A distinct molecular profile associated with mucinous epithelial ovarian cancer

Mucinous epithelial ovarian cancers (MOC) are clinically and morphologically distinct from the other histological subtypes of ovarian cancer. To determine the genetic basis of MOC and to identify potential tumour markers, gene expression profiling of 49 primary ovarian cancers of different histological subtypes was performed using a customised oligonucleotide microarray containing > 59,000 probesets. The results show that MOC express a genetic profile that both differs and overlaps with other subtypes of epithelial ovarian cancer. Concordant with its histological phenotype, MOC express genes characteristic of mucinous carcinomas of varying epithelial origin, including intestinal carcinomas. Differences in gene expression between MOC and other histological subtypes of ovarian cancer were confirmed by RT–PCR and/or immunohistochemistry. In particular, galectin 4 (LGALS4) was highly and specifically expressed in MOC, but expressed at lower levels in benign mucinous cysts and borderline (atypical proliferative) tumours, supporting a malignant progression model of MOC. Hence LGALS4 may have application as an early and differential diagnostic marker of MOC.

Carcinomas arising from the epithelial cells of the ovary are the fifth most common malignancy in women and the leading cause of death from gynaecological cancers. Epithelial ovarian cancers comprise a group of related but distinct carcinomas that likely arise from a common epithelial cell type but develop via differentiation pathways and differ in their clinical presentation and aetiology. They are currently classified into different histological subtypes (including serous, endometrioid, mucinous and clear cell) based on their morphological resemblance to normal epithelia in the gynaecological and intestinal tracts; however the genetic basis underlying their divergence is poorly understood.

The majority of mucinous ovarian cancers (MOC) are diagnosed at an early stage, either as borderline (atypical proliferative) tumours or low-grade carcinomas, and have an excellent prognosis (Sherman et al, 2004). Although less common, advanced MOC is associated with a very poor survival that surpasses the poor prognosis for women with advanced stage serous ovarian cancer (Sherman et al, 2004). Accumulating pathological and epidemiological evidence supports a progression model of MOC, from benign cysts to borderline tumours to invasive adenocarcinoma (Feeley and Wells, 2001; Shih and Kurman, 2004). However, it can be difficult to identify invasion which may only be focally present and thus such tumours, particularly those of large size, must be extensively sampled for accurate diagnosis (Riopel et al, 1999; Lee and Young, 2003; Seidman et al, 2003; Ronnett et al, 2004). Moreover, it can be very difficult to differentiate primary MOC from secondary mucinous carcinomas from other sites, in particular the gastrointestinal tract (Hart, 2005). Indeed it is thought that many carcinomas diagnosed as primary mucinous epithelial ovarian cancer are likely metastatic disease, and that the true frequency of mucinous carcinoma arising in the ovary is < 3% of all ovarian carcinomas (Gilks, 2004; Seidman et al, 2004). Hence early diagnosis and accurate classification of MOC, including the ability to identify patients who are likely to progress to invasive disease, is critical to patient prognosis and treatment (Hart, 2005).

The molecular basis of MOC, including the genetic events that initiate the development of disease and those leading to malignant progression, are largely unknown. One genetic abnormality characteristic of MOC is a high frequency of mutations in KRAS, thought to occur early in the development of MOC (Feeley and Wells, 2001). Unlike serous ovarian carcinomas, mutations in p53 are rarely observed in MOC (Shih and Kurman, 2004). In our laboratory, we have successfully applied transcript profiling of whole tissue as a screening tool to determine molecular changes underlying cancer, which has led to the identification of several potential markers for prostate, ovarian and pancreatic cancer (Henshall et al, 2003a, b; Heinzelman-Schwarz et al, 2004; Segara et al, 2005). In the current study, we have determined the gene

**Keywords:** ovarian cancer; mucinous; microarray; immunohistochemistry; diagnosis
expression profiles of mucinous borderline tumours and MOC using oligonucleotide microarrays representing over 90% of the expressed human genome. By comparing the results to transcript profiles of the other histological subtypes of ovarian cancer, we aimed to determine the molecular basis of mucinous ovarian tumours and to identify potential tumour markers. Following validation of the transcript profiling results using RT–PCR analysis on ovarian cancer extracts of varying histological subtypes, we determined the protein expression of one such candidate tumour marker, galectin 4 (LGALS4), in primary ovarian tissue (normal surface epithelium, benign mucinous cysts, mucinous borderline tumours and ovarian carcinomas) using high-throughput immunohistochemistry based on tissue microarrays.

