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Expression analysis of stage III serous ovarian adenocarcinoma distinguishes a sub-group of survivors

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ABSTRACT

It is difficult to predict the clinical outcome for patients with ovarian cancer. However, the use of biomarkers as additional prognostic factors may improve the outcome for these patients. In order to find novel candidate biomarkers, differences in gene expressions were analysed in 54 stage III serous ovarian adenocarcinomas with oligonucleotide microarrays containing 27,000 unique probes. The microarray data was verified with quantitative real-time polymerase chain reaction for the genes *TACC1*, *MUC5B* and *PRAME*. Using hierarchical cluster analysis we detected a sub-group that included 60% of the survivors. The gene expressions in tumours from patients in this sub-group of survivors were compared with the remaining tumours, and 204 genes were found to be differently expressed. We conclude that the sub-group of survivors might represent patients with favourable tumour biology and sensitivity to treatment. A selection of the 204 genes might be used as a predictive model to distinguish patients within and outside of this group. Alternative chemotherapy strategies could then be offered as first-line treatment, which may lead to improvements in the clinical outcome for these patients.

1. Introduction

Ovarian carcinoma is one of the most common types of gynaecological cancers, ranking fifth in incidence of cancer deaths in women.[1] This is due partly to the long asymptomatic phase of the disease and partly to the fact that no specific screening method for early detection is available. More than 70% of the affected women are diagnosed with advanced disease, stage III or IV, according to the International Federation of Gynaecology and Obstetrics (FIGO) classification. The most common type is epithelial ovarian cancer, which has several histopathological subtypes, including serous, mucinous, endometrioid and clear cell carcinoma. Among these different types, serous papillary adenocarcinoma is the most common form, accounting for 50% of all cases.[2] The treatment of ovarian carcinoma patients is governed by various prognostic factors, such as surgical stage, volume of residual tumour after primary surgery and histological grade. Despite this, the clinical outcome can be difficult to predict in an individual patient with advanced disease. Some patients will be cured

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with surgery followed by chemotherapy, while others will relapse. If these different patient groups could be identified before therapy, then alternative treatments or strategies might be used instead of standard treatment.

The use of biomarkers as prognostic factors may facilitate identification of patients who are likely to relapse and die of the disease. Microarray analyses of ovarian tumours have successfully studied the relationship between gene expression and survival or chemotherapy resistance.[3–7] However, most studies compare normal ovarian tissue with ovarian tumours, early/low malignant potential (LMP) tumours with late-stage tumours, or tumour groups with different histological subtypes and stages.[8–10] Since some differences in expression could depend on tumour type and stage, it is important to study homogeneous tumour groups in survival analyses. Therefore, we wanted to investigate gene expression differences in stage III serous ovarian adenocarcinoma. We aimed to define gene expression patterns associated with survival in order to identify profiles and/or molecular markers that could have prognostic relevance.

In this study, a hierarchical cluster analysis of the microarray results identified a cluster including 60% (12/20) of the survivors. The gene expressions of the 12 tumours from patients in the sub-group of survivors were compared with the remaining 42 tumours. This comparison showed that 204 genes were significantly differently expressed. Several of the differently expressed genes were cancer-related, including *TACC1* (transforming acidic coiled-coil containing protein 1), *MUC5B* (mucin 5 subtype B) and *PRAME* (preferentially expressed antigen in melanoma). These genes might be of interest for use as molecular markers, and were selected to verify our microarray data with quantitative real-time polymerase chain reaction (QPCR).

2. Material and methods

2.1. *Patient and tumour material*

In the microarray analysis 54 stage III serous papillary adenocarcinomas of the ovary were collected from patients diagnosed between 1993 and 2000 at the Department of Oncology, Sahlgrenska University Hospital, Göteborg, Sweden (Table 1). The median age of the patients at first diagnosis was 59.5 years (range 35–84 years). The tumours were removed at primary surgery and secured for pathological examination and RNA extraction. Twenty-two of the tumours were classified as well- or moderately-differentiated. Among the patients, 13 had no macroscopic residual tumour left after primary surgery. Clinical staging of the tumours was performed according to FIGO standards and the 3 stage IIIA and 16 stage IIIB tumours were considered as a single group. After surgery, patients were treated with a combination of Farmorubicine® (epirubicin), carboplatin and cyclophosphamide. Blood from all patients was taken postoperatively before chemotherapy for evaluation of CA-125 tumour marker level. Values >35 IU/ml were considered positive. Twenty patients survived 5 years or more after first diagnosis and were considered survivors. The remaining 34 patients succumbed to cancer. All tumours were reviewed by the same pathologist according to the treatment program for West Sweden. Samples were evaluated for tumour cell content and only tumours containing at least 50% tumour cells were included.

