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Gene expression signatures differentiate ovarian/peritoneal serous carcinoma from breast carcinoma in effusions

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Abstract

Ovarian/primary peritoneal carcinoma and breast carcinoma are the gynaecological cancers that most frequently involve the serosal cavities. With the objective of improving on the limited diagnostic panel currently available for the differential diagnosis of these two malignancies, as well as to define tumour-specific biological targets, we compared their global gene expression patterns. Gene expression profiles of 10 serous ovarian/peritoneal and eight ductal breast carcinoma effusions were analysed using the HumanRef-8 BeadChip from Illumina. Differentially expressed candidate genes were validated using quantitative real-time PCR and immunohistochemistry. Unsupervised hierarchical clustering using all 54,675 genes in the array separated ovarian from breast carcinoma samples. We identified 288 unique probes that were significantly differentially expressed in the two cancers by greater than 3.5-fold, of which 81 and 207 were overexpressed in breast and ovarian/peritoneal carcinoma, respectively. SAM analysis identified 1078 differentially expressed probes with false discovery rate less than 0.05. Genes overexpressed in breast carcinoma included *TFF1, TFF3, FOXA1*, *CA12*, *GATA3*, *SDC1*, *PITX1, TH, EHFD1, EFEMP1, TOB1* and *KLF2*. Genes overexpressed in ovarian/peritoneal carcinoma included *SPON1*, *RBP1*, *MFGE8*, *TM4SF12*, *MMP7*, *KLK5/6/7*, *FOLR1/3*, *PAX8*, *APOL2* and *NRCAM*. The differential expression of 14 genes was validated by quantitative real-time PCR, and differences in 5 gene products were confirmed by immunohistochemistry. Expression profiling distinguishes ovarian/peritoneal carcinoma from breast carcinoma and identifies genes that are differentially expressed in these two tumour types. The molecular signatures unique to these cancers may facilitate their differential diagnosis and may provide a molecular basis for therapeutic target discovery.

Keywords: gene expression array • breast carcinoma • ovarian carcinoma • serous effusions

Introduction

Breast cancer is by far the most common malignancy (23% of all cancers) and the leading cause of cancer mortality in women (14% of female cancer deaths). In 2002, the estimated number of new cases was 1.15 million worldwide [1]. The prognosis of breast cancer is relatively good, with 5-year survival averaging 73% in developed countries and 57% in developing countries [1]. In the United States, 61% of new breast cancer cases are diagnosed while localized, 31% are diagnosed in a regional stage and 6% have already metastasized to distant sites at diagnosis (stage unknown in the remaining 2%) [2]. Breast cancer metastasizes most often to axillary lymph nodes, but may involve any organ. Metastasis to serosal surfaces, which occurs in approximately 50% of patients, involves primarily the pleural cavity [3, 4], and occasionally the pericardial and peritoneal cavities [5, 6]. Pleural effusions may occur at any point during the clinical course and may be the sole manifestation of metastatic disease [3, 4, 7]. The presence of pleural effusion is

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associated with poor prognosis, with median survival of 6 and 13 months reported in two independent studies [3, 4].

Ovarian cancer is the leading cause of death from gynaecological cancer and is the sixth most common cancer in women throughout the world, with approximately 204,000 new cases and 125,000 disease-related deaths estimated in 2002 [1]. Incidence rates are highest in developed countries [1]. Ovarian carcinoma (OC) and the closely related and morphologically indistinguishable primary peritoneal carcinoma (PPC) involve the peritoneal surface in the majority of cases, with the formation of solid nodules and the accumulation of ascites fluid [8, 9]. Metastasis to the pleural space is frequent at both diagnosis and recurrence and constitutes the most common manifestation of FIGO stage IV disease [10–12]. Treatment by combined surgery and chemotherapy consisting of platinum compounds and taxanes results in an initial response rate of 75%. However, the majority of patients with advanced-stage disease develop recurrence within the first 2–3 years, resulting in a 5-year survival rate of 30% for these patients [13].

Although the presence of cancer cells in effusions marks the presence of advanced-stage disease and is generally associated with poor prognosis, there are obvious therapeutic and prognostic implications related to the differentiation of OC/PPC from breast carcinoma, as diagnosis of the latter in effusions universally defines stage IV disease and portends extremely poor prognosis. Beyond the difficulty in diagnosing the origin of metastatic carcinoma in general, the differential diagnosis of breast carcinoma and OC/PPC is particularly challenging. Both tumours may express hormone receptors, epithelial markers (e.g. cytokeratin 7, Ber-EP4), growth factor receptors (*e.g.* Her2/neu) and other surface molecules (*e.g.* CA 125); both occur as part of hereditary syndromes related to mutations in the BRCA1 and BRCA2 genes [14] and both are able to metastasize to the other organ, although this occurs more frequently in the direction of breast cancer spreading to the ovaries [15]. Identification of new markers may aid in improved diagnosis of these tumours and may provide therapeutic targets specific for each type of cancer.

