# Original Article

# Methylation Analysis of Tumour Suppressor Genes in Ovarian Cancer Using MS-MLPA

(MS-MLPA / DNA methylation / ovarian cancer / CDH13 / epigenetics)

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**Abstract. Epigenetic changes are considered to be a frequent event during tumour development. Hypermethylation of promoter CpG islands represents an alternative mechanism for inactivation of tumour suppressor genes, DNA repair genes, cell cycle regulators and transcription factors. The aim of this study was to investigate promoter methylation of specific genes in ovarian cancer by comparison with normal ovarian tissue. To search for epigenetic events we used methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) to compare the methylation status of 69 tissue samples of ovarian cancer with 40 control samples. Using a 15% cut-off for methylation, we observed significantly higher methylation in genes *MGMT*, *PAX5*, *CDH13*, *WT1*, *THBS1*, *GATA5* in the ovarian cancer group, while in the *ESR1* gene we observed significantly higher methylation in the control group compared with the ovarian cancer group. These findings could potentially be used in screening of ovarian cancer and may have implications for future chemotherapy based on epigenetic changes.**

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Abbreviations: EDTA – ethylenediamine tetraacetic acid, ERα – oestrogen receptor α, FFPE – formalin-fixed, paraffin-embedded, MGMT – O-6-methylguanine-DNA methyltransferase, MS-HRM – methylation-sensitive high-resolution melting, MS-MLPA – methylation-specific multiplex ligation-dependent probe amplification, MSP – methylation-specific PCR, *PAX5* – paired box gene 5, PCR – polymerase chain reaction, THBS1 – thrombospondin 1, WHO – World Health Organization, *WT1* – Wilms tumour suppressor 1 gene.

## Introduction

Ovarian cancer is a leading cause of death from gynaecologic tumours due to its aggressive nature and the fact that the majority of patients are diagnosed in advanced stages of the disease. Five-year overall survival is strongly stage-dependent and is higher in women with stage I ovarian cancer, who have a 5-year survival rate of over 90 % (Karlan, 2009); however, only 25 % of women with advanced ovarian cancer survive five years after diagnosis. More than 85 % of patients with advanced disease relapse after cessation of primary therapy, despite an initially good response. It has generally been accepted that diagnosis of ovarian cancer at an early stage would result in a significant improvement in survival.

The role of epigenetics in cancer is undisputed. Aberrant methylation of normally unmethylated CpG islands, located in the 5' promoter region of genes, has been associated with transcriptional inactivation of several genes in human cancer, and presents as an alternative to mutational inactivation (Jones and Baylin, 2007).

Aberrant methylation of multiple CpG islands is a frequent event in epithelial ovarian cancer. CpG island hypermethylation of tumour suppressor genes such as *BRCA1* (Chan et al., 2002), *RASSF1A* (Yoon et al., 2001), *MLH1* (Gras et al., 2001), *ARH1* (Feng et al., 2008) and *OPMLC* (Mei et al., 2006), among others, is a known event in ovarian tumorigenesis. However, the importance of epigenetic changes in tumour suppressor genes in ovarian cancer remains largely unknown, and it is possible that more genes will be identified as being frequently inactivated through DNA methylation, and thus involved in the pathogenesis of ovarian cancer.

A number of methods have been developed for detection of methylation alterations in tumours, such as methylation-specific PCR (MSP), methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), methylation-sensitive high-resolution melting (MS-HRM), DNA sequencing, microarrays and others (Chmelařová and Palička, 2011). MS-MLPA

(Nygren et al., 2005) represents a rather novel cost-effective and time-efficient method. MS-MLPA is an ideal technique to use in formalin-fixed, paraffin-embedded (FFPE) samples. It permits simultaneous identification of epigenetic alterations in a predefined set of up to 25 genes. The present study applies the MS-MLPA analysis to ovarian cancer.