Table 1 – Clinicopathological features of the patients

Case number	Age at diagnosis (years)	Stage	Grade	Radically operated	Five-year survival
26	41	IIIC	3	no	no
45	43	IIIA	2	yes	yes
46	43	IIIB	1	yes	yes
47	56	IIIC	3	no	no
76	76	IIIC	2	no	no
83	58	IIIC	3	no	yes
99	72	IIIC	3	yes	yes
123	56	IIIC	3	no	no
134	60	IIIC	3	no	yes
164	73	IIIC	3	no	no
193	59	IIIC	2	no	no
198	67	IIIC	3	no	no
202	59	IIIC	3	no	no
211	70	IIIC	3	no	no
272	54	IIIC	3	no	no
405	50	IIIB	1	no	yes
436	56	IIIC	3	no	no
452	69	IIIC	3	no	no
454	43	IIIC	2	no	yes
462	59	IIIB	3	no	no
480	73	IIIC	3	yes	no
489	64	IIIC	3	no	no
505	74	IIIB	3	no	no
541	64	IIIC	3	no	no
559	75	IIIC	3	no	no
563	62	IIIA	1	no	yes
626	54	IIIC	3	no	no
662	65	IIIC	2	no	no
719	77	IIIC	3	no	no
742	49	IIIC	3	yes	yes
755	77	IIIC	2	no	yes
759	52	IIIC	3	no	no
789	63	IIIC	2	no	no
918	70	IIIB	1	yes	no
988	52	IIIC	3	yes	yes
1035	36	IIIA	1	yes	yes
1047	44	IIIB	2	no	yes
1059	84	IIIB	2	yes	yes
1177	55	IIIB	3	no	no
1178	60	IIIB	2	yes	yes
1180	43	IIIB	3	no	no
1186	65	IIIB	3	yes	no
1242	70	IIIC	1	yes	yes
1274	65	IIIC	3	no	yes
1426	54	IIIB	2	no	yes
1487	47	IIIB	3	no	no
1528	50	IIIC	2	no	no
1538	62	IIIC	2	no	no
1567	68	IIIB	3	no	no
1568	51	IIIC	3	no	no
1574	35	IIIC	2	yes	yes
1658	70	IIIC	2	no	no
1760	40	IIIB	2	no	yes
1805	82	IIIB	3	no	no

2.2. *Microarray*

Total RNA was isolated from frozen tumours by homogenisation with TRIzol Reagent (Invitrogen, Carlsbad, CA, United

States of America (USA)) in a Mikro-Dismembrator S and then extracted with RNeasy mini kit (Qiagen, Valencia, CA, USA). The RNeasy step was repeated to purify the RNA further. Since RNA is extremely unstable and degrades quickly, the quality of the RNA was verified with Agilent 2100 bioanalyser (Agilent Biotechnologies, Palo Alto, CA, USA). Fluorescence-labelled cDNA probes were made from the RNA by reverse transcription. Tumour RNA was labelled with Cy3-dCTP and a universal human reference RNA (Stratagene, La Jolla, CA, USA) with Cy5-dCTP (Amersham, Buckinghamshire, UK). The use of a common reference probe allowed us to treat the fluorescent ratios as measurements of the relative expression level of each gene across all samples. The probes were cleaned up using Pronto! Plus Systems (Promega, Madison, WI, USA), according to the manufacturers' recommendations. The probes were hybridised to oligonucleotide microarrays containing 27,000 unique probes on a glass slide provided by Swegene DNA Microarray Resource Centre, Lund, Sweden (http://swegene.onk.lu.se). All arrays used were from the same print run. Microarray slides were scanned with Agilent microarray scanner G2505B (Agilent technologies, Palo Alto, CA, USA) and image processing was performed with GenePix software 6.0.0.74 (Axon Instruments, Union City, CA, USA).

For analysis and visualisation of the results, the generated raw intensity files were transferred to R (version 2.1.1) used with the package Limma.[11,12] More information about the computer programs is available at http://www.R-project.org and http://www.bioconductor.org. The background intensities were removed by local background subtraction. Low and negative values were increased to 20 in order to reduce identification of false-positive differently expressed genes. Spots with concurrent low red and green signals (<20) and flagged spots were removed. $\log_2$(ratio) values were computed and loess normalisation was applied to each array in order to remove intensity-dependent effects in the values.[13] Only genes with a valid $\log_2$(ratio) value in at least 40 of the 54 tumours (16,976 genes) were used in the analysis. The microarray raw data and $\log_2$(ratio) values is available at http://www.oncology.gu.se/forskning/opublicerad_data/MArawData_KPartheen06 and http://www.oncology.gu.se/forskning/opublicerad_data/MAlog2value_KPartheen06.

