Overexpression of the Cell Adhesion Molecules DDR1, Claudin 3, and Ep-CAM in Metaplastic Ovarian Epithelium and Ovarian Cancer

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ABSTRACT

Purpose: **A better understanding of the molecular pathways underlying the development of epithelial ovarian cancer (EOC) is critical to identify ovarian tumor markers for use in diagnostic or therapeutic applications. The aims of this study were to integrate the results from 14 transcript profiling studies of EOC to identify novel biomarkers and to examine their expression in early and late stages of the disease.**

Experimental Design: **A database incorporating genes identified as being highly up-regulated in each study was constructed. Candidate tumor markers were selected from genes that overlapped between studies and by evidence of surface membrane or secreted expression. The expression patterns of three integral membrane proteins, discoidin domain receptor 1 (DDR1), claudin 3 (CLDN3), and epithelial cell adhesion molecule, all of which are involved in cell adhesion, were evaluated in a cohort of 158 primary EOC using immunohistochemistry.**

Results: **We confirmed that these genes are highly overexpressed in all histological subtypes of EOC compared with normal ovarian surface epithelium, identifying DDR1 and CLDN3 as new biomarkers of EOC. Furthermore, we determined that these genes are also expressed in ovarian epithelial inclusion cysts, a site of metaplastic changes within the normal ovary, in borderline tumors and in low-grade and stage cancer. A trend toward an association between low CLDN3 expression and poor patient outcome was also observed.**

Conclusions: **These results suggest that up-regulation of DDR1, CLDN3, and epithelial cell adhesion molecule are early events in the development of EOC and have potential application in the early detection of disease.**

INTRODUCTION

The most common malignant tumors arising from the ovary are epithelial ovarian cancers (EOC). Thought to arise from ovarian surface epithelium (OSE), EOC exhibits different histological phenotypes that appear to be genetically and biologically distinct diseases (1). EOC is the fifth most common cause of death from all cancers occurring in women and the leading cause of death from gynecological malignancies (2). Over 75% of women present with locally advanced or disseminated disease, typically characterized by a gradual invasion of the surrounding organs. Despite aggressive treatment, the 5-year survival rate is only 30–50% (2). This poor overall prognosis results from a lack of early symptoms and early diagnosis, ineffective therapy for advanced disease, and from limited understanding of the early-initiating events and early stages of ovarian cancer development.

A major challenge in ovarian cancer research remains the need to identify tumor markers to aid in diagnosis, as prognostic indicators and as targets for new therapeutic strategies. The only currently available clinical marker, CA125, can predict the persistence of ovarian cancer with >95% accuracy; however, it lacks specificity and sensitivity for the initial diagnosis of disease (3).

A deeper understanding of the genetic pathways underlying development and progression is critical to new tumor marker identification. To this end, a number of research groups have applied mRNA expression profiling techniques to identify genes that are abnormally regulated in EOC (4–16).[6] However, there is a need to compare the results from individual studies to determine differentially expressed genes free of artifacts due to

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[6] S. M. Henshall, *et al.*, unpublished data.

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Table 1 EOC transcription profiling studies included in the OLOV database[a]

Authors	Method	No. of genes queried	No. of genes up-regulated	Tissue/Cell source	Ref.
Henshall *et al.*[6]	Oligonucleotide microarray	59,618	271	51 EOC; 4 normal ovaries	Unpublished
Schummer *et al.*	cDNA array	21,500	134	10 EOC; 6 normal ovaries	4
Wang *et al.*	cDNA array	5,718	295	7 EOC	5
Hough *et al.*	SAGE	56,000	45	3 EOC; 10 EOC cell lines; 1 HOSE	6
Ismail *et al.*	cDNA array	255	16	10 EOC cell lines; 5 HOSE	7
Martoglio *et al.*	cDNA array	332	33	4 SOC; 5 normal ovaries	8
Ono *et al.*	cDNA array	9,121	55	9 EOC; 9 benign EOC	9
Shridhar *et al.*	cDNA array	25,000	16	14 EOC	10
Tapper *et al.*	cDNA array	588	38	6 SOC; 1 serous cystadenoma	11
Tonin *et al.*	Oligonucleotide microarray	6,416	1,815	4 EOC cell lines; 1EOC; 1 normal OSE	12
Welsh *et al.*	Oligonucleotide microarray	6,000	1,243	27 EOC; 3 normal ovaries	13
Bayani *et al.*	cDNA array	1,718	194	3 EOC; 1 normal ovary	14
Matai *et al.*	Oligonucleotide microarray	12,600	111	21 EOC; 9 HOSE	15
Sawiris *et al.*	cDNA array	516	25	11 SOC; 7 EOC cell lines	16

[a] EOC, epithelial ovarian cancer; OLOV, overlapping ovarian; SAGE, serial analysis of gene expression; HOSE, human ovarian surface epithelial cells.

differences in protocols, microarray platforms, and analysis techniques. Our approach has been to devise a method based on an automated overlapping ovarian (OLOV) database to integrate and intervalidate the results of our own oligonucleotide microarray-based transcription profiling study of 51 primary ovarian tumors with 13 published expression profiling studies of EOC.

We identified three adhesion molecules that were overexpressed in at least two studies and validated these results by immunohistochemistry using tissue microarrays of a large cohort of 158 ovarian tumors of varying histological subtypes. The selected molecules were the discoidin domain receptor 1 (DDR1), a tyrosine receptor kinase activated by collagen and involved in cell-matrix communication (17), claudin 3 (CLDN3), a component of epithelial cell tight junctions, which are critical to the maintenance of cell polarity and permeability (18), and the epithelial cell adhesion molecule (Ep-CAM), a member of the CAM family, which includes the epithelial-specific cadherin, E-cadherin (CDH1; Ref. 19).