## Material and Methods

### *Tissue samples*

Formalin-fixed, paraffin-embedded tissue samples of ovarian adenocarcinomas and normal ovarian tissue were obtained from 109 women treated at the Department of Obstetrics and Gynaecology, University Hospital Hradec Králové, Czech Republic: 69 patients with ovarian cancer and 40 patients with normal ovaries. The samples of normal ovary were obtained from patients surgically treated for a non-malignant diagnosis (such as descent of uterus with adnexectomy, uterine myomas, etc.). The paraffin blocks were retrieved from the archive of the Fingerland Department of Pathology, University Hospital Hradec Králové. All slides were reviewed by an experienced pathologist and the carcinomas classified according to the current WHO classification of tumours of the female genital organs (Tavassoli and Devilee, 2003). The study was approved by the Ethics Committee of University Hospital Hradec Králové.

DNA was extracted from FFPE samples using a Qiagen DNA extraction kit (Qiagen GmbH, Hilden, Germany).

### *Methylation-specific multiplex ligation--dependent probe amplification (MS-MLPA)*

The present study used the MS-MLPA probe set ME002-B1 (MRC-Holland, Amsterdam, The Netherlands), which can simultaneously check for aberrant methylation in 25 tumour suppressor genes (Table 1). Probe sequences, gene loci and chromosome locations can be found at http://www.mlpa.com. Individual genes were evaluated by two probes, which recognized different *Hha*1 restriction sites in their regions. The experimental procedure was carried out according to the manufacturer's instructions, with minor modifications.

In short, DNA (100 ng) was dissolved in up to 5 µl TE-buffer (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0), denatured and subsequently cooled down to 25 °C. After adding the probe mix, the probes were allowed to hybridize (overnight at 60 °C). Subsequently, the samples were divided into two parts: in one half, the samples were directly ligated, while in the other half ligation was combined with the *Hha*I restriction enzyme digestion. This digestion resulted in ligation of the methylated sequences only. PCR was performed with all the samples using a standard thermal cycler (GeneAmp 9700, Applied Biosystems, Foster City, CA), with 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min with final extension of 20 min at 72 °C. Aliquots of 0.6 µl of the PCR reaction were combined with 0.2 µl LIZ-labelled internal size standard (Applied Biosystems) and 9.0 µl deionized formamide. After denaturation, fragments

*Table 1. Genes in the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) KIT ME002 Tumour suppressor-2 (MRC Holland)*

| Gene | Name | Probes | Chromosomal location |
|---|---|---|---|
| ***BRCA1*** | Breast cancer 1 | 03296-L01269 | 17q21.3 |
| ***BRCA2*** | Breast cancer 2 | 02285-L01776 | 13q13.1 |
| ***ATM*** | Ataxia telangiectasia mutated | 03023-L02413 | 11q23 |
| ***TP53*** | Tumour protein p53 | 02374-L02530 | 17p13.1 |
| ***PTEN*** | Phosphatase and tensin homologue | 03808-L02169 | 10q23.3 |
| ***MGMTa*** | O-6-methylguanine-DNA methyltransferase | 05670-L05146 | 10q26.3 |
| ***PAX5*** | Paired box gene 5 | 03750-L03210 | 9p13 |
| ***CDH13*** | Cadherin 13, H-cadherin | 02257-L01742 | 16q23.3 |
| ***TP73*** | Tumour protein p73 | 01684-L01264 | 1p36.3 |
| ***WT1*** | Wilms tumour 1 | 02755-L02204 | 11p13 |
| ***VHL*** | von Hippel-Lindau tumour suppressor | 03818-L03850 | 3p25.3 |
| ***GSTP1*** | Glutathione S-transferase pi 1 | 02747-L02174 | 11q13 |
| ***CHFR*** | Checkpoint with forkhead and ring finger domains | 02737-L02164 | 12q24.3 |
| ***ESR1*** | Oestrogen receptor 1 | 02746-L02173 | 6q25.1 |
| ***RB1a*** | Retinoblastoma 1 | 02734-L02161 | 13q14.2 |
| ***MSH6*** | MutS homologue 6 | 01250-L00798 | 2p16.3 |
| ***MGMTb*** | O-6-methylguanine-DNA methyltransferase | 13716-L15582 | 10q26.3 |
| ***THBS1*** | Thrombospondin 1 | 01678-L17140 | 15q15 |
| ***CADM1*** | Cell adhesion molecule 1 | 03816-L17141 | 11q23 |
| ***STK1*** | Serine/threonine protein kinase | 06783-L17143 | 19q13.3 |
| ***PYCARD*** | PYD and CARD domain containing | 02252-L01737 | 16p11.2 |
| ***PAX6*** | Paired box gene 6 | 03749-L03209 | 11p13 |
| ***CDKN2A*** | Cyclin-dependent kinase inhibitor 2A | 01530-L00955 | 9p21.3 |
| ***GATA5*** | GATA-binding protein 5 | 03752-L06199 | 20q13.3 |
| ***RARB*** | Retinoic acid receptor, β | 04046-L02172 | 3p24.2 |
| ***CD44*** | CD44 molecule (Indian blood group) | 04500-L02761 | 11p12 |
| ***RB1b*** | Retinoblastoma 1 | 04502-L02199 | 13q14.2 |

