RESEARCH PAPER

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Epigenetic analysis of sporadic and Lynch-associated ovarian cancers reveals histology-specific patterns of DNA methylation

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Abbreviations: Dm, methylation dosage ratio; miRNAs, microRNAs; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; LS, Lynch syndrome; TSG, tumor suppressor gene; WT1, Wilms tumor suppressor 1 sense; WT1-AS, Wilms tumor suppressor 1 antisense

Diagnosis and treatment of epithelial ovarian cancer is challenging due to the poor understanding of the pathogenesis of the disease. Our aim was to investigate epigenetic mechanisms in ovarian tumorigenesis and, especially, whether tumors with different histological subtypes or hereditary background (Lynch syndrome) exhibit differential susceptibility to epigenetic inactivation of growth regulatory genes. Gene candidates for epigenetic regulation were identified from the literature and by expression profiling of ovarian and endometrial cancer cell lines treated with demethylating agents. Thirteen genes were chosen for methylation-specific multiplex ligation-dependent probe amplification assays on 104 (85 sporadic and 19 Lynch syndrome-associated) ovarian carcinomas. Increased methylation (i.e., hypermethylation) of variable degree was characteristic of ovarian carcinomas relative to the corresponding normal tissues, and hypermethylation was consistently more prominent in non-serous than serous tumors for individual genes and gene sets investigated. Lynch syndrome-associated clear cell carcinomas showed the highest frequencies of hypermethylation. Among endometrioid ovarian carcinomas, lower levels of promoter methylation of *RSK4*, *SPARC*, and *HOXA9* were significantly associated with higher tumor grade; thus, the methylation patterns showed a shift to the direction of high-grade serous tumors. In conclusion, we provide evidence of a frequent epigenetic inactivation of *RSK4*, *SPARC*, *PROM1*, *HOXA10*, *HOXA9*, *WT1-AS*, *SFRP2*, *SFRP5*, *OPCML*, and MIR34B in the development of non-serous ovarian carcinomas of Lynch and sporadic origin, as compared to serous tumors. Our findings shed light on the role of epigenetic mechanisms in ovarian tumorigenesis and identify potential targets for translational applications.

Introduction

Epithelial ovarian cancer is the most lethal gynecological malignancy due to late diagnosis of the disease.[1] Heredity is a major risk factor; at least 1 ovarian cancer in 10 is estimated to develop as the result of autosomal dominant predisposition with high penetrance.[2] Lynch syndrome (LS), which is associated with germline mutations in 1 of 4 DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*), is the third most common cause of inherited ovarian cancer after *BRCA1* and *BRCA2* mutations. The estimated lifetime risk of ovarian carcinoma in women with LS is up to 12%.[3,4]

Epithelial ovarian cancer is classified into 5 major histotypes (serous, endometrioid, clear cell, mucinous, and undifferentiated), each histology displaying remarkable differences in their molecular pathogenesis and clinical outcome.[5] In addition to the

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type of differentiation, ovarian carcinomas can be classified based on the degree of differentiation, with grade 1 corresponding to low grade and grades 2 and 3 corresponding to high grade.[6] Histology and tumor grade stratify ovarian carcinomas into 2 broad categories, type I and type II, which correlate with molecular and clinical features. Type I tumors (low-grade serous, low-grade endometrioid, clear cell, and mucinous carcinomas) are thought to develop in a stepwise manner from borderline tumors or endometriosis, and have frequent mutations in *KRAS/BRAF* and Wnt signaling pathway genes, *PIK3CA* and *ARID1A* mutations especially in clear cell carcinoma), and occasional microsatellite instability (MSI).[7-9] in Clear cell and endometrioid ovarian cancers account for approximately 15–20% of epithelial ovarian cancers in Western countries and are the predominant types in LS.[8] Type II tumors (high-grade serous, high-grade endometrioid, malignant mixed mesodermal and undifferentiated tumors) may arise *de novo* or from precursor lesions remaining to be reliably identified, with fallopian tube as the likely origin for high-grade serous carcinoma.[8] Molecularly, type II tumors are characterized by *TP53* alterations, positive *WT1* expression (in high-grade serous carcinoma), and chromosomal instability.[7-9] High-grade serous cancers are the most common type of ovarian cancer overall (70%) and the predominant type in *BRCA1/2*-associated ovarian cancer.[8] The prognosis of high-grade serous ovarian carcinoma is generally poor, compared to intermediate prognosis for clear cell ovarian carcinoma, and favorable prognosis for endometrioid ovarian carcinoma.[8]

Besides genetic changes, epigenetic events are important for cancer initiation and progression, and may provide molecular tools to detect and manage cancer. DNA methylation of promoter CpG islands is the best established epigenetic modification capable of silencing conventional tumor suppressor genes and tumor suppressive microRNAs (miRNAs) (hypermethylation) or activating oncogenes and oncogenic miRNAs (hypomethylation).[10] While yet to be incorporated in ovarian cancer classifications, genome-wide studies have identified DNA methylation signatures associated with histological subtype of epithelial ovarian cancer[11,12] and disease progression,[13] and ovarian cancer is viewed as a model of translational applications of epigenetic alterations.[14,15] Epigenetically silenced genes may offer new targets for therapeutic intervention, based on re-expression of tumor suppressor genes via demethylating drugs.[16]

We took advantage of expression profiling of ovarian and endometrial cancer cell lines treated with demethylating agents to identify candidates for epigenetically silenced tumor suppressor genes in epithelial ovarian cancer. The extent to which epigenetic dysregulation of tumor suppressor genes is histology-specific in ovarian cancer is incompletely understood; moreover, it is unknown if the origin of ovarian cancer as hereditary vs. sporadic disease influences the epigenetic patterns. We find that inactivation of tumor suppressor genes is selective and depends on the histology and clinical category of ovarian cancer, as well as the individual genes in question, to be described in detail below.