MATERIALS AND METHODS

Tissue and clinicopathological data

Tissue specimens (fresh/frozen and formalin-fixed paraffin-embedded samples) collected from patients undergoing primary laparotomy at the Gynaecological Cancer Centre, Royal Hospital for Women, Sydney, and the Royal North Shore Hospital, Sydney, between 1990 and 2003 were included in this study following informed consent and approval by the appropriate hospital research ethics committee. The histological classification at diagnosis was independently confirmed by a gynaecological pathologist for all tissue specimens before inclusion in the study. Normal ovaries were obtained from patients undergoing surgery for benign gynaecological conditions or unrelated malignancies. Patients exhibiting clinical, morphological or microscopic features suggesting metastatic mucinous ovarian carcinoma rather than primary MOC, including concurrent gastrointestinal carcinomas, the presence of Pseudomyxoma peritonei/ovarii, bilateral disease, Krukenberg tumours, and advanced stage borderline tumours (Lee and Young, 2003; Seidman et al., 2003; Hart, 2005), were excluded from the study. The clinical and pathological details of the tissue cohort used in this study are shown in Table 1.

Molecular profiling and data analysis

Transcript profiling was performed as previously described (Heinzelmann-Schwarz et al., 2004) using the Eos Hu03, a customised Affymetrix GeneChip oligonucleotide microarray containing over 59 000 probesets for the interrogation of approximately 46 000 unique sequences (Eos Biotechnology/Protein Design Labs, Fremont, CA, USA; Platzer et al., 2002) using total RNA extracted from three MOC (stage I), four mucinous borderline tumours, eight endometrioid ovarian cancers, 31 serous ovarian cancers, three serous borderline tumours, and four normal ovaries. Only those tumour samples containing >75% of borderline or invasive cancer were used for transcript profiling. Following normalisation as described (Henshall et al., 2003a), data was log-transformed before further analysis. In addition, prior to hierarchical clustering or principal components analysis, the data were scaled to ensure that each gene exhibited the same mean and variance. Principal components analysis was used to provide a visual demonstration of the variation in gene expression of the top ranked between ovarian cancer histological subtypes using the Stats package in R (http://www.r-project.org; Smyth, 2004). Hierarchical clustering of genes and samples was performed using an euclidean distance metric with average linkage (Spotfire DecisionSite 8.0). A penalised t-test (Lönnstedt and Speed, 2002; Smyth, 2004) was used to identify genes differentially regulated between MOC and other subtypes of ovarian cancer. P-values were adjusted for multiple testing using the Benjamini–Yekutieli method (Benjamini and Yekutieli, 2001). Genes with an adjusted P-value <0.01 can be interpreted as having a false discovery rate of 1%. Genes were assigned to functional categories (molecular function, biochemical process, cellular localisation, chromosome) using Gene Ontology (http://vortex.cs.wayne.edu/projects.htm; Draghici et al., 2003) and GenMAPP (www.genmapp.org) analysis (Dahlquist et al., 2002).

RT–PCR

RNA (2 μg) was treated with DNase then reverse-transcribed using the Reverse Transcription System (Promega, Australia) according to the manufacturer’s instructions. Semi-quantitative RT–PCR was performed by the amplification of selected gene transcripts using 2 μl of the resulting cDNA in a 25 μl reaction volume incorporating 200 μM of dNTPs (Roche, Australia), 2.5 mM MgCl2, 1.5 U of AmpliTag Gold (Promega), and 1 μM of each oligonucleotide pair. Oligonucleotide primers and PCR product size for each gene were as follows: LGALS4: forward 5’ GCTCAACGTTGGGAAATGTCCTGGTTTAG, reverse 5’ TTAGTATGCTCTAGCCAGAGGATATG (260 bp); MGC2871: TGAATGCCGGACCTTGCGAG, TCTGCCCAAAAACAGTATCA (260 bp); MUCDHL: AATGTGGGACCCAGCCACCA, CAGGTTCCTCCCCTAAGATGCTT (270 bp); CDH17: TCCACATCTCCGTGTCTCTTTATGG, GCCGTCCAGGCTACTGTT (270 bp); MEPA: GGTTCCTCCTCCCCTCAAGAGCTAATG (240 bp); MUC13: GTAGGCTGGCCAGATGCTCAGTGGTCCCTCATC (240 bp); FABP1: GAGCCACCGAACTTTGAGCC, TGTTGATTATGTCGCCTGTGATGC (300 bp); C19orf12: CCAACGGCAGATGAGAACCT.