To implement a hierarchical clustering, the gene expression values were transferred to the cluster software Hierarchical Clustering Explorer (HLE), available at http://www.cs.umd.edu/hcil/multi-cluster/. Low variable genes, with a standard deviation (SD) across all samples of less than 1, were filtered out. Thus, genes with similar expression in all tumours have less influence on the result than differently expressed genes. The hierarchical clustering was performed with the linkage methods Complete and Average, and the distance measures Euclidean and Pearson correlation coefficient in different combinations to determine if the same result was accomplished with different tests.

We aimed to study differences between: (i) tumours from survivors and deceased patients; (ii) tumours from patients in the sub-group of survivors detected by hierarchical clustering and the remaining tumours; (iii) tumours from patients with and without macroscopic residual tumour left after primary surgery; and (iv) stage IIIa/b and stage IIIc tumours. Differences in $\log_2$(ratio) values between groups were tested for each gene using two-sample *t*-tests with a moderated *t* statistic.[12] *P*-values were adjusted to control the false discovery rate (FDR; i.e. the expected proportion of false positive findings among all positive findings). A gene with an adjusted *P*-value <0.05 and at least a two-fold change between groups was classified as significantly differently expressed.

2.3. *Verification with QPCR*

Twenty RNA samples, 10 from survivors and 10 from deceased patients, were selected to verify microarray data and to validate the expression of 3 differently expressed genes, *TACC1*, *MUC5B* and *PRAME*. Two reference genes found to be stably expressed in the microarray experiments, *GAPDH* and β-actin, were used for normalisation.

From each tumour sample 0.5 μg of total RNA was reverse transcribed in duplicate with a mixture of random hexamers and oligo(dT) primers, using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions.

Each cDNA sample was analysed in duplicate by real-time PCR, giving a total of four data points for each tumour sample. All real-time PCR reactions contained 0.8 U JumpStart™ Taq polymerase (Sigma-Aldrich, St Louis, MO, USA), 0.4x SYBR Green I and cDNA corresponding to 20 ng total RNA. Real-time PCR was performed on a Stratagene Mx3005p (Stratagene Corp., La Jolla, CA, USA) with 3 min initial denaturation at 95 °C, followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. A melt curve analysis was performed after each run to verify specific amplification. Primer sequences for the target genes were as follows: *TACC1* forward 5′-AACTCCCCACCCCTCTCTT, reverse 5′-CTTCTTCACCTTACAGCCACTC, *MUC5B* forward 5′-TGCCCCTTGTTCTGTGACTT, reverse 5′-ACGCACTTCATCTGGTCCTC and *PRAME* forward 5′-TATCGCCCAGTTCACCTCTC, reverse 5′-GGGACTTACATCGGTCAGCA. The reference gene assays were obtained from the Human Endogenous Control Gene Panel (TATAA Biocenter, http://www.tataa.com). The efficiency of each QPCR assay was estimated from the slope of a standard curve, generated from the serial dilution of purified PCR products. All assays showed PCR efficiencies close to 90%, and this value was used for subsequent calculations. For each assay the average Ct value for each tumour sample was converted to relative copy numbers according to the equation:

$$\frac{N_0^A}{N_0^B} = (1+E)^{(CT_B - CT_A)}.$$

$\lfloor N_0 \rfloor_A$ and $\lfloor N_0 \rfloor_B$ are the initial numbers of template molecules in samples A and B, CT_A and CT_B are the corresponding CT values, and *E* is the PCR efficiency. Using a PCR efficiency of 90% the equation becomes $\frac{N_0^A}{N_0^B} = 1.9^{(CT_B - CT_A)}$. The data was then normalised by the geometric average of the two reference genes.[14]

3. Results

3.1. *Microarray*

In the present investigation, hierarchical clustering with different combinations of linkage methods and distance

measures were used, which generated similar results in the different tests. The most striking cluster similarity between the tests included 12 of 20 (60%) of the survivors. The 12 survivors composed one large cluster together with only one deceased patient (193DC) when using Pearson correlation coefficient and average linkage (Fig. 1). In the test with Euclidean as distance measure and average linkage, 2 deceased patients were included (193DC and 662DC) in the cluster with survivors. All tumours from patients in the cluster except one (134SC) were well or moderately differentiated. The remaining 8 tumours from survivors that did not cluster were distributed more varyingly in the analysis depending on which tests were used, and only 3 were well or moderately differentiated (Fig. 1). We analysed our cluster results in relation to pre-operative CA-125 levels and recurrence in survivors, but found no correlation with our clustering data (data not shown). However, concerning decrease of CA-125 levels after the first course of chemotherapy, we found negative values in 7 of 12 clustered patients, whereas negative values after the first course were found in 2 of 8 survivors who did not cluster. Moreover, 2 patients in each of these survival groups had negative CA-125 values preoperatively. Furthermore, the clustering did not separate the tumours according to sub-stages or residual tumour after primary surgery. All 3 stage IIIA tumours and 6 of the 13 tumours from patients with no macroscopic residual disease were found in the recurrent cluster mentioned above. Nevertheless, the number of tumours in these groups was low which unfortunately weakens the significance of the results.

