

# Whole genome expression profiling of advance stage papillary serous ovarian cancer reveals activated pathways

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Ovarian cancer is the most lethal type of gynecologic cancer in the Western world. The high case fatality rate is due in part because most ovarian cancer patients present with advanced stage disease which is essentially incurable. In order to obtain a whole genome assessment of aberrant gene expression in advanced ovarian cancer, we used oligonucleotide microarrays comprising over 40 000 features to profile 37 advanced stage papillary serous primary carcinomas. We identified 1191 genes that were significantly ( $P < 0.001$ ) differentially regulated between the ovarian cancer specimens and normal ovarian surface epithelium. The microarray data were validated using real time RT-PCR on 14 randomly selected differentially regulated genes. The list of differentially expressed genes includes ones that are involved in cell growth, differentiation, adhesion, apoptosis and migration. In addition, numerous genes whose function remains to be elucidated were also identified. The microarray data were imported into PathwayAssist software to identify signaling pathways involved in ovarian cancer tumorigenesis. Based on our expression results, a signaling pathway associated with tumor cell migration, spread and invasion was identified as being activated in advanced ovarian cancer. The data generated in this study represent a comprehensive list of genes aberrantly expressed in serous papillary ovarian adenocarcinoma and may be useful for the identification of potentially new and novel markers and therapeutic targets for ovarian cancer.

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## Introduction

Ovarian cancer is the fifth most common malignancy in women as well as the fifth leading cause of cancer deaths in women in the United States (Jemal *et al.*, 2003). In

2004, 24 400 women in the United States will be diagnosed with the disease, and an estimated 14 300 will die from it (Jemal *et al.*, 2003). It is the leading cause of death from gynecologic cancers in the United States and is the most lethal type of gynecologic cancer in the Western world. This high case fatality rate is due in part to the fact that most ovarian cancer patients present with advanced stage disease where the disease is more difficult to treat. Although patients presenting with stage I ovarian cancer have a 5-year survival rate of over 85%, only 25% of patients presenting with advanced stage disease survive to 5 years after initial diagnosis (Friedlander, 1998).

There is substantial evidence that the majority of ovarian cancers comprise carcinomas arising from the surface epithelium (Godwin *et al.*, 1992; Testa *et al.*, 1994; Nap *et al.*, 1996; Auersperg *et al.*, 1999). Of the four main histologic subtypes, serous papillary, mucinous, endometrioid and clear cell, the serous adenocarcinomas are the most common subtype and comprise approximately 50% of all ovarian carcinomas (Scully *et al.*, 1998), with the endometrioid subtype accounting for 20–25% of ovarian cancer. Thus, the majority of poor prognosis ovarian cancers comprise the serous subtype.

Ovarian cancers are highly aneuploid and genetically complex tumors that develop in a multistep process involving alterations of numerous genes. With the advent of SAGE and DNA microarray technology, it is now possible to study gene expression profiles of large numbers of tumor samples and to determine the characteristic gene expression patterns associated with those tumors. To date, a number of studies utilizing these technologies to determine gene expression profiles of ovarian cancer have been reported (Schummer *et al.*, 1999; Wang *et al.*, 1999; Hough *et al.*, 2000; Ismail *et al.*, 2000; Ono *et al.*, 2000; Tapper *et al.*, 2001; Tonin *et al.*, 2001; Welsh *et al.*, 2001; Wong *et al.*, 2001; Schwartz *et al.*, 2002; Schaner *et al.*, 2003; Adib *et al.*, 2004), however, only a few of these studies compare papillary serous ovarian cancer to normal ovarian epithelium (Schummer *et al.*, 1999; Ono *et al.*, 2000; Welsh *et al.*, 2001; Schaner *et al.*, 2003; Adib *et al.*, 2004). Furthermore, only one of these studies exclusively analyses serous ovarian cancer to normal ovary (Welsh *et al.*,

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2001). The data generated from these studies have provided important information. These studies, however, have scientific limitations such as small numbers of tumors analysed, exclusive analysis of cell lines as opposed to primary tumors and normal epithelium, as well as limited numbers of features on the microarrays used in the studies. Thus, to date, a substantial analysis of the gene expression pattern of papillary serous ovarian cancer as compared to the normal ovarian epithelium has not been undertaken.

In the present study, we used oligonucleotide microarrays containing over 40 000 features to profile 37 advanced stage papillary serous ovarian adenocarcinomas and compare their gene expression profiles with normal ovarian surface epithelium. Our microarray analysis identified 1191 genes that were differentially regulated by 1.5-fold or greater between normal ovarian surface epithelium and papillary serous ovarian carcinoma ( $P < 0.001$ ). Fourteen genes from this list were randomly chosen and used to validate the arrays by quantitative real-time RT-PCR (qRT-PCR). In addition, the microarray data were imported into PathwayAssist software and signaling pathways potentially involved in ovarian cancer tumorigenesis were identified. The data generated in this study represent a comprehensive list of genes aberrantly expressed in serous papillary ovarian adenocarcinoma and may be useful for the identification of potentially new and novel markers and therapeutic targets for ovarian cancer.

## Results

### *Whole genome expression profiles of papillary serous ovarian adenocarcinoma versus normal ovarian epithelium*

Global gene expression profiles were examined for 37 papillary serous ovarian adenocarcinomas and six normal ovarian surface epithelium cytobrushings using an oligonucleotide array consisting of 47 000 transcript sequences. After initial filtering of the data, an informative data set consisting of 22 579 sequences was generated. Comparison of the gene expression profiles of the cancer specimens with the normal ovary brushings revealed 1191 differentially expressed sequences that differed by 1.5-fold or greater with a significance of  $P < 0.001$ . A representative list of differentially regulated genes comprising those with the highest fold increase and decrease in each biologic category is shown in Table 1. The complete list of 1191 differentially regulated genes is detailed in the supplementary information.

Of the 1191 differentially regulated genes, slightly more were underexpressed (54%) in ovarian cancer compared to normal ovary brushings than overexpressed (46%). Over half (56%) of the differentially expressed genes code for proteins whose functions have not been characterized and 95 of these are as yet not associated with any protein. The remaining 44% of the genes encode proteins involved in numerous biologic functions as assessed by Gene Ontology (GO), including

cell adhesion, apoptosis, growth and differentiation (Figure 1). Since the cancer specimens were not microdissected, it is possible that some of these genes are expressed in stromal cells rather than the epithelial tumor cells.