In addition, we evaluated their expression pattern in early- and late-stage disease, in borderline or low malignant potential tumors (BL), in OSE, and in inclusion cysts (IC), which contain small foci of serous metaplastic epithelium that are frequently found in the cortex of normal ovaries and proposed as the site of EOC initiation (20). The expression of these genes was then correlated to patient outcome using clinicopathological follow-up data for each patient. Finally, we compared the expression pattern of these markers to that of other biomarkers of EOC: CA125; mucin 1 (MUC1), which is highly up-regulated in all adenocarcinomas (21), and CDH1, a marker of metaplastic changes in ovarian epithelium, which is associated with invasion and metastasis in diverse human cancers (20, 22).

MATERIALS AND METHODS

Tissue and Clinicopathological Data. Tissue specimens (fresh/frozen and formalin-fixed, paraffin-embedded samples) were collected from patients undergoing primary laparotomy at the Gynaecological Cancer Centre, Royal Hospital for Women, Sydney, Australia, following informed consent. Clinical, pathology and outcome data on each patient were collected and archived. All experimental procedures were approved by the Research Ethics Committee of the Sydney South East Area Hospital (00/115).

Transcription Profiling. Expression profiling was performed on a cohort of 51 epithelial ovarian tumors, comprising 8 endometrioid ovarian cancers, 4 mucinous ovarian cancers, and 31 serous ovarian cancers (including 12 corresponding omental deposits), 8 BL tumors, and 4 ovaries removed for benign conditions (normal). The histopathological diagnosis of each tissue sample was independently reconfirmed by a second pathologist (J. Kench and L. Edwards) on H&E-stained sections of fresh frozen tissue. Only those tumor samples containing >75% of BL or invasive cancer were used for transcript profiling. Total RNA was extracted and transcription profiling performed as previously described (23) using the Eos Hu03, a customized Affymetrix GeneChip oligonucleotide microarray (Affymetrix, Santa Clara, CA) containing >59,000 probe sets for the interrogation of ~46,000 unique sequences (Protein Design Lab, San Francisco, CA). After normalization of the data (23), we used a ranked penalized t-statistic with P values adjusted for multiple testing using the Holm procedure (LIMMA package in Bioconductor)[7] to identify 271 up-regulated and 184 down-regulated genes in EOC compared with gene expression in the normal ovaries ($P \leq 0.01$).

Construction of the Database OLOV. The OLOV database was designed using Microsoft Access software to identify genes that overlapped in our own and at least one other published transcription profiling study. Genes identified in our study as being up-regulated ($n = 271$) were entered in the database, along with genes from 13 previously published gene expression profiling studies of EOC (Table 1). Genes from published studies only included those that were published as a list in the reference article or were available as supplementary material on a linked web site. Each gene was entered as an individual entry into the database. To ensure that the database

[7] Internet address: http://bioconductor.org.

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would have the capacity to identify overlapping genes, it was necessary to enter all known designated identifiers for each gene sourced from online gene database tools, including the National Center for Biotechnology Information suite,[8] Gene Cards,[9] and SOURCE,[10] including gene name(s) and symbol(s), nucleotide accession number(s), and Unigene cluster. In addition, information on putative function, cellular localization, chromosomal location, and normal body expression, where available, was entered into the database. This information could not be included for uncharacterized or hypothetical genes, including expression sequence tags, precluding their selection as potential tumor markers in this study. An interface and data entry/retrieval form was included to administer the database and to run specific queries. Using independent database queries in OLOV, we identified nucleotide sequence accession numbers, Unigene clusters, or gene symbols that overlapped with genes overexpressed in our transcription profiling study.

Immunohistochemistry. Archival tissue from 158 tumors removed at primary laparotomy (34 BL and 124 EOC) and 12 normal ovaries, removed during surgery for benign conditions, were included in the cohort. H&E-stained sections of each sample were reviewed by two pathologists (J. Scurry and R. Scolyer) and areas corresponding to tumor tissue marked. Tissue core biopsies of 1.0 or 2.0 mm were incorporated into medium-density tissue microarrays (Beecher Instruments, Silver Spring, MD). Each patient was represented by two to five cores sampled from different areas of the tumor. Sections from each array were stained with H&E to confirm the inclusion of tumor tissue in each core, and cores containing no tumor were excluded from the study.

Four-μm sections were mounted on Superfrost Plus adhesion slides (Lomb Scientific, Sydney, Australia) and heated in a convection oven at 75°C for 2 h to promote adherence. Sections were dewaxed and rehydrated according to standard protocols, followed by an antigen unmasking procedure, either EDTA/citrate buffer (DDR1 and CA125), high pH target retrieval solution (DAKO Corporation, Carpinteria, CA) (CLDN3), low pH target retrieval solution (DAKO) (CDH1) or proteinase K digestion (DAKO) (Ep-CAM, MUC1). The following primary antibodies were used: anti-CA125 (1:50; Abcam Limited, Cambridge, United Kingdom); anti-MUC1 (1:1000; Abcam); anti-CDH1 (1:200; DAKO); anti-DDR1 (C-20, 1:30; Santa Cruz Biotechnology, Santa Cruz, CA); anti-Ep-CAM (1:5; Abcam); and anti-CLDN3 (1:100, ZYMED Laboratories, South San Francisco, CA). Bound antibody was detected using DAKO LINK/LABEL or LINK/EnVision using 3,3′-diaminobenzidine Plus (DAKO) as a substrate. Negative controls omitted the primary antibody, and a positive and negative control tissue for each antibody was identified from electronic Northern blot data[9] or the published literature. Counterstaining was performed with hematoxylin and 1% acid alcohol. Immunostaining was scored by the percentage of cells staining (0–100% of cells stained within one core). Scoring was independently assessed by a gynecologist (V. Heinzelmann-Schwarz) and a gynecological pathologist (J. Scurry and R. Scolyer), and discrepancies were resolved by consensus. The results from all cores from one patient were averaged. Differences in protein expression were determined using a Mann-Whitney U test. P of <0.05 was required for significance. All statistical analyses were performed using Statview 4.5 software (Abacus Systems, Berkeley, CA).

