Cancer Letters 318 (2012) 76–85

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Integrative genome-wide expression and promoter DNA methylation profiling identifies a potential novel panel of ovarian cancer epigenetic biomarkers

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ARTICLE INFO

Article history:
Received 16 August 2011
Received in revised form 11 November 2011
Accepted 2 December 2011

Keywords:
Ovarian cancer
Epigenetics
DNA methylation
Biomarkers
Microarray analysis

ABSTRACT

To identify epigenetic-based biomarkers for diagnosis of ovarian cancer we performed MeDIP-Chip in A2780 and CaOV3 ovarian cancer cell lines. Validation by Sequenom massARRAY methylation analysis confirmed a panel of six gene promoters (*ARMCX1*, *ICAM4*, *LOC134466*, *PEG3*, *PYCARD & SGNE1*) where hypermethylation discriminated 27 serous ovarian cancer clinical samples versus 12 normal ovarian surface epithelial cells (OSE) (ROC of 0.98). Notably, CpG sites across the transcription start site of a potential long-intergenic non-coding RNA (lincRNA) gene (*LOC134466*), was shown to be hypermethylated in 81% of serous EOC and could differentiate tumours from OSE ($p < 0.05$). We propose that this potential biomarker panel holds great promise as a diagnostic test for high-grade (Type II) serous ovarian cancer.

1. Introduction

Ovarian cancer is a heterogeneous disease of the female reproductive tract which, despite its relatively low incidence in developed countries, carries a poor prognosis as the 5th leading cause of cancer death in women. Epithelial ovarian cancer (EOC) comprises 90% of all ovarian cancer cases [1]. Type I EOC primarily consists of low-grade serous, mucinous, endometrioid and clear cell subtypes, and is characterised as slow growing with intact DNA repair machinery. Type II EOC, also known as high grade serous morphology tumour, comprises 70% of EOC cases [2] and is characterised by rapid growth with no identified precursor lesions and genome instability (p53 loss) [3]. The molecular events underlying Type II EOC remain poorly understood and despite initial response to chemotherapy, these tumours often recur with chemoresistance. Due to rapid growth and non-specific clinical symptoms, EOC is typically diagnosed at a late stage, when the tumour has spread beyond the pelvis. Despite recent advances in surgery and adjuvant chemotherapeutics, EOC still carries only a 40% survival rate over 5 years. However if diagnosed early, when the lesion is still confined to the ovaries, EOC has a 80–90% survival rate over 5 years [4]. To overcome the non-specific clinical manifestation of EOC and to increase overall survival, molecular markers of preclinical or early stage EOC tumours are required. Existing biochemical markers such as CA125 are suitable as markers for tumour recurrence, but the high false positive rate makes the test unsuitable as an EOC diagnostic marker [5]. It is likely that a panel of molecular markers may be required to increase the specificity of a molecular EOC test [6].

DNA methylation is an epigenetic mark that shows considerable promise in early cancer diagnostics [7], including as a marker of EOC [8]. Aberrant hypermethylation of CpG dinucleotides in CpG island-associated promoters is commonly linked with gene repression and can occur early in cancer cells. Candidate genes, such as *P16*, *MLH1*, *RASSF1*, *PYCARD*, *BRCA1* and *TCEAL* are reported to be hypermethylated in EOC [9–11]. However few studies to date have identified global changes in DNA methylation in EOC [12–14]. Here, we integrated publicly available Type II EOC expression profiles, comparing primary tumours to OSE, together with Type II EOC cell line expression and DNA methylation profiles, to identify common ovarian cancer DNA methylation lesions. We identified a novel six-gene panel, where promoter methylation could differentiate EOC from OSE. In addition, we identified a long intergenic non-coding (linc) RNA gene (*LOC134466*, also known as *ZFN300P1*), that was down-regulated in EOC and the CpG island

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doi:10.1016/j.canlet.2011.12.003

spanning the transcription start site was methylated in approximately 80% of serous ovarian tumours.

2. Materials and methods

2.1. Cell line, tissue and OSE collection and processing

Eight cancer cell lines derived from various subtypes of EOC (IGROV1, OV90, SKOV3, OVCAR3, COLO316, EFO27, TOV112D and TOV21G) along with two human immortalised OSE (HOSE 6.3 and HOSE 17.1) cell lines (Supplementary Table 2) were obtained and cultured as described previously [15]. Cell lines were authenticated by short tandem repeat polymorphism, single nucleotide polymorphism, and fingerprint analyses and passaged for less than 6 months. Forty-six fresh frozen tissue (FFT) tumour samples were obtained with informed consent from women undergoing debulking surgery for EOC at the Royal Hospital for Women (RHW, Sydney Australia), snap frozen in liquid nitrogen and stored at −80 °C. One hundred archival formalin-fixed paraffin embedded (FFPE) tumour samples were processed at RHW and blocks were acquired for DNA methylation analysis. A section from each tumour sample was stained with Haematoxylin and Eosin and regions containing >80% tumour were marked and the corresponding piece (10–25 mg) removed, by macrodissection in the case of FFT and tissue coring with FFPE, for DNA extraction. All tumour samples were obtained prior to any chemotherapeutic treatment. Neoadjuvant chemotherapy was designated as an exclusion criterion. Seventeen pathologically normal OSE were obtained with consent, by scraping the ovary during surgery for non-ovarian gynaecological malignancies followed by establishment of epithelial cells in culture. Cultures were evaluated for purity by staining for high molecular weight cytokeratin to exclude stromal contamination and maintained in culture as previously described [16]. Cell pellets from passage three or less were processed for DNA. Experimental procedures were approved by the Human Research Ethics Committee of the Sydney South East Area Hospital Service, Northern Section (00/115).

2.2. Nucleic acid extraction and processing

Total RNA for RT-PCR was extracted with Qiagen RNeasy mini kit (Qiagen, Almeda CA, USA). One microgram total RNA for RT-PCR was DNase treated (Ambion, Austin TX, USA) and reverse transcribed using oligo dT primers (Promega, Alexandria NSW Australia). Genomic DNA was extracted from tumour and OSE with Qiagen QiaAMP mini kits (Qiagen, Almeda CA, USA), from archival FFPE tissue with Gentra Puregene DNA isolation kit (Qiagen) and from cell lines with the Stratagene DNA extraction Kit (Agilent, Santa Clara CA, USA). One to two micrograms genomic control DNA (Roche Applied Sciences, Indianapolis IN, USA) *in vitro* methylated DNA (Chemicon International, Temecula CA, USA) and RNase treated cell line and tumour DNA was bisulphite converted either using the Epitect kit (Qiagen, Almeda CA, USA) or as previously described [17,18].