Results

Selection of epigenetic markers

The study design is illustrated in **Figure 1**. Ovarian and endometrial cancer cell lines (Suppl. **Table 1**, Suppl. **Fig. 1**) were treated with 5-aza-CdR and TSA, resulting in the identification of 1 to 5 thousand significantly up-regulated genes depending on the cell line. Genes specific to non-serous vs. serous cancers as well as genes shared by non-serous and serous cancers were detected (Suppl. **Fig. 2**; data to be published in detail separately). Since our aim was to address histology- and patient group-specificity of tumor suppressor gene inactivation by epigenetic mechanisms, genes with known function in pathways relevant to ovarian tumorigenesis (see below and ref.[17]) and with available experimental and/or literature evidence of methylation-sensitivity were prioritized in marker selection. Accordingly, 5 genes from the expression microarrays (*RSK4*, *SPARC*, *PROM1*, *HOXA9* and *HOXA10*) were combined with 8 genes from the literature (*CABLES1*, *WT1-AS*, *WT1*, *SFRP2*, *SFRP5*, *OPCML*, MIR34B, and let-7a-3) to compile an informative epigenetic marker panel for custom-made methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assays on patient samples (Suppl. **Table 2A**, Suppl. **Table 2B**, Suppl. **Fig. 3**, Suppl. **Fig. 4**). Among genes identified through our expression profiling experiments, *RSK4* showed statistically significant upregulation after treatment in the non-serous cancer cell lines ES2 (2-fold) and AN3CA (2 – 3-fold depending on the probe), *HOXA9-HOXA10* in several non-serous (ES2 2-fold, HEC59 2-fold) and serous cell lines (CAOV3 2-fold, SKOV3 2 – 3-fold), *SPARC* in HEC59 (non-serous, 5 – 15-fold) and *PROM1* in CAOV3 (serous, 3-fold).

Analysis of tumor and normal tissues for promoter methylation

MS-MLPA was our method of choice as it allows for multiplex, quantitative analysis of methylation in archival formalin-fixed paraffin-embedded (FFPE) specimens without the need of bisulphite conversion (Suppl. **Fig. 3B**). The clinical series (Table 1) comprised ovarian cancers of the clear cell and endometrioid subtypes of sporadic (n = 65) and LS-associated cases (n = 19). Sporadic serous (n = 20) samples were also analyzed. Based on the assumed tissue of origin (see Introduction), normal (unrelated) endometrium was used as a reference for endometrioid and clear cell ovarian carcinomas, whereas specimens of normal (unrelated) fallopian tubes served as a reference for serous tumors. With the exception of *WT1* and *CABLES1* (with low degree of methylation in both tumor and normal tissues) and let-7a-3 (with high degree of methylation in tumor and normal tissues), the remaining genes displayed low levels of methylation in normal endometrium and fallopian tubes and increased methylation of variable degree in ovarian carcinomas (**Fig. 2**, Suppl. **Table 3**).

Hypermethylation frequencies in non-serous versus serous ovarian carcinomas

As evident from **Figure 2** and Suppl. **Table 3**, some genes showed relatively high levels of methylation in normal tissues