Association between Gene Expression in EOC and Patient Outcome. The immunohistochemistry results were correlated to patient outcome using comprehensive clinical follow-up data for each patient. Patients with BL tumors and those who had died of reasons unrelated to their malignancy were excluded from the survival analyses (total, $n = 115$; Table 3). Relapse-free time was measured from the date of diagnosis to the date of last follow-up (for disease-free patients) or to disease relapse, defined as either reappearance of clinical symptoms or a rising serum CA125 level. Patients with progressive disease were excluded from the relapse-free survival analysis. The length of survival was defined from the date of diagnosis to the date of patient death or the most recent follow-up date. An association between clinicopathological parameters, gene expression, and outcome was determined using a Kaplan-Meier analysis and a Cox proportional hazards model. For continuous variables, we used interquartile range comparing 25th and 75th percentile expression values to define hazard ratios.

RESULTS

Identification of Up-Regulated Genes Overexpressed in EOC. Using OLOV, we identified 69 genes common to our study and at least one other (Table 2). Several of these have been previously shown to be up-regulated in EOC, including *MUC1* (21) and *Ep-CAM* (19), both identified in six transcription profiling studies: *HE4* (Ref. 24, overlapping in eight studies) and osteopontin (*SSP1*; Ref. 25, overlapping in four studies), which are currently under investigation as potential secreted biomarkers of EOC (24, 25); and *HER3* (26) and *SLPI* (Ref. 27, overlapping in three studies). None of the other genes in this list are known to be involved in EOC. However, several of the genes have been associated with carcinogenesis, *e.g.,* the *Src* family tyrosine kinase *LYN* (28), *LLGL2*, the human homologue of the *Drosophila* lethal giant larvae tumor suppressor gene (29), *BCL2* (30), *MEST*/*PEG1*, an imprinted gene involved in progression of breast cancer (31), *ELF3* (*ESX*), an epithelial-specific transcription factor (32), *KLF5*, a transcription factor and putative tumor suppressor gene (33, 34), and *RGS19* and its inhibitor *RGS19IP1* (*GIPC1*), which are involved in G-protein signaling (35). The ovarian tumor marker *CA125* was noticeably absent from this list of genes. Although there was a trend to higher expression in EOC (Fig. 1), this was not statistically significant at the 1% level and was therefore not included in the overexpressed genes entered into the database.

Selection of Candidate Tumor Markers. We used the OLOV database to search for specific structural and functional characteristics to identify gene products with potential as cell-surface therapeutic targets or serum biomarkers of EOC. From the list of 69 overlapping genes, we identified genes that fulfilled the following criteria: a low P value (EOC *versus* normal ovary) in our transcription profiling study; predicted to be

[8] Internet address: http://www.ncbi.nlm.nih.gov.
[9] Internet address: http://www.bioinformatics.weizmann.ac.il/cards.
[10] Internet address: http://www5.stanford.edu.

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Table 2 Genes overlapping between our own study and other published expression profiling studies of EOC ($n = 69$)[a]
All listed genes have P values $\leq$ 0.0001 in our study.