2.3. Pharmacological reactivation of methylated genes

Experiments were performed in triplicate using ovarian cancer cell lines CaOV3 and A2780, derived from poorly differentiated primary ovarian adeoncarcinomas [19–22]. The rationale for selection was based on a screen for responsiveness to 5-aza-dC treatment including gene re-expression and cellular toxicity in multiple EOC cell lines (data not shown). Cell lines were treated at 30% confluence with 5 μM and 2.5 μM respectively (concentrations previously optimised to minimise cellular toxicity, data not shown), with the DNA methyltransferase inhibitor 5-aza-2′deoxycytidine (5-Aza-dC), (Sigma Aldrich, St. Louis MO, USA) for 24 h. Cells were then expanded to 90% confluency, media changed every 24h before extracting RNA (RNeasy, Qiagen). As a positive control, re-expression and DNA demethylation for the methylated gene *DLEC1* was verified by qPCR and clonal bisulphite sequencing (Supplementary Fig. 1A and B).

2.4. DNA methylation analysis

2.4.1. Sequenom massARRAY quantitative methylation analysis

T7 tagged Sequenom methylation PCR assays were designed according to Coolen et al. [23] for bisulphite converted DNA specificity, and tested for bias using a thermal gradient on mixes of 50% methylated:unmethylated template. Assays were performed in triplicate, as per conditions indicated in Supplementary Table 1, and SYBR heat dissociation curves using ABI 7900HT to ensure appropriate amplification. Replicates were pooled, SAP treated, reverse transcribed, cleaved and applied to spectrochips according to manufacturer's instructions for MALDI-TOF analysis (Sequenom, San Diego CA, USA) and results analysed using epityper software, the R [24] package RseqMeth [25] and Microsoft Excel™. CpG methylation levels were averaged across the amplicon and average methylation levels greater than 25% were called positive. Clonal bisulphite sequencing analysis was performed on selected pooled Sequenom PCR products, as previously described [18].

2.4.2. Methylation specific headloop suppression PCR assay (MSH-PCR)

Methylation specific headloop suppression assay (MSH-PCR) was designed as previously described [26], with MSH-PCR directed against CpG methylation at the transcriptional start site (TSS), as determined by refSeq, of *LOC134466* (Supplementary Fig. 2A). MSH-PCR reaction conditions were optimised to distinguish methylated from unmethylated DNA (Supplementary Fig. 2B), using fully methylated (Chemicon International, Temecula CA, USA) and unmethylated control (OV90) DNA. Triplicate MSH-PCRs were performed on bisulphite converted DNA, from 100 FFPE EOC and 13 EOC. The melting temperature (Tm) of the amplicon was calculated from the derivative SYBR signal during a heat dissociation cycle. Samples were considered methylated with a Tm of >78 °C as compared to the fully methylated control DNA (Supplementary Fig. 2C).

2.5. mRNA expression analysis

Total RNA from A2780 and CaOV3 cell lines, plus and minus treatment with 5-Aza-dC was characterised on the Agilent bioanalyzer RNA nano chip (Agilent, Foster City CA, USA) for an RNA integrity number (RIN) of >9.0, labelled and hybridised to Affymetrix human genome U133 plus 2 GeneChips according to manufacturer's instructions. The GeneChip scans were analysed by GCOS (Affymetrix) and resulting probeset intensities normalised by the robust multi-array average (RMA) method [27]. Principle components analysis (PCA) indicated that the two cell lines' expression profiles were distinct (data not shown). The data were normalised separately for each cell line and robust multichip average (RMA) values for analysis were imported into Genespring GX 7.3. Genes re-expressed by 5-Aza-dC were identified as "absent" (undetectable) in all three replicate untreated samples and "present" or "marginal" (detectable) in two of three replicate 5-Aza-dC treated samples. Unsupervised hierarchical clustering was used to show that the array expression results for genes identified as of interest were capable of distinguishing 5-Aza-dC treated from untreated cell lines (Supplementary Fig. 3). qRT-PCR was performed in triplicate using the ABI 7900HT Real-Time PCR System (Applied Biosystems, Foster City CA, USA), under standard thermocycling with ABI Power SYBR mastermix (Applied Biosystems), diluted cDNA. Primer sequences are shown in Supplementary Table 1.

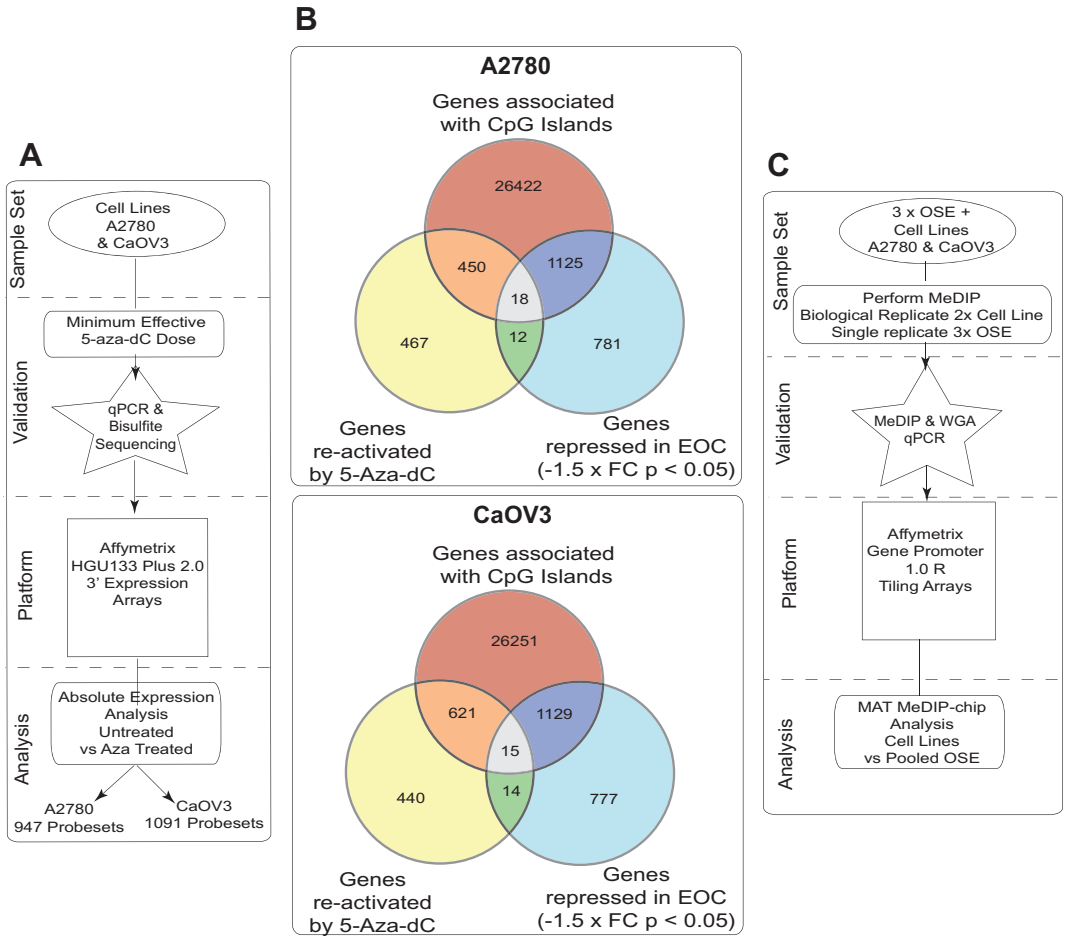
2.6. DNA methylation profiling and analysis

