes for each cohort. Abbreviations: R, chemoresistant tumours; S, chemosensitive tumours. $*P<0.05$, $**P<0.01$. The colour reproduction of this figure is available at the *Britsh Journal of Cancer* online.

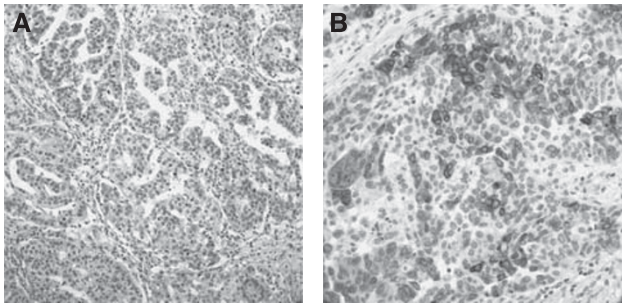

Figure 3 Representative immunohistochemical staining for GRIA2 protein. (**A**) Negative expression (original magnification ×200). (**B**) Strong expression for GRIA2 protein on the membranes of the malignant cells (original magnification ×400).

of biomarkers in cancers, Trinh *et al* (2011) have suggested an interesting opposite confounding of prognostic *vs* predictive factors. The study analysed the oncogenic pathway profiling in advanced serous ovarian carcinomas. Unexpectedly, it was revealed that invasive gene signature and wound healing response were increased in chemosensitive tumours and not in the chemoresistant ones. *GRIA2* might be one of the examples of such a confounding factor.

In this study, downregulation of GRIA2 was found in cases with a poorer prognosis. Among the many mechanisms, epigenetic silencing can cause downregulation of genes. Interestingly, Carmen *et al* have examined an Epigenetic-Aging-Signature, and GRIA2 hypermethylation was found to be associated with aging (Koch and Wagner, 2011). Although we could not find studies about the downregulation of GRIA2 in drug-resistant tumours, GRIA2 have been reported to be repressed by oestrogen. Greathouse *et al* (2008) have identified GRIA2 as oestrogen-responsive genes in uterine leiomyoma.

Table 4 Distribution of GRIA2 expression according to clinicopathological characteristics of the serous papillary ovarian carcinoma ($n=48$)

		GRIA2 expression		
Characteristics	**No. (%)**	**Positive**	**Negative**	***P*-value**
Total		6 (12.5%)	42 (87.5%)	
Age, years				
Median (range)		56 (50–75)	53 (31–78)	0.407
CA-125 level (U ml^{-1})				
Mean ± s.d.		3545 ± 4053	1889 ± 3201	0.257
Histological grade				
2	10 (20.8)	3	7	0.095
3	38 (79.2)	3	35	
Stage				
II	6 (12.5)	2	4	0.157
III/IV	42 (87.5)	4	38	
Optimal debulking				
Optimal	22 (45.8)	1	21	0.199
Suboptimal	26 (54.2)	5	21	
Response to chemotherapy				
Sensitive	40 (83.3)	6	34	0.571
Resistant	8 (16.7)	0	8	
Survival status				
Died of disease	19 (39.6)	0	19	0.149
Died of another pathology	1 (2.1)	0	1	
Alive with disease	12 (25.0)	2	10	
Alive without disease	16 (33.3)	4	12	

Of the five genes identified, three genes (GRIA2, CHL1, and FGBP) showed same prognostic significance in our data and the TCGA data. FCGBP is Fc fragment of IgG-binding protein and there are no studies in ovarian cancer. CHL1 is an adhesion molecule with homology to L1CAM (close homologue of L1) and there are some reports in ovarian cancer. Senchenko *et al*, 2011 have reported that CHL1 could act as a putative tumour suppressor during the primary tumour growth. However, they also suggested that re-expression of the gene on the edge of tumour mass might promote local invasive growth and enable further metastatic spread in ovary, colon, and breast cancer. Wolterink *et al* (2010) also have reported that mAbs to the CHL1 prolonged survival and reduced tumour burden in nude mice. The role of CHL1 in ovarian cancer warrants further investigation.

In this study 57 genes were classified as differentially expressed between chemoresistant and chemosensitive tumours, which strengthens the theory that biological differences exist according to chemosensitivity. Further analysis of the genes differentially expressed among these tumours is of great interest to find candidate genes that may be used as prognostic markers in patients with ovarian cancer. The identification of *GRIA2* among the most differentially expressed genes provides interesting information on the prognosis of patients with serous papillary ovarian adenocarcinoma.