### *Validation of microarray data*

To ensure the accuracy of our microarray, we performed an 'electronic validation' by comparing our list of differentially expressed genes to those genes that have previously been documented to be differentially regulated in advanced stage papillary serous ovarian cancer specimens. In all, 12 of the differentially expressed genes identified from our analysis have previously been reported to be differentially expressed (Table 2). Those genes previously identified as being overexpressed in serous ovarian cancer showed a similar pattern of expression in our study and, similarly, genes that were underexpressed in our study showed the same trend as that reported in previously published findings.

To further validate the microarray results, 14 genes differentially expressed between the cancer and normal specimens were selected for qRT-PCR analysis on 10 of the cancer specimens and four normal samples. The expression differences for both the over expressed genes (Figure 2a) and underexpressed genes (Figure 2b) in cancers as compared to normal samples were quite apparent, and mirrored the microarray data. Although the quantitative change for each gene did not exactly correlate between the qRT-PCR and microarray analyses, the general trend of being either overexpressed or underexpressed was consistent between the two techniques.

### *Identification of signaling pathways contributing to ovarian tumorigenesis*

To identify signaling pathways that are associated with ovarian tumorigenesis and that may contribute to tumor progression and metastasis, we analysed our microarray expression data using PathwayAssist (Iobion Informatics, LLC). This software utilizes a proprietary database containing over 140 000 references on protein interactions obtained from PubMed to generate a biological association network (BAN) of known protein interactions. By overlaying microarray expression data onto the BAN, co-regulated genes that define a specific signaling pathway can be identified. Thus, we used this software to characterize signaling pathways associated with ovarian cancer based solely on the expression data we obtained by microarray analysis. After importing the 1191 differentially expressed genes into the PathwayAssist, we identified a signaling pathway associated with advanced ovarian cancer. Figure 3 shows this pathway with the solid symbols representing genes as down-regulated in cancer, the open symbols representing upregulated genes and the gray symbols are genes whose expression did not change significantly between cancer and normal samples. Our expression data identified the PAR1, PAR2, MT-SP1, SNX1, GPRK5,

**Table 1** Summary and functional categorization of select genes displaying the largest fold change in advanced papillary serous ovarian carcinomas ( $P \leq 0.00001$ )

Gene	GenBank accession	P-value	Fold change	Chromosome location
<i>Cell communication</i>				
COL15A1	NM_001855	8.17E-12	4.9	9q21-q22
LRP8	D50678	5.34E-14	3.2	1p34
ARL7	BC001051	7.32E-08	2.6	2q37.2
PAMCI	NM_005447	3.86E-09	2.3	12q21.32
GPR92	AW183080	3.45E-06	-3.8 <sup>a</sup>	12p13.31
TXNIP	NM_006472	7.02E-08	-5.2	1q21.2
EPHB6	D83492	8.44E-07	-6.1	7q33-q35
FLRT2	AB007865	2.68E-11	-15.3	14q24-q32
<i>Cell death</i>				
BCL10	AA994334	7.02E-07	1.9	1p22
MGC13096	AL574186	8.75E-09	1.8	19q13.12
CTNNA1	AF006070	4.31E-06	-2.3	9q31.2
COP	NM_052889	1.32E-07	-2.5	11
<i>Cell differentiation</i>				
NOTCH4	U95299	9.31E-10	1.9	6p21.3
<i>Cell growth and/or maintenance</i>				
ATP8B1	BG290908	1.19E-10	7.7	18q21-q22
RGC32	AF036549	1.82E-10	7.4	13q13.3
LCN2	NM_005564	2.56E-09	4.8	9q34
HIST2H2AA	AI313324	5.75E-10	4.5	1q21.3
ENPEP	L14721	6.17E-13	3.4	4q25
H2AFX	BC004915	2.64E-08	2.8	11q23.2-q23.3
FZD3	AY005130	3.45E-10	2.8	8p21
SMC4L1	AK002200	5.62E-09	2.7	3q26.1
PDGFRA	M21574	3.83E-06	-2.9	4q11-q13
SEMA3C	AB000220	1.05E-08	-2.9	7q21-q31
MET	J02958	3.20E-11	-3.1	7q31
LRP2	U33837	9.87E-06	-3.6	2q24-q31
KCNK6	BC004367	3.97E-15	-3.7	19q13.1
DAB2	U39050	3.25E-08	-5.0	5p13
ARHI	AK021882	4.84E-17	-7.9	1p31
ABCA8	AB020629	9.43E-09	-9.4	17q24
<i>Cell motility</i>				
MAGP2	U37283	2.34E-09	6.2	12p13.1-p12.3
VAV3	AF067817	3.96E-10	4.5	1p13.3
JAG2	Y14330	2.22E-06	1.7	14q32
FLII	BG421186	1.11E-08	-1.6	17p11.2
DTNA	BC005300	1.02E-06	-1.9	18q12
SLIT2	AF055585	1.76E-06	-1.9	4p15.2
PPAP2A	AF014403	5.19E-08	-2.6	5q11
C4A	NM_000592	3.50E-16	-6.7	6p21.3
<i>Hemostasis</i>				
TFPI2	BC005330	3.88E-11	-5.4	7q22
<i>Invasive growth</i>				
HTLF	BF590117	5.03E-06	1.9	2p22-p16
NDP52	BC004130	1.49E-08	-1.6	17q21.33
<i>Metabolism</i>				
LOC148203	BE966267	1.35E-08	3.8	19p13.11
KIAA1804	A1809005	4.14E-07	3.0	1q42
LOC221061	AU150943	8.60E-19	2.9	10p13
HBXAP	BF512183	6.29E-14	2.6	11q13.4
IBRDC2	A1953847	6.95E-07	2.4	6p22.3
ZNF138	AA114243	1.59E-06	2.4	7q11.21-q11.23
OCN	U49184	3.79E-10	2.3	5q13.1
KIAA1630	BC002477	5.01E-09	2.3	10p14
TRA2A	AA831170	1.24E-07	-2.9	7p15.3
ZFPM2	NM_012082	1.17E-07	-2.9	8q23
DPP4	M80536	2.14E-07	-3.0	2q24.3
GSTM5	NM_000851	3.90E-10	-3.2	1p13.3
TM4SF7	BC000389	9.45E-07	-3.2	11p15.5
KIAA1055	BF195608	3.39E-06	-3.8	15q24.1