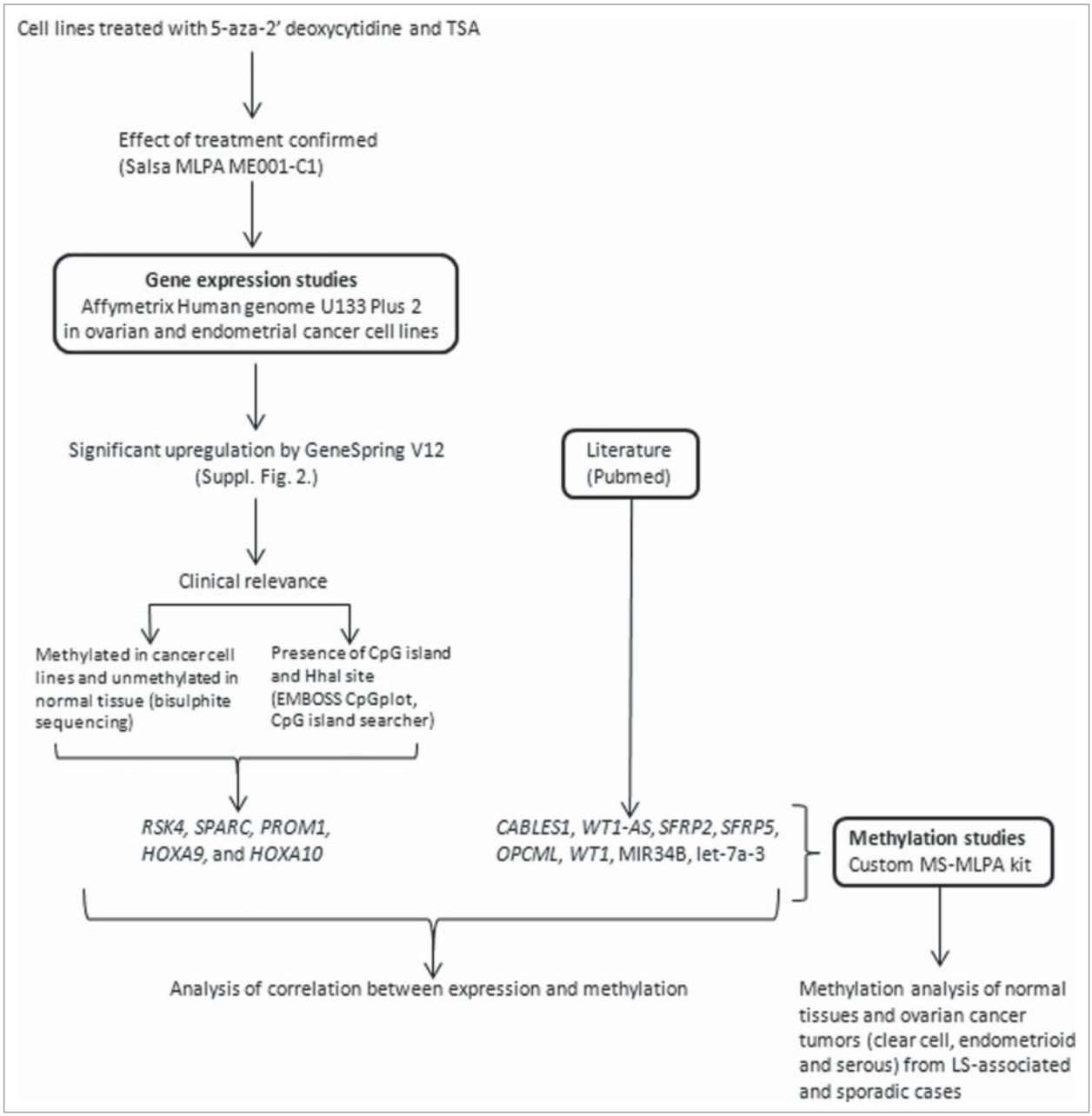

Figure 1. Flowchart of this investigation.

already (e.g., *RSK4* and let-7a-3 with methylation dosage ratio (Dm) values in normal tissues clearly above the detection threshold for methylation of Dm = 0.20, see Materials and Methods). Additionally, some genes showed significant differences between the normal reference tissues (e.g. *SPARC* with average Dm = 0.23 for normal endometrium vs. Dm = 0.13 for normal fallopian tubes, $P < 0.000$). For meaningful comparisons across markers and patient groups, we adopted the concept of *hyper*methylation to describe increased methylation in tumor relative to normal DNA, and determined gene-specific thresholds based on Dm values in the respective normal reference tissues (Materials and Methods, Suppl. **Table 4**). The percentages of tumors with hypermethylation are given in **Table 2**. Non-serous vs. serous comparisons were restricted to sporadic ovarian cancers since there was no serous group among the LS-associated ovarian carcinomas. *SPARC* (77% vs. 15%, $P < 0.001$), *HOXA10* (72% vs. 30%, $P < 0.05$), *HOXA9* (88% vs. 35%, $P < 0.001$), *WT1-AS* (81% vs. 30%, $P < 0.001$), and *OPCML* (80% vs. 30%, $P < 0.001$) showed significantly higher frequencies of tumors with hypermethylation in the combined non-serous (endometrioid + clear cell) vs. serous group (the indicated P values include correction for multiple testing).

Table 1. Clinicopathological data of sporadic and Lynch-associated ovarian carcinoma

	Sporadic				Lynch-associated[c]		
	Clear cell	Endometrioid[a]	Serous	Total	Clear cell	Endometrioid	Total
No. of cases	36	28	20	84	7	12	19
Grade							
G1	NA	11	0	11	NA	6	6
G2 and G3	NA	17	20	37	NA	5	5
Stage							
I and II	22	14	3	39	6	9	15
III and IV	14	14	17	45	1	3	4
Overall MMR status							
MMR deficient	6 (17%)	4 (14%)	1 (5%)	11 (13%)	7 (100%)	11 (92%)	18 (95%)
MMR proficient	30 (83%)	24 (86%)	19 (95%)	73 (87%)	0 (0%)	1 (8%)	1 (5%)
Average no. of TSGs methylated out of 24[b]	2.5	3.0	0.6		5.0	3.8	
Average no. of TSGs methylated out of 13[c]	6.7	6.4	2.8		8.3	6.4	

[a]One of the Lynch endometrioid tumors is borderline tumor, which is not graded.
[b]Niskakoski et al.[18]
[c]The predisposing mutation affected *MLH1* in 15 and *MSH2* in 4 cases (see Niskakoski et al.[18] for details).

Hypermethylation in LS-associated vs. sporadic ovarian cancer

Among all ovarian cancer groups investigated, LS-associated clear cell tumors showed the highest frequencies of hypermethylation (**Table 2**). *RSK4* was hypermethylated in 7/7 (100%) LS-clear cell carcinomas vs. 21/36 (58%) in sporadic clear cell carcinomas (borderline significant with $P = 0.077$ by Fisher's exact test). The corresponding frequencies were 5/7 (71%) vs. 10/36 (28%) for *PROM1* ($P = 0.040$) and 5/7 (71%) vs. 8/36 (22%) ($P = 0.019$) for MIR34B. None of the above differences, however, remained significant after correction for multiple testing. Hypermethylation frequencies in LS-associated endometrioid ovarian carcinomas were comparable to sporadic endometrioid carcinomas.

Hypermethylation with 13 "ovarian-cancer-related" genes vs. 24 "general" tumor suppressor genes (TSGs)

The number of hypermethylated genes out of 13 was determined for each individual tumor and the average values calculated for each ovarian cancer group. The average values from the highest to the lowest were 8.3 for LS-clear cell, 6.7 for sporadic clear cell, 6.4 for LS-endometrioid, 6.4 for sporadic endometrioid, and 2.7 for sporadic serous ovarian carcinomas. The values provided further support to the observations noted above, namely, higher frequencies of hypermethylation in non-serous vs. serous ovarian tumors ($P < 0.0001$ by *t*- test for independent samples) and higher frequencies of hypermethylation in clear cell carcinomas from LS-associated vs. sporadic cases ($P = 0.052$). We have previously[18] determined the TSG methylator phenotype for the tumors using 24 TSGs that are commonly methylated in various cancers (**Table 1**). The average fraction of hypermethylated genes was higher for the set of 13 vs. 24 genes in all patient groups (**Table 1**). In a sample-specific comparison of the proportions of hypermethylated genes, the present 13 "ovarian cancer-related" genes showed a positive correlation with the 24 "general" TSGs in each individual patient group, with the P-value reaching statistical significance for sporadic clear cell ovarian carcinoma ($P = 0.031$ by Spearman correlation analysis).