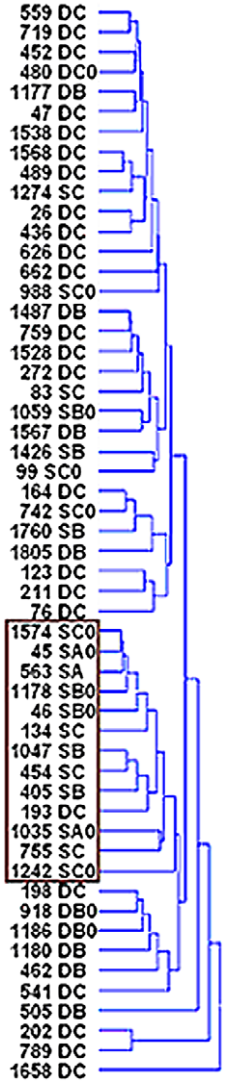

Fig. 1 – Hierarchical clustering using Pearson correlation coefficient and average linkage. The tumours are named with tumour ID, Survivor (S) or Deceased (D), stage (A, B or C), and radically operated (0). The sub-group of tumours from survivors clustered together is marked with a square.

Subsequently, we compared the 12 tumours from patients in the sub-group of survivors that clustered together with the 42 remaining tumours with a moderated *t*-test. The comparison showed that 204 genes could be classified as significantly differently expressed using our criteria (genes with an adjusted *P*-value <0.05 and at least a two-fold change between groups). Genes more expressed in the sub-group of survivors are shown in Table 2 and less expressed genes in Table 3. When tumours from all survivors were compared with tumours from deceased patients, 2 genes were differently expressed, *TACC1* and *CDH3*. The difference in *CDH3* expression was caused by low or absent intensity values for the tumours and was therefore not further evaluated. Among the differently expressed genes, the most prominent candidates for tumour markers are *TACC1*, *MUC5B* and *PRAME*. These genes had low *P*-values and displayed the largest change in the 2 moderated *t*-tests. To further visualise differences in gene expression between tumours from survivors and deceased patients, a hierarchical clustering of the genes with a *P*-value <0.1 (36 genes) was performed. The 21 genes less expressed among survivors and the 15 genes more expressed were separated into two clusters (Fig. 2). Furthermore, no genes were differently expressed between tumours divided according to sub-stage and residual tumour after primary surgery.

3.2. *Verification with QPCR*

Three differently expressed genes, *TACC1*, *MUC5B* and *PRAME* were selected for verification of the microarray data with QPCR analysis. The QPCR data of the three gene products were highly correlated with the microarray data ($r = 0.87$, 0.90 and 0.85, respectively), confirming the reliability of our expression data. The relative copy numbers in four samples (47DC, 76DC, 480DC0 and 1059SB0) for *MUC5B* were extremely low and therefore excluded. The relative copy numbers as $\log_2$ values of the three genes compared with the relative expression as $\log_2$(ratio) values measured by microarray for the 20 tumours are shown in Fig. 3. Although we selected tumours that expressed all three genes in the microarray analysis, the expected trend is evident, with higher expression of *TACC1* and *MUC5B* and lower expression of *PRAME* in tumours from survivors.

4. Discussion

It is difficult to predict the clinical outcome for individual patients with advanced ovarian cancer. The prognostic factors for the patients available today need to be improved and en-

Table 2 – Genes more expressed in tumours from patients in the sub-group of survivors compared with the remaining tumours, with at least two-fold change and adjusted P-value <0.05, and in tumours from all survivors compared with tumours from deceased patients with adjusted P-value <0.2