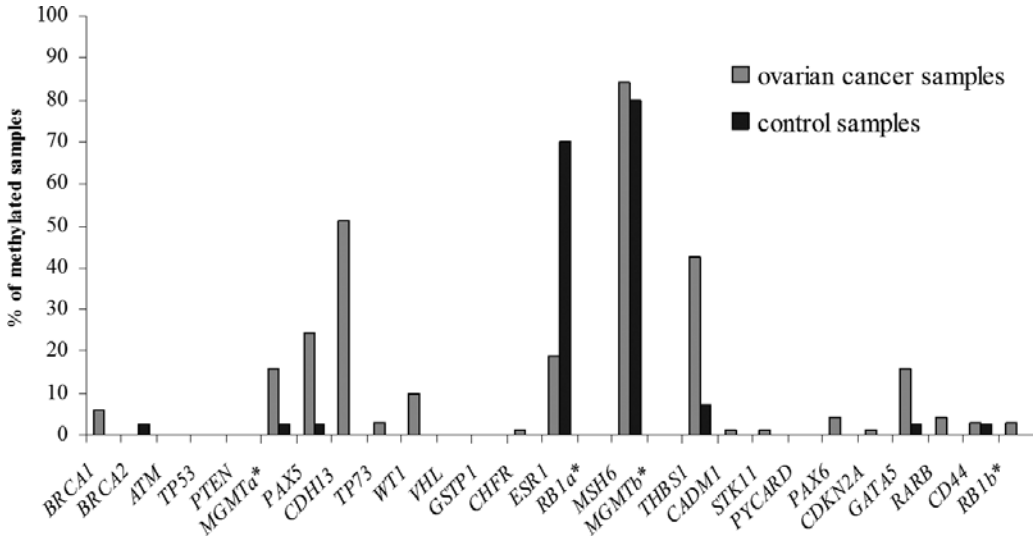


Fig. 1. Methylation of specific genes in ovarian cancer samples and control samples.
Comparison of methylation frequencies (cut-off value 15%) of the 25 analysed genes in ovarian cancer and control samples. * Two CpG loci (a and b) were analysed.

were separated and quantified by electrophoresis in an ABI 3130 capillary sequencer and analysed using GeneMapper4.0 (both Applied Biosystems). Peak identification with values corresponding to the peak size in base pairs (bp) and peak areas were used for further data processing. Methylation dosage ratio was obtained by the following calculation: Dm = $(P_x/P_{ctrl})$Dig/ $(P_x/P_{ctrl})$ Undig, where Dm is the methylation dosage ratio, $P_x$ is the peak area of a given probe, $P_{ctrl}$ is the sum of the peak areas of all control probes, Dig stands for *Hha*I-digested sample and Undig for undigested sample. Dm can vary between 0 and 1.0 (corresponding to 0–100% of methylated DNA). Based on previous experiments, we considered a promoter to show methylation if the methylation dosage ratio was ≥ 0.15, which corresponds to 15 % of methylated DNA (Moelans et al., 2011).

CpG universal methylated and unmethylated DNA (Chemicon International, Temecula, CA) were used in every run as controls.