Clinical correlations

CpG island methylation of the 13 genes of interest showed no association with the clinical stage of ovarian cancer. Grade analysis was restricted to endometrioid ovarian carcinomas since clear cell carcinomas are not graded and all serous carcinomas were of high grade (**Table 1**). Interestingly, Dm values for *RSK4*, *SPARC*, and *HOXA9* decreased with increasing grade (grade 1 compared with grades 2 and 3 combined) (**Fig. 3**). Thus, the methylation pattern of high-grade endometrioid ovarian carcinomas showed a trend toward lower methylation levels characteristic of high-grade serous tumors.

Correlation of methylation with expression in cancer cell lines

DNA methylation (Dm values) showed significant inverse correlation with expression for *RSK4, SPARC, PROM1, CABLES1*, *HOXA10, HOXA9,* and MIR34B in the cancer cell lines and normal tissues investigated (**Fig. 4**). The observed patterns as a whole suggested that loss of expression was likely linked to promoter hypermethylation of these genes. Future studies are warranted to confirm the cell line data in patient specimens. While no RNA was available from the present archival samples for expression studies, the available literature does provide evidence of inverse correlation between methylation and expression for the genes in question in clinical specimens of ovarian and endometrial cancer.[19-22]

Discussion

This study was undertaken to investigate the ability of epigenetic markers to stratify epithelial ovarian carcinomas according to histological subtype and hereditary background (Lynch

syndrome). In regard to genes ascertained through expression profiling of cancer cell lines, *RSK4* encodes a putative suppressor of RAS/ERK signaling,[23] the product of *SPARC* modulates cell-matrix interactions,[24] *PROM1/CD133* codes for a cancer stem cell marker,[20] and the homeobox genes *HOXA9* and *HOXA10* control differentiation of the Müllerian ducts into the fallopian tubes, uterus, and cervix.[25] As for genes ascertained through the literature, Wilms tumor suppressor 1 sense (*WT1*) and antisense (*WT1-AS*) expression are markers of serous ovarian cancer,[7] *CABLES1* encodes a cyclin-dependent kinase,[26] *SFRP2* and *SFRP5* code for secreted antagonists of Wnt signaling,[27] and the product of *OPCML/OBCAM* is a cell adhesion molecule initially identified as a tumor suppressor for epithelial ovarian cancer.[28] Finally, the panel of protein-coding genes was supplemented with 2 ovarian cancer-associated miRNAs: MIR34B, a tumor suppressor[22] and let-7a-3, a putative oncogene.[29]

One of the major findings of this study was a high frequency of hypermethylation in both LS-associated and sporadic non-serous types as compared with sporadic serous ovarian cancer (**Table 2**). We observed low frequencies of hypermethylation for serous ovarian cancer even for genes with high rates of methylation previously reported for the serous subtype, including *SPARC*,[30] *HOXA10*,[31] *HOXA9*,[32] *OPCML*,[33] and MIR34B.[22] While the CpG islands investigated were generally the same, different methods for methylation analyses, definitions for hypermethylation, and tissues used for reference may in part explain the observed discrepancies.