Table 1 Continued

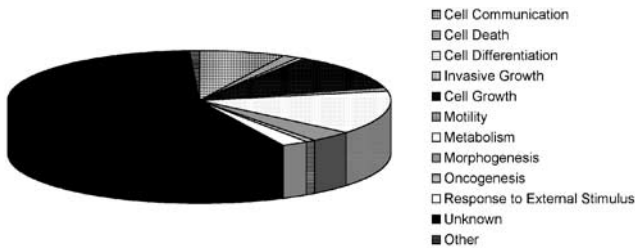
Gene	GenBank accession	P-value	Fold change	Chromosome location
KLK11	AB012917	2.32E-08	-5.5	19q13.3-q13.4
HSD11B1	NM_005525	9.19E-10	-5.6	1q32-q41
<i>Morphogenesis</i>				
F2R	BC002464	6.42E-08	5.8	5q13
MCAM	M28882	4.04E-10	5.3	11q23.3
FGF18	AI798863	1.01E-10	3.8	5q34
CACNA1A	AA769818	2.78E-09	3.3	19p13.2-p13.1
PAPSS2	AF150754	3.31E-10	-3.9	10q23-q24
GATA6	U66075	1.74E-08	-3.9	18q11.1-q11.2
IGFBP4	NM_001552	2.32E-09	-4.2	17q12-q21.1
BNC	L03427	3.27E-07	-23.5	15q25.1
<i>Oncogenesis</i>				
FGFR1OP	NM_007045	2.52E-16	2.6	6q27
BCR	T30183	1.72E-11	-2.1	22q11
ALDH1A2	NM_003888	2.98E-11	-12.7	15q21.2
ANXA8	BC004376	5.56E-13	-34.4	10q11.2
<i>Organogenesis</i>				
LR8	AF115384	2.60E-06	-3.3	7q36.1
<i>Pathogenesis</i>				
C19orf2	AB006572	5.54E-06	1.8	19q12
USP7	BF591638	9.15E-06	-1.6	16p13.3
<i>Pregnancy</i>				
TRO	AF349719	6.93E-06	-2.0	Xp11.22-p11.21
<i>Response to external stimulus</i>				
CD24	AK000168	4.30E-10	56.2	6q21
CKLFSF8	AW080832	3.74E-10	3.5	3p22.3
IFITM2	NM_006435	3.07E-12	-2.5	11p15.5
CKLFSF4	AA191708	5.06E-09	-2.5	16q22.1
HF1	X04697	3.52E-06	-2.8	1q32
SERPING1	M13690	1.42E-14	-4.0	11q12-q13.1
<i>Function unknown</i>				
HPS3	AI922198	1.88E-10	18.7	3q24
Unknown	AI343467	6.97E-11	6.3	
HSPC195	BC006428	3.46E-08	6.2	5q31.3
Unknown	N63377	3.55E-08	5.6	
KIAA0186	NM_021067	3.36E-17	5.5	20p11.21
LRIG1	AB050468	3.11E-14	4.5	3
MGC13057	BE645119	6.28E-10	4.3	2q32.3
SLC39A4	BC001688	4.04E-13	4.3	8q24.3
DUFD1	AL138828	4.89E-14	4.3	6q23.2
SORT1	BE742268	1.84E-11	4.0	1p21.3-p13.1
FLJ33516	AI967987	1.39E-07	-5.9	Xq22.3
Unknown	BE220209	9.70E-09	-7.8	
C20orf75	BC019612	5.95E-11	-8.2	20p13
C11orf9	BC004938	1.49E-07	-8.5	11q12-q13.1
RPESP	AW451999	2.70E-12	-8.8	8q13.3
Unknown	BG413606	7.07E-06	-14.6	
NY-REN-7	AW514267	9.34E-20	-27.5	5q35.3
UPK3B	BC004304	4.67E-15	-40.1	7q11.2
ITLN1	AB036706	3.12E-06	-43.7	1
CALB2	NM_001740	2.19E-13	-57.8	16q22.2

<sup>a</sup>Downregulated in cancer specimens compared to normal ovary epithelium

MAGP2, HEF1, FAK, VAV3, YES, CDC42, RECK, ET-1, IAP and MT1-MMP genes as coordinately differentially regulated between cancer and normal. These genes encode for proteins that are part of a signaling pathway associated with tumor cell migration, adhesion and invasion. This pathway is linked to cell

cycle progression by multiple other genes, including ARHI, GATA6, CCND1 and DOC-2 (DAB-2), which were also identified as differentially expressed.

PAR1, PAR2, HEF1, VAV3, CDC42, MAGP2, RECK, SNX1 and GPRK5 have not been previously identified as being dysregulated in serous ovarian



**Figure 1** Biologic functions of the 1191 genes differentially regulated between normal surface ovarian epithelium and advanced stage serous ovarian cancer. Other represents genes involved in drug resistance, extracellular matrix biogenesis, hemostasis, organogenesis, pathogenesis, pregnancy, reproduction, rhythmic behavior and small molecule transport

**Table 2** Genes previously identified as differentially regulated in ovarian cancer tissue specimens by microarray analysis