### *Statistical analysis*

Proportions were compared by two-tailed Fisher's exact test. Associations with P value < 0.05 were considered to be significant.

## Results

### *Promoter methylation using a 15% cut-off*

In the present study we used the MS-MLPA probe set ME002 (MRC-Holland) to analyse samples from 69 patients with ovarian cancer and 40 control samples. Using a 15% cut-off for methylation we observed statistically significant higher methylation in the genes *MGMT* (P = 0.05), *PAX5* (P = 0.002), *CDH13* (P < 0.001), *WT1* (P = 0.045), *THBS1* (P = 0.048), and *GATA5* (P = 0.05) in ovarian cancer patients than in the control group. Conversely, in gene *ESR1* we observed a statistically significantly (P < 0.001) higher methylation in the control group than in the ovarian cancer group. For gene *MSH6* we observed high methylation (about 80%) in both ovarian cancer and control samples. For genes *ATM*, *TP53*, *PTEN*, *VLH*, *GSTP1*, *RB1*, *MGMTb* and *PYCARD*, the methylation rate did not exceed the 15% threshold; the other genes also showed relevant differences in methylation between ovarian cancer and control samples (Fig. 1).

### *Correlation with clinicopathological features*

The mean (median) age of patients at the time of diagnosis was 54 years (range 21–79 years) in the carcinoma group and 57.5 years (range 40–84 years) in the control group. Table 2 shows the baseline clinicopathological characteristics. The methylation results from the ovarian cancer specimens were compared with clinicopathological characteristics including age, histological type, tumour stage and histological grade (Table 2).

Genes *MGMTa*, *PAX5*, *CDH13*, *THSB1* and *GATA5* showed a significantly (P < 0.05) higher methylation in endometrioid type compared with the serous histological type of ovarian cancer (Table 3). There was also significantly higher methylation of *WT1* (P = 0.006) in the early stages than in the late stages of ovarian cancer. No significant differences in methylation were observed between histological grades of ovarian cancer.

## Discussion

The biological features of ovarian cancer are determined by the underlying molecular alterations of the tumour cells, including the epigenetic inactivation of tumour suppressor genes as well as mutations and deletions. It is now clear that *de novo* promoter methylation is a common mechanism for inactivation of tumour suppressor genes. The promoter methylation status has been reported in several human neoplasms. The purpose

*Table 2. Clinicopathological characteristics vs. methylation of specific genes*

| characteristic | | number of samples | % of samples | % of methylated samples in specific genes | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *BRCA1* | *MGMTa* | *PAX5* | *CDH13* | *WT1* | *ESR1* | *THSB1* | *GATA5* |
| **age** | **≤ 45 years** | 10 | 14.5 | 0 | 10 | 20 | 60 | 20 | 10 | 10 | 10 |
| | **> 45 years** | 59 | 85.5 | 6.8 | 17 | 25.4 | 45.8 | 8.5 | 20.3 | 27.1 | 17 |
| **stage** | **I. + II.** | 23 | 33.3 | 0 | 8.3 | 25 | 62.5 | 25 | 16.7 | 20.8 | 16.7 |
| | **III. + IV.** | 46 | 66.7 | 8.9 | 20 | 24.4 | 40 | 2.2 | 20 | 26.7 | 15.6 |
| **histology** | **serous** | 48 | 69.6 | 6.3 | 10.4 | 18.8 | 37.8 | 6.3 | 14.6 | 16.7 | 8.3 |
| | **endometrioid** | 14 | 20.3 | 7.1 | 35.7 | 50 | 78.6 | 14.3 | 28.6 | 50 | 42.9 |
| | **clear cell** | 2 | 2.9 | 0 | 50 | 50 | 50 | 0 | 50 | 50 | 50 |
| | **mucinous** | 5 | 7.2 | 0 | 0 | 0 | 80 | 40 | 20 | 20 | 20 |
| **grade** | **1** | 13 | 18.8 | 0 | 7.7 | 23.1 | 53.9 | 15.4 | 15.4 | 7.7 | 7.7 |
| | **2** | 16 | 23.2 | 6.3 | 25 | 31.3 | 50 | 18.8 | 25 | 31.3 | 25 |
| | **3** | 38 | 55.1 | 7.9 | 15.8 | 2.1 | 42.1 | 5.3 | 15.8 | 23.7 | 13.2 |
| | **dedifferentiated** | 2 | 2.9 | 0 | 0 | 50 | 100 | 0 | 50 | 100 | 50 |