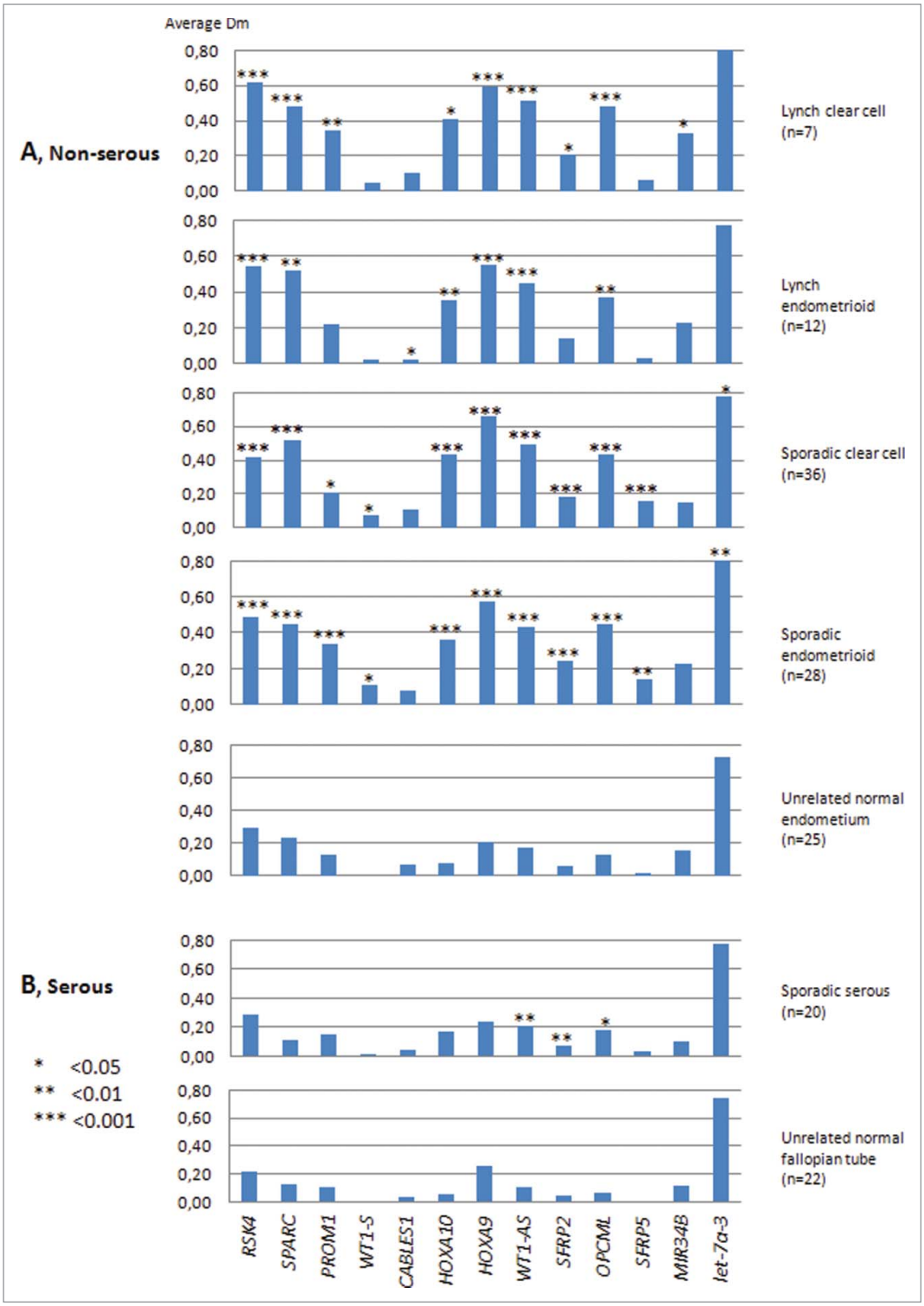

Figure 2. Average Dm values from MS-MLPA analyses on non-serous and serous ovarian carcinomas and the corresponding normal tissue references. Asterisks denote significantly elevated methylation in tumor vs. normal tissue by *t*-test for independent samples. The average Dm values of tumor DNAs may in fact be somewhat higher than those shown if possible "contamination" with normal cells is taken into account (see Materials and Methods).

Table 2. Hypermethylation frequencies in different patient groups

		Percentage of hypermethylated tumors[a]												
Tumor category	**Number of tumors**	***RSK4***	***SPARC***	***PROM1***	***WT1***	***CABLES1***	***HOXA10***	***HOXA9***	***WT1-AS***	***SFRP2***	***OPCML***	***SFRP5***	**MIR34B**	**let-7a-3**
(a) Lynch clear cell	7	100	86	71	0	14	71	100	100	43	100	14	71	57
(b) Lynch endometrioid	12	83	75	33	8	0	67	92	92	25	83	8	42	33
(c) Sporadic clear cell	36	58	86	28	8	8	78	94	94	33	86	31	22	39
(d) Sporadic endometrioid	28	64	64	54	21	4	64	79	64	39	71	25	36	57
(e) Sporadic serous	20	65	15	20	0	0	30	35	30	5	30	5	5	35
(f) Lynch non-serous (= a + b)	19	89	79	47	5	5	68	95	95	32	89	11	53	42
(g) Sporadic non-serous (= c + d)	64	61	77	39	14	6	72	56	81	36	80	28	28	47
P value [b]														
I Sporadic versus hereditary														
(a) vs. (c)		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
(b) versus (d)		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
(f) vs. (g)		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
II Sporadic non-serous versus serous														
(g) vs. (e)		ns	< 0.001	ns	ns	ns	<0.05	<0.001	<0.001	ns	<0.001	ns	ns	ns
III Sporadic and Lynch non-serous versus sporadic serous														
(f + g) vs. (e)		ns	< 0.001	ns	ns	ns	<0.05	<0.001	<0.001	ns	<0.001	ns	ns	ns

[a] Using cutoffs determined by methylation in the respective normal tissues (Suppl. **Table 4.**)
[b] Determined by Fisher exact test and corrected for multiple testing
ns = Nonsignificant