No. of overlaps	Symbol	GenBank accession no.	Cellular localization	Chromosome
8	*HE4*	NM_006103	SEC	20q13.12
6[b]	*Ep-CAM*	NM_002354	MB	2p21
6[b]	*MUC1*	NM_182741	MB	1q21
5	*KRT8*	NM_002273	CYT	12q13
4	*CRIP1*	NM_001311	CYT	7q11.23
4	*OPN*	NM_000582	SEC	4q21-q25
4	*SPINT2*	NM_021102	MB	19q13.1
4	*S100A11*	NM_005620	N/CYT	1q21
4	*KIAA0101*	NM_014736	NK	15q22.1
4	*CKS2*	NM_001827	N	9q22
4	*CD9*	NM_001769	MB	12p13.3
3	*SLC25A5*	NM_001152	MIT	Xq24-q26
3	*KRT19*	NM_002276	CYT	17q21.2
3[b]	*CLDN3*	NM_001306	MB	7q11.23
3	*GAPD*	NM_002046	CYT	12p13
3	*TPX2*	NM_012112	N	20q11.2
3	*KLF5*	NM_001730	N	13q21-q22
3	*HER3*	NM_001982	MB	12q13.3
3	*CPSF3*	NM_016207	N	2p25.2
3	*CXADR*	NM_001338	MB	21q21.1
3	*SLPI*	NM_003064	SEC	20q12
3	*SLC2A1*	NM_006516	MB	1p35-p31.3
3	*NME1*	NM_000269	N/CYT	17q21.3
3	*IFI30*	NM_006332	LYS	19p13.1
3	*UBCH10*	NM_007019	N/CYT	20q13.12
3	*CD24*	NM_013230	MB	6q21
3	*IFI27*	NM_005532	MB	14q32
3	*APRT*	NM_000485	CYT	16q24.3
3	*PTPRF*	NM_002840	MB	1p34
2	*MYL6*	NM_079425	CYT	12q13.13
2	*VAMP8*	NM_003761	GA	2p12–11.2
2	*DSP*	NM_004415	MB	6p24
2	*MELK*	NM_014791	N	9p13.1
2	*LISCH7*	NM_015925	N	19q13.13
2	*UCP2*	NM_003355	MIT	11q13
2	*BCL2*	NM_000633	MIT/N/ER	18q21.33
2	*TRIM26*	NM_003449	CYT	6p21.3
2	*SPINT1*	NM_181642	SEC	15q14
2	*RGS19*	NM_005873	MB	20q13.3
2	*COX7B*	NM_001866	MIT	Xq13.3
2	*CYCS*	NM_018947	MIT	7p15.2
2	*TPI1*	NM_000365	CYT	12p13
2	*ELF3*	NM_004433	N	1q32.2
2	*RGS19IP*	NM_005716	MB/CYT	19p13.1
2	*HSPE1*	NM_002157	MIT	2q33.1
2	*YWHAZ*	NM_145690	CYT	8q23.1
2	*MEST*	NM_002402	CYT/MIT	7q32
2	*ARF1*	NM_001658	GA	1q42
2	*CNNB2*	NM_004701	N	15q21.3
2	*KPNA2*	NM_002266	N/CYT	17q23.1
2[b]	*DDR1*	NM_013994	MB	6p21.3
2	*MTHFD2*	NM_006636	MIT	2p13.1
2	*FLJ20171*	NM_017697	NK	8q22.1
2	*MAL2*	NM_052886	ER	8q24.12
2	*HERC1*	NM_003922	GA	15q22
2	*HM13*	NM_178582	ER	20q11.21
2	*COX7A2*	NM_001865	MIT	6q12
2	*CTSC*	NM_001814	LYS	11q14.1-q14.3
2	*SEC61A1*	NM_013336	ER	3q21.3
2	*LLGL2*	NM_004524	MB	17q24-q25
2	*GUK1*	NM_000858	MIT	1q32-q41
2	*SYNGR2*	NM_004710	MB	17q25.3
2	*ARPC1B*	NM_005720	CYT	7q22.1
2	*FABP5*	NM_001444	CYT	8q21.13
2	*PFN1*	NM_005022	CYT	17p13.3
2	*ATP5G3*	NM_001689	MIT	2q31.2
2	*MGC14697*	NM_032747	CYT	10q24.33
2	*ARHE*	NM_005168	CYT	2q23.3
2	*LYN*	NM_002350	N/CYT	8q13

[a] EOC, epithelial ovarian cancer; SEC, secreted; MB, surface membrane; CYT, cytoplasm; N, nucleus; NK, not known; MIT, mitochondria; ER, endoplasmic reticulum; GA, golgi apparatus; LYS, lysosomes.
[b] Genes were selected for additional follow-up.

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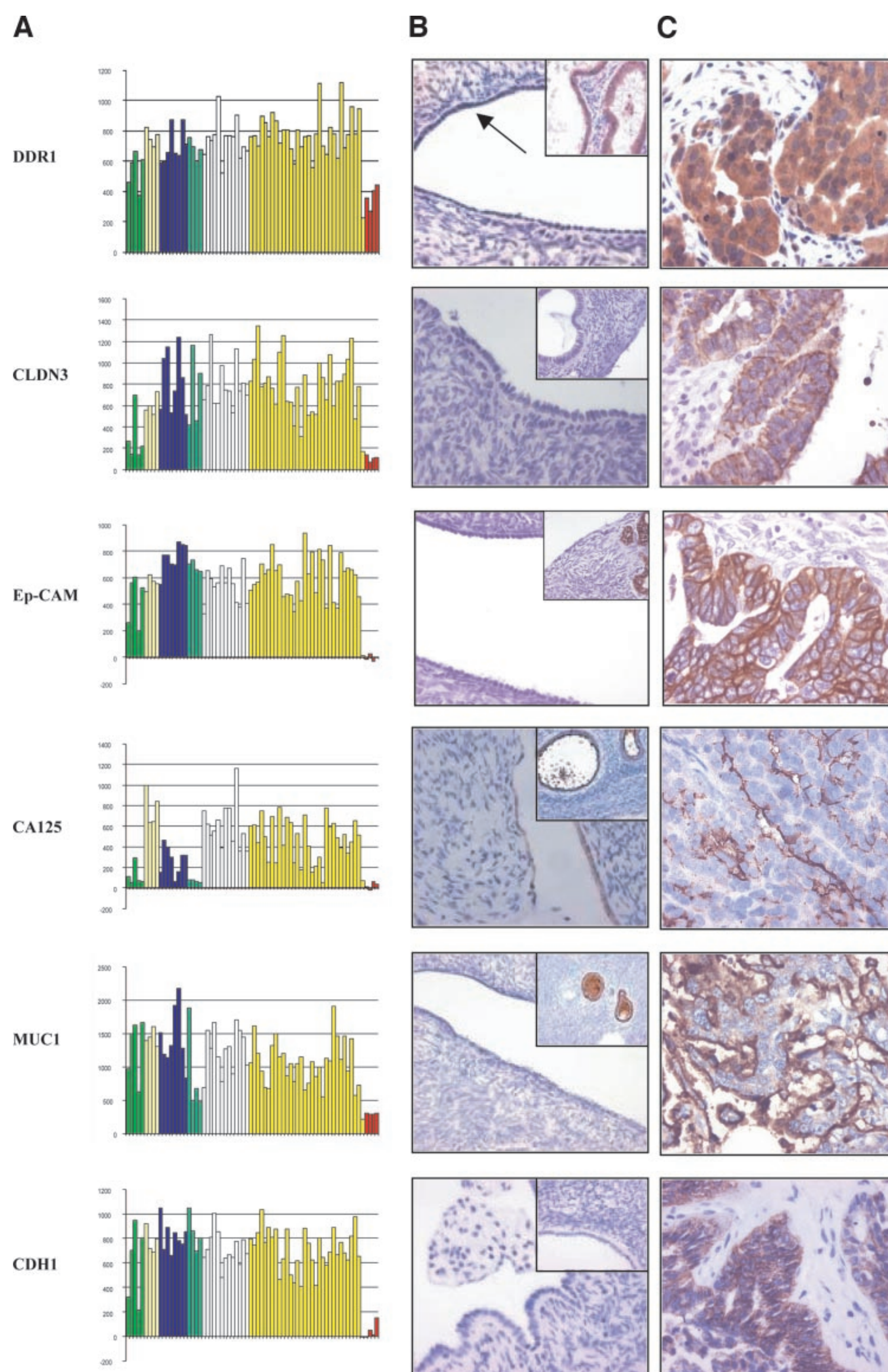