Whole genome DNA methylation profiling was performed on EOC cell lines A2780 & CaOV3 (in duplicate) and from three short-term primary cultures of normal ovarian surface epithelium samples, as previously described [28]. Briefly, 4 μg genomic DNA was fragmented to a mean fragment length of ~400 bp and immunoprecipitation (IP) of methylated DNA was performed using a mouse anti-5-methylcytosine MAb (Millipore, Billerica MA, USA) and enriching on protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz CA, USA). IP and total genomic DNA were purified by phenol: chloroform extraction and subjected to whole genome amplification (WGA) in duplicate (WGA2+, Sigma Aldrich, St. Louis MO, USA). SYBR qPCR was performed to ensure the ratio of enrichment of methylated DNA in the IP to input was retained through WGA (Supplementary Fig. 1C) before amplified samples were fragmented, labelled and applied to Affymetrix whole genome promoter 1.0R tiling arrays and scanned. Array CEL files were normalised and analysed using MAT [29] for aroma.affymetrix [30] with a smoothing window of 600 bp (Supplementary Fig. 1D). Output was visualised in the integrated genome browser (IGB, Affymetrix) and interrogated using the Repitools package [25]. The Repitools function 'significancePlot' was used to visualise averaged promoter methylation of multiple genes, whereby normalised MeDIP-Chip output at a selection of gene promoters was averaged and compared to the output of 1000 random gene selections of the same size. To assess the extent of copy number aberrations in the samples, promoter tiling profiles of total DNA inputs were compared to a genomic reference. Large scale copy number variations (CNV) were observed in the cancer cell lines, particularly the p53 mutant CaOV3 (Supplementary Fig. 4), consistent with SNP data from the cancer genome project (http://www.sanger.ac.uk/), whereas no large scale CNV were observed in OSE. To address the potential impact of copy number differences on readout, all array data was normalised to inputs before comparisons were made between samples.

3. Results

3.2. Discovery pipeline of methylated genes in EOC

Three steps were implemented to identify aberrantly silenced and methylated genes in EOC (Fig. 1A–C). First, to identify genes potentially silenced by DNA methylation, genome wide epigenetic re-expression profiles were generated for two ovarian cancer cell lines (Fig. 1A). A2780 and CaOV3 cells were treated with DNA methyltransferase inhibitor 5-Aza-deoxycytidine (5-Aza-dC), under conditions that induce re-expression and demethylation of the methylated gene *DLEC1* (Supplementary Fig. 1A and B) and



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	Gene Symbol	Probe-set ID	Fold Induction by 5-Aza-dC	Fold Repression in EOC	Additional Inclusion Criteria
A2780	ZNF177	207417_s_at	1.61	- 1.75	
	ZNF542	239250_at	2.35	- 2.11	
	SCIN1	1552365_at	1.28	- 2.26	
	PARVA	222454_s_at	1.78	- 2.51	
	SPOCK2	202524_s_at	1.41	- 2.99	N/A
	LTBP2	223690_at	1.57	- 3.02	
	HSPA2	211538_s_at	33.78	- 3.29	
	PAPLN	226435_at	2.78	- 4.2	
	PEG3	209242_at	3.87	- 16.13	
	PYCARD	221666_s_at	3.72	NA	[37]
	SOCS1	210001_s_at	4.80	NA	[38]
	ARMCX1	218694_at	1.19	- 4.44	[34]
	SGNE1	203889_at	4.15	- 4.55	[39]
	IL18	206295_at	1.20	- 4.72	[35]
CaOV3	LOC134466	244289_at	1.18	- 2.92	
	PTGER	213933_at	1.32	- 4.69	N/A
	TLE4	204872_at	1.20	- 6.49	
	KLF4	220266_s_at	3.78	- 8.85	
	BMP6	206176_at	1.25	NA	[36]
	CST6	231248_at	1.96	NA	[36]
	ICAM4	207194_s_at	1.89	NA	[45]

Fig. 1. Pipeline to identify candidate genes aberrantly silenced by methylation in Type II EOC. (A) Identification of genes that were induced after 5-aza-dC treatment of Type II EOC cell lines. (B) Integration of re-expression cell line data, with publicly available data of repressed genes in HGOC relative to OSE and promoter association with CpG islands. (C) Identification of genes using direct profiling of gene promoter DNA methylation using MeDIP-Chip. (D) Candidate genes chosen for validation as potential methylated biomarkers of EOC. The white, orange and green shading correspond to the shading from (B) with white denoting genes displaying all three properties addressed in (B). The orange and green shading highlight additional candidate genes that exhibited two of the three properties addressed in (B) and published evidence of a role in cancer (additional inclusion criteria). (See above-mentioned references for further information.)

mRNA expression profiles were generated using Affymetrix whole genome HGU133 plus 2.0 arrays. Genes showing potential methylation-based silencing were identified if they were undetectable in the untreated cells, but re-expressed by 5-Aza-dC treatments. Using these criteria we identified 947 and 1091 probesets in A2780 and CaOV3 respectively, that were reactivated (Fig. 1A).

Second, to identify potentially methylated genes that were also down-regulated in clinical EOC, we analysed publicly available

gene annotations and mRNA expression profiles in 54 Type II EOC compared with 10 OSE tissue samples (>1.5 negative fold change, corrected *p*-value >0.05) [31] (Fig. 1B). Genes corresponding to the 5-Aza-dC re-expressed probesets were filtered based on alignment to a promoter associated CpG island [32]. The resulting gene lists were then compared with probesets consistently down-regulated in Type II EOC, in order to enrich for genes likely to have a function *in vivo*. Genes displaying multiple criteria (i.e. re-expressed by 5-Aza-dC in either cell line and down regulated in EOC and/or associated with CpG islands) formed the basis for selection of candidates for further validation.