| Variable | No. patients (%) |
|----------|------------------|
| Histological type |                       |
| Serous | 55 (44.4) |
| Mucinous | 39 (31.4) |
| Endometrioid | 22 (17.7) |
| Clear cell | 8 (6.5) |
| FIGO stage |                           |
| I | 27 (28.4) |
| II | 5 (5.3) |
| III | 55 (57.9) |
| IV | 8 (8.4) |
| Grade |                 |
| Borderline (mucinous only) | 29 (24.8) |
| 1 | 20 (17.1) |
| 2 | 35 (29.9) |
| 3 | 33 (28.2) |
| Age |                    |
| < 60 | 62 (50.0) |
| ≥ 60 | 62 (50.0) |
| Residual disease |                      |
| ≤ 1 | 92 (74.2) |
| > 1 | 32 (25.8) |
| CA125 |            |
| < 500 | 58 (60.0) |
| > 500 | 39 (40.0) |
| Outcome |                        |
| Alive | 62 (53.0) |
| Death (related to malignancy) | 48 (41.0) |
| Death (unrelated or unknown cause) | 7 (6.0) |

*Unless otherwise stated. bCarcinomas only; cn = 117. n = 97.
Molecular Diagnostics

Immunohistochemistry

Protein expression of LGALS4 was determined in a cohort of fixed tissue from 124 patients with ovarian cancer (comprising 10 MOC (independent of the samples that were transcript profiled), 55 serous ovarian cancers, 22 endometrioid ovarian cancers, eight clear cell ovarian cancers, and 29 mucinous borderline tumours; Table 1). In addition, eight benign mucinous cysts and 14 normal ovaries, located in regions of genomic amplification in cancer. 20q13 have been previously associated with a high frequency of loss of heterozygosity in MOC (Feltmate 2005). Together these data suggest chromosomal amplification affecting these genomic loci in MOC. Moreover, both 3p21.3 (Byrd and Bresalier, 2004; Hart, 2005) and 20q13 (Ep-CAM), CDH1 (E-cadherin), KLF5 (Kruppel-like factor 5) and ERB-B3 (Dari et al, 1997; Balzar et al, 1999; Maihle et al, 2002; Heinzelmann-Schwarz et al, 2004). Combining the two analyses, we identified that 13 of these 40 genes overlap with those that are upregulated in MOC compared to the other subtypes of ovarian cancer (highlighted in Table 3). Only four genes were identified as down-regulated in MOC compared to normal ovaries (adjusted P<0.01) (Table 3), all of which are also reduced in the other subtypes of ovarian cancer.

We next clustered the upregulated genes in MOC compared to the other subtypes by their chromosomal location and identified several genomic regions that appeared to be over-represented in MOC, including 3p21.3 (VILL, MST1R, SLCL26A6, GLYCFT, FLJ20209), 7q22 (MUC3B, ACHC, MUC17, CLDN15, LOC55971), 11p15 (USHC1, MUCDHL, MUC2, SLCL22A18), 11q13 (STATD10, PLCB3, MOGAT2), 11q24 (CTXL, KIAA1201, LOC120224, RICS), 15q14-15 (PPPI4R1D, ITPKA, CKMT1, NMS1), 19p13.3 (FUT3, FLEKHIJ, C19orf21, LOC284422, GNA11), 19q13.1-13.4 (CYP2S1, FXIYD3, LGALS4, CEACAM5, CEACAM6, FLJ20200, PTPRH), and 20q13 (HNF4A, BCAS1, PTX6). Genes that were not microdissected before RNA extraction and profiling and therefore contain a high proportion of stromal tissue compared to epithelial cells, these genes likely reflect epithelial-specific genes expressed in MOC. Nonetheless, the majority of these genes are common to all subtypes of ovarian cancer, and several have been previously implicated in its pathogenesis, including TACSTD1 (Ep-CAM), CDH1 (E-cadherin), KLF5 (Kruppel-like factor 5) and ERB-B3 (Dari et al, 1997; Balzar et al, 1999; Maihle et al, 2002; Heinzelmann-Schwarz et al, 2004). Combining the two analyses, we identified that 13 of these 40 genes overlap with those that are upregulated in MOC compared to the other subtypes of ovarian cancer (highlighted in Table 3). Only four genes were identified as down-regulated in MOC compared to normal ovaries (adjusted P<0.01) (Table 3), all of which are also reduced in the other subtypes of ovarian cancer.