Fig. 1 *A*, transcription profiling of EOC. Each *bar* represents the signal intensity from one tumor. Legend: *green*, mucinous borderline or low malignant potential tumors; *yellow*, serous borderline or low malignant potential tumors; *blue*, EnOC; *jade*, MOC; *white*, matched omentum of serous ovarian cancer (SOC) patients; *bright yellow*, SOC; and *red*, normal ovaries. *B*, representative immunohistochemistry staining of gene expression in ovarian surface epithelium (*arrow*), inclusion cysts (*inset*) and SOC (*C*). Magnification, ×40.

membrane-expressed or extracellularly (secreted); and with limited expression in normal ovaries. This query identified 21 genes as candidate tumor markers of EOC, including *MUC1*, *HE4*, and *osteopontin*, along with several novel candidate tumor markers. Several adhesion molecules were identified in this query, including *desmoplakin*, a component of desmosomes, and *protein-tyrosine phosphatase receptor type* F, which is involved in the regulation of epithelial cell-cell contacts at adhering junctions. We selected three of candidates for additional investigation: *DDR1* (17); *Ep-CAM* (19); and *CLDN3* (18), all of which

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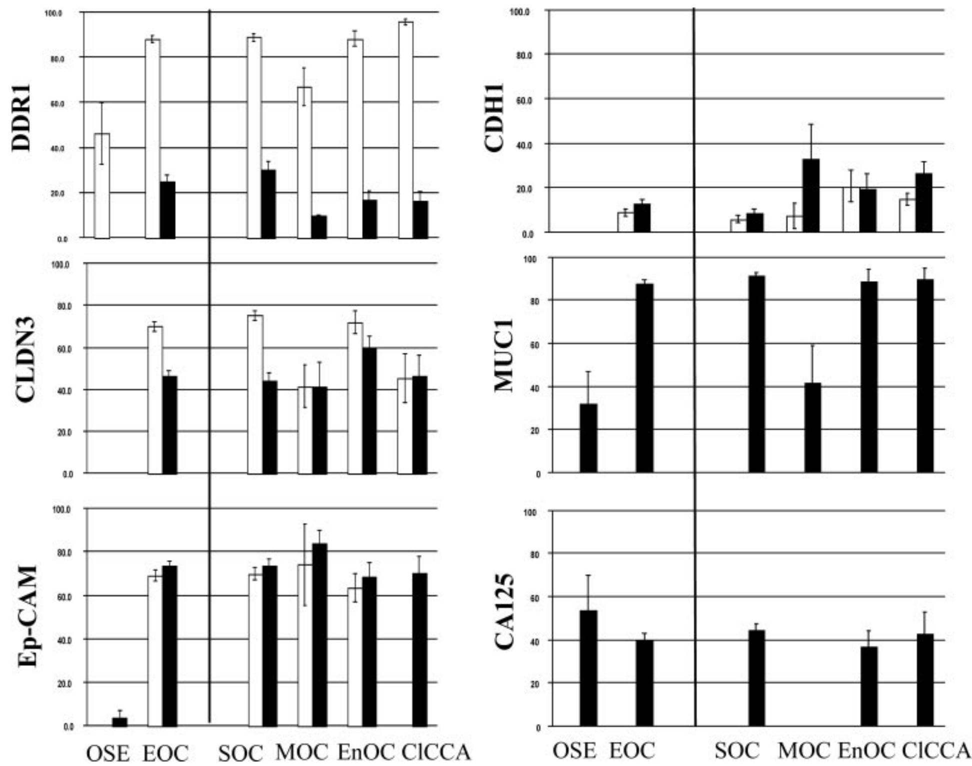

Fig. 2 Gene expression (immunohistochemistry) in epithelial ovarian cancer (EOC). Mean percentage of cells (±SE) expressing each gene in ovarian surface epithelium (OSE; n = 10), all EOC (n = 115), and in subtypes of EOC: serous ovarian cancer (SOC; n = 79); mucinous ovarian cancer (MOC; n = 9); endometrioid ovarian cancer (EnOC; n = 19); clear cell ovarian cancer (ClCCA; n = 7). □, cytoplasmic; ■, membrane expression.

encode epithelial-specific integral membrane proteins involved in cellular adhesion. Appropriate antibodies were available for these three genes (Table 2), and at the time, no immunohistochemistry study on EOC had been performed on these candidates.

DDR1, CLDN3, and Ep-CAM Are Highly Overexpressed in EOC of all Histological Subtypes. Expression of CLDN3, Ep-CAM, and CDH1 was predominantly localized to the cell membrane in control tissues, with a lower proportion of cytoplasmic staining (data not shown). A similar staining pattern was observed in the tumor cells (Fig. 1). DDR1 expression was predominantly cytoplasmic in control tissues, OSE and EOC. Membrane expression of DDR1 was only found in cancer specimens. Expression of the secreted molecules CA125 and MUC1 was confined to the apical membrane surface in both normal and tumor tissues. As predicted, all of the genes were only expressed in the tumor cells and not the stroma, confirming their epithelial specificity.