Third, genome-wide methylation profiles were generated by methylated DNA immunoprecipitation, followed by promoter tiling array analysis (MeDIP-Chip) for A2780, CaOV3 and three normal OSE samples (Fig. 1C). Direct measurement of DNA methylation was performed on candidate genes, by MeDIP-Chip and Sequenom massARRAY gene promoters, to assess differential levels of DNA methylation (Supplementary Fig. 1D). Averaged promoter methylation levels were plotted (−2000 bp to +500 bp relative to the transcription start site (TSS) as determined by refSeq) of the candidate gene list versus random gene selections in the cancer cell lines and compared to pooled OSE (Fig. 2). Both ovarian cancer cell lines showed an increase in methylation at the TSS compared to OSE, consistent with CpG island methylation being a hallmark of cancer [33]. A2780 cells displayed hypermethylation at the TSS in all genes re-expressed by 5-Aza-dC, and a modest increase when limited to genes with CpG islands (Fig. 2A). Interestingly in CaOV3, minimal hypermethylation was observed for 5-Aza-dC responsive genes (Fig. 2B), suggesting that fewer genes are hypermethylated in CaOV3 relative to A2780.

3.3. Validation of candidate gene methylation and expression in multiple EOC cell lines

From the genes identified in the discovery pipeline (Fig. 1A), 21 genes that were re-expressed by 5-Aza-dC were chosen for further evaluation as they also met at least two of the following criteria: (1) contained a CpG island associated promoter, as determined by the USCS genome browser (www.genome.uscs.edu, human genome build 18); (2) were down-regulated in ovarian cancer, according to the Bonome et al. 2005 study that compared 54 Type II EOC and 10 OSE [31]; (3) had a potential role in cancer as ascertained from the NCBI entrez gene reference into function (www.ncbi.nlm.nih.gov/gene) and PubMeth (www.pubmeth.org) (Fig. 1D). Validation of methylation of these 21 gene promoters was performed using Sequenom massARRAY assays on A2780 and CaOV3 DNA. Methylation states between MeDIP-Chip and Sequenom assays were compared on a gene-by-gene basis and mapped relative to the TSS, with seven examples shown in Fig. 3A (methylation of remaining genes shown in Supplementary Fig. 5). The correlation between the two methylation assays; MeDIP-Chip and Sequenom was assessed for all 21 genes in both cell lines and was found to be 0.5604 and 0.6677 respectively (Fig. 3B). Sequenom assays confirmed that 16/21 (76%) genes were methylated in at least one of the ovarian cancer cell lines. Methylation frequency of these 16 genes was then tested in an expanded cohort of three OSE, two immortalised OSE cell lines (HOSE17.1 and HOSE6.3) and multiple EOC subtypes, including serous (IGROV1, OV90, SKOV3, OVCAR3 & COLO316), mucinous (EFO27), endometrioid (TOV112D) and clear cell (TOV21G) subtypes (Fig. 4A). 15/16 genes showed hypermethylation in the broad panel of EOC subtypes and a lack of methylation

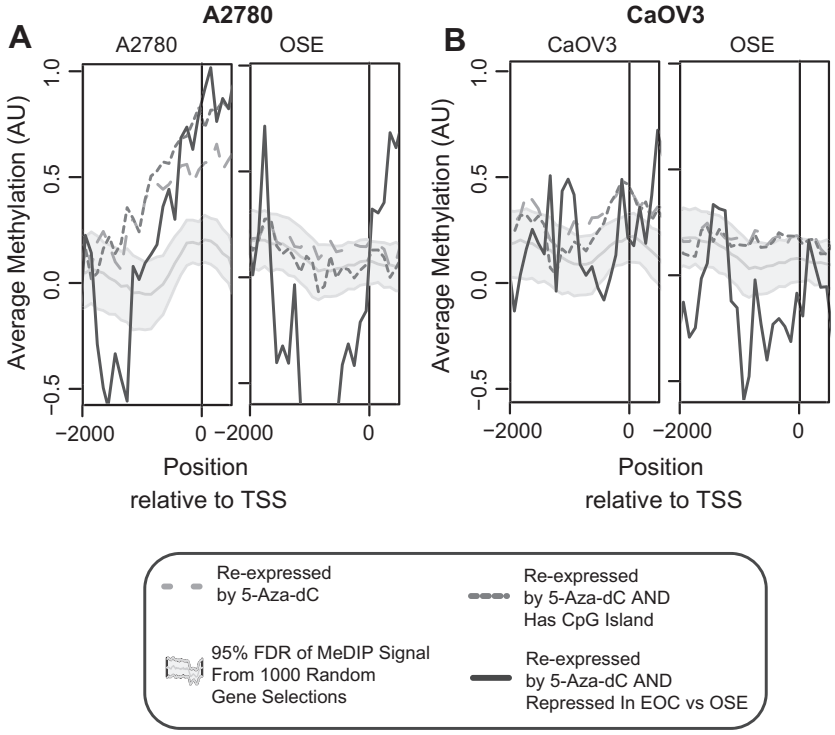

Fig. 2. Methylation profiles of gene candidate lists in cancer cell lines and OSE relative to random gene selections. (A) Averaged MeDIP-Chip signals from candidate genes identified from A2780 cells ($n = 947$ genes re-expressed by 5-aza-dC (light grey broken line) of which either 468 have a CpG island (dark grey broken line) or 30 are downregulated in Type II EOC versus OSE (solid black line). (B) Averaged MeDIP-Chip signals from candidate genes identified from CaOV3 cells ($n = 1091$ genes re-expressed by 5-aza-dC (light grey broken line) of which either 635 have a CpG island (dark grey broken line) or 30 are downregulated in Type II EOC versus OSE (solid black Line). Averaged MeDIP-Chip signal (*y* axes) of gene candidate lists over the promoter (2 kb upstream) of the TSS (*x*-axes) in EOC cell lines A2780, CaOV3 and OSE. Methylation distributions of 1000 random selections of genes were plotted (grey shading) for comparison.