To date, only one study comparing the gene expression signatures of breast carcinoma and OC/PPC has been published, in which Schaner *et al.* identified 61 genes that were differentially expressed in these cancers [16]. However, the OC/PPC that were analysed consisted of tumours of different histological types and included tumours of both primary and metastatic origin. We have previously shown that primary OC/PPC and metastases from this cancer in effusions have different gene expression signatures [17] and have demonstrated in multiple studies that tumour cells at these different anatomic sites are biologically distinct (reviewed in Ref. [18]). To elucidate molecular differences between breast carcinoma and OC/PPC cells in effusions, in this study we have performed a gene expression analysis of 18 effusions. OC/PPC specimens were all of the serous type, as serous carcinomas constitute >90% of metastatic OC/PPC in effusions. Breast carcinomas were uniformly of the ductal type. We identified a set of genes that are differentially expressed in breast carcinoma and OC/PPC, which may facilitate our understanding of the biology of metastasis in these two tumour types, and may provide new diagnostic markers.

Material and methods

Patients and material

The clinical material consisted of 10 OC/PPC and 8 breast carcinoma effusions submitted to the Department of Pathology at the Norwegian Radium Hospital during the period 1999–2004. OC/PPC effusions were all peritoneal, whereas the breast carcinoma effusions were from the pleural ($n = 6$), peritoneal ($n = 1$) and pericardial ($n = 1$) space.

All OC/PPC were of the high-grade serous type, and all breast carcinoma specimens consisted of infiltrating duct carcinoma. OC/PPC effusions were from patients aged 53–76 (mean = 66) years, diagnosed at FIGO stage II ($n = 1$), III ($n = 8$) or IV ($n = 1$). Residual disease volume at primary operation was ≤1 cm in five cases, >1 cm in four cases and unknown in one case.

Breast carcinomas were from patients aged 42–76 years (mean = 59 years). Two patients were diagnosed at TNM stage I, three at stage II, one at stage III and two at stage IV. Six patients were operated and received no chemotherapy at that time, one was operated following neo-adjuvant chemotherapy and one was inoperable. Four tumours were receptor-negative an three were receptor-positive.

Effusions were processed immediately after tapping with centrifugation for 10 min at 2000 rpm. The resulting pellet was used for routine cytological diagnosis and evaluation of specimen adequacy. The remaining material was diluted at 1:1 ratio in RPMI 1640 medium containing 50% FCS and 20% DMSO and aliquoted for freezing at −70°C. Cell blocks were prepared using the thrombin clot method. Diagnoses were established based on morphology and immunohistochemistry (IHC) [19]. Only specimens containing >50% tumour cells were selected for the study. The majority of samples contained 80–100% tumour cells. The study was approved by the Regional Committee for Medical Research Ethics in Norway.

The material analysed using quantitative real-time PCR consisted of eight OC/PPC and seven breast carcinomas ($n = 15$) from the above-described series of 18 specimens.

The material analysed using IHC consisted of 47 serous OC/PPC and 29 breast carcinoma (27 ductal, 2 lobular) effusions. Clinicopathologic data were available for all OC/PPC and for 21/29 breast carcinoma patients. OC/PPC consisted of 40 OC and 7 PPC. Effusions were from patients aged 38–76 years (mean = 60 years). Grading using the FIGO system was follows: grade 1:7 patients; grade 2:9 patients; grade 3:27 patients; not graded (inoperable patients where only biopsy was obtained): 4 patients. One effusion was from a patient diagnosed with FIGO stage II disease, 32 were diagnosed at stage III and 14 were at stage IV. Residual disease volume at primary operation was ≤1 cm in 16 cases, >1 cm in 26 cases, unknown in 5 cases (inoperable patients or not registered). Twenty effusions were pre-chemotherapy specimens obtained at diagnosis, whereas the remaining 27 were tapped at disease recurrence.

The 21 breast carcinomas were from patients aged 33–86 years (mean = 58 years). Five patients were diagnosed at TNM stage I, nine at stage II, four at stage III and three at stage IV. Twelve patients were operated and received no chemotherapy at that time, five were operated following neo-adjuvant chemotherapy and four were inoperable. Ten patients received anti-hormonal therapy and 10 did not (data unavailable for one patient). Radiation was applied in nine cases and was not used in nine (data unavailable for two patients).