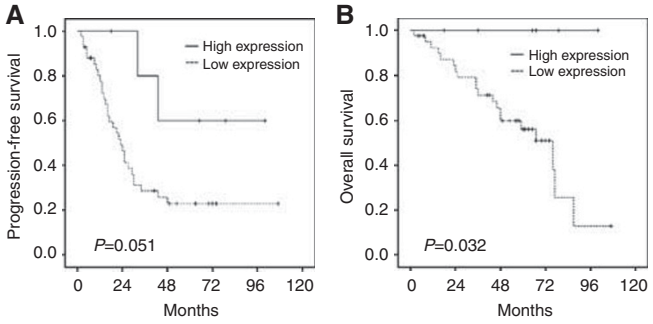

Figure 4 Correlations between GRIA2 protein expression and progression-free (**A**) and overall (**B**) survival. The survival was longer in six patients with GRIA2-positive tumours than in 42 patients with GRIA2-negative tumours.

Table 5 Univariate and multivariate analysis for progression-free survival ($n = 48$)

	Univariate			Multivariate		
Variables	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Age (years): >50 *vs* ⩽50	1.45	0.71–2.94	0.306	1.69	0.82–3.50	0.159
CA125: >674 *vs* ⩽674	1.58	0.78–3.18	0.205	1.47	0.73–2.98	0.284
Surgery: optimal *vs* suboptimal	0.77	0.38–1.55	0.461	0.70	0.34–1.43	0.326
GRIA2 expression	0.27	0.06–1.12	0.072	0.19	0.04–0.82	0.026

Abbreviations: CI = confidence interval; HR = hazard ratio.

Conflict of interest

The authors declare no conflict of interest.

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Conflict of interest

The authors declare no conflict of interest.

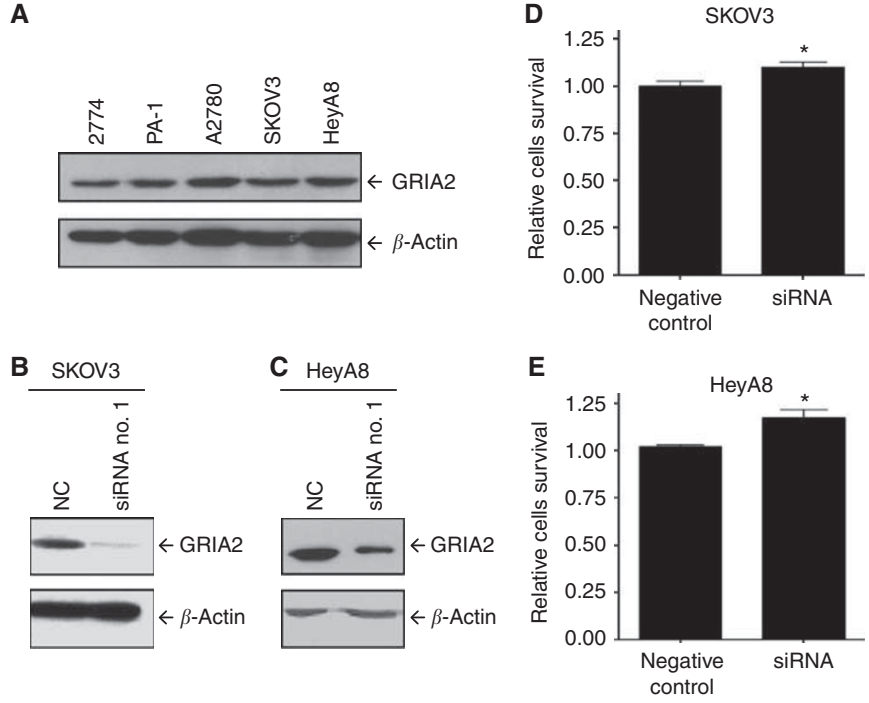

Figure 5 Effects of GRIA2 expression on the cell survival of epithelial ovarian cancer cells *in vitro*. (**A**) Expression of GRIA2 in epithelial ovarian cancer cell lines. Transfection of GIRA2 siRNA (100 nM) for 72 h reduced GRIA2 protein expression of SKOV3 (**B**) and HeyA8 (**C**) cells compared with negative control. Transfection of GIRA2 siRNA (100 nM) for 72 h increased cell survival of SKOV3 (**D**) and HeyA8 (**E**) cells compared with controls as assessed by MTT assay. Columns, mean of three independent experiments; bars, s.e. (*$P < 0.05$).

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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