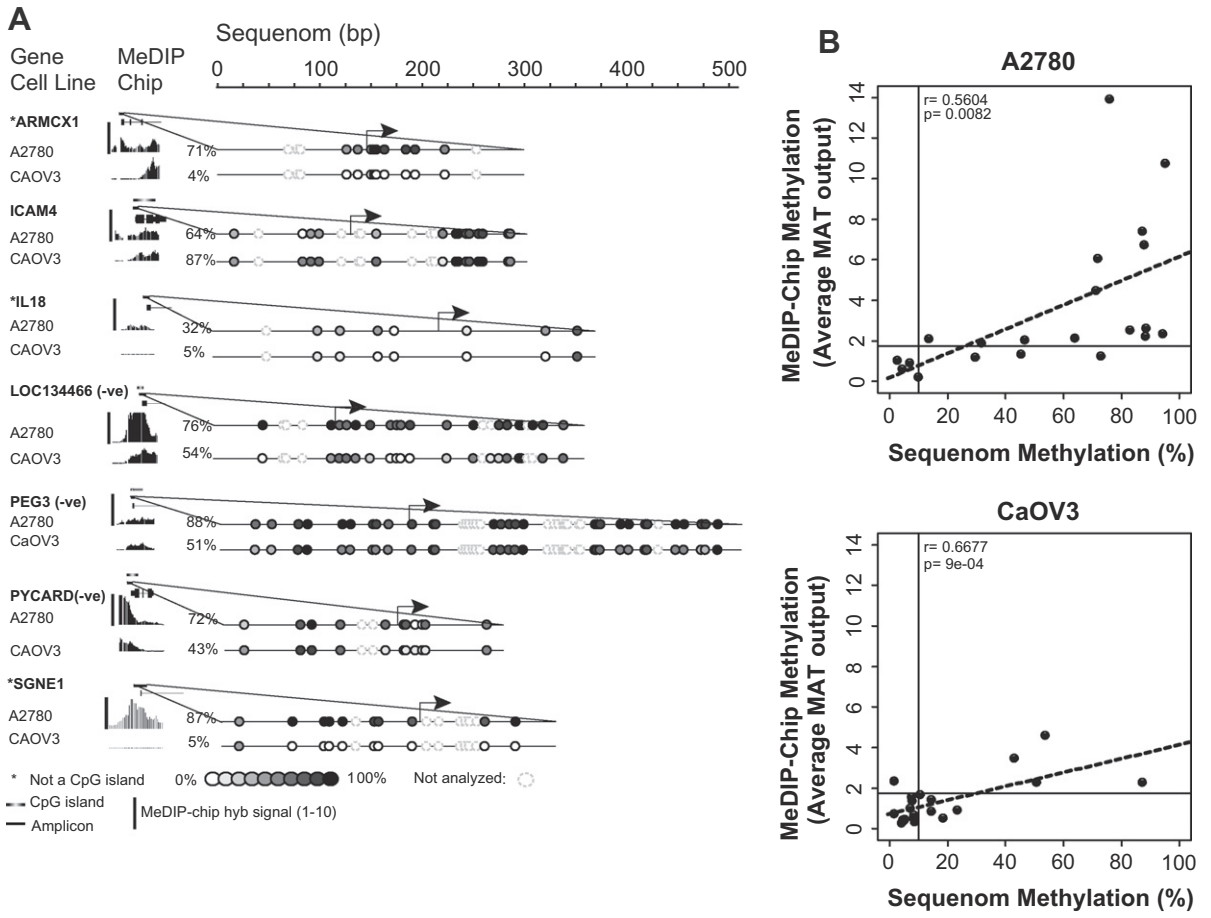

Fig. 3. Validation of gene candidate methylation by Sequenom in A2780 and CaOV3 cell lines. (A) Sequenom massARRAY data for seven example candidate gene promoters in A2780 and CaOV3, with corresponding MeDIP-Chip profile (remaining 14/21 genes are shown in Supplementary Fig. 5). Sequenom data was used to calculate individual informative CpG methylation levels (grayscale) and an average promoter methylation value (percentage). Informative CpG units were identified by excluding overlapping peaks and CpG dinucleotides within primer sequences. TSS are shown as arrows, * indicates non-canonical CpG island and −ve indicates genes on the negative DNA strand. (B) Direct comparison of MeDIP-Chip (*y*-axis) with average Sequenom (*x*-axis) correlation of average DNA methylation measured by Sequenom (*x*-axis) and MAT analysis for the 21 gene promoters in A2780 (top panel) and CaOV3 (bottom panel), Pearson's correlation coefficient (*r*) is calculated and line of best fit is plotted (dotted line).

in normal OSE cells. *SCIN* was methylated in only one cancer cell line and was omitted from further analysis. To investigate if DNA methylation was associated with gene repression, eight genes that displayed some methylation in the cell lines were tested by qPCR in two immortalised OSE and eight EOC cell lines. Hypermethylation was qualitatively associated with gene repression, as evidenced by a negative slope in the line of best fit, for the majority of genes (*ARMCX1*, *HSPA2*, *PEG3*, *PYCARD*, *ICAM4*). However some genes (*IL18* and *ZNF177*) showed little association, or were already repressed (*SCIN*) in nearly all cell lines (Fig. 4B).

3.4. Gene methylation in primary serous ovarian cancer and normal tissues

Next we measured promoter methylation of the 15 genes using Sequenom massARRAY in a panel of 19 serous EOC patient samples (Fig. 5A). Hierarchical clustering by DNA methylation levels revealed that eight of these genes (*ARMCX1*, *ICAM4*, *IL18*, *LOC134466*, *PEG3*, *PYCARD*, *SGNE1 & ZNF177*) were hypermethylated in more than one tumour (Fig. 5A). Promoter methylation levels for these eight genes were then compared between an expanded panel of cancer tissue ($n = 27$), as well as OSE ($n = 12$), by plotting a receiver operating characteristic (ROC), and calculating the area under the curve (AUC) of methylation levels in cancer versus normal (Fig. 5B). To evaluate the performance of the eight genes as a panel, a logistic regression model was fitted to the gene methylation data. To identify methylated genes that could potentially contribute to a cancer diagnostic, feature selection was performed by stepwise removal of the least significant gene from the model followed by analysis of variance (ANOVA). This approach revealed that methylation of a panel of six genes (*ARMCX1*, *ICAM4*, *LOC134466*, *PEG3*, *PYCARD* and *SGNE1*) was a potent discriminator of cancer versus normal, with a high AUC (0.98). In addition, methylation of *LOC134466* a putative pseudogene of *ZNF300*, located at chromosome 5q33.1, was identified as an individual discriminator (AUC = 0.72), indicating that this gene may be a potential new independent marker of EOC, especially as there was no evidence of genetic deletion across this locus in A2780 or CaOV3 (Supplementary Fig. 4B).

3.5. LOC134466 is a novel gene commonly hypermethylated in EOC

A more detailed analysis of *LOC134466* methylation was performed using bisulphite clonal sequencing in five EOC and four OSE patient samples. We found an enrichment of methylation at four CpG dinucleotides at the TSS in tumours, relative to OSE DNA (73.6% and 10.3% respectively, chi squared $p > 0.0001$) (Fig. 6A). In addition, methylation of the four CpG dinucleotides was commonly associated with gene repression in immortalised OSE, and EOC cell lines (Fig. 6B). *LOC134466* methylation was quantified by Sequenom massARRAY for an additional 42 cancers (comprised of DNA from 19