In accordance with the transcription profiling results, membrane expression of DDR1 (P = 0.03), CLDN3 (P < 0.0001), Ep-CAM (P < 0.0001), MUC1 (P = 0.001), and CDH1 (P = 0.03) was significantly higher in cancer compared with OSE (Fig. 2). CA125 expression was not significantly different between OSE and EOC (P = 0.34). As Ep-CAM, CLDN3, and CDH1 showed either low levels or no expression in OSE, they appear to be specifically activated during tumorigenesis. In general, CLDN3 and Ep-CAM were expressed on the cell surface and in the cytoplasm of EOC. Cytoplasmic expression of DDR1 was detectable in the OSE but was significantly higher in EOC (P = 0.008), whereas membrane expression of DDR1 was absent in OSE and up-regulated in EOC (P = 0.03).

Unlike CA125 (3), which is not expressed in mucinous ovarian cancer, CLDN3 and Ep-CAM were expressed in all histological subtypes of EOC (Fig. 2). When the level of membrane expression was compared with that in OSE, this up-regulation was significant in all subtypes (CLDN3 P = 0.03 mucinous ovarian cancer, $P \leq 0.002$ all other subtypes; Ep-CAM $P \leq 0.005$ for all subtypes). Both membrane and cytoplasmic expression of DDR1 showed a similar trend toward overexpression in all subtypes, which was significant in endometrioid ovarian cancer ($P \leq 0.02$) and clear cell ovarian cancer (membrane expression, P = 0.004); and serous ovarian cancer and endometrioid ovarian cancer (cytoplasmic expression, P = 0.05 and P = 0.008, respectively).

DDR1, CLDN3, and Ep-CAM Are Expressed in IC, BL, and Low-Grade and Stage EOC. DDR1, CLDN3, Ep-CAM, CDH1, and MUC1 exhibited elevated membrane expression compared with OSE in both low and high grade and stage of EOC (Fig. 3). This overexpression was significant with the exception of membrane DDR1 (P = 0.2) and MUC1 (P = 0.08) expression in low-grade (G1) tumors and CDH1 expression in grade 1 (P = 0.09) and grade 2 (P = 0.06) cancers. Moreover, CDH1 expression was significantly higher in International Federation of Gynecologists and Obstetricians (FIGO) stage I/II EOC compared with OSE (P = 0.01) but not in stage III/IV (P = 0.06). This may reflect our observed trend toward lower expression in high stage as compared with low-stage EOC (Fig. 3).

DDR1, CLDN3, Ep-CAM, and CDH1 were also expressed at higher levels in BL tumors than in OSE. This overexpression was significant except for DDR1 (both membrane and cytoplasmic expression, P = 0.07 and P = 0.27, respectively). As in

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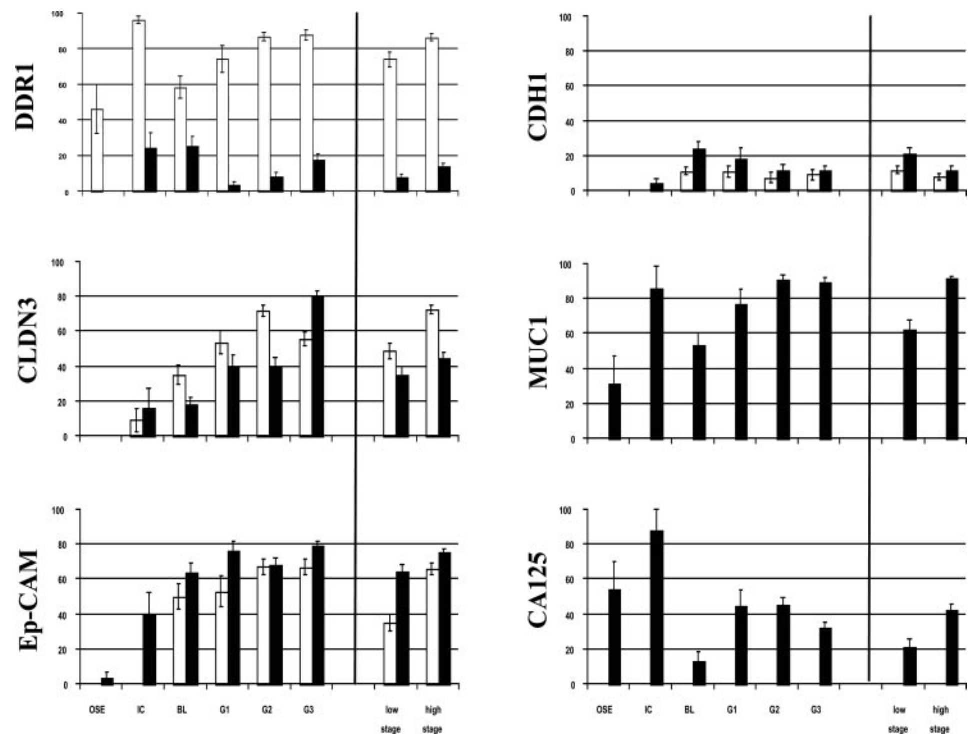

Fig. 3 Gene expression (immunohistochemistry) in inclusion cysts (IC), borderline or low malignant potential tumors, and increasing grade and stage epithelial ovarian cancer (EOC). Mean percentage of cells [(±SE) expressing each gene in OSE, IC (n = 8), borderline or low malignant potential tumors (n = 34), grade 1 (G1, n = 18), grade 2 (G2, n = 45), grade 3 (G3, n = 44) cancers, and low (I/II; n = 28) and high III/IV; (n = 84) stage EOC]. □, cytoplasmic; ■, membrane expression.