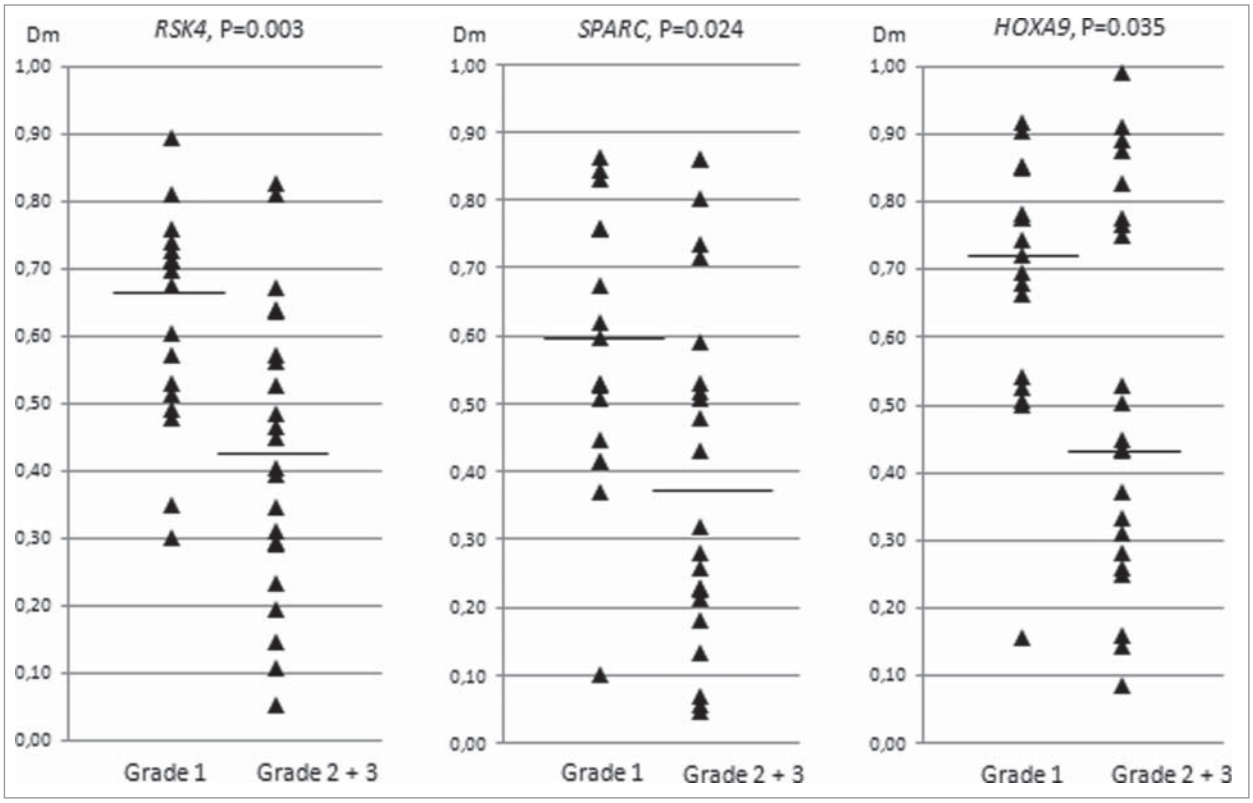

Figure 3. Distribution of methylation dosage ratios (Dm values) for *RSK4*, *SPARC*, and *HOXA9* in endometrioid ovarian carcinomas (sporadic and Lynch-associated combined) stratified by grade (low refers to grade 1 and high to grades 2 and 3). The horizontal line denotes the median and each triangle represents the Dm value of individual data point. Significance values by *t*-test for independent samples are shown.

The expression of a number of genes from our gene panel has been associated with specific histology of ovarian cancer in the literature. For example, *WT1* is expressed in serous, but not in endometrioid, clear cell, or mucinous types.[7] Moreover, *WT1* sense and antisense mRNAs show a similar expression pattern relative to each other in each tissue.[34] These findings are in agreement with our methylation data showing higher frequencies of inactivation by hypermethylation (of mainly WT1-AS) in non-serous vs. serous tumors (**Table 2**). The *HOXA* gene cluster coordinates the patterning of the Müllerian system, with *HOXA9* normally expressed in fallopian tubes, *HOXA10* in endometrium, and *HOXA11* in endocervix. Ectopic expression of *HOXA9* is postulated to give rise to serous ovarian carcinoma, that of *HOXA10* to endometrioid ovarian carcinoma, and that of *HOXA11* to mucinous ovarian carcinoma.[25] In reality, regulation of ovarian cancer development by the HOX system appears complex and the above delineated patterns were poorly reflected by our observations of equally low methylation of *HOXA9* and *HOXA10* in fallopian tubes vs. endometrium and *HOXA9* and *HOXA10* both showing significantly more frequent inactivation by hypermethylation in non-serous than serous ovarian cancers. Our finding of lower methylation – a serous-like shift – in *HOXA9* associated with high-grade endometrioid ovarian carcinomas (**Fig. 3**) however fits the hypothesis of ectopic expression of *HOXA9* accompanying the development of serous ovarian carcinoma.

Comparison of Lynch-associated ovarian carcinomas with their sporadic counterparts revealed less striking differences than the non-serous vs. serous comparison described above. Clear cell carcinomas from LS patients showed the highest frequencies of hypermethylation among all subgroups of ovarian carcinoma we have investigated to date, irrespective of the gene set (13 vs. 24 genes) used. In particular, MIR34B was hypermethylated in 71% of LS-associated vs. 22% of sporadic clear cell carcinomas (**Table 2**). Studies have shown that MIR34B is induced by p53 and upon overexpression mediates apoptosis or growth arrest in response to cellular stress, whereas reduction of miR-34 attenuates these functions.[35] While p53 expression is abnormal in a majority of serous and a considerable proportion of endometrioid and clear cell ovarian carcinomas from sporadic cases, LS-associated ovarian carcinomas (endometrioid and clear cell) lack p53 aberrations.[18] Our finding of a more frequent inactivation of MIR34B by hypermethylation in LS-associated than sporadic clear cell carcinomas, together with the absence of abnormal p53 in LS-associated as opposed to sporadic clear cell tumors, supports the idea that MIR34B inactivation and abnormal p53 in part function independently to achieve the same tumorigenic purpose. Indeed, p53-independent regulation for MIR34B was recently reported.[36]

Our study illustrates well the known advantages and disadvantages of the "expression-based" strategy to identify epigenetic markers.[37] The method allows for large-scale screening of