Gene symbol	GenBank Accession	Reference(s)
CCND1	BC000076	Dhar <i>et al.</i> (1999) Barbieri <i>et al.</i> (1999) Adib <i>et al.</i> (2004)
LCN2	NM_005564	Adib <i>et al.</i> (2004)
IAP	NM_001777	Adib <i>et al.</i> (2004)
JAG2	AF020201	Adib <i>et al.</i> (2004)
TACSTD1	NM_002354	Adib <i>et al.</i> (2004)
VEGF	AF024710	Adib <i>et al.</i> (2004)
MUC1	AF348143	Schummer <i>et al.</i> (1999) Hough <i>et al.</i> (2000) Welsh <i>et al.</i> (2001)
CD24	AK000168	Welsh <i>et al.</i> (2001) Adib <i>et al.</i> (2004)
COL15A1	NM_001855	Ismail <i>et al.</i> (2000)
SEPP1	NM_005410	Wang <i>et al.</i> (1999)
GSTM5	NM_000851	Wang <i>et al.</i> (1999)
IGFBP4	AY442346	Tapper <i>et al.</i> (2001)

cancers. Independent studies using Northern or Western blot analysis as well as immunohistochemical techniques have also identified CCND1 (Barbieri *et al.*, 1999), ARHI (Yu *et al.*, 2003), DOC-2/DAB-2 (Mok *et al.*, 1998), GATA-6 (Capo-chichi *et al.*, 2003), ET-1 (Bagnato *et al.*, 1999), YES1 (Wiener *et al.*, 2003), FAK (Judson *et al.*, 1999) and MT-SP1 (Oberst *et al.*, 2002) as being differentially regulated in serous ovarian carcinomas. MT1-MMP has been shown to be upregulated in serous effusions (Baciu *et al.*, 2003) but not in primary ovarian cancer specimens, and IAP has been identified by microarray analysis as upregulated in ovarian cancer specimens (Adib *et al.*, 2004). Thus, we have identified a series of interacting genes (Table 3) that constitute a potentially important signaling pathway involved in ovarian cancer.

## Discussion

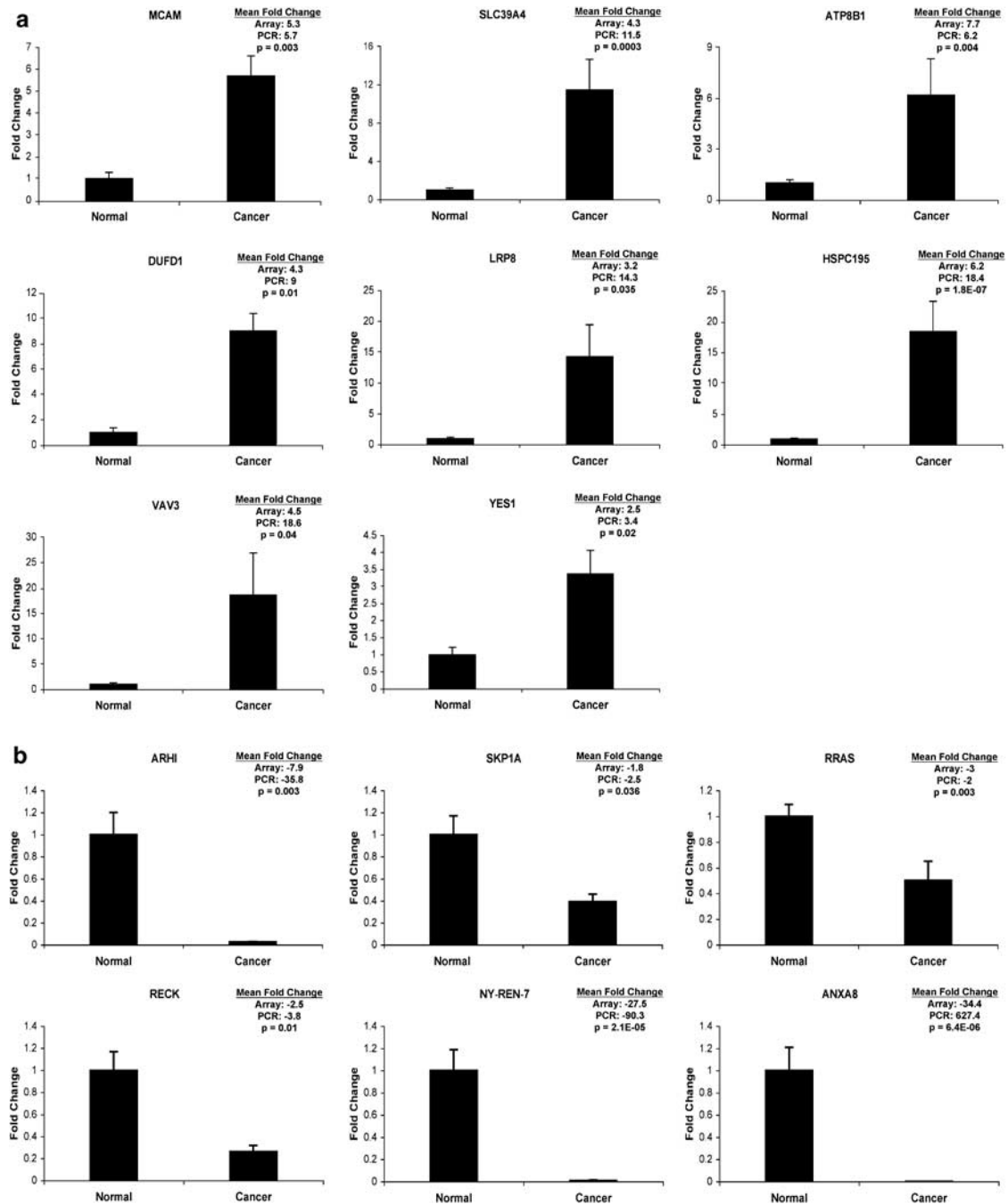
Gene expression profiling is a powerful tool for identifying aberrant gene expression patterns associated with the process of tumorigenesis. A number of studies

have been published reporting the use of microarray analysis to identify genes that are differentially regulated in ovarian cancer compared to normal ovarian epithelium (Schummer *et al.*, 1999; Ismail *et al.*, 2000; Ono *et al.*, 2000; Welsh *et al.*, 2001; Wong *et al.*, 2001). These studies, although providing useful information, have been limited by the use of ovarian cancer cell lines as opposed to primary cancer specimens, as well as sample size and number of features on the microarrays used. To determine a more global assessment of differential gene expression, we have performed a genome wide gene expression profile of papillary serous ovarian carcinoma using an oligonucleotide array containing 47 000 transcripts and have identified 1191 sequences that are differentially regulated between cancer and normal specimens with a significance of  $P < 0.001$ .