*Table 3. Promoter methylation using 15% cut-off value in specific genes in endometrioid and serous types of ovarian cancer*

| gene | endometrioid (N = 14) | serous (N = 48) | P value |
|---|---|---|---|
| ***BRCA1*** | 7.1 % | 6.3 % | 1 |
| ***MGMTa*** | 35.7 % | 10.4 % | 0.037 |
| ***PAX5*** | 50.0 % | 18.8 % | 0.034 |
| ***CDH13*** | 78.6 % | 37.8 % | 0.006 |
| ***WT1*** | 14.3 % | 6.3 % | 0.58 |
| ***ESR1*** | 28.6 % | 14.6 % | 0.25 |
| ***THSB1*** | 50.0 % | 16.7 % | 0.017 |
| ***GATA5*** | 42.9 % | 8.3 % | 0.006 |

of this study was to investigate promoter methylation of a set of common tumour suppressor genes in 69 ovarian cancer and 40 control samples. We used MS-MLPA, and a threshold of 15% methylation was applied based on previous experiments (Moelans et al., 2011).

Genes *ATM*, *TP53*, *PTEN*, *VLH*, *GSTP1*, *RB1a*, *MGMTb* and *PYCARD* never showed methylation above this threshold, suggesting that promoter methylation of CpG loci of these tumour suppressor genes may not play an important role in ovarian carcinogenenesis. On the other hand, *BRCA1*, *BRCA2*, *MGMTa*, *PAX5*, *CDH13*, *TP73*, *WT1*, *CHFR*, *ESR1*, *MSH6*, *THSB1*, *CADM1*, *STK11*, *PAX6*, *CDKN2A*, *GATA5*, *RARB*, *CD44* and *RB1b* did show promoter methylation to a varying extent above the 15% threshold.

*MSH6* was previously shown to be frequently methylated in breast cancer and also in normal breast tissue (Moelans et al., 2011). In the present study, *MSH6* methylation was very frequent in both ovarian cancer and normal ovarian tissue (Fig. 1). These findings suggest that *MSH6* methylation could be specific for ovarian and breast tissue.

We observed significantly higher methylation in genes *MGMTa*, *PAX5*, *CDH13*, *WT1*, *THBS1* and *GATA5* in the cancer group than in the control group, indicating that promoter methylation of these tumour suppressor genes plays an important role in ovarian carcinogenesis. These genes could be used in future screening for ovarian cancer, because methylated DNA has been detected in body fluids of ovarian cancer patients, for example in the plasma, and the level correlated reasonably well with methylation levels in tumour tissue (Ibanez de Caceres et al., 2004). This finding could also have implications for future chemotherapy based on epigenetic changes, because platinum resistance is strongly associated with methylation-induced silencing of various drug response genes and pathways (Asadollahi et al., 2010).

O-6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that prevents DNA crosslinking by removing cytotoxic adducts from O-6-methylguanine in DNA. Using MSP, Roh et al. (2011) found promoter methylation of this gene in 14.7 % of mucinous and clear-cell ovarian carcinomas. Our data using MS-MLPA correlate with this finding.

Our present study is the first study to demonstrate methylation of paired box gene 5 (*PAX5*) and thrombospondin 1 (*THBS1*) in ovarian cancer. In other human cancers the methylation status is 0–6 % for the gene *PAX5* (Palmisano et al., 2003) and 2–43 % for the gene *THBS1* (Bonazzi et al., 2011).