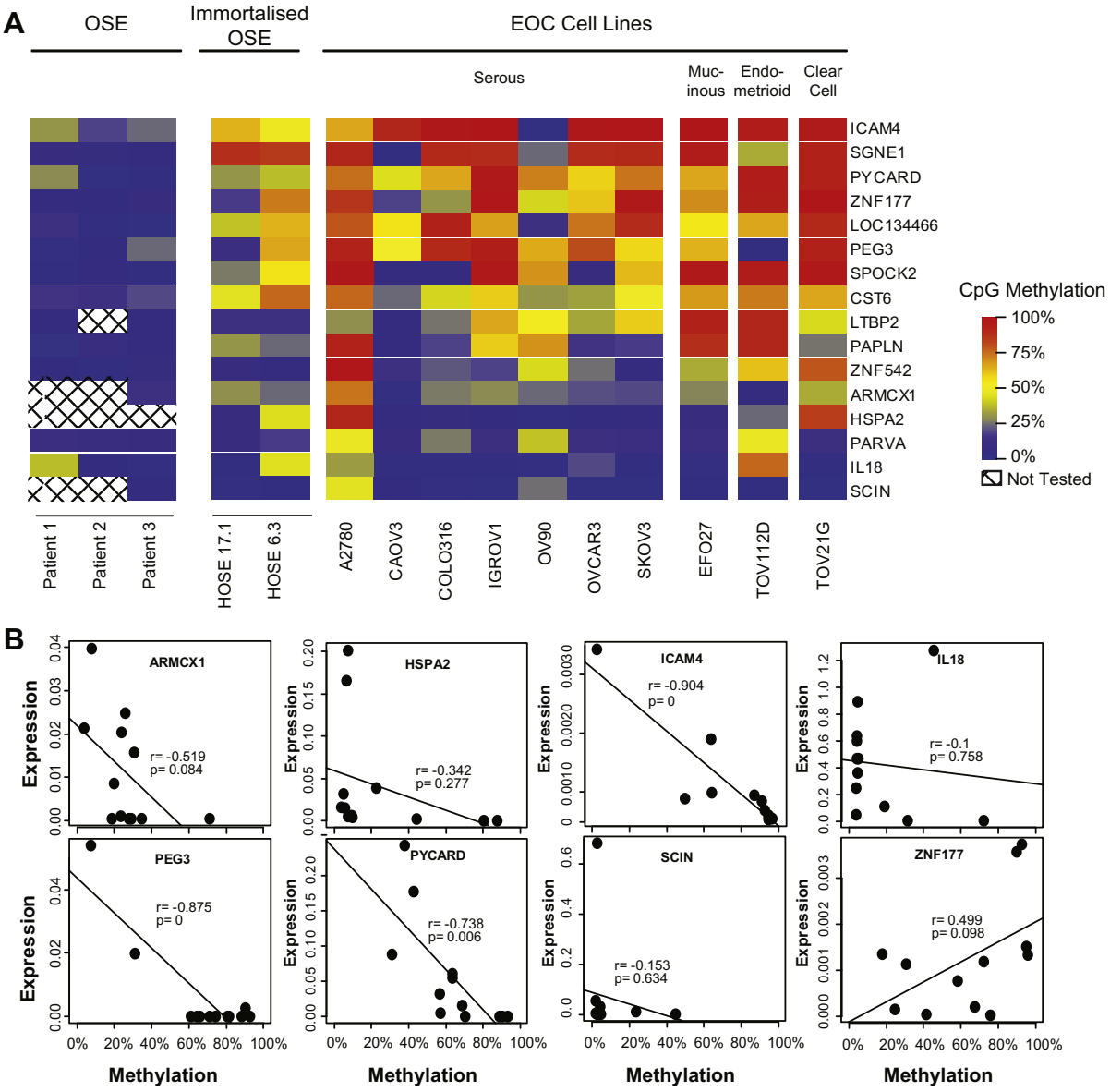

Fig. 4. Analysis of methylation in primary EOC and OSE (A) Average promoter methylation, as determined by Sequenom analysis of 16 candidate genes in a panel of primary OSE, immortalised OSE (HOSE) and EOC cell lines. (B) Relative expression (normalised to 18S) of eight methylated genes versus average promoter methylation. A line of best fit is plotted and Pearson's correlation coefficient (r) and p-value shown.

FFT and 23 FFPE) and two OSE, giving a total cohort of 69 cancers and 14 OSE (Table 1). Unsupervised hierarchical clustering (Fig. 6C) revealed that a high proportion (34/69, 49%) of cancers were hypermethylated at these four CpG sites, relative to OSE (0/14; chi squared 13.25, $p = 0.0005$). Averaged CpG methylation at these sites was significantly higher in cancers than OSE ($p < 0.005$, Fig. 6D) and enabled discrimination of cancers versus OSE by ROC curve (AUC = 0.74, Fig. 6E). To examine the potential of hypermethylation of the *LOC134466* locus for detection of ovarian cancer, we developed a methylation specific headloop suppression assay (MSH-PCR) [26,40], to specifically interrogate the methylation status of the four TSS-associated CpG sites in archival formalin-fixed paraffin-embedded EOC, relative to OSE (Fig. 6F and Table 2). Hypermethylation was observed in 81% (81/100) of cancers relative to 7.7% of OSE (1/13). Fisher's exact test was performed in the cancer cohort of 100 patients, to investigate if DNA methylation of *LOC134466* was associated with several clinicopathological parameters. *LOC134466* methylation was found to not be associated with age (>65 versus <65, $p = 1$), stage (I + II versus III + IV, $p = 1$), grade (1 versus 2 + 3 $p = 0.58$), ascites ($p = 0.78$) or complete clinical response (no evidence of residual disease and/or biochemically negative three months later, $p = 0.21$).

4. Discussion

Type II EOC carries a high mortality rate due to a lack of clinical signs and molecular markers of early stage disease. Since aberrant DNA methylation occurs early in cancer progression and can be readily detected in clinical samples, this modification provides great potential for an early stage ovarian cancer biomarker [8]. However, it is becoming increasing clear that single gene markers will be less effective in cancer detection and a panel of methylated markers will be needed to provide increased sensitivity. In order to uncover potential methylated biomarkers of ovarian cancer, we employed an integrative approach that combined whole genome methylation and gene expression profiles from ovarian cancer cell lines with primary tissue expression profiles. Epigenetic