We validated our microarray analysis comparing our differentially regulated genes with those from other microarray studies on serous ovarian cancer. Our results were consistent with those where the data sets overlapped. In addition, we identified a number of genes that have been independently identified as differentially expressed in ovarian cancer specimens and/or cell lines by Western, Northern and immunohistochemical techniques, including ARHI (Yu *et al.*, 1999), DOC-2/DAB-2 (Mok *et al.*, 1998), GATA-6 (Capo-chichi *et al.*, 2003), ALEX1 (Kurochkin *et al.*, 2001) and YES1 (Wiener *et al.*, 2003), and again our results were consistent with these findings.

The gold standard for validation is real-time PCR. There was an excellent correlation between the microarray and RT-PCR analyses even though there was no precise quantitative agreement in gene expression between the two techniques. It has been documented that agreement between the two techniques is dependent on the separation between the PCR primers and the microarray probes, as well as the number of absent calls by the microarray software (Etienne *et al.*, 2004). Since our choice of PCR primers varied from gene to gene with respect to the position of the microarray probe, the discrepancy in the absolute fold changes between the microarray analysis and qRT-PCR is not totally unexpected.

Many of the differentially regulated genes are well characterized and have widely varying functions, including cell cycle progression, cellular proliferation, apoptosis and motility. Clearly, there are a number of genes identified that have been associated with tumorigenesis, such as ARHI (Yu *et al.*, 1999, 2003; Peng *et al.*, 2000; Bao *et al.*, 2002; Luo *et al.*, 2003; Wang *et al.*, 2003) that is downregulated in breast and ovarian cancers, and the protease activated receptor 1 (PAR1), which has been implicated in epithelial tumor cell motility and invasion for breast, and colon cancer (Sambrano and Coughlin, 1999; Even-Ram *et al.*, 2001; Darmoul *et al.*, 2003; Yin *et al.*, 2003; Booden *et al.*, 2004). However, we have also identified a number of genes whose role in oncogenesis has not yet been confirmed, such as TRO, which functions in pregnancy, or LR8, which plays a role in organogenesis (Table 1). While it is quite possible that the differential regulation

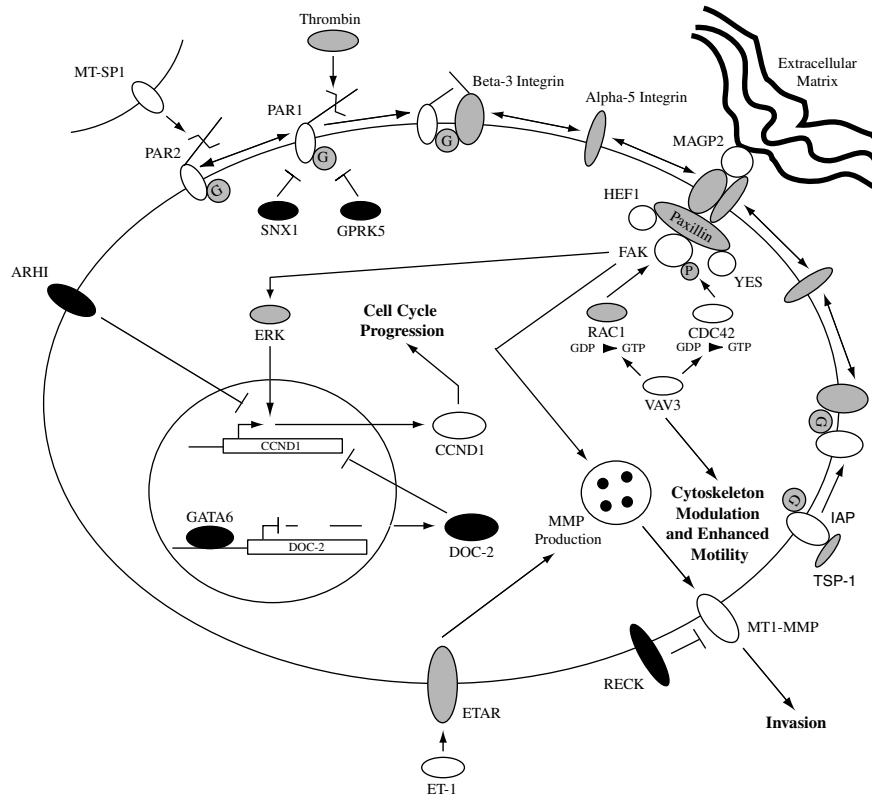


**Figure 2** Validation of microarray analysis. qRT-PCR was used to validate the overexpression (a) or underexpression (b) of 14 randomly selected genes (see Table 4) in cancer specimens compared to normal ovarian epithelium identified using microarray analysis. The fold change was calculated by determining the expression of each gene in each sample relative to  $\beta$ -actin expression and then comparing this relative gene expression with the mean expression for each gene in the four normal samples as described in Materials and methods. Error bars represent s.e.m. and mean fold changes as determined by microarray and RT-PCR are shown in the top corner of each panel

of these genes between ovarian cancer and normal specimens may be entirely inconsequential, the possibility exists that some of these genes have novel activities that may serve a more direct role in tumor development.

The mechanism(s) of differential regulation of the genes identified in this study (Table 1 and supplementary data) remains unknown. Certainly, many of the

genes are differentially expressed due to transcriptional activity, but some may be the result of amplification or deletion since they reside at chromosomal locations that have previously been reported to be altered in advanced papillary serous ovarian carcinoma. Examples of these upregulated genes include SKIL, APG3, AAT1 and DDEF1, which are located at 3q26, 3q13.2, 3q12-q13.3



**Figure 3** Schematic representation of potential signaling pathways involved in ovarian cancer. Pathways were identified by incorporating the microarray results (genes which are differentially expressed between normal and malignant ovarian epithelial cells) into PathwayAssist. Open symbols represent genes that are upregulated in cancer compared to normal ovarian epithelium, solid symbols are genes that are downregulated in cancer specimens compared to normal and gray shaded symbols represent genes that did not show a significant difference between cancer and normal specimens

and 8q24.1-q24.2, respectively, all chromosomal locations reported to be amplified in ovarian cancer (Iwabuchi *et al.*, 1995; Bayani *et al.*, 2002; Hauptmann *et al.*, 2002; Hu *et al.*, 2003; Israeli *et al.*, 2003). Similarly, MGC8721, ANAPC4, RECK and SLIT2 located at 8p12, 4p15.31, 9p13-p12 and 4p15.2, respectively, are examples of downregulated genes that occur at chromosomal locations previously reported to be frequently lost in ovarian carcinomas (Iwabuchi *et al.*, 1995; Bayani *et al.*, 2002; Hauptmann *et al.*, 2002; Hu *et al.*, 2003; Israeli *et al.*, 2003). Determining the precise mechanism of dysregulation of these genes will require CGH and LOH analysis on the same samples subjected to expression profiling. Interestingly, immune-related genes including HLA-G, HLA-B, HLA-C and HLA-F (all MHC class I genes) and TAP1 (required for MHC class I assembly) are all downregulated in tumor specimens compared to normal ovarian surface epithelium, and all of these genes reside on chromosome 6p21.3. Coordinate downregulation of these genes may be one mechanism that ovarian tumors evade detection by the host immune system.