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MOC express genes associated with mucin production and intestinal-type epithelium

Using Gene Ontology classifiers, we grouped the genes with upregulated expression in MOC compared to the other subtypes to identify biological processes that may specifically underlie the development and progression of MOC. Consistent with its morphological phenotype, we identified genes encoding several mucins including MUC2, MUC3A (MUC3) and MUC17 but not MUC16 (CA125). This mucin profile is similar to that of mucinous colon carcinomas, in particular the presence of MUC2 and absence of MUC5A (Byrd and Bresalier, 2004; Hart, 2005). Several mucin-related molecules involved in carbohydrate metabolism and protein glycosylation were identified including FUT3, GCNT3, SI,
FBP1, UGT1A9; and TFF1, an estrogen-regulated member of the trefoil factor family of secreted peptides associated with mucin production and frequently overexpressed in other mucinous adenocarcinomas (Emami et al., 2004). We also identified a number of genes associated with intestinal expression including the caudal type homeobox transcription factors CDX1 and CDX2. CDX transcription factors are essential in intestinal epithelial development, and are also associated with oncogenesis via the modulation of various cellular processes including proliferation, apoptosis, and cell adhesion (Guo et al., 2004). Moreover, two CDX2 intestinal-specific targets were identified: sucrase isomaltase (SI), a critical gene in intestinal development (Guo et al., 2004) and CDH17, an enterocyte-specific cell adhesion molecule (Hinoi et al., 2002). Other intestinal-type cell adhesion molecules included LGALS4, a member of the galectin family of carbohydrate-binding molecules (Huflejt and Leffler, 2004); three members of the transmembrane 4 (tetraspanin) superfamily (TM4SF4/IL-TMP, TM4SF5/L6H and TM4SF3/CO-029) associated with cellular proliferation, adhesion, motility, and tumour cell metastasis (Wright et al., 2000); and two members of the carcinoembryonic antigen family, CEACAM6 and CEACAM5 (CEA), frequently expressed by at least a subset of MOC (McCluggage, 2000). The identification of intestinal-type adhesion factors suggests that altered cell adhesion is a feature of MOC.

Figure 1  (A) Principal components analysis based on 500 genes with the most variable signal intensities (based on variance) separates the histological subtypes of EOC. MOC (n = 3) are circled; (B) Hierarchical clustering and heat map of differentially expressed genes (n = 167 upregulated and n = 18 down-regulated) in MOC compared to serous and endometrioid ovarian cancers. Clustering was performed on all transcript profiled samples (n = 3 MOC; n = 4 mucinous borderline tumours; n = 8 endometrioid ovarian cancers (endo); n = 3 serous borderline tumours; n = 31 serous ovarian cancers (unlabelled columns); and four normal ovaries) as described in the Materials and Methods. Expression levels are colour coded with red, green and black corresponding to an increase, a decrease, and no change in gene expression, respectively.
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Similar to other histological subtypes of ovarian cancer (Heinzelmann-Schwarz et al, 2004). Moreover, several of these adhesion factors have been previously implicated in carcinogenesis, including TM4SF and CEACAM family members (Scholzel et al, 2000; Wright et al, 2000; Iliantzis et al, 2002), CDH17 (Grotzinger et al, 2000; Ilantzis et al, 2000; Maggiora et al, 2003) and LGALS4 (Huflejt and Leffler, 2004). Moreover, several of these adhesion factors have been previously implicated in carcinogenesis, including TM4SF and CEACAM family members (Scholzel et al, 2000; Wright et al, 2000; Iliantzis et al, 2002), CDH17 (Grotzinger et al, 2000; Ilantzis et al, 2000; Maggiora et al, 2003) and LGALS4 (Huflejt and Leffler, 2004).

Cellular pathways underlying MOC development
Gene Ontology analysis identified a number of genes involved in cellular processes associated with cancer, including cell adhesion, signalling, proliferation, and apoptosis (Table 4). Several putative oncoproteins were differentially expressed in MOC, including the breast tumour kinase BRK (PTK6) (Barker et al, 1997) not previously implicated in ovarian cancer pathogenesis; and MSTIR/ RON, a receptor tyrosine kinase associated with proliferation and motility of cancer cells including ovarian carcinoma (Hess et al, 2003; Maggiora et al, 2003; Wang et al, 2003).

Although KRAS mutations are associated with MOC (Shih and Kurman, 2004), we did not find any evidence of increased KRAS activity at the transcriptional level. Using GenMAPP analysis, we examined if any probesets corresponding to other members of the mitogen activated protein (MAP) kinase cascade were differentially expressed in MOC compared to the other subtypes of ovarian cancer. This revealed a slight increase in ERK1 (1.19-fold change, adjusted \( P < 0.01 \)).