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Table 1 Primers used in quantitative PCR analysis

Gene symbol	Forward primer	Reverse primer	Ref. seq. number
TFF1	GGCCCAGACAGAGACGTGTA	GGGACGTCGATGGTATTAGG	NM_003225
TFF3	CCTTGCTGTCCTCCAGCTCT	CAGGGATCCTGGAGTCAAAG	NM_003226
PITX1	GTCGTCTGACACGGAGCTG	AACTGCTGGCTTGTGAAGTG	NM_002653
CA12	GTGCTCCTGCTGGTGATCTT	AGGATGTCACTGTGCAGGTCT	NM_001218
TH	CCGTGCTAAACCTGCTCTTC	CGCACGAAGTACTCCAGGT	NM_000360
EFHD1	TCAAGGAGGTGGATGAGGAC	TTCTTGGCACCTTTGACACC	NM_025202
EFEMP1	GATATCCAGGAGGGCACTGA	CAGCAGGCTACGAGCAAAGT	NM_004105
TOB1	TTGATGATGTTCGTGGCAAT	TGGAGAGCTGGACACTGATG	NM_005749
RBP1	CTCATCACCCTCGATCCACT	GAGGAGGATCTGACAGGCAT	NM_002899
SPON1	CTGTCCACGGAAAATTCAGC	CCGTGAAAATGGAGGAAGAA	NM_006108
MFGE8	TGCTGTTATTCTTCAGGCCC	ACCTGTTTGAGACCCCTGTG	NM_005928
TSPAN12	AACGCCACAAGCCAGTTCTA	ATCCGGTCATGATTGCTGTT	NM_012338
APOL2	CTCACTCTCACACCAAGGCA	CGGAGGACGTGTCTGGTTAT	NM_145637
NRCAM	TTGTGAAACGTTGTGTGCAA	GAGGTGTCTAGCCCAGTGGA	NM_005010

Microarray expression and GeneChip analysis

RNA was prepared from tumour samples using a Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). Illumina HumanRef-8 BeadChip arrays were used to analyse gene expression in both tumours. The BeadChip includes ~24,500 well-annotated transcripts with up-to-date content derived from the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) database (Build 36.2, Release 22). RNA labelling, hybridization and scanning for the arrays were performed using the standard protocols in the JHMI Microarray Core. Treeview, developed by the Eisen group (http://rana.lbl.gov/eisen/) was used to perform unsupervised clustering analysis using all probes spotted on the HumanRef-8 BeadChip. Comparison of gene expression levels between the two cancer types was performed using both T-test and SAM analyses.

Quantitative real-time RT-PCR

Among the above-detailed differentially expressed genes, we selected 14 for validation using quantitative real-time PCR. These consisted of some genes that were overexpressed in breast carcinoma (*TFF1, TFF3*, *CA12*, *PITX1, TH, EHFD1, EFEMP1* and *TOB*) and some that were overexpressed in OC/PPC (*SPON1*, *RBP1*, *MFGE8*, *TM4SF12*, *APOL2* and *NRCAM*). Genes were chosen based on their potential relevance for ovarian or breast cancer biology and clinical behaviour, as judged by the two senior authors of this manuscript (BD and TLW). Quantitative real-time PCR was performed to determine gene expression levels in all OC/PPC and breast carcinomas using a protocol previously described [20]. Primers were designed to test the performance in quantitative real-time PCR and those generating robust and specific PCR products without detectable primer dimers were selected for analysis (Table 1). Approximately 16–100 ng of cDNA was included in the real-time PCR, which was performed using an iCycler. Threshold cycle numbers were obtained using the iCycler Optical system interface software (Bio-Rad Lab, Hercules, CA, USA). Averages in the threshold cycle number (Ct) of duplicate measurements were obtained. The results were expressed as the difference between the Ct of the gene of interest and the Ct of a control gene (APP) for which expression is relatively constant among previously analysed SAGE libraries. In cases where no gene expression was observed, a cutoff Ct value of 45 cycles was used.

Immunohistochemistry

The choice of genes chosen for validation using IHC was based on the availability of antibodies with adequate performance in formalin-fixed paraffin-embedded material, as well as on biological relevance. Protein expression of matrix metalloproteinase-7 (MMP-7), trefoil factor 1 (TFF1), trefoil factor 3 (TFF3), forkhead box A1 (FOXA1) and carbonic anhydrase XII (CA XII) was analysed.

The mouse monoclonal MMP-7 antibody (Novus Biologicals, Littleton, CO, USA) was applied 1:100 with pre-treatment in Tris/EDTA buffer. Rabbit polyclonal TFF1 and CA XII antibodies (Atlas Antibodies, Stockholm, Sweden) were applied 1:200 with pre-treatment in Citrate buffer and 1:100 with pre-treatment in

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Tris/EDTA buffer, respectively. The mouse monoclonal TFF3 and rabbit polyclonal FOXA1 antibodies (Abcam, Cambridge, UK) were applied 1:100 with pre-treatment in Citrate and Tris/EDTA buffer, respectively. IHC was performed using the EnVision^{TM+} + peroxidase system (Dako, Glostrup, Denmark). Appropriate positive and negative controls were used.

The extent of staining was scored by an experienced cytopathologist (BD); cases were grouped according to the percentage of immunoreactive tumour cells as 0%, 1–5%, 6–25%, 26–75% and 76–100%. This scoring system was applied because we have used it in our previous publications dealing with breast and ovarian carcinoma effusions, thereby simplifying future studies of the association with other cancer-associated proteins.