One of the challenging aspects of expression profiling is determining the biologic interactions and relevance of large numbers of differentially expressed genes. We used the newly developed PathwayAssist software to identify clusters of interacting genes that were coordinately up-

or downregulated, and thus identify signaling pathways that contribute to ovarian carcinogenesis (Figure 3). We constrained our analysis to genes that were expressed in epithelial cells, thus ensuring that pathways identified were likely to be expressed in ovarian tumor cells. Our microarray data showed that the HEF1, FAK, YES, CDC42 and membrane type 1 matrix metalloproteinase (MT1-MMP) genes are upregulated in ovarian cancer specimens compared to normal ovarian epithelium. FAK has been previously identified as a marker in papillary serous ovarian carcinoma (Judson *et al.*, 1999) and activated FAK phosphorylates a number of substrates, including HEF1 (Law *et al.*, 1996; Schaller *et al.*, 1999). In MCF7 breast cancer cells, increased HEF1 expression results in enhanced expression of a number of genes involved in motility and invasion, including MT1-MMP (Fashena *et al.*, 2002). This matrix metalloproteinase modifies the extracellular matrix (Imai *et al.*, 1996; Sato and Seiki, 1996; Ohuchi *et al.*, 1997) and can activate other latent MMPs (Sato *et al.*, 1994; Sato and Seiki, 1996). It has been implicated in ovarian cancer cell invasion (Ellerbroek *et al.*, 1999) and is associated with poor outcome (Davidson *et al.*, 1999, 2001). It also promotes tumor growth and angiogenesis through upregulation of VEGF (Sounni *et al.*, 2002). MT1-MMP activity is inhibited by the membrane-bound glycoprotein RECK (Oh *et al.*, 2001)

**Table 3** Genes up- or downregulated by  $\geq 1.5$ -fold ( $P \leq 0.001$ ) identified using PathwayAssist software to be involved in signaling pathways contributing to papillary serous ovarian tumorigenesis

GeneBank Accession	Gene symbol	Chromosomal location	Function	Description	Reference(s)
BC002464 <i>Upregulated genes</i>	PAR1	5q13	Motility and invasion	Involved in integrin signaling.	Even-Ram <i>et al.</i> (2001)
U20428	MT-SP1 <sup>a,b</sup>	11q24-11q25	Motility and invasion	Membrane bound protease identified in ovarian cancers.	Yin <i>et al.</i> (2003) Oberst <i>et al.</i> (2001)
BE965369	PAR2	5q13	Motility and invasion	MT-SP1 substrate. PAR2 can also be activated by cleaved PAR1.	Oberst <i>et al.</i> (2002) Takeda <i>et al.</i> (2000)
NM_001777	IAP <sup>c</sup>	3q13.1-q13.2	Motility and invasion	Identified in ovarian cancer. Implicated in integrin mediated phosphorylation of FAK.	Sasaki <i>et al.</i> (2000) Campbell <i>et al.</i> (1992) Mawby <i>et al.</i> (1994) Gao <i>et al.</i> (1996)
U37283	MAGP2 Microfibril-associated Glycoprotein-2	12p13.1-p12.3	Motility and invasion	Extracellular matrix protein, which associates with microfilaments. Stimulates focal adhesion formation and FAK phosphorylation.	Gibson <i>et al.</i> (1998)
AA912743	FAK <sup>a</sup>	8q24-qter	Motility and invasion	Identified in ovarian cancer. Implicated in migration, matrix protease regulation, apoptosis, and cell cycle progression.	Judson <i>et al.</i> (1999)
L43821	HEF1	6p25-p24	Motility and invasion	FAK substrate which enhances motility and expression of invasion and matrix protease related transcripts.	Fashena <i>et al.</i> (2002) Irby and Yearman, (2000)
NM_005433	YES1 <sup>b</sup>	18p11.31-p11.21	Motility and invasion	Src protein family member identified in ovarian cancer and implicated in cancer progression.	Wiener <i>et al.</i> (2003) Schmitz <i>et al.</i> (2000) Lozano <i>et al.</i> (2003)
AF067817	VAV3	1p13.3	Motility and invasion	Regulator of cytoskeleton, motility, and invasion.	Zeng <i>et al.</i> (2000)
U41078	MT1-MMP <sup>b,c</sup>	14q11-q12	Invasion, (MMP activation, and integrin processing)	Membrane bound MMP involved in ovarian cancer cell invasion.	Sachdev <i>et al.</i> (2002) Fishman <i>et al.</i> (1996)
BC000076	CND1 <sup>a,c</sup>	11q13	Cell cycle progression	Over expressed in ovarian cancers. Up-regulated by FAK via integrin signaling.	Davidson <i>et al.</i> (2001) Batu <i>et al.</i> (2003) Dhar <i>et al.</i> (1999)
NM_001955	ET-1 <sup>a,c</sup>	6p24.1	Invasion	Highly expressed and secreted by ovarian cancers. Enhances MT1-MMP secretion and FAK phosphorylation.	Barbieri <i>et al.</i> (1999) Zhao <i>et al.</i> (2001) Bagnato <i>et al.</i> (1999)