Table 2: Genes (n = 50 of 167 probesets*) identified as upregulated in MOC compared to other histological subtypes of ovarian cancer (ranked by adjusted \( P < 0.01 \)).

| Rank | Symbol* | Name | Unigene* | Locus link | Location |
|------|---------|------|----------|-----------|----------|
| 1    | LGALS4  | Lectin, galactoside-binding, soluble, 4 (galactin 4) | Hs.5302 | 3960 | 19q13.2 |
| 2    | cDNA clone IMAGE:5759948, partial cds | | Hs.49437 | NA | 15q15.1 |
| 3    | MUCDHL  | Mucin and cadherin-like | Hs.166519 | 53841 | 11p15.5 |
| 4    | apobec-1 complementation factor | | Hs.8349 | 29974 | 10q21.1 |
| 5    | CDH17   | Cadherin 17, L1 cadherin (liver-intestine) | Hs.19190 | 10q21.1 | 1p32 |
| 6    | MEPIA   | Meprin A, alpha (PABA peptide hydrolase) | Hs.125608 | 461546 | 9q32.1 |
| 7    | MUC13   | Mucin 13, epithelial transmembrane | Hs.5940 | 56667 | 3q21 |
| 8    | FABP1   | Fatty acid binding protein 1, liver | Hs.380305 | 2168 | 2p11 |
| 9    | MUC3B   | Mucin 3B, intestinal | Hs.489354 | NA | 7q22 |
| 10   | CEACAM5 | CEA-related cell adhesion molecule 5 (CEA) | Hs.220529 | 1048 | 19q13.2 |
| 11   | PDZK2   | PDZ domain containing 2 | Hs.374726 | 79841 | 1q23.3 |
| 12   | GPA33   | Glycoprotein A33 (transmembrane) | Hs.53864 | 10223 | 1q24.1 |
| 13   | RNF128  | Ring finger protein 128 | Hs.496542 | 79589 | Xq22.3 |
| 14   | MUCDHL  | Mucin and cadherin-like | Hs.166519 | 53841 | 11p15.5 |
| 15   | CEACAM5 | CEA-related cell adhesion molecule 5 (CEA) | Hs.220529 | 1048 | 19q13.2 |
| 16   | CDH17   | Cadherin 17, L1 cadherin (liver-intestine) | Hs.19190 | 10q21.1 | 1p32 |
| 17   | MEPIA   | Meprin A, alpha (PABA peptide hydrolase) | Hs.125608 | 461546 | 9q32.1 |
| 18   | MUC13   | Mucin 13, epithelial transmembrane | Hs.5940 | 56667 | 3q21 |
| 19   | FABP1   | Fatty acid binding protein 1, liver | Hs.380305 | 2168 | 2p11 |
| 20   | MUC3B   | Mucin 3B, intestinal | Hs.489354 | NA | 7q22 |
| 21   | CEACAM5 | CEA-related cell adhesion molecule 5 (CEA) | Hs.220529 | 1048 | 19q13.2 |
| 22   | PDZK2   | PDZ domain containing 2 | Hs.374726 | 79841 | 1q23.3 |
| 23   | GPA33   | Glycoprotein A33 (transmembrane) | Hs.53864 | 10223 | 1q24.1 |
| 24   | RNF128  | Ring finger protein 128 | Hs.496542 | 79589 | Xq22.3 |

*Full list of genes are listed in Supplementary Data. *HUGO. *Unigene identifiers were derived from the UniGene Build #176 (October 2004).
Table 3: Genes identified as (A) up-regulated (n = 40) and (B) down-regulated (n = 4) in MOC compared to normal ovaries (ranked by adjusted \( P < 0.01 \)). Genes highlighted in bold (n = 13) are also up-regulated in MOC compared to other subtypes of ovarian cancer.