Cytoplasmic staining was scored as positive for MMP7, TFF1 and TFF3. CA XII staining was scored at the membrane and cytoplasm. For FOXA1, intense nuclear staining was regarded as positive. No specimen contained less than 100 tumour cells. Differences in protein expression between breast carcinoma and OC/PPC cases were analysed by Mann– Whitney U-test using the SPSS program (version 15.0, Chicago, IL, USA).

Results

Unsupervised hierarchical clustering distinguishes OC/PPC and breast carcinoma as separate groups

All OC/PPC effusions clustered under one branch, whereas seven of eight breast carcinoma effusions clustered under a separate branch. The remaining breast carcinoma clustered under a major branch (Fig. 1). Supervised analysis was performed to identify genes with the highest difference in expression between the breast carcinoma and OC/PPC groups. Using T-test with a cutoff ratio of at least a 3.5-fold difference between these two tumour types ($P < 0.05$), we identified 288 genes that were differentially expressed, of which 81 and 207 were overexpressed in breast carcinoma and OC/PPC, respectively. In addition, we used SAM analysis to identify differentially expressed genes. Using this analysis, 1078 differentially expressed genes with false discovery rate less than 0.05 were identified (Fig. 2). Genes overexpressed in breast carcinoma included *TFF1, TFF3*, *FOXA1*, *CA12*, *GATA3*, *SDC1*, *PITX1, TH, EHFD1, EFEMP1, TOB1* and *KLF2*. Genes overexpressed in OC/PPC included *SPON1*, *RBP1*, *MFGE8*, *TM4SF12*, *MMP7*, *KLK5/6/7*, *FOLR1/3*, *NRCAM*, *APOL2*, *WT1*, *MSLN* and *PAX8*. The full gene list is available at: https://jshare.johnshopkins.edu/twang16/Norway%20Ascites/.

The cluster and graph based on 1078 genes identified by SAM analysis are shown in Fig. 2A and B.

Validation experiments

Expression levels of the 14 selected transcripts were analysed using hierarchical clustering. As shown in Fig. 3, the levels of OC/PPC markers were significantly higher in OC/PPC samples than in breast carcinoma samples ($P < 0.005$). Similarly, the levels of breast carcinoma markers were higher in breast carcinoma specimens than in OC/PPC specimens, with *TFF1* and *TFF3* having the most significant *P*-value ($P < 0.005$).

Analysis by IHC of five proteins confirmed the array findings for all five genes, revealing statistically significant differences between OC/PPC and breast carcinoma effusions in the expression of all five analysed proteins (Fig. 4, Table 2). Analysis of the association between CA12, TFF1, TFF3 and FOXA1 protein expression and receptor status in the effusion specimens did not show significant differences between hormone receptor-positive and receptor-negative patients (data not shown), although these data were regarded as preliminary because of sample size.

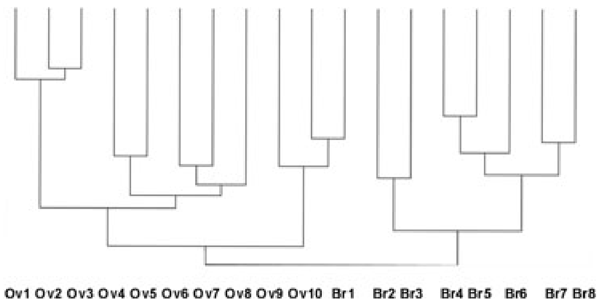

Fig. 1 Unsupervised cluster analysis of gene expression profiling of ovarian/primary peritoneal serous carcinoma (OC/PPC) and breast carcinoma. Based on the analysis of all probes available on the HumanRef-8 BeadChip, all 10 OC/PPCs cluster close to each other and are distant from the breast carcinomas.

Discussion

Differentiation of OC/PPC from breast carcinoma has obvious diagnostic, therapeutic and prognostic implications. To date, only a few markers have been evaluated for their ability to differentiate OC/PPC from breast carcinoma. WT-1, the Wilms tumour gene product, has been shown to be frequently expressed in serous OC/PPC, with rare expression in breast carcinoma [21–23]. Gross cystic disease fluid protein-15 (GCDFP-15) and mammaglobin are highly specific markers of breast carcinoma in this differential diagnosis, but have variable sensitivity [22–27]. Two additional markers of OC/PPC were recently described. Mesothelin was reported to be a specific marker for ovarian and uterine serous carcinomas. However, staining was focal in many specimens [27]. PAX8 was shown to stain 108/124 OC/PPC, predominantly of the serous type, whereas absent in 243 breast carcinomas [28].