Table 3 Continued

GenBank Accession	Gene symbol	Chromosomal location	Function	Description	Reference(s)
					Rosano <i>et al.</i> (2001) Bagnato <i>et al.</i> (1997)
<i>Downregulated genes</i>					
AI052536	SNX1	15q22.1	Receptor regulation	Involved in agonist induced degradation of PAR1.	Wang <i>et al.</i> (2002)
L15388	GPRK5	10q24-qter	Receptor regulation	Phosphorylation of PAR1 by GPRK5 causes receptor desensitization.	Tiruppathi <i>et al.</i> (2000)
NM_021111	RECK	9p13-p12	MMP inhibitor	Membrane anchored protein capable of inhibiting MT1-MMP.	Oh <i>et al.</i> (2001) Rhee and Coussens, (2002)
AK021882	ARH1 <sup>b</sup>	1p31	Tumor suppressor	Down-regulated in ovarian cancers. Growth suppression associated with decreased cyclin D1 promoter activity.	Yu <i>et al.</i> (1999)
U66075	GATA6 <sup>a,c</sup>	18q11.1-q11.2	Tumor suppressor regulation	Transcription factor that is under expressed in ovarian cancers. Implicated in regulation of DOC-2.	Yu <i>et al.</i> (2003) Capo-chichi <i>et al.</i> (2003)
U39050	DOC-2 <sup>a,c</sup>	5p13	Tumor suppressor	Down-regulated in ovarian cancers. Deregulation may enhance cyclin D1 levels.	Mok <i>et al.</i> (1998)
					Hocevar <i>et al.</i> (2003)

<sup>a</sup>Identified as upregulated in clinical ovarian cancer specimens at the transcript level. <sup>b</sup>Identified as upregulated in clinical ovarian cancer specimens at the protein level. <sup>c</sup>Identified and/or analysed in ovarian cancer cell line(s)

and RECK expression is associated with a good clinical outcome in breast cancer patients (Span *et al.*, 2003). In our study HEF1, an activator of MT1-MMP, was upregulated and RECK, an inhibitor of MT1-MMP activity, was downregulated. Another positive regulator of MT1-MMP, endothelin-1 (ET-1) (Rosano *et al.*, 2003), was identified as being upregulated in cancer specimens by our microarray analysis. This pathway has also been implicated in increased cell motility. Activation of Rho GTPase family members, including CDC42, leads to localized actin polymerization and lamellipodia formation (Nobes and Hall, 1995), and this activity is enhanced by the vav-3 oncogene (VAV3), which functions as a guanine exchange factor for CDC42 and RAC1, and is able to induce morphological changes in cells (Zeng *et al.*, 2000). VAV3 can also stimulate increased tyrosine phosphorylation of FAK (Sachdev *et al.*, 2002). Both VAV3 and CDC42 have not previously been associated with serous ovarian cancer and, given their functions, may be attractive candidates as therapeutic targets using small-molecule inhibitors. A number of other genes associated with this pathway have not previously been characterized in serous ovarian cancer, including PAR1, PAR2, MAGP2, SNX1 and GPRK5. Thus, our data suggest that positive signaling through FAK coupled to the downregulation of RECK enhances MT1-MMP activity resulting in increased invasion. It is likely that the deregulation of this pathway is important for the development of advanced stage ovarian epithelial cancer. It is important to note that not every gene involved in this pathway is differentially regulated between normal and cancer in every case, as evidenced by the RT-PCR data (Figure 2). It is likely that dysregulation of subsets of these genes is sufficient to activate this signaling pathway within individual tumors. The analysis of a large sample size (37 serous ovarian tumor specimens) and the sum of that data allowed us to identify all of the key genes involved and therefore the pathway itself.

Selecting our specimens to represent the two ends of the pathologic spectrum, normal ovarian epithelium and advanced stage ovarian cancers most likely increased the probability of identifying this pathway. Expression profiling of early-stage tumors will be necessary to determine the precise clinical contributions of the different elements of this pathway. Invasion and dissemination of ovarian tumor cells throughout the peritoneal cavity converts a surgically treatable and potentially curable tumor into one that is incurable. Intraperitoneal dissemination is one of the major reasons for the poor survival of ovarian cancer patients, and we speculate that a possible mechanism for this spread is activation of the pathway described above. Further, it is important to note that many of the genes identified within this pathway are potential targets for novel therapeutic agents.

In summary, by utilizing a 47 000 feature oligonucleotide array, we have performed a global analysis of gene expression profiles between normal ovarian surface epithelium and advanced stage papillary serous ovarian cancer and identified 1191 sequences that are statistically

significantly differentially regulated. Combining gene expression profiles and the PathwayAssist software provides a powerful tool for the identification of signaling pathways. We provide direct evidence for one such pathway that is present in advanced ovarian cancer. The size of our microarray chip allowed us to identify members of this pathway that have not previously been associated with serous ovarian cancer. Combining our data with already published observations enabled us to identify this pathway as being potentially biological meaningful in serous ovarian cancer. Signaling pathways associated with other biologic processes such as cell cycle progression and motility can similarly be identified by combining our microarray data with the PathwayAssist software. In addition, identification of these genes and pathways provides further insight into the disease and may aid investigators in the study of potentially new and interesting targets that need to be characterized and assessed for their relevance as markers or therapeutic targets for ovarian cancer.

## Materials and methods

### Tissue samples

In all, 37 stage III or IV fresh frozen papillary serous ovarian cancers from the Cooperative Human Tissue Network (CHTN) and the Gynecologic Oncology Group (GOG) tissue bank and six normal ovary surface epithelium (OSE) cytobrushings obtained from the Brigham and Women's Hospital, Boston, MA were analysed. All normal ovarian samples were obtained from post-menopausal women. Although the cancer specimens were not microdissected, all samples were determined to contain at least 80% tumor. Specimens were procured under IRB-approved protocols. All tissue samples were stored at  $-140^{\circ}\text{C}$  until processed

### RNA isolation

Total RNA from each sample was extracted using Trizol (Life Technologies, Inc., Gaithersburg, MD, USA) as per the manufacturer's instructions, followed by purification using RNeasy Mini columns (Qiagen, Inc., Valencia, CA, USA). To enhance the total RNA yield, OSE samples were purified on an RNeasy Micro column (Qiagen, Inc., Valencia, CA, USA) after Trizol extraction.