Methylation has been previously described in Wilms tumour suppressor 1 gene (*WT1*) and GATA5 transcription factor (Kaneuchi et al., 2005, Wakana et al., 2006, Montavon et al., 2012). Using MSP Kaneuchi et al. (2005) reported methylation in 88.2 % of ovarian clear-cell carcinomas and in 24 % of serous carcinomas. Our data using MS-MLPA demonstrate methylation in ovarian cancer in 10 % cases (clear-cell 0 %, serous 6.3 %). This difference is caused by searching for DNA methylation in different CpG loci in the promoter region. This demonstrates the importance of the location of the CpG dinucleotide, which is discussed by van Vlodrop et al. (2011).

*CDH13* is frequently methylated in both hereditary and sporadic ovarian cancer (Bol et al., 2010). Bol et al. (2010) described the importance of methylation of *CDH13* using MS-MLPA kit ME001B (MRC-Holland), and so they investigated CpG loci different from those in our study. In both of these regions there is important methylation in ovarian cancer samples. Our study also confirms that methylation of H-cadherin (*CDH13*) is an important event in ovarian carcinogenesis (methylated in more than 50 % of ovarian cancer samples).

Higher methylation of *ESR1* was statistically significant ($P < 0.001$) in the control group compared to the ovarian cancer group. *ESR1* was previously shown to be widely methylated in breast cancer and there was a statistically significant association with lower expression of oestrogen receptor α (ERα) (Gaudet et al., 2009). This finding could indicate lower ERα levels in normal healthy ovarian tissue than in ovarian cancer tissue because of promoter methylation.

In conclusion, our study showed that there are significant differences in promoter methylation in *MGMTa*, *PAX5*, *CDH13*, *WT1*, *THBS1*, *GATA5* and *ESR1* genes between ovarian cancer and control samples, suggesting the importance of epigenetic changes of these genes in ovarian carcinogenesis. These epigenetic characteristics could have implications for an ovarian cancer screening programme and for therapeutic strategies, especially chemotherapy based on epigenetic changes.

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The authors declare that there are no conflicts of interest.

## References

Asadollahi, R., Hyde, C. A., Zhong, X. Y. (2010) Epigenetics of ovarian cancer: from the lab to the clinic. *Gynecol. Oncol*. **118**, 81-87.

Bol, G. M., Suijkerbuijk, K. P., Bart, J., Vooijs, M., van der Wall, E., van Diest, P. J. (2010) Methylation profiles of hereditary and sporadic ovarian cancer. *Histopathology* **57**, 363-370.

Bonazzi, V. F., Nancarrow, D. J., Stark, M. S., Moser, R. J., Boyle, G. M., Aoude, L. G., Schmidt, C., Hayward, N. K. (2011) Cross-platform array screening identifies COL1A2, THBS1, TNFRSF10D and UCHL1 as genes frequently silenced by methylation in melanoma. *PLoS One* **6**, e26121.

Chan, K. Y., Ozçelik, H., Cheung, A. N., Ngan, H. Y., Khoo, U. S. (2002) Epigenetic factors controlling the BRCA1 and BRCA2 genes in sporadic ovarian cancer. *Cancer Res*. **62**, 4151-4156.

Chmelařová, M., Palička, V. (2011) The most frequent methods used for DNA methylation analysis. *Cas. Lek. Cesk*. **150**, 442-445. (in Czech)

Feng, W., Marquez, R. T., Lu, Z., Liu, J., Lu, K. H., Issa, J. P., Fishman, D. M., Yu, Y., Bast Jr. R. C. (2008) Imprinted tumor suppressor genes ARHI and PEG3 are the most frequently down-regulated in human ovarian cancers by loss of heterozygosity and promoter methylation. *Cancer* **112**, 1489-1502.

Gras, E., Catasus, L., Argüelles, R., Moreno-Bueno, G., Palacios, J., Gamallo, C., Matias-Guiu, X., Prat, J. (2001) Microsatellite instability, MLH-1 promoter hypermethylation, and frameshift mutations at coding mononucleotide repeat microsatellites in ovarian tumors. *Cancer* **92**, 2829-2836.