EOC, CA125 expression was significantly lower in BL tumors compared with OSE (P = 0.009).

We also detected expression of each gene in IC, with DDR1 (membrane P = 0.049 and cytoplasmic P = 0.006) and Ep-CAM ($P \leq 0.0001$) being significantly up-regulated compared with OSE (Fig. 3). CLDN3 showed the same trend but was not significant (P = 0.26). Unlike BL and EOC, Ep-CAM expression in IC was confined to the surface membrane. As predicted (20), we did observe CDH1 expression in IC, but this was not significantly higher than in OSE (P = 0.42).

DDR1, CLDN3, and Ep-CAM Expression Is Not Associated with Outcome in EOC. The clinicopathological characteristics of the EOC patient cohort included in the outcome study are shown in Table 3. The mean age of the patients was 60 years at diagnosis (range, 27.3–86.4 years). Most patients presented with advanced stage III or IV = 75%) and high-grade (grade 2 or 3 = 83%) disease, and the majority of tumors (62%) was classified as serous ovarian cancer. The mean follow-up time of the cohort was 35.5 months (range, 0–158.5 months) with a mean relapse-free survival of 27.9 months and a mean disease-specific survival of 35.5 months. In our cohort, 64% of those patients who had initially responded to therapy (n = 89) relapsed and 61% of the total EOC cohort (n = 115) died of their malignancy.

As the clinicopathological behavior of BL and EOC is different, only patients with EOC were included in the outcome analyses. Univariate analysis using a Cox proportional hazards model demonstrated that tumor stage and volume of residual disease were predictors of shorter relapse-free survival. Tumor stage, residual disease, and patient performance status were predictors of shorter disease-specific survival in patients with EOC, whereas grade and CA125 serum levels were not significant (Table 4). When gene expression and patient outcome were analyzed, none of the genes under study were associated with relapse-free survival or disease-specific survival, although patients with low CLDN3 expression showed a trend toward earlier death (P = 0.068; Table 4).

DISCUSSION

Epithelial cell adhesions, including intercellular (junctional) and cell-extracellular matrix adhesions, are critical to the maintenance of structural integrity, polarity, and cell-cell communication, and their expression is tightly regulated in normal cells. Loss of cell adhesion is frequently observed in tumor cells concordant with a breakdown of cellular organization, causing an uncontrolled leakage of nutrients and other factors necessary for the survival and growth of tumor cells and loss of cell-cell contact inhibition leading to increased cell motility. As such, their loss is normally associated with progression to a more malignant phenotype. For example, CDH1 expression is often reduced or lost in tumors, which correlates with progression to invasive and metastatic disease (20, 22). In contrast, we determined that DDR1, CLDN3, and Ep-CAM are highly overexpressed in all histological subtypes of EOC. Overexpression of adhesion molecules has been observed in other tumors, including CLDN3 and CLDN4 in prostate and pancreatic cancer (36), and p120, a component of the CDH1 complex, in gastric and pancreatic cancer (37, 38). However, as in EOC, the functional significance of their overexpression is unclear. Several possibilities can be envisaged that are not mutually exclusive. First, the OSE is a modified mesothelium that exhibits characteristics of

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Table 3 Clinicopathological characteristics of the EOC patient cohort[a,b]

Variable	No. of patients	% of patients
Age (yrs)		
<50	23	20
≥50	92	80
Tumor stage (FIGO) (n = 112)		
I	25	22
II	3	3
III	69	62
IV	15	13
Tumor grade (n = 107)		
G1	18	17
G2	45	42
G3	44	41
Histological type		
Serous	79	69
Mucinous	9	8
Endometrioid	19	17
Clear cell	7	6
Mixed	1	0.01
Residual disease (n = 114)		
<1 cm	57	50
≥1 cm	57	50
Preoperative CA125 (n = 94)		
<500 units/ml	42	45
≥500 units/ml	52	55
Performance status (n = 103)		
<1	70	68
≥1	33	32
Chemotherapy (n = 96)		
Neoadjuvant	4	4
Adjuvant	92	96
Outcome relapse		
Relapse[c]	57	64
Outcome death		
Death in relation to malignancy	71	61
Death unrelated to malignancy	9	8
Alive with progressive disease	3	3
Alive without disease	32	28

[a] EOC, epithelial ovarian cancer; FIGO, International Federation of Gynecologists and Obstetricians.

[b] n = 115, except where stated otherwise.

[c] Percentage of patients with relapse (defined in "Materials and Methods") was calculated using only those patients that had initially responded well to treatment (n = 89).

both mesothelial and epithelial cells. In carcinogenesis, the OSE becomes more committed to a epithelial phenotype, correlating with expression of CDH1 (20, 22). The expression of CLDN3 and Ep-CAM may similarly reflect conversion to an epithelial phenotype. Secondly, increased expression may be an attempt to overcompensate for the loss of cell adhesion due to other molecular changes. Indeed, the characteristic spread of ovarian tumors within the abdomen may reflect a specific pattern of adhesion molecules peculiar to EOC. Thirdly, the high expression levels of these genes may indicate another function in tumorigenesis unrelated to cell adhesion such as intracellular signaling (39). We observed a trend toward increased cytoplasmic expression in EOC, which differs between histological subtypes. Expression of DDR1 was predominantly cytoplasmic, although it is possible that we did not detect all membrane-localized DDR1 due to strong cytoplasmic staining or to specific cleavage from the cell surface (40). At least for CLDN3, increased cytoplasmic expression is not associated with functional tight junctions in EOC cell lines (36). It should be noted that disruption of cell adhesions themselves might cause mislocalization of these proteins.