| Rank | Symbol | Name | Unigene | Locus Link | Location |
|------|--------|------|---------|------------|----------|
| (A) Upregulated | | | | | |
| 1 | LGALS4 | Lectin, galactoside-binding, soluble, 4 (galectin 4) | Hs.5302 | 3960 | 19q13.2 |
| 2 | TACSTD1 | Hypothetical protein FJ20171 | Hs.487471 | 54845 | 8p22.1 |
| 3 | ERBB3 | V-erb-b2 erythroblast leukemia viral oncogene homolog 3 | Hs.306251 | 2065 | 12q13 |
| 4 | cDNA clone IMAGE:5759948, partial cds | Ets homologous factor | Hs.447537 | 4072 | 2p21 |
| 5 | BCMP1 | Breast cancer membrane protein 1 | Hs.100686 | 155465 | 7p21.1 |
| 6 | CXXC5 | CXXC finger 5 | Hs.189119 | 51523 | 5q31.2 |
| 7 | EHF | Ets homologous factor | Hs.502036 | 26298 | 11p12 |
| 8 | STARD10 | START domain containing 10 | Hs.188606 | 10809 | 11q13 |
| 9 | C19orf21 | Chromosome 19 open reading frame 21 | Hs.439180 | 126353 | 19p13.3 |
| 10 | MUC13 | Mucin 13, epithelial transmembrane | Hs.5940 | 56667 | 3q21.2 |
| 11 | PROM1 | Prominin 1 | Hs.479220 | 8842 | 4p15.32 |
| 12 | MUCDHL | Mucin and cadherin-like | Hs.5302 | 113452 | 1p35-p34 |
| 13 | CEACAM5 | Carcinoembryonic antigen-related cell adhesion molecule 5 (CEA) | Hs.320151 | 10555 | 9q34.3 |
| 14 | TACSTD1 | Tumor-associated calcium signal transducer 1 (Ep-CAM) | Hs.692 | 4072 | 2p21.3 |
| 15 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 16 | BCMP1 | Breast cancer membrane protein 1 | Hs.692 | 4072 | 2p21.3 |
| 17 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 18 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 19 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 20 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 21 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 22 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 23 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 24 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 25 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 26 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 27 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 28 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 29 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 30 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 31 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 32 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 33 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 34 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 35 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 36 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 37 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 38 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 39 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |
| 40 | MUC13 | Mucin 13, epithelial transmembrane | Hs.692 | 4072 | 2p21.3 |

| Rank | Symbol | Name | Unigene | Locus Link | Location |
|------|--------|------|---------|------------|----------|
| (B) Downregulated | | | | | |
| 1 | LGALS4 | Lectin, galactoside-binding, soluble, 4 (galectin 4) | Hs.5302 | 3960 | 19q13.2 |
| 2 | MGC32871 | | | | |
| 3 | CDH17 | | | | |
| 4 | CDH17 | | | | |
| 5 | CDH17 | | | | |
| 6 | CDH17 | | | | |
| 7 | CDH17 | | | | |
| 8 | CDH17 | | | | |
| 9 | CDH17 | | | | |
| 10 | CDH17 | | | | |
| 11 | CDH17 | | | | |
| 12 | CDH17 | | | | |
| 13 | CDH17 | | | | |
| 14 | CDH17 | | | | |
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| 21 | CDH17 | | | | |
| 22 | CDH17 | | | | |
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| 24 | CDH17 | | | | |
| 25 | CDH17 | | | | |
| 26 | CDH17 | | | | |
| 27 | CDH17 | | | | |
| 28 | CDH17 | | | | |
| 29 | CDH17 | | | | |
| 30 | CDH17 | | | | |
| 31 | CDH17 | | | | |
| 32 | CDH17 | | | | |
| 33 | CDH17 | | | | |
| 34 | CDH17 | | | | |
| 35 | CDH17 | | | | |
| 36 | CDH17 | | | | |
| 37 | CDH17 | | | | |
| 38 | CDH17 | | | | |
| 39 | CDH17 | | | | |
| 40 | CDH17 | | | | |

LGALS4 is specifically expressed in MOC

LGALS4 is an intestinal cell surface adhesion molecule that is overexpressed in intestinal carcinomas (Grotzinger et al., 2001). The results of the transcript profiling experiment suggested that LGALS4 was also highly overexpressed in MOC (Table 2, Figure 2).