Gene symbol	Gene name	P-value sub-group of survivors	P-value survivors
MUC5B	Mucin 5 subtype B. tracheobronchial	<0.001	0.063
ITGB3	Integrin beta 3 (platelet glycoprotein IIIa antigen CD61)	<0.001	0.097
DDX59	DEAD (Asp-Glu-Ala-Asp) box polypeptide 59	<0.001	0.064
ALS2	Amyotrophic lateral sclerosis 2 (juvenile)	<0.001	0.160
SLC1A1	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter system Xag) member 1	<0.001	0.040
PSD	Pleckstrin and Sec7 domain protein	<0.001	0.064
EPPB9	B9 protein	<0.001	0.097
NULL	Homo sapiens cDNA FLJ13268 fis clone OVARC1000971	<0.001	0.142
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	<0.001	0.176
TACC1	Transforming acidic coiled-coil containing protein 1	<0.001	0.025
CAPS	Calcyphosine	0.001	0.101
KIAA1698	KIAA1698 protein	0.001	0.064
DAF	Decay accelerating factor for complement (CD55 Cromer blood group system)	0.001	0.064
ZNF482	Zinc finger protein 482	0.001	0.101
SLC7A2	Solute carrier family 7 (cationic amino acid transporter. y+ system). member 2	0.001	0.157
ITGB4	Integrin beta 4	0.003	0.101
SORL1	Sortilin-related receptor L(DLR class) A repeats-containing	0.003	0.160
C16orf45	Chromosome 16 open reading frame 45	0.003	0.097
MUM1	Melanoma associated antigen (mutated) 1	0.005	0.160
TIGA1	TIGA1	0.005	0.152
CDH3	Cadherin 3 type 1 P-cadherin (placental)	0.005	0.040
SEMA3B	Sema domain immunoglobulin domain (Ig) short basic domain secreted (semaphorin) 3B	0.005	0.192
CLU	clusterin (complement lysis inhibitor. SP-40.40. sulfated glycoprotein 2. testosterone-repressed prostate message 2. apolipoprotein J)	0.006	0.064
DUX4	Double homeobox, 4	0.012	0.112
PLK2	Polo-like kinase 2 (Drosophila)	0.015	0.137
EGR1	Early growth response 1	0.016	0.166
P2RY5	Purinergic receptor P2Y. G-protein coupled, 5	0.017	0.124
NULL	FLJ22528 fis clone HRC12825	0.027	0.166
DHCR7	7-dehydrocholesterol reductase	0.027	0.193
SCARA3	Scavenger receptor class A. member 3	0.028	0.188

larged. In this study, we used oligonucleotide microarrays to analyse the expression of 27,000 genes to identify differences in expression levels in 54 stage III ovarian serous papillary adenocarcinomas. This homogeneous tumour group was subjected to hierarchical cluster analysis. From this, we detected a sub-group comprised of as many as 60% of survivors, infiltrated by only 1 or 2 deceased patients. Several previous attempts to classify tumours according to survival have been performed with different results. Lancaster and colleagues[3] compared the expression in relation to long-term and short-term survival among patients with stage III and IV tumours. The samples did not segregate in the clustering of total gene expression profiles, as we found in our study, but could be identified with a small set of genes. In another study, cluster analysis did not separate tumours based on response to chemotherapy in the total material.[15] Instead, a subset of tumour samples exhibiting the most extreme differences in CA-125 level was clustered and this subset was stratified into a resistant and a sensitive group. Although the number of survivors is limited in our study, the observed trend of better biological response, controlled by multiple CA-125 analyses, suggests possible differences in sensitivity to chemotherapy between the survivors within and outside the sub-group. We also tried to find other explanations for why the survivors clustered together. The other correlation we found was with the tumours' differentiation grade, with over-representation of well- or moderately-differentiated tumours in the sub-group of survivors. The fact that all tumours from survivors did not cluster together is probably caused by biological heterogeneity within this homogeneous group of tumours. The sub-group of survivors from the cluster result may represent a subset of tumours with a specific genetic signature that is favourable regarding tumour progression and/or might be more sensitive to chemotherapy treatment.

Furthermore, 204 genes were expressed differently between the 12 tumours from patients in the sub-group of survivors and the remaining 42 tumours. Several of these genes are known to be involved in cancer development and progression. A combination of the results from our two survival analyses and the available information about the gene functions could be used to select genes of interest. Then, a selection of the 204 genes may be used as molecular markers to identify patterns associated with better survival. In accordance with our results, 2 previous microarray studies started

Table 3 – Genes expressed less in tumours from patients in the sub-group of survivors compared with the remaining tumours, with at least two-fold change and adjusted P-value <0.05, and in tumours from all survivors compared with tumours from deceased patients with adjusted P-value <0.2