In this study, which we believe to be first to compare the gene expression profile of metastatic serous OC/PPC and breast carcinoma, we observed that specimens from these two cancers cluster separately and identified a large number of genes that are

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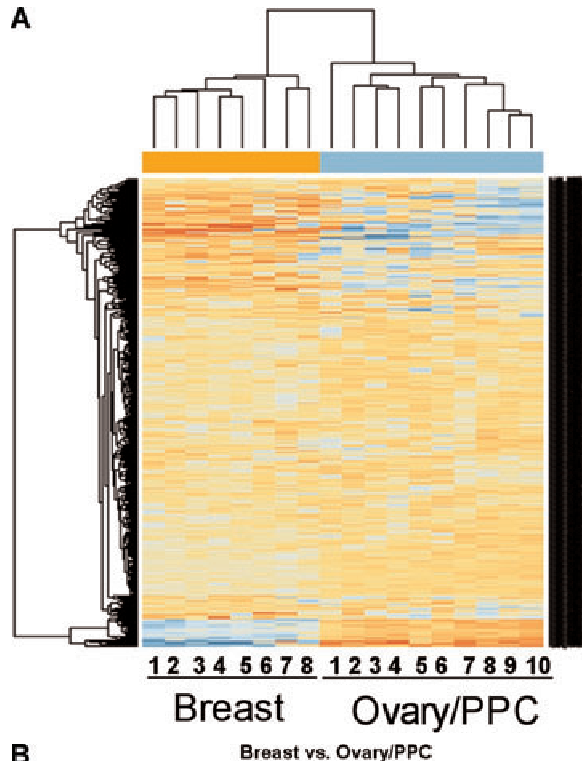

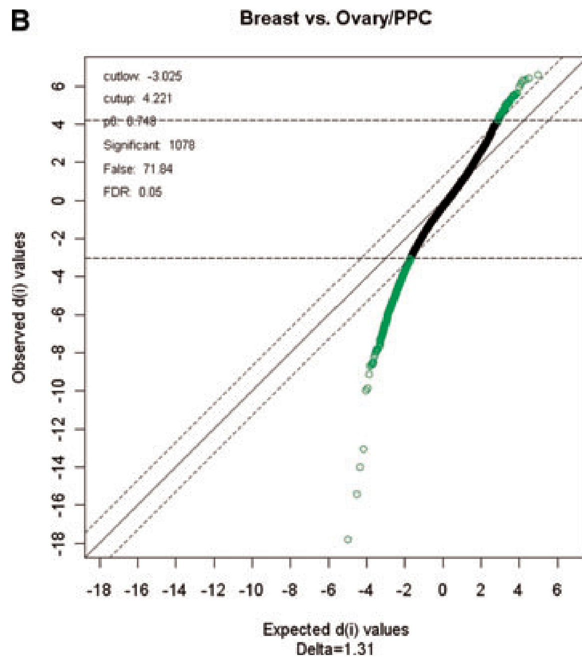

Fig. 2 (**A**) Cluster analysis shows two distinct groups of samples (horizontal tree) corresponding to breast carcinoma (8 specimens) and OC/PPC (10 specimens). The dendrogram of the vertical tree demonstrates a total of 1078 genes that are preferentially expressed in either the breast carcinoma group or the OC/PPC group. The expression levels are expressed by the increased gradient of orange-to-red intensity. (**B**) SAM analysis demonstrated 1078 differentially expressed genes at 5% FDR cutoff between OC/PPC and breast carcinomas

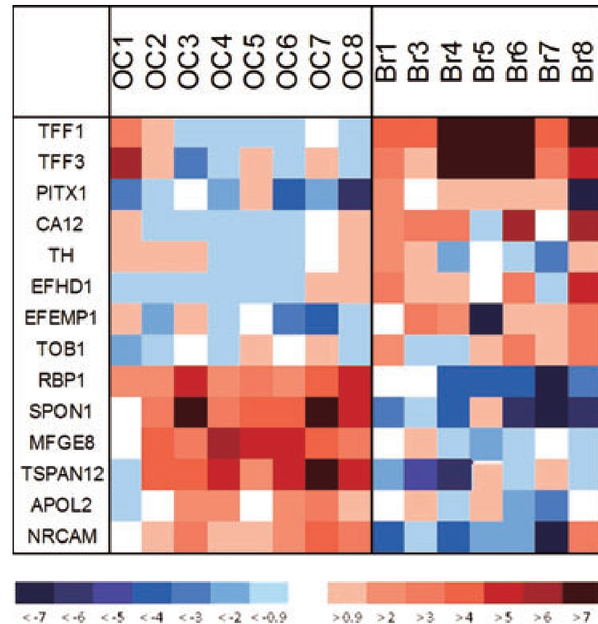

Fig. 3 Quantitative real-time PCR was performed to validate 14 differentially expressed genes. The expression level of each gene in individual specimen is shown as a pseudo-colour gradient based on the relative expression level of a given gene to the average value derived from the control gene, APP. The first eight genes are more highly expressed in breast carcinoma, whereas the remaining six are more highly expressed in OC/PPC.

differentially expressed in OC/PPC and breast carcinoma. One of the breast carcinomas clustered separately from the remaining seven effusions, possibly because of differences in the reactive cell content of this effusion (50% tumour cell population *versus* 80–100% in the latter seven specimens). Interestingly, the short gene list (see https://jshare.johnshopkins.edu/twang16/Norway%20Ascites/) included the genes for WT-1, mesothelin and PAX8, but not those for GCDFP-15 and mammaglobin. In addition, the list of genes overexpressed in OC/PPC included several genes that code for proteins that we have previously shown to be overexpressed in OC/PPC compared to breast carcinoma, including kallikreins [29], folate receptors [30] and IGF-2 and IGFBP-3, which are part of the insulin growth factor signalling pathway [31]. Consequently, we chose to validate more breast cancer-specific than OC/PPC-specific genes in this study.