Figure 2: Semi-quantitative RT–PCR analysis of RNA expression in normal ovaries (n = 2), mucinous borderline tumours (n = 3), mucinous ovarian cancers (n = 3) and serous ovarian cancers (n = 3). RT-, no reverse transcriptase control; water, no cDNA. For gene descriptions, see Table 2 and Supplementary Data.
Moreover, LGALS4 is located at 19q13.3, a region associated with a high frequency of loss of heterozygosity in MOC (Feltmate et al., 2005) and where we identified a cluster of upregulated genes. We therefore examined the expression of LGALS4 in ovarian carcinoma using immunohistochemistry (Table 1, Figure 3B). In accordance with the transcript profiling results, expression of LGALS4 was highly and specifically expressed in MOC (median expression 72% of cells staining positive) compared to the other ovarian carcinoma subtypes (serous and endometroid, \( P < 0.001 \); clear cell \( P = 0.002 \)) and to normal ovarian surface epithelium (\( P = 0.002 \), all of which had a median expression equivalent to zero (Figure 4A). To identify if LGALS4 expression occurs early in disease onset, we examined its expression in benign mucinous cysts and mucinous borderline tumours, in addition to low- and high-stage MOC. LGALS4 expression was detected at a median expression level of approximately 30% of cells staining in benign mucinous cysts, increasing in borderline tumours to similar levels of expression as in MOC (\( > 70\% \) of cells; Figure 4B). There was no significant difference in expression between borderline tumours and low-grade MOC (\( P = 0.47 \)), and although a decrease in expression from low- to high-stage MOC was observed, this was not statistically significant (\( P = 0.21 \)). Statistical analysis did not reveal any correlation between LGALS4 expression and clinicopathological parameters (age, grade, stage, outcome; Table 1) in the ovarian mucinous tumour cohort (data not shown).

**DISCUSSION**

Mucinous ovarian cancers are one of the less common histological subtypes of ovarian carcinoma. Combined with the difficulty in accurate diagnosis of primary disease, its relative rarity has contributed to the lack of knowledge regarding the molecular basis of this malignancy.
Mucinous ovarian cancer, normal ovarian surface epithelium, and are associated with altered biological properties including loss of organ specificity, are a common feature of cancer. Alterations in expression of mucins, such as LGALS4, are consistent with activation of expression early in MOC development (Huflejt and Wells, 2001). A variety of mucin molecules are logical phenotype, including intestinal-specific genes, which likely represents the signal intensity of the 15th percentile of the gene expression in normal body tissues (Henshall et al., 2003a); (B) representative immunohistochemistry staining for LGALS4 in MOC, serous ovarian cancer, normal ovarian surface epithelium (arrowed) and epithelial inclusion cysts (inset); ×40 magnification.

Figure 3 (A) mRNA transcript profile for LGALS4. The dashed line represents the signal intensity of the 15th percentile of the gene expression in normal body tissues (Henshall et al., 2003a); (B) representative immunohistochemistry staining for LGALS4 in MOC, serous ovarian cancer, normal ovarian surface epithelium (arrowed) and epithelial inclusion cysts (inset); ×40 magnification.

Figure 4 Box plots showing distribution of expression of LGALS4 in (A) normal ovarian surface epithelium (n = 14) and in different histological subtypes of ovarian carcinoma: MOC (n = 10); endometrioid ovarian cancer (n = 22); serous ovarian cancer (n = 55); clear cell ovarian cancer (n = 8); and (B) in epithelial ovarian inclusion cysts (n = 8); benign mucinous cysts (n = 8); mucinous borderline tumours (n = 29); low-(grade I; n = 6) and high-grade (grade II–III; n = 4) MOC; and low- (grade I; n = 5) and high-stage MOC (stage II–III; n = 5). For explanation of box plots, see Materials and Methods.

Table 2

| Gene          | Expression Level |
|---------------|-----------------|
| LGALS4        | Consistently high |
| REG4          | Low               |
| TM4SF4        | Low               |
| FABP1         | Low               |
| CTSE          | Low               |
| CEACAM5       | Low               |
| CEACAM6       | Low               |
| REG4          | Low               |
| FABP1         | Low               |
| CTSE          | Low               |
| CEACAM5       | Low               |
| CEACAM6       | Low               |
| REG4          | Low               |
| FABP1         | Low               |
| CTSE          | Low               |
| CEACAM5       | Low               |
| CEACAM6       | Low               |
| REG4          | Low               |
| FABP1         | Low               |
| CTSE          | Low               |
| CEACAM5       | Low               |
| CEACAM6       | Low               |
| REG4          | Low               |
| FABP1         | Low               |
| CTSE          | Low               |
| CEACAM5       | Low               |
| CEACAM6       | Low               |
| REG4          | Low               |
| FABP1         | Low               |
| CTSE          | Low               |

Progression from borderline tumours and low-stage carcinoma to advanced MOC is associated with a poor outcome; hence the identification of tumour markers that can detect early disease, together with those that can predict patients likely to progress to advanced stage MOC, would have a major impact on patient prognosis. We determined that LGALS4 is not expressed in normal ovarian surface epithelium but is expressed at high levels in mucinous borderline tumours and in benign mucinous cysts, consistent with activation of expression early in MOC development. We did not identify any genes including LGALS4 that were significantly differentially expressed between mucinous borderline tumours and MOC, suggesting that there may be very few or only subtle changes in gene expression between mucinous borderline tumours and low-stage MOC (which were used in the transcript profiling analysis), concordant with their similar outcomes. A study with sufficient power to compare high-stage MOC to borderline and low-stage MOC may reveal gene expression changes that correlate with the poor prognosis in these patients.