We determined that DDR1, CLDN3, and Ep-CAM were expressed in low grade and stage EOC and in BL tumors. Although it is unclear if all EOC follow a classical stepwise progression pathway (41), our results suggest that at least some changes in gene expression detected in high-grade cancer are also found in low-grade cancer and BL tumors. In addition, we found evidence that these genes are expressed in IC, representing metaplastic ovarian epithelium. EOC are proposed to arise from IC within the ovarian cortex, where due to the absence of the tunica albuginea, a structural barrier separating the OSE from the ovarian stroma, the epithelium is subject to high concentrations of growth factors and hormones in the microenvironment (20). Gene expression in IC may reflect early changes associated with progression to a neoplastic phenotype, as shown for CDH1 (20, 22). As with CDH1, we found that DDR1, CLDN3, and Ep-CAM were expressed in IC. Thus, dysregulation of expression of genes involved in cell adhesion may occur at an early stage in ovarian tumorigenesis.

Expression of DDR1, CLDN3, and Ep-CAM did not correlate with relapse-free or disease-specific survival of patients, although those patients with low expression of CLDN3 showed a trend toward earlier death, which may become significant when a larger cohort of patients is studied. Expression of CA125, MUC1, and CDH1 was also not associated with patient outcome. An association between loss of CDH1 expression and shorter patient survival has been reported in a small study of 20 BL and 20 EOC patients (42). Although we observed a trend toward loss of CDH1 expression in high-stage EOC, this was not significant. There are several conflicting studies on the expression of CDH1 in high-stage EOC (20, 22, 43). Our results support the hypothesis that CDH1 expression is activated early in ovarian tumorigenesis but is reduced in high-stage disease, corresponding with an invasive phenotype.

Regardless of their role in tumorigenesis, these markers have potential clinical application for the treatment and detection of EOC. The overexpression of Ep-CAM on the cell surface of adenocarcinomas of different origin, including breast, ovary, colon, and lung is well described (19). Several therapeutic strategies based on Ep-CAM targeting in cancer, including ovarian tumors, are under investigation (44, 45); however, to our knowledge, this study and another recent article are the first to examine Ep-CAM expression in EOC (46). A high level of CLDN3 expression on the surface of ovarian cancer cells suggests that the development of similar therapeutic approaches targeting CLDN3 in EOC is warranted. This has been recently confirmed by a similar investigation of CLDN3 expression in a cohort of 70 ovarian tumors published during the course of this study (36). DDR1 is overexpressed in several tumors, including high-grade brain, esophageal, and breast cancers (47). Although originally cloned from an ovarian cancer cell line, this is the first reported study of DDR1 expression in ovarian tumors and identifies it as a new biomarker of EOC. A low level of membrane expression may limit DDR1 as an antibody-directed therapeutic target; however, along with Ep-CAM and CLDN3, it has potential use as a diagnostic marker of both low- and high-grade

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Table 4 Univariate analysis of an association between clinicopathological parameters and candidate tumor marker expression with patient outcome in EOC[a]

	Relapse-free survival		Disease-specific survival	
	HR	*P*	HR	*P*
Tumor stage[b]				
1–2 *versus* 3–4	3.8 (1.5–9.6)	0.005	13.2 (3.2–54.7)	0.0004
Tumor grade				
Grade 1–2 *versus* grade 3	1.4 (0.8–2.4)	0.25	1.3 (0.8–2.2)	0.26
Residual disease				
<1 cm *versus* ≥1 cm	3.0 (1.7–5.3)	0.0002	3.3 (2.0–5.3)	<0.0001
CA125				
<500 *versus* ≥500 units/ml	1.3 (0.7–2.5)	0.44	1.2 (0.7–2.2)	0.45
Performance status				
<1 *versus* >1	1.3 (0.4–4.4)	0.62	3.5 (1.7–7.1)	0.0008
DDR1 expression[c], membrane	0.99 (0.55–1.77)	0.96	1.13 (0.70–1.80)	0.63
CLDN3 expression, membrane	0.79 (0.45–1.40)	0.43	0.63 (0.40–1.0)	0.068
Ep-CAM expression, membrane	0.93 (0.64–1.34)	0.76	0.83 (0.62–1.11)	0.25
CDH1 expression, membrane	0.96 (0.75–1.24)	0.76	0.86 (0.67–1.11)	0.22
MUC1 expression, membrane apical	0.97 (0.79–1.19)	0.82	1.03 (0.84–1.27)	0.73
CA125 expression, membrane apical	0.69 (0.43–1.09)	0.14	0.87 (0.55–1.38)	0.57

[a] EOC, epithelial ovarian cancer; HR, hazards ratio.
[b] Protein expression for all clinical variables are dichotomized.
[c] Protein expression for all markers are continuous. In this case, HR refers to the interquartile range HR.

EOC. As membrane-bound molecules are frequently shed from the surface of tumors or, as with DDR1, may be specifically cleaved (40), these biomarkers could be found circulating in the serum of EOC patients. Furthermore, it may be possible to detect circulating antibodies induced against the membrane-bound proteins. Indeed, antibodies against Ep-CAM have recently been reported in the serum of patients with EOC (46).

Using the overlapping database described in this study, we identified an approach to compare transcript profiling results, regardless of limits caused by incomplete data provided by each study or differences in methodologies. Our results suggest that up-regulation of DDR1, CLDN3, and Ep-CAM are early events in the development of EOC and have potential application in the early detection of disease. Analysis of the remaining genes identified as being differentially up-regulated in combined transcription profiling studies may reveal other cellular processes disrupted in EOC, leading to the identification of additional novel biomarkers and potential therapeutic targets and providing insight into the molecular basis of disease progression.

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