Among genes that were overexpressed in breast carcinoma, we focused on *TFF1*, *TFF3*, *PITX1*, *CA12*, *TH*, *EHFD1*, *EFEMP1*, *FOXA1* and *TOB1*, and confirmed the array data using qPCR and/or IHC. *TFF1* and *TFF3* are members of the trefoil factor family, small (11–22 kD) proteins that contain trefoil domains with cysteine residues and disulphide bridges, and which are involved in epithelial protection and restitution of mucous membranes, predominantly in the gastrointestinal tract [32]. TFF1 and TFF3 expression was previously reported in breast cancer [33], and TFF1 was shown to be regulated by estrogen in breast cancer cell lines [34]. Detection of TFF1 mRNA in the bone marrow of breast

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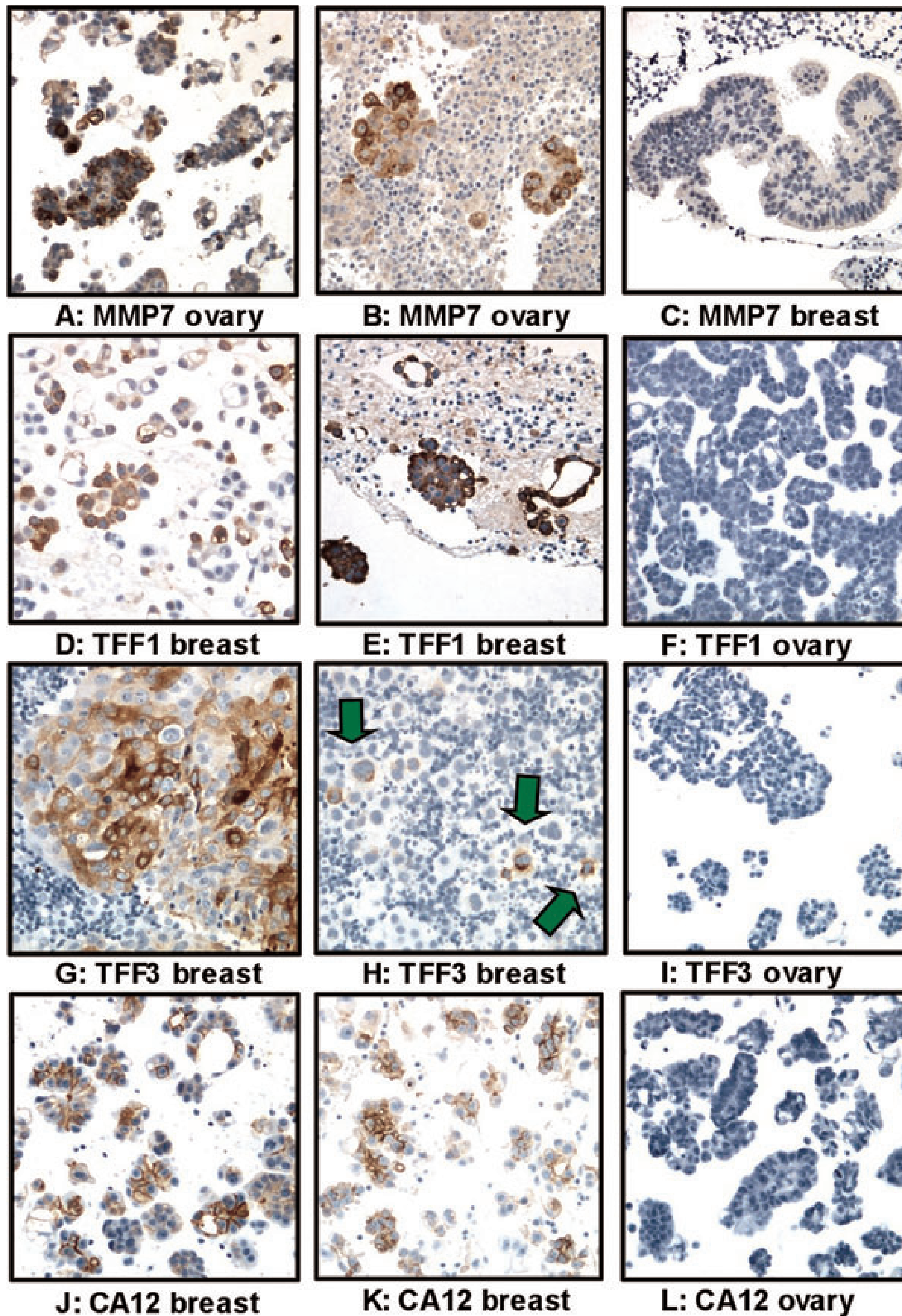