Gaudet, M. M., Campan, M., Figueroa, J. D., Yang, X. R., Lissowska, J., Peplonska, B., Brinton, L. A., Rimm, D. L., Laird, P. W., Garcia-Closas, M., Sherman, M. E. (2009) DNA hypermethylation of ESR1 and PGR in breast cancer: pathologic and epidemiologic associations. *Cancer Epidemiol. Biomarkers Prev*. **18**, 3036-3043.

Ibanez de Caceres, I., Battagli, C., Esteller, M., Herman, J. G., Dulaimi, E., Edelson, M. I., Bergman, C., Ehya, H., Eisenberg, B. L., Cairns, P. (2004) Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. *Cancer Res*. **64**, 6476-6481.

Jones, P. A., Baylin, S. B. (2007) The epigenomics of cancer. *Cell* **128**, 683-692.

Kaneuchi, M., Sasaki, M., Tanaka, Y., Shiina, H., Yamada, H., Yamamoto, R., Sakuragi, N., Enokida, H., Verma, M., Dahiya, R. (2005) WT1 and WT1-AS genes are inactivated by promoter methylation in ovarian clear cell adenocarcinoma. *Cancer* **104**, 1924-1930.

Karlan B. Y. (2009) Patients at high risk for ovarian cancer should undergo routine screening. *Clinical Ovarian Cancer* **5**, 83-89.

Mei, F. C., Young, T. W., Liu, J., Cheng, X. (2006) RAS-mediated epigenetic inactivation of OPCML in oncogenic transformation of human ovarian surface epithelial cells. *FASEB J*. **20**, 497-499.

Moelans, C. B., Verschuur-Maes, A. H., van Diest, P. J. (2011) Frequent promoter hypermethylation of BRCA2, CDH13, MSH6, PAX5, PAX6 and WT1 in ductal carcinoma in situ and invasive breast cancer. *J. Pathol*. **225**, 222-231.

Montavon, C., Gloss, B. S., Warton, K., Barton, C. A., Statham, A. L., Scurry, J. P., Tabor, B., Nguyen, T. V., Qu, W., Samimi, G., Hacker, N. F., Sutherland, R. L., Clark, S. J., O'Brien, P. M. (2012) Prognostic and diagnostic significance of DNA methylation patterns in high grade serous ovarian cancer. *Gynecol. Oncol.* **124**, 582-888

Nygren, A. O., Ameziane, N., Duarte, H. M., Vijzelaar, R. N., Waisfisz, Q., Hess, C. J., Schouten, J. P., Errami, A. (2005) Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Res*. **33**, e128.

Palmisano, W. A., Crume, K. P., Grimes, M. J., Winters, S. A., Toyota, M., Esteller, M., Joste, N., Baylin, S. B., Belinsky, S. A. (2003) Aberrant promoter methylation of the transcription factor genes PAX5 α and β in human cancers. *Cancer Res*. **63**, 4620-4625.

Roh, H. J., Suh, D. S., Choi, K. U., Yoo, H. J., Joo, W. D., Yoon, M. S. (2011) Inactivation of O6-methyguanine-DNA methyltransferase by promoter hypermethylation: association of epithelial ovarian carcinogenesis in specific histological types. *J. Obstet. Gynaecol. Res*. **37**, 851-860.

Tavassoli, F. A, Devilee, P. (eds.) (2003) *World Health Organization. Classification of Tumors*. Pathology and genetics: tumors of the breast and female genital organs. pp. 113-202. IARC Press, Lyon.

van Vlodrop, I. J., Niessen, H. E., Derks, S., Baldewijns, M. M., van Criekinge, W., Herman, J. G., van Engeland, M. (2011) Analysis of promoter CpG island hypermethylation in cancer: location, location, location! *Clin. Cancer Res*. **17**, 4225-4231.

Wakana, K., Akiyama, Y., Aso, T., Yuasa, Y. (2006) Involvement of GATA-4/-5 transcription factors in ovarian carcinogenesis. *Cancer Lett*. **241**, 281-288.

Yoon, J. H., Dammann, R., Pfeifer, G. P. (2001) Hypermethylation of the CpG island of the RASSF1A gene in ovarian and renal cell carcinomas. *Int. J. Cancer* **94**, 212-217.