Gene symbol	Gene name	P-value sub-group of survivors	P-value survivors
SCYL2	SCY1-like 2 (*S. cerevisiae*)	<0.001	0.101
PSMA7	Proteasome (prosome macropain) subunit alpha type 7	<0.001	0.105
JUND	Jun D proto-oncogene	<0.001	0.064
CALR	Calreticulin	<0.001	0.147
HIST2H2AA	Histone 2. H2aa	<0.001	0.064
CST3	Cystatin C (amyloid angiopathy and cerebral haemorrhage)	<0.001	0.064
CAPG	Capping protein (actin filament) gelsolin-like	<0.001	0.101
BCL2L11	BCL2-like 11 (apoptosis facilitator)	<0.001	0.112
C6orf106	Chromosome 6 open reading frame 106	<0.001	0.097
HIST2H4	Histone 2. H4	0.001	0.064
DNAJB11	DnaJ (Hsp40) homolog subfamily B member 11	0.001	0.097
SLC2A4RG	SLC2A4 regulator	0.001	0.097
G1P2	Interferon alpha-inducible protein (clone IFI-15K)	0.001	0.179
TCF3	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	0.001	0.167
KLHDC3	Kelch domain containing 3	0.001	0.064
HIST1H2BM	Histone 1. H2bm	0.001	0.129
MGC10433	Hypothetical protein MGC10433	0.001	0.064
KLHL24	Kelch-like 24 (Drosophila)	0.001	0.097
FADS2	Fatty acid desaturase 2	0.001	0.196
HIST1H2AJ	Histone 1. H2aj	0.001	0.151
C1QG	Complement component 1. q subcomponent. gamma polypeptide	0.002	0.101
TP53I11	Tumour protein p53 inducible protein 11	0.002	0.198
MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	0.002	0.175
HIST1H2AM	Histone 1, H2am	0.002	0.127
CENPF	Centromere protein F (350/400 kD mitosin)	0.002	0.113
RALA	V-ral simian leukaemia viral oncogene homolog A (ras related)	0.002	0.199
SNRPB	Small nuclear ribonucleoprotein polypeptides B and B1	0.002	0.064
RHEB2	Ras homolog enriched in brain 2	0.002	0.199
UBL5	Ubiquitin-like 5	0.003	0.101
MFAP2	Microfibrillar-associated protein 2	0.003	0.121
SERPING1	Serpin peptidase inhibitor. clade G (C1 inhibitor). member 1. (angioedema. hereditary)	0.003	0.127
PEPD	Peptidase D	0.003	0.199
FMNL3	Formin-like 3	0.003	0.157
NDUFB9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 9 22kD	0.003	0.131
PRAME	Preferentially expressed antigen in melanoma	0.004	0.064
TROAP	Trophinin associated protein (tastin)	0.005	0.101
SPINT2	Serine peptidase inhibitor Kunitz type 2	0.006	0.101
HMG4L	High-mobility group (non-histone chromosomal) protein 4-like	0.007	0.157
PLD3	Phospholipase D family. member 3	0.008	0.171
PSMC4	Proteasome (prosome macropain) 26S subunit ATPase 4	0.009	0.111
GCDH	Glutaryl-Coenzyme A dehydrogenase	0.011	0.085
SATB2	SATB family member 2	0.011	0.151
EIF3S12	Eukaryotic translation initiation factor 3. subunit 12	0.015	0.196
ALPL	Alkaline phosphatase liver/bone/kidney	0.042	0.064
EVI1	Ecotropic viral integration site 1	0.044	0.157

with a training set of tumours. The first developed a 14-gene predictive model to distinguish patients with short versus long time to recurrence,[4] and the second found a 115-gene signature that could distinguish between short- and long-time survivors.[6] The predictive models were then evaluated with new tumour samples and the models correlated well, indicating that it is possible to predict the clinical outcome with a gene expression profile. Therefore, further evaluating the genes we have found as possible molecular markers is of great interest, to draw conclusions about their applicability as prognostic markers as well as their use in a predictive model.

Several of the differently expressed genes are of interest for further evaluation as possible candidate biomarkers to distinguish between survivors and deceased patients. In the present study we focused on *TACC1*, *MUC5B* and *PRAME*, since these genes had low *P*-values and displayed the largest-fold change out of the genes that previously have been associated with cancer. We found that *TACC1* was expressed significantly more, both in tumours from survivors compared with deceased patients, and in tumours from patients in the sub-group of survivors compared with the remaining tumours. Down-regulation of the gene has previously been shown in several tumour types, including ovarian and breast cancer.[16]