Fig. 4 Immunohistochemistry analysis using MMP7, TFF1, TFF3, CA12 and FOXA1 as markers for the differentiation of OC/PPC from breast carcinoma. MMP7: MMP7 (cytoplasmic immunoreactivity) expression in two OC/PPC effusions (**A**, **B**), but no expression in breast carcinoma (**C**). TFF1: TFF1 expression in two breast carcinoma effusions (**D**, **E**), but no expression in OC/PPC (**F**). Staining is cytoplasmic. TFF3: TFF3 immunoreactivity (cytoplasmic staining) in two breast carcinoma effusions, one with large cohesive cell groups (**G**), the other with numerous single tumour cells (**H**; arrows). A TFF3-negative OC/PPC specimen is shown in (**I**). CA12: Tumour cells in two breast carcinoma effusions express CA12 (**J**, **K**). OC/PPC cells (**L**) are negative. FOXA1: Tumour cells in two breast carcinoma effusions express FOXA1 at the nucleus (**M**, **N**). OC/PPC cells (**O**) are negative.

cancer patients was recently shown to be associated with a high risk of relapse [35]. Pituitary homeobox 1 (PITX1) is involved in embryonic development of the pituitary, the hind limb and the brancial arches, but has additionally been shown to negatively regulate the Ras oncogene [36]. Its expression is reduced in various cancers (e.g. lung carcinoma) compared to corresponding normal tissue [37]. The CA family consists of 14 zinc metalloenzymes, of which 11 have CA activity, facilitating the transport of CO_2 and protons in the intracellular and extracellular space [38]. Two of the membrane-associated members of the family, CA IX and CA XII, are cancer-associated proteins that are considered to be potential therapeutic targets [38]. CA XII is regulated by estrogen *in vitro*

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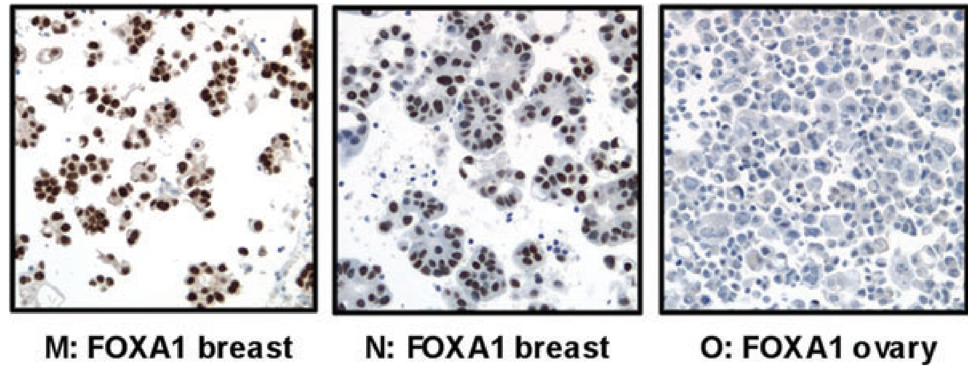

Fig. 4 Continued.

Table 2 Immunohistochemistry results

Diagnosis	**MMP7 staining extent**					
	0%	1–5%	6–25%	26–75%	76–100%	*P*-value
Ovarian AC (47)	28 (60%)	12 (26%)	4 (8%)	3 (6%)	0 (0%)	0.034
Breast AC (29)	24 (83%)	4 (14%)	0 (0%)	0 (0%)	1 (3%)	
Diagnosis	**TFF1 staining extent**					
	0%	1–5%	6–25%	26–75%	76–100%	*P*-value
Ovarian AC (47)	46 (98%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	$<$0.001
Breast AC (29)	9 (31%)	6 (21%)	2 (7%)	7 (24%)	5 (17%)	
Diagnosis	**TFF3 staining extent**					
	0%	1–5%	6–25%	26–75%	76–100%	*P*-value
Ovarian AC (47)	41 (87%)	4 (9%)	1 (2%)	1 (2%)	0 (0%)	$<$0.001
Breast AC (29)	8 (28%)	4 (14%)	3 (10%)	5 (17%)	9 (31%)	
Diagnosis	**CA XII staining extent**					
	0%	1–5%	6–25%	26–75%	76–100%	*P*-value
Ovarian AC (47)	24 (51%)	18 (38%)	4 (9%)	1 (2%)	0 (0%)	$<$0.001
Breast AC (29)	5 (17%)	4 (14%)	5 (17%)	8 (28%)	7 (24%)	
Diagnosis	**FOXA1 staining extent**					
	0%	1–5%	6–25%	26–75%	76–100%	*P*-value
Ovarian AC (47)	45 (96%)	0 (0%)	0 (0%)	1 (2%)	1 (2%)	$<$0.001
Breast AC (29)	6 (21%)	0 (0%)	2 (7%)	0 (0%)	21 (72%)	