### Hybridization of RNAs to oligonucleotide arrays

Human Genome U133A Plus 2.0. GeneChip oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA) representing 47 000 transcripts and variants, including 38 500 well-characterized human genes, were used in this study. Biotin-labeled cRNA was prepared as described in the Affymetrix Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA). Briefly, 5  $\mu\text{g}$  of purified total RNA template was reverse transcribed to generate double-stranded cDNA using HPLC-purified T7-(dT)<sub>24</sub> primer (Midland Certified Reagent Company, Inc., Midland, TX, USA) and Superscript II<sup>TM</sup> RNase H<sup>-</sup> reverse transcriptase (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA). Following second-strand cDNA synthesis and clean-up, biotinylated antisense RNA (aRNA) was generated by *in vitro* transcription using the

Bioarray, High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA). In all, 15  $\mu\text{g}$  of each RNA preparation was fragmented and combined with a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD and Cre). Hybridization to the oligonucleotide arrays and subsequent washing and detection was performed as recommended by the manufacturer. Array images were acquired using a GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA) and analysed with Genechip<sup>®</sup> Operating Software (GCOS). The image from each GeneChip was scaled such that the average intensity value for all of the arrays was adjusted to a target intensity of 500.

### Microarray data analysis

Genechip images and data sets were uploaded into the National Cancer Institute's Microarray Analysis Database (mAdb) for evaluation (<http://nciarray.nci.nih.gov/index.shtml>). Of the 47 000 transcript sequences represented on the array, hybridization control sequences and sequences scored as absent (A) or marginal (M) in all 40 samples were excluded from the analysis. Furthermore, only those transcripts possessing an expression value for at least one OSE specimen and  $\geq 20\%$  of the ovarian cancers were considered. The resulting data set contained 22 579 informative sequences. Sequences that were either 1.5-fold overexpressed or under-expressed in the tumor group compared to the OSE group and had a *t*-test  $P < 0.001$  were considered to be differentially regulated.

To control for multiple comparisons, we reported as significant genes only those that reached significance at level  $P = 0.001$ . Testing 22 579 probes at this significance level, we expect that the average number of spuriously significant (false positive) results will be 23 or less. We found that 1716 probes reached the stringent significance level of 0.001, far exceeding

**Table 4** Primer sequences for the genes used in qRT-PCR analysis

Gene	Primer sequences
NY-REN-7	Sense: 5'-TGGGTCTCTCCTTGTATATTTG-3' Antisense: 5'-TAACCACCACAACAATAAGAAC-3'
HSPC195	Sense: 5'-GCAGCAGTTGTAGGAATCG-3' Antisense: 5'-AAGCATCACCTTCTCCAGAG-3'
ARHI	Sense: 5'-CAGGAGCCCCGAGAAGAAATC-3' Antisense: 5'-CTACACGTACAGGATAGGAG-3'
DUFD1	Sense: 5'-GTATCAGCAACACAAGCCTTC-3' Antisense: 5'-AGCCTTTAACAGTCCAAATCAG-3'
LRP8	Sense: 5'-GTGCTCAGAAAGTCAAGATAGG-3' Antisense: 5'-CTTCAGGGTGCTTCAGAGG-3'
SLC39A4	Sense: 5'-TTGGAGTCAGCGAGGAGAG-3' Antisense: 5'-ACGTTGTGCAGCAGGAAG-3'
ANXA8	Sense: 5'-AACCGAGATGCCATTAACATTC-3' Antisense: 5'-CACAGTCTCCTTGGGTCAG-3'
ATP8B1	Sense: 5'-ATTATGGAACTCCTCAAAGAG-3' Antisense: 5'-AGGACTGATATTGTGAACATTC-3'
MCAM	Sense: 5'-GAGGTCTACACTGTCTTTCATG-3' Antisense: 5'-GCCACTAAGTACCATTCTC-3'
RRAS	Sense: 5'-AGAGAAGCAACCACCACAAG-3' Antisense: 5'-GGAGACCCAGATGAGGAAATG-3'
SKP1A	Sense: 5'-AATACTAGTTGCACTGCTCTG-3' Antisense: 5'-AACTACACATGCAATGAGGAC-3'
VAV3	Sense: 5'-ACAAGGAGCCAGAACATTCAG-3' Antisense: 5'-GAAGTCATACCGCGATGG-3'
YES1	Sense: 5'-GCCGAGTGCCATTCAG-3' Antisense: 5'-GTCCTTCTTCCAACACAGATTC-3'
RECK	Sense: 5'-CCCAGATTATTGCCAGAGAC-3' Antisense: 5'-CCAGTTCACAGCAGCCTAAG-3'

the average number of false positive. Using the false discovery rate controlling procedure of Benjamin and Hochberg (1995), the false discovery rate for these 1716 probes is only 1.32%. We reported 1191 of the 1716 significant probes that were unique and differentially regulated by 1.5-fold or greater between normal ovarian surface epithelium and papillary serous ovarian carcinoma.

Under the conservative assumption that genes with at least 1.5-fold change and genes with less than 1.5-fold change have equal chances of being false positive, the false discovery rate for these 1191 genes is no greater than 1.32%.

#### Identification of signaling pathways

The gene list generated by microarray analysis was analysed using PathwayAssist software (Iobion Informatics, LLC, La Jolla, CA, USA) to identify and group these genes into specific cellular pathways. PathwayAssist is a software tool for biological pathway analysis. It allows for the identification and visualization of pathways, gene regulation networks and protein interaction maps. The software program utilizes a natural language processor to extract information from databases such as Pub Med to provide direct associations. For microarray analysis, expression data can be imported into

Pathway Assist and it will graphically identify all known relationships between the differentially expressed genes.

#### Quantitative real-time PCR

qRT-PCR was used to validate the differential expression of selected genes. RNA from 10 tumor specimens used for microarray analysis and four OSE samples that were not part of the OSE specimens analysed by microarray were used. qRT-PCR was performed using an iCycler Real-Time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the Quantitect SYBR Green RT-PCR Kit (Qiagen, Inc., Valencia, CA, USA) as per the manufacturer's instructions. The fold change for each target gene was calculated using the  $2^{-\Delta\Delta CT}$  method as previously described (Livak and Schmittgen, 2001) with  $\beta$ -actin as the reference gene. The primers for each gene validated by qRT-PCR are shown in Table 4. Sequences of the  $\beta$ -actin primers have been previously described (Kreuzer *et al.*, 1999).

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