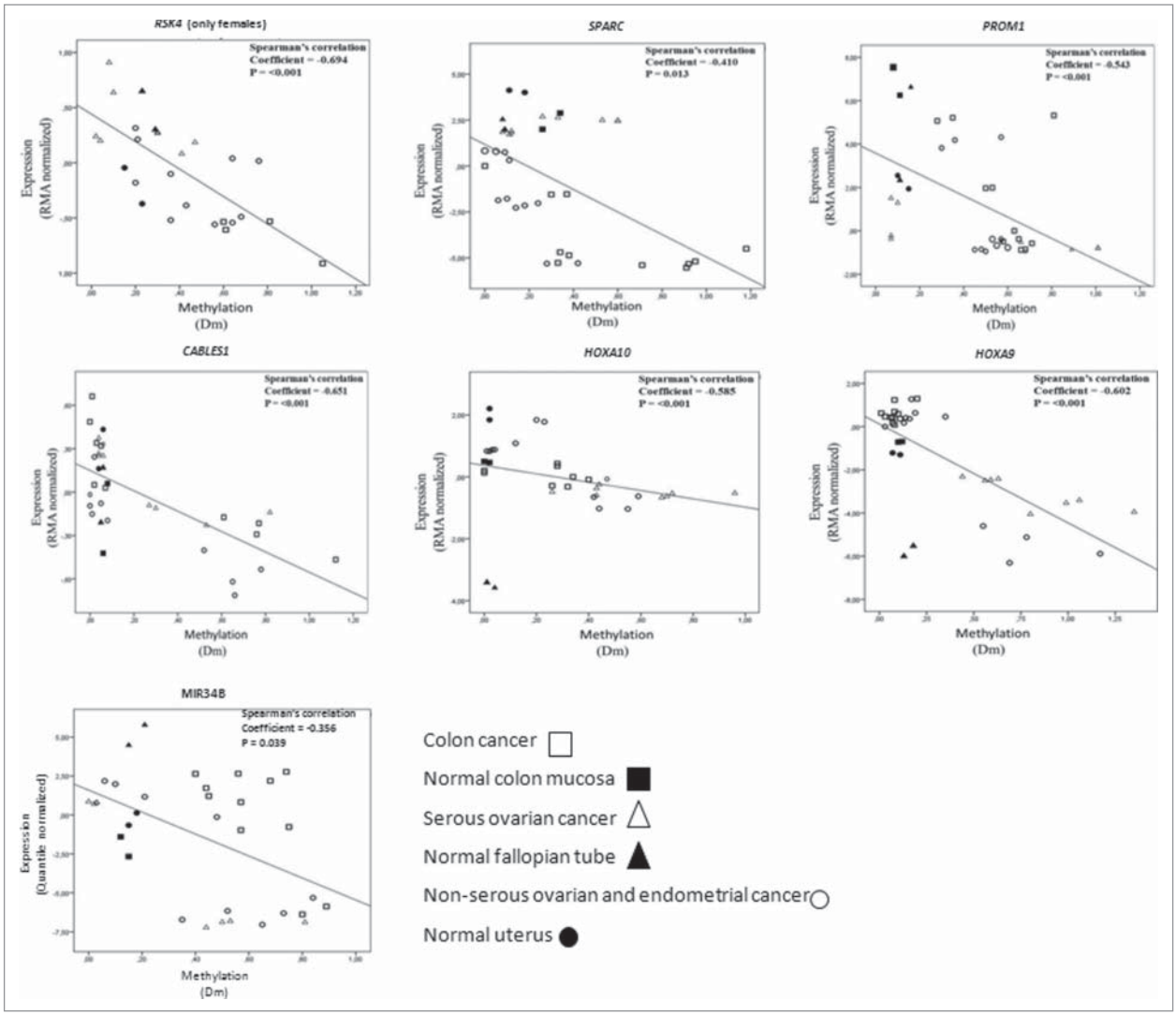

Figure 4. Correlation analysis of expression (Y-axis, RMA normalized values for protein coding genes and quantile normalized values for MIR34B from arrays) and methylation (X-axis, Dm values from MS-MLPA). The analysis includes cancer cell lines and normal tissue references for which high molecular weight DNA and RNA were available. Data points for normal tissues predominantly clustered in the left top quadrants, compatible with low methylation and high expression. Cancer cells with high degree of methylation often showed low expression (hence, were located in the right bottom quadrants), whereas cancer cells with low methylation showed high or low expression depending on the intrinsic properties of the genes and tissue types in question. While methylation for all these genes significantly correlated with transcriptional repression overall, subsets of specimens occasionally showed transcriptional regulation apparently unrelated to methylation (see, e.g., *MIR34B*).

differentially expressed genes, and re-expression of silenced genes after treatment can be taken as evidence of functional importance of methylation. An important disadvantage is that treatments with demethylating agents can only be performed on cell lines, whereas primary tumors remain to be investigated for methylation and expression by different methods. Several lines of evidence support functional significance of the methylation findings we describe. First, methylation was inversely correlated with mRNA expression in cell lines (**Fig. 4**). Second, promoter methylation of the 13 genes of interest was part of a more generalized TSG methylator phenotype. Third, our cell line studies and available literature suggest that these genes are likely to be involved (via epigenetic or other mechanisms) in the pathogenesis of several other common cancers beyond ovarian cancer (e.g., colorectal cancer). Fourth, lower levels of promoter methylation of *RSK4*, *SPARC*, and *HOXA9* (**Fig. 3**) may identify a more aggressive (high-grade) subgroup among endometrioid ovarian cancers that are generally considered to be associated with a favorable prognosis (see Introduction). These 3 genes have been reported to have both oncogenic and tumor suppressor properties, and their increased expression (the predicted result of reduced promoter methylation) promotes tumor growth.[38-40]

Among the 13 ovarian cancer-related genes investigated, *WT1-S*, *WT1-AS*, *SFRP2*, *OPCML*, *SFRP5*, and *let-7a-3* did not show significant inverse correlation between methylation and

expression in our cell lines (**Fig. 4**), raising a question about the functional significance of promoter methylation observed in these genes in the different patient groups (**Fig. 2**, **Table 2**). It is possible that the methylation changes reflected a generalized CpG island methylator (CIMP) phenotype where a majority of methylation events may represent "passenger" methylation.[41] On the other hand, for at least some of the genes listed above, other investigations provide evidence of significant inverse correlation between methylation and expression in ovarian cancer.[28,42]

In conclusion, our data demonstrate that aberrant DNA methylation of *RSK4*, *SPARC*, *PROM1*, *HOXA10*, *HOXA9*, *WT1-AS*, *SFRP2*, *SFRP5*, *OPCML*, and MIR34B is a frequent and selective event in ovarian tumorigenesis (**Fig. 2**, **Table 2**). Our findings increase the understanding of the significance of epigenetic mechanisms in ovarian tumorigenesis and identify possible targets to be evaluated for diagnostic and/or therapeutic purposes.

Materials and Methods

Patient material

The study material consisted of normal and tumor samples of all available LS-associated ovarian carcinomas in Finland, combined with sporadic cases of corresponding histological types (**Table 1**).[18] DNA was extracted from formalin-fixed paraffin-embedded (FFPE) samples from representative tumor sections shown to contain > 60% tumor epithelium. The average tumor percentages for the different patient groups were 80% for Lynch associated, 85% for sporadic clear cell, 77% for sporadic endometrioid and 76% for sporadic serous ovarian carcinomas. The Institutional Review Boards of the Departments of Surgery (466/E6/01) and the Obstetrics and Gynecology (040/95) of the Helsinki University Central Hospital (Helsinki, Finland) and that of the Jyväskylä Central Hospital (Jyväskylä, Finland) (Dnro 5/2007) as well as the National Authority for Medicolegal Affairs (Dnro 1272/04/044/07) approved this study.