[39], and its expression in clinical specimens is associated with low histological grade, estrogen receptor expression and lower risk of relapse [40]. EF-hand domain family member 1 (EFHD1) is an EF-hand domain-containing protein that is increasingly expressed during neuronal differentiation [41] and was shown to be regulated by the transcription factor hepatocyte nuclear factor 4α in renal cell carcinoma [42]. Its expression in breast carcinoma has not been studied to date. The EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), also known as fibulin 3, is an extracellular matrix protein that mediates cell–cell and cell–matrix communication. Fibulin 3 inhibits angiogenesis and its expression is reduced in different tumours, including both ovarian and breast carcinomas [43]. Its downregulation in breast carcinoma was recently shown to occur through promoter methylation [44]. Our

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group previously found that the *EFEMP1* gene is overexpressed in peritoneal mesothelioma compared to OC/PPC [45], suggesting that OC/PPC is the strongest repressor of EFEMP1 expression among cancers affecting the serosal cavities. TOB1 is member of the Btg/Tob family, proteins that inhibit cell proliferation, and is downregulated and inactivated through phosphorylation in several cancers [46]. In breast cancer, c-ErbB2 binds TOB1, a cellular interaction inhibiting its anti-proliferative effect [47]. Higher TOB1 mRNA expression was recently shown to correlate with shorter distant metastasis-free survival in breast carcinoma, and TOB1 protein phosphorylation was associated with EGF and EGFR expression and with higher proliferation [48].

Among genes that were overexpressed in OC/PPC, *SPON1*, *RBP1*, *MFGE8*, *TM4SF12*, *APOL2*, *NRCAM* and *MMP7* were chosen for further validation. Spon1, also known as F-spondin, is part of the thrombospondin type-1 receptor family, and is involved in neuronal differentiation and regulation of angiogenesis [49]. Spon1 is highly expressed in OC [50], particularly of the high-grade serous type [51]. Cellular retinol-binding protein 1 (RBP1) binds retinoic acid, a major effector of differentiation and apoptosis, thereby regulating its activity [52]. RBP1 expression was found to be reduced in OC/PPC cell lines compared to ovarian surface epithelium [53], but its clinical and diagnostic role in this tumour has not been established to date. Milk fat globule-EGF factor 8 protein (MFGE8), also known as SED1, lactadherin and BA46, is a 53 kD protein initially isolated from the apical part of mammary epithelial cells during lactation. Its main physiological roles are removal of apoptotic debris and maintenance of epithelium in various organs. However, MFGE8 contains EGF repeats, the second of which includes an RGD integrin-binding motif involved in binding of $\alpha v\beta 3/5$ integrins and mediation of adhesion and integrin-related signal transduction [54]. MFGE8 blockage leads to destruction of tumour cells in a mouse model [55]. Although MFGE8 is expressed at high levels in breast carcinoma [56], we unexpectedly found still higher levels of this gene in OC/PPC, a finding that has not been reported to date and merits further research. TM4SF12 (tetraspanin 12; TSPAN12), is member of the Tetraspanin family, transmembrane molecules that interact with other membrane proteins, including immunoglobulin superfamily members, integrins and claudins, as well as intracellular signalling proteins. Tetraspanins are involved in a myriad of physiological and pathological processes, including cancer, and both tumour suppressor and tumour promoter roles have been reported [57]. Tetraspanin 12 has not been studied in OC to date. However, two other tetraspanins, KAI1 and CD9, have been shown to be inhibitors of integrin expression and function in OC, thereby negatively regulating metastasis and proliferation [58, 59]. APOL2 has not been studied in OC to date, but we previously reported on the biological role of APOE, another apolipoprotein family member, in OC [60]. Nr-CAM, member of the CAM family, is an adhesion molecule that is normally expressed only in neuronal cells, and may be involved in neurite growth [61]. However, Nr-CAM is expressed in gliomas [62], as well as in non-neuronal tumours, for example pancreas and thyroid carcinoma [63, 64]. Its expression in OC/PPC is of interest in view of our previous observation that expression of another neuron-associated molecule, the nerve growth factor receptor TrkA, is associated with poor survival in OC/PPC [65]. We previously showed that *MMP7*, a member of the matrix metalloproteinase family, is overexpressed in OC/PPC compared to peritoneal mesothelioma [45], and this protein was recently suggested to be as an early diagnosis serum marker in OC [66, 67].

In conclusion, gene expression array analysis identified many genes that are differentially expressed in OC/PPC and breast carcinoma effusions. This may aid in the diagnostic setting and provide new potential candidates for prognostic and therapeutic characterization of these tumours. Focusing on metastatic disease may aid patients with advanced or recurrent disease who critically depend on biological approaches for prolonging survival.

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