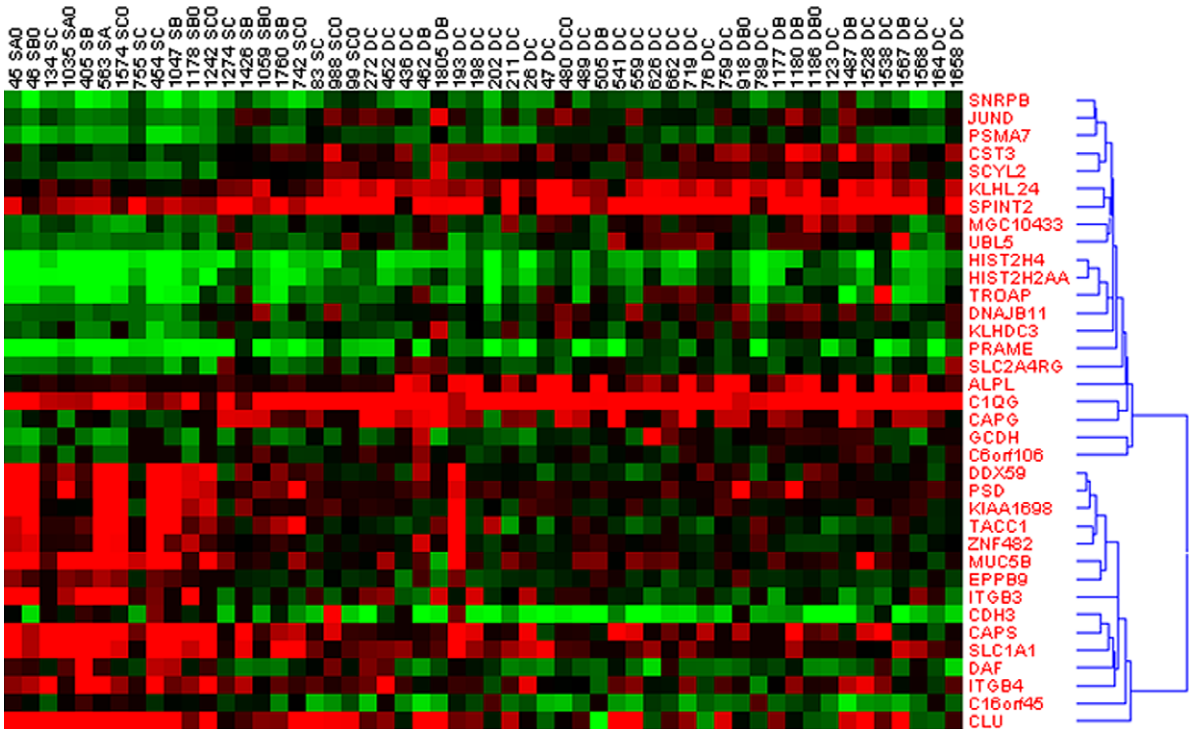

Fig. 2 – Genes differently expressed between tumours from survivors and deceased patients with an adjusted *P*-value <0.1 using a two-fold threshold. Log_2(ratio) (cy3/cy5) colour scale ranges from green < 0 through black = 0 to red > 0.

The gene is located at chromosome region 8p11, a region frequently deleted in different tumour types, and *TACC1* might be one of the cancer-related genes of interest in this region.[17–19] The normal function of TACC1 is not precisely known, but observations have shown that the protein is concentrated at centrosomes during mitosis and may play a role in cytokinesis.[20,21] Moreover, the protein may be involved in the control of mRNA metabolism.[16] The gene has, to our knowledge, not previously been described as differently expressed in microarray studies of ovarian cancer. In an immunohistochemical analysis by Lauffart and colleagues,[22] TACC1 expression was absent or minimal in 36.9% of the ovarian tumours, with decreasing expression in stage I (85.7%), stage II (72.2%) and stage III (64.7%) serous papillary carcinomas. These circumstances, with altered protein levels of TACC1 during tumour progression and the differences in expression between survivals and deceased patients, strengthen *TACC1* as a target for further evaluation as a prognostic marker for ovarian cancer.

The gene *MUC5B* was significantly more expressed in tumours from patients in the sub-group of survivors compared with the remaining tumours in our study. The gene belongs to the mucin family of high-molecular-weight glycoproteins found in human epithelial cells. MUC5B, a secreted gel forming mucin, has been studied and found to be abnormally expressed in several tumour types, such as gastric carcinoma and breast cancer.[23,24] In ovarian cancer studies, higher expressions of *MUC5B* in LMP and G1 tumours compared with G2 and G3 tumours have been described.[25] In addition, Gilks and colleagues[26] compared LMP tumours and serous carcinomas and found that *MUC5B* and the gene *CLU* were expressed in higher levels in LMP. In the present study, the higher expressions of *MUC5B* and *CLU* in tumours from survivors compared with deceased patients support these data. This finding corresponds with the theory that early-stage cancers share expression patterns with the advanced-stage long-term survivors, suggesting a shared favourable biology.[5]

In contrast with the other two genes discussed, *PRAME* was expressed less in tumours from patients in the sub-group of survivors compared with the remaining tumours in our study. In previous microarray studies, *PRAME* has been described as up-regulated in malignant ovarian tumours compared with normal ovarian tissue.[27–30] In a study by Lancaster and colleagues[3] *PRAME* was up-regulated in ovarian cancer samples compared with normal ovarian surface epithelium, but did not separate tumours according to survival, as we did in our study. Further, the gene *PRAME* is frequently expressed in a variety of cancers, such as melanoma and neuroblastoma, and is evaluated as a potential marker for advanced disease[31,32] The function of *PRAME* in normal tissue is still unknown, but it encodes an antigen recognised by autologous cytolytic T lymphocytes and its expression is absent or low in normal adult tissue, except male germ cells.[31] Because of the high expression in several different cancer types and the low or absent expression in normal tissues, evaluation of *PRAME* as a target for immunotherapeutic strategies is of great interest.[33]

In the present study, we compared stage IIIA/B tumours with stage IIIC tumours, without finding any significant differences. This result indicates that some tumours in stage IIIA/B may already have the same alterations in expression as stage IIIC tumours. On the other hand, some clinical