Bisulphite Modification and Sequencing

Cancer cell line (Suppl. Table 1) and normal samples (600 ng of DNA) were bisulphite converted using EZ DNA Methylation-Direct™ Kit (Zymo Research, Orange, CA, USA). Bisulphite modified DNA was amplified with methylation-unbiased primers (Suppl. **Table 2A**) and sequenced either directly or after cloning (Suppl. **Fig. 3**). For the latter purpose, amplification products were cloned into a pCR2.1 TOPO vector by using the TOPO TA Cloning System (Invitrogen, Carlsbad, CA, USA). All resulting white colonies were used for the purification of DNA for sequencing. Gene promoter regions were identified by the EMBOSS CpGplot[1] and CpG island searcher[2] programs, and promoter information from the literature was taken into account when appropriate (Suppl. **Fig. 4**).

[1]http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot

[2]http://ccat.hcs.usc.edu/cpgislands2

Custom methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) for methylation analysis

The methylation profiles of CpG sites within the CpG-islands in genes under investigation were detected by bisulphite sequencing in cancer cell lines and normal tissues. Those CpG dinucleotides that were part of the methylation-sensitive enzyme HhaI restriction site (GCGC) were chosen for the probe-design for custom-made MS-MLPA (Suppl. **Table 2B**). When the CpG dinucleotide is methylated, HhaI enzyme cannot recognize the site and a PCR product together with a signal peak will be generated. To complete MS-MLPA assay, Salsa MLPA kit P-300-A1 human DNA reference-2 (Lot 0408, Amsterdam, the Netherlands) was added to the custom MS-MLPA probe mix. MS-MLPA was carried out following the manufacturer´s instructions (http://www.mrc-holland.com) using 100 to 200 ng of DNA. The PCR products were separated by capillary electrophoresis (ABI 3730 Automatic DNA Sequencer, Applied Biosystems, Carlsbad, CA, USA) and analyzed by GeneMapper4.0 genotyping software (Applied Biosystems).

The methylation dosage ratio (Dm) was calculated as described.[43] Dm varies from 0 to 1.0, which corresponds to the percentage of methylated DNA. Based on comparison with bisulphite sequencing results, Dm values of ≥ 0.20 were considered to reliably indicate methylation. Thresholds for hypermethylation that distinguish tumor from normal DNA in patient samples were specified for each gene on the basis of methylation values in normal DNAs of the tissue of origin (26 specimens of unrelated normal endometrium and 22 unrelated normal fallopian tubes) as described.[18]

Tumor suppressor gene (TSG) methylator phenotype

The TSG methylator phenotype was established for the tumors previously[18] and utilized the SALSA MS-MLPA ME001-C1 Tumor suppressor-1 kit (MRC-Holland, Amsterdam, the Netherlands) for 24 commonly methylated TSGs.

Cell culturing and epigenetic drug treatments

Ovarian cancer (CAOV3, SKOV3, and ES2), colon cancer (RKO, HCT15 and HCT116) and endometrial cancer cell lines (HEC59 and AN3CA) were cultured according to the supplier's protocol (ATCC, Rockville, MD, USA). Drug treatments were done according to Derks et al.[44] cells were treated with 1 μM of global genomic DNA demethylating agent 5-aza-2'deoxycytidine (Sigma, A3656) and 300 nM of trichostatin A (Sigma, T1952) for 96 h and 18 h, respectively. All treatments were performed in duplicates. DNA was isolated using standard protocols and total RNA extracted with miRNeasy mini kit (Qiagen, Valencia, CA, USA). The efficiency of the drug treatments was confirmed by methylation (SALSA MS-MLPA ME001-C1 Tumor suppressor -1 kit, MRC-Holland, Amsterdam, the Netherlands) analyses of selected tumor suppressor genes.

Genome-wide gene expression analysis

mRNA gene expression was analyzed using Affymetrix Human Genome U133 Plus 2.0 GeneChip® microarrays (Affymetrix, Santa Clara, CA), containing over 54,000 probe sets

covering 47,000 transcripts. Samples of RNA from the cell lines shown in Suppl. **Table 1** (treated and untreated) and respective normal tissues (purchased from Amsbio, Abingdon, UK or fresh-frozen tissues obtained from local hospitals) were amplified, labeled and hybridized as described.[45] Array image was analyzed using the GeneChip operating software (GCOS; Affymetrix, Santa Clara, CA) and comparison analysis was done according to the manufacturer's instructions. After image acquisition, raw fluorescent signal (cel. file) from Affymetrix GeneChip Operating Software (GCOS) was used for analysis.

Agilent's human miRNA microarrays (8 × 15 K from Agilent Technologies, G4470B), containing 723 human and 76 human viral miRNAs sourced from the Sanger miRBase v. 10.1, were used to analyze miRNA gene expression. Signal intensities of fluorescence were calculated by Agilent's Feature Extraction software version 10.7.3.1.

Analysis of microarray data

GeneSpring GX software, version 12 (Agilent Technologies) was used for microarray data analysis. RMA normalization was used for mRNA data and quantile normalization for miRNA data. Statistically significant differentially expressed genes identified by moderated t-Test combined with the Benjamini and Hochberg correction for multiple testing and using filters based on *P*-value cut-off 0.05 and fold change cut-off +/-1.5.

Statistical analysis

Statistical analyses of gene expression data were performed as described above. Statistical analysis of other data was performed with the software SPSS 20.0 (SPSS, Chicago, IL, USA). For methylation and expression correlation analysis, Pearson product-moment correlation coefficient (r) or Spearman rank correlation coefficient (rho) was used (depending on whether or not the data were normally distributed, as evaluated by Shapiro-Wilk test). Significance for the differences between groups was determined using Student's *t* test (for independent samples) or Fisher's exact test as appropriate. P values < 0.05 (2-tailed) were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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