There are currently no specific or sensitive serum markers for the diagnosis of MOC (Rapkiewicz et al., 2004). MOC often fail to express the ovarian cancer serum marker CA125 (MUC16), which is frequently elevated in the serum of patients with nonmucinous ovarian carcinoma. Although a cell surface adhesion molecule, LGALS4 has at least a partial extracellular component (Huflejt and Wells, 2001), and can be mediated by the differential expression of glycosylation enzymes including fucosyltransferases and sialyltransferases. We identified several such enzymes, suggesting specific mucin glycosylation patterns are a feature of MOC.

It is likely that many carcinomas diagnosed as primary mucinous ovarian cancer are more likely to be metastatic disease originating in the gastrointestinal tract (Gilks, 2004; Seidman et al., 2004). In this study, we were particularly careful to only include patients that clearly fit with current clinical and histological guidelines as primary MOC rather than metastatic disease (Seidman et al., 2003; Hart, 2005). However, these strict selection criteria, combined with the relative rarity of MOC, resulted in a small sample number both for the transcript profiling and validation experiments. In addition, although comprising at least 75% tumour cells, the tissue samples used in the transcript profiling experiments were not microdissected and therefore may contain a small proportion of stromal elements. Therefore, our results remain to be validated in independent studies. To this end, several published studies have reported similar findings in regard to the genetic profile of MOC. First, Schwartz et al. (2002) used principal components analysis to show that gene expression profiles could distinguish MOC from serous ovarian cancer, with some overlap with endometrioid ovarian cancer. Secondly, using cDNA arrays incorporating 9121 elements, Ono et al. (2000) identified 115 genes that were differentially regulated between serous ovarian carcinomas and MOC. By comparing the Unigene/Locus Link identifiers corresponding to the GenBank accessions in the Ono study to the gene identifiers in our study (Table 2 and Supplementary Data), we identified only one gene (TUBB2; tubulin beta 2) that overlaps between our lists of differentially expressed genes. A more recent study reported 46 genes that were overexpressed in MOC compared to the other histological subtypes of ovarian carcinoma and to normal ovarian surface epithelium (Marquez et al., 2005). Fifteen of those genes (TM4SF3, S100P, TM4SF4, CEACAM6, LGALS4, CEACAM5, TUBB, CTSE, GCTN, REG4, FABP1, SDCBP2, TFF1, RNFI28, PLAC8) were also identified in our study. Moreover, we also showed that LGALS4 is consistently highly expressed in MOC but is absent in the other histological subtypes of ovarian cancer and normal ovaries using immunohistochemistry, thus confirming the transcript profiling results.
presence in serum. Serum antibodies against LGALS4 have, however, been reported in a patient with colorectal cancer (Scanlan et al, 1998). Given the high level of LGALS4 expression in MOC, one might predict that similar antibodies could be detected in patients with MOC, which is currently under investigation. Hence LGALS4 may have application as an early serum diagnostic marker of MOC, either alone or in combination with other markers such as CEA (CEACAM5) and CA19.9 (Rapkiewicz et al, 2004; Hart, 2005).

In addition, the high level of LGALS4 expression in MOC may aid in the histological differentiation of primary MOC from metastatic ovarian carcinoma arising at other sites (Heinzelmann-Schwarz et al; manuscript submitted for publication).

As previously suggested (Hess et al, 2004), the obvious genetic similarities of MOC with mucinous-type intestinal carcinomas support a move toward the use of a therapeutic approach tailored to the molecular characteristics of MOC rather than the tissue of origin. Patients with advanced stage MOC generally receive the same adjuvant chemotherapy as the other subtypes of ovarian carcinoma, normally a platinum-based approach combined with paclitaxel. The poor survival associated with advanced MOC may reflect a failure to respond to this regime (Hess et al, 2004). Hence, alternative combination chemotherapy regimes that target both the ovarian and mucinous intestinal genetic components of MOC, such as a platin compound combined with 5-fluorouracil, commonly used in the treatment of intestinal carcinomas, may prove to be more efficacious for MOC. This, however, remains to be tested in appropriate clinical trials.

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