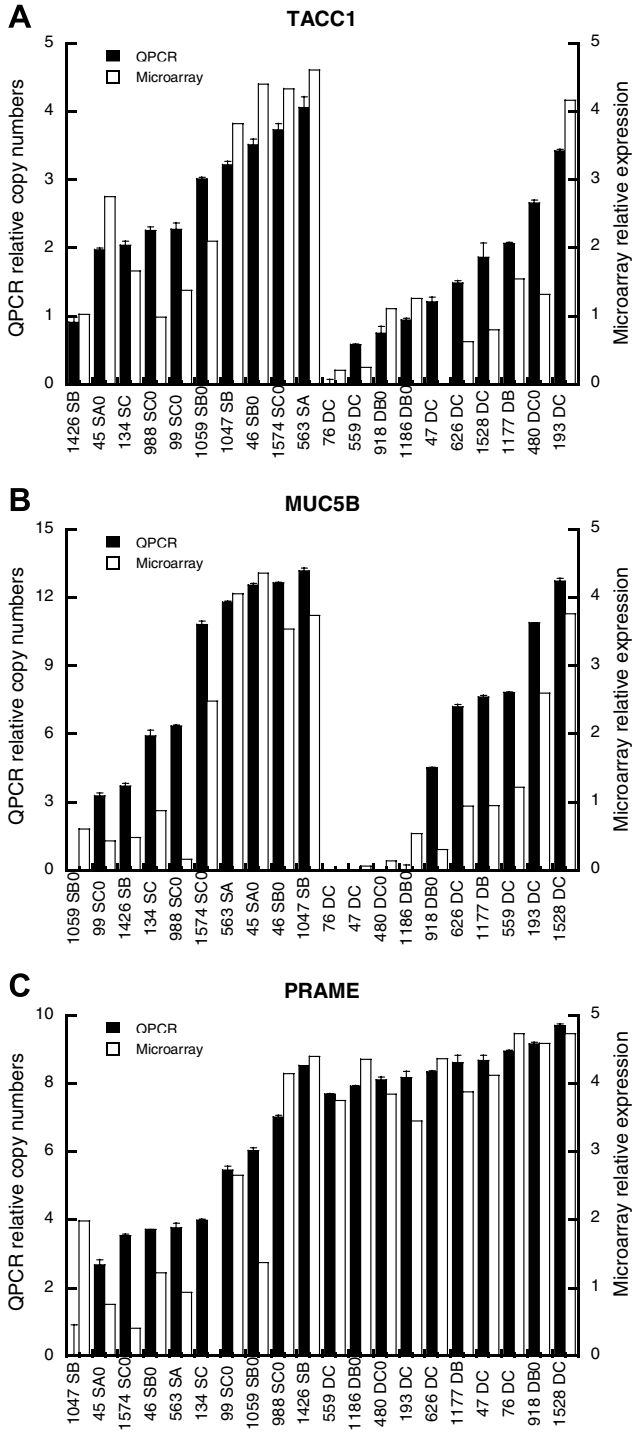

Fig. 3 – Relative expression as log_2values based on the microarray data and relative copy numbers as log_2 values based on the QPCR analysis for (A) *TACC1*, (B) *MUC5B* and (C) *PRAME*. The tumours are named with tumour ID, Survivor (S) or Deceased (D), stage (A, B or C), and radically operated (0). QPCR data for 47DC, 76DC, 480DC0 and 1059SB0 for *MUC5B* were extremely low and therefore excluded.

stage IIIC tumours may have been surgically removed in an earlier biological stage of tumour progression. If so, our findings suggest discrepancies between biological and clinical tumour progression. Moreover, no significant differences were found between patients with or without residual tumours after primary surgery. However, in our previous comparative genomic hybridisation (CGH) study of 98 stage III serous papillary adenocarcinomas, we found a significant correlation between specific chromosomal alterations and residual tumour after primary surgery.[17] Differences in tumour biology probably exist between tumours from patients with or without residual tumour after primary surgery, but this question must be further investigated with other methods.

In conclusion, with a hierarchical cluster analysis of stage III serous papillary adenocarcinomas, we detected a sub-group composed of 60% of the survivors. To our knowledge such a finding has never before been achieved. With a moderated *t*-test, 204 genes could be classified as differently expressed between tumours from patients in the sub-group of survivors and the remaining tumours. This result strengthens the theory that biological differences exist between tumours from survivors and deceased patients. Moreover, our results suggest that, in the future, it may be possible biologically to characterise a sub-group of patients who could be treated postoperatively by alternative chemotherapy and thereby improve the clinical outcome. Further analysis of the genes differently expressed among these tumours is of great interest, in order to find candidate genes to be used as prognostic markers in ovarian cancer.

Conflict of interest statement

None declared.

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