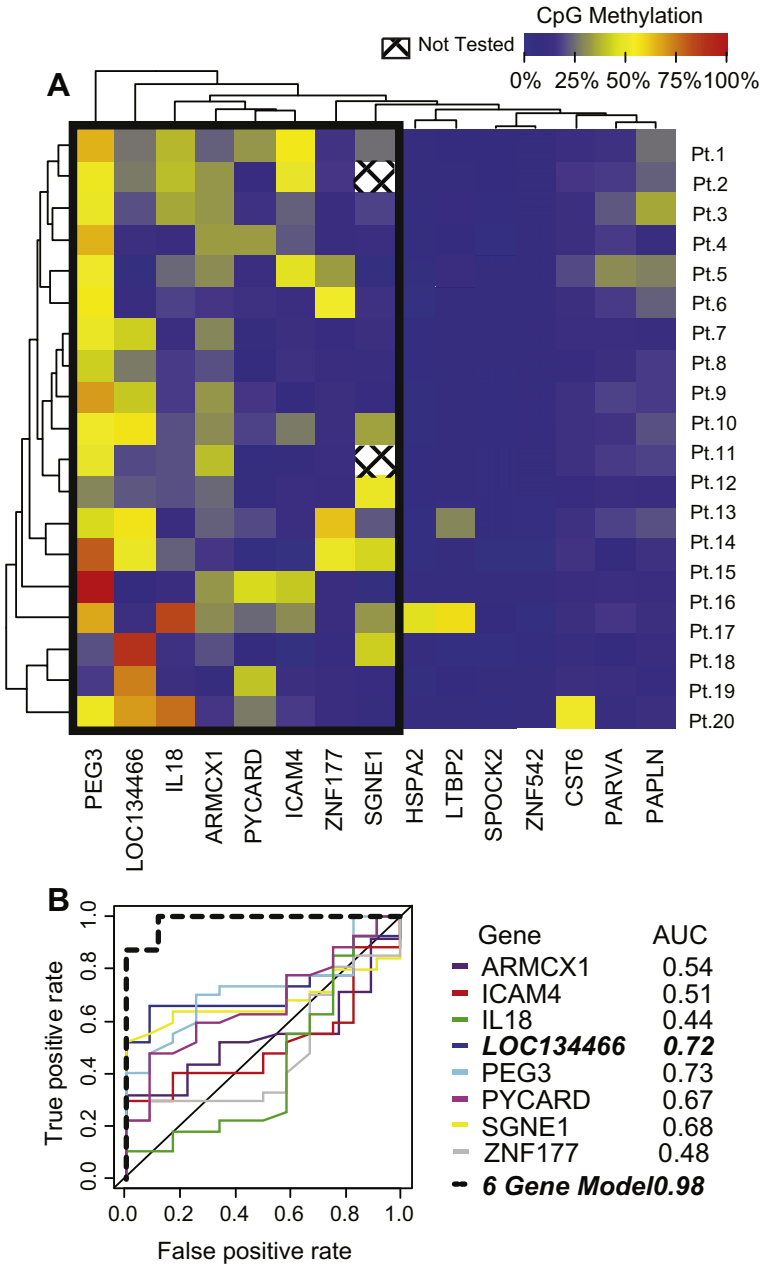

Fig. 5. Methylation in primary tumors and associated ROC curves (A) Hierarchical clustering of average promoter methylation for 15 candidate genes in a panel of 19 serous EOC. Eight genes (enclosed by box) exhibit evidence of methylation in multiple tumor samples. (B) Receiver operating characteristic (ROC) curves, generated from methylation from an extended panel of cancer (n = 27) and normal OSE (n = 12). Area under the curve (AUC) for the eight genes identified in (A) and logistic regression model of six genes identified as best fitted to the data by ANOVA (black broken line).

re-expression profiles in cancer cell lines provide a valuable tool in uncovering genes that are potentially repressed by promoter DNA methylation. However, dissecting specific effects of 5-Aza-dC treatment that result in DNA demethylation, from global effects of 5-Aza-dC treatment on cellular toxicity and cell death, alterations in chromatin [41], or secondary effects of gene reactivation can be difficult. Limiting the re-expression lists to genes that are associated with promoter CpG islands we reasoned would likely remove some, but not all, of the off-target effects of 5-Aza-dC treatment. We therefore directly measured DNA methylation to help identify genes silenced by DNA methylation in ovarian cancer cells. In addition, we incorporated genes consistently repressed in expression profiles from Type II EOC versus OSE to uncover genes that were commonly repressed in ovarian cancer and thus likely to be important in EOC development. The high proportion (76%) of genes showing hypermethylation in either or both ovarian cell lines validated the incorporation of multiple sources of data and filtering to discover aberrantly methylated cancer associated genes.

Studies of single gene candidate methylation frequencies in EOC have shown varying results [8]. Genes commonly methylated in multiple cancers, such as *BRCA1*, *RASSF1A* and *MLH1*, have been shown to be methylated in less than 50% of EOC. Fewer genes, such as *OPCML* [42] and *TCEAL* [9], have shown methylation frequencies in excess of 80%. The high degree of gene methylation variation observed in EOC has indicated the need to identify EOC specific methylation patterns to better characterise the molecular changes underlying the development of the disease. These changes can then be utilised to develop early diagnostic tests. However, there is accumulating evidence that the fallopian tube epithelium may give

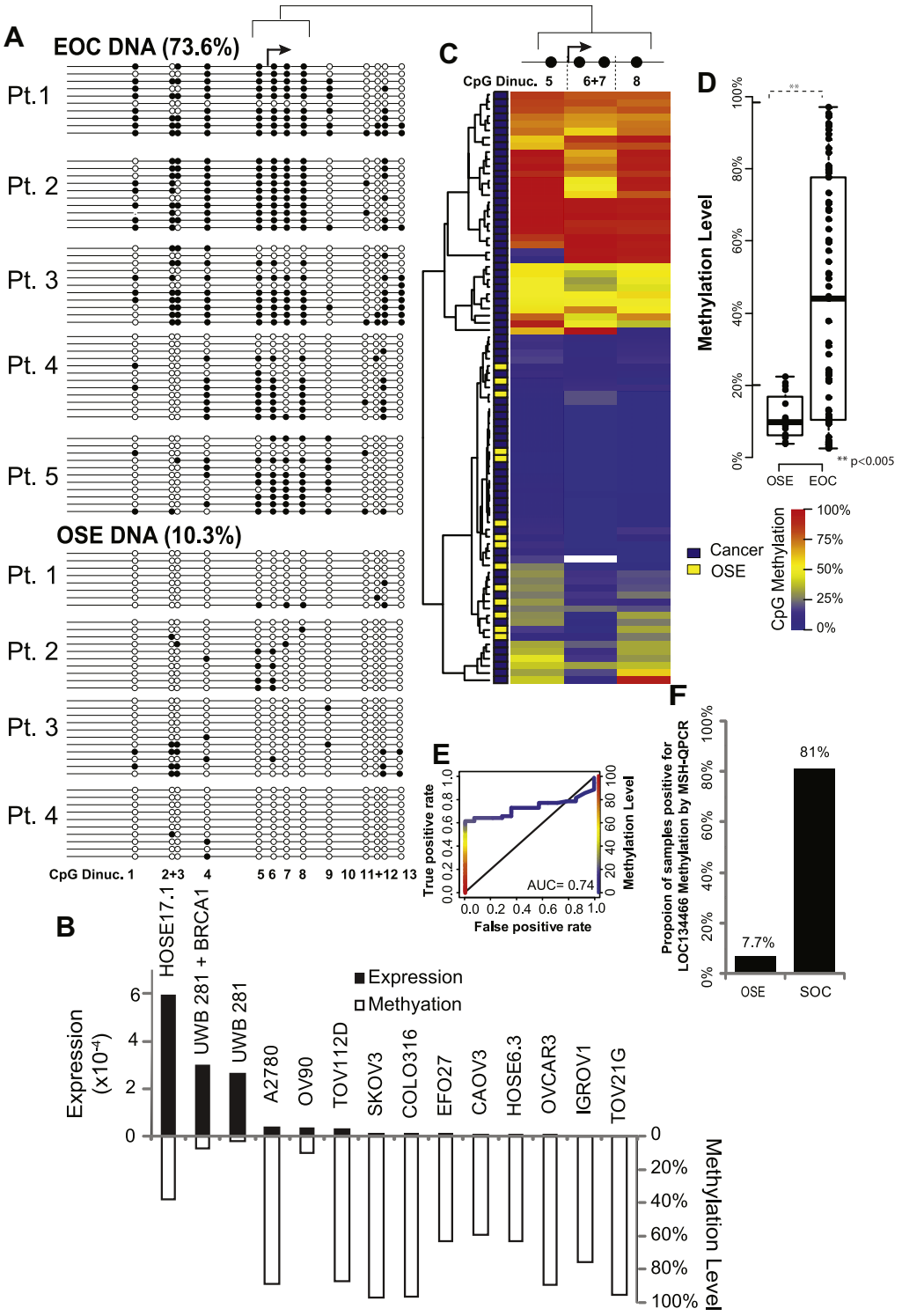

Fig. 6. Detailed analysis of LOC134466 (A) Bisulphite sequencing of *LOC134466* promoter DNA from five TYPE II EOC tumors and four OSE. Each line represents a clone with CpG dinucleotides in circles. Open circles indicate unmethylated and closed indicate methylated. Arrow represents the TSS of the gene. (B) Expression of *LOC134466* (relative to 18S) in cell lines compared with average methylation of CpG units at the TSS of the gene. (C) Hierarchical clustering of Sequenom methylation levels at the three CpG units (corresponding to the four CpG dinucleotides identified in A) flanking the TSS for 69 Type II EOC tumors and 14 OSE. Averaged methylation for this region is significantly higher in tumor samples (D) as tested by Mann–Whitney U test and a low false-positive rate for cancer discrimination at high methylation levels by ROC curve (E). (F) Percentage of samples positive for headloop suppression PCR assay designed to interrogate CpGs at the TSS for 13 OSE and 100 archival FFPE Type II EOC samples.

rise to some Type II EOC [43]. Therefore, to develop a diagnostic test based on these results, more testing is required on normal tissues, including cells derived from fallopian tube.

Using quantitative gene specific DNA methylation assays we identified a novel six-gene methylation panel that could discriminate EOC from normal OSE. Interestingly, many of these genes have previously been reported to be involved in cancer. *ARMCX1* is an armadillo repeat containing tumour suppressor gene (TSG) that is inactivated in ovarian, bladder and colorectal cancer [34,44]. *ICAM4* is an intracellular adhesion molecule capable of binding alpha (V) integrins and is located in a breast and prostate susceptibility locus (19q 13.2) [45]. *PEG3* has previously been shown to be a

Table1
Cohort details.

Age	EOC (n = 147)	OSE (n = 17)
	Median = 59.6 Range = 24–85.8	Median = 47 Range = 36.1–61
Characteristic		Number of EOC patients
FIGO stage	I/II	17
	III/IV	130
Tumour grade	G1	4
	G2	58
	G3	85
Ascites present at surgery		91
Complete clinical response		55

TSG with aberrant methylation changes in a small proportion of ovarian cancers [46]. *SGNE1* is a molecular chaperone protein important in neural function and has been shown to be hypermethylated in medulloblastoma [39]. *PYCARD*, a potent TSG has previously been shown to be methylated in ~19% of EOC [37]. These genes are diverse in molecular function, suggesting that there may be a cumulative effect or multiple pathways involved in deregulation of these genes contributing to ovarian cancer pathogenesis. By combining these genes in a methylation biomarker panel, there is the potential to improve levels of ovarian cancer specificity, by detecting multiple molecular aberrations evident in the disease [6].

Interestingly, we also identified a novel methylated marker *LOC134466*, also known as *ZFN300P1*, a pseudogene of *ZNF300*, which is highly expressed in normal ovary [47]. Our results demonstrate that *LOC134466* is commonly repressed in EOC, and is hypermethylated in the CpG island spanning the TSS in approximately 80% of ovarian cancer DNA. Furthermore, CNV analysis of the Type II EOC cell lines showed no evidence of genetic deletion (Supplementary Fig. 4B), our results represent the first evidence of deregulation of *LOC134466* in cancer and shows that DNA methylation is a potential mechanism associated with decreased RNA expression in a high proportion of Type II EOC tumours. Notably, the *LOC134466* transcript has been recently identified as a putative large intergenic non-coding RNA (lincRNA), localised to the cell nuclei and is associated with targeting the epigenetic repression complexes PRC2 and coREST to many genomic regions [48]. In addition, lincRNAs have been implicated in cancer metastasis [49] and have also been shown to mediate global gene responses to p53 [50], suggesting that methylation associated silencing of *LOC134466* may be a critical step in the oncogenic progression of a high proportion of Type II EOC. Due to the high frequency of hypermethylation in EOC and potential role in carcinogenesis, further investigation is underway to elucidate the potential role of this transcript in EOC development.

From an integrated approach to candidate discovery, we have been able to identify a six-gene panel of potential biomarkers that exhibit hypermethylation in Type II EOC. The biomarker panel holds great promise to provide the basis for an early stage diagnostic test, which may greatly improve outcomes for patients, however more testing is required on larger cohorts to validate its clinical utility. Moreover, we have demonstrated that the putative lincRNA *LOC134466* is repressed and hypermethylated in a large proportion of Type II EOC. Due to the high level of promoter methylation of this gene in EOC, further investigation is underway to elucidate the role of the *LOC134466* non-coding RNA transcript in ovarian cancer development and progression.

Acknowledgements

We thank Clare Stirzaker and Jenny Song for help with MeDIP training and troubleshooting, Rebecca Hinshelwood for help with developing and interpreting bisulphite analyses, Marcel Coolen for scientific discussions and help with Sequenom, Warran Kaplan for help with experimental design of re-expression arrays and Genespring, Mark Cowley for help with flags analysis, Aaron Statham for help with R programming. This work was supported by funding from NHMRC (SJC), Cancer Australia Fellowship (PMO), Cancer Institute NSW, Gynaecological Oncology Fund and an Australian Postgraduate Award (BSG).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2011.12.003.

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