Original Article

Promoter Methylation of *GATA4*, *WIF1*, *NTRK1* and Other Selected Tumour Suppressor Genes in Ovarian Cancer

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

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Abstract. Ovarian cancer is the leading cause of death from gynaecologic tumours, but the molecular and especially epigenetic events underlying the transformation are poorly understood. Various methylation changes have been identified and show promise as potential cancer biomarkers. The aim of this study was to investigate promoter methylation of selected tumour suppressor genes in ovarian cancer by comparison with normal ovarian tissue. To search for epigenetic events we used methylation-specific multiplex ligation-dependent probe amplification to compare the methylation status of 44 tissue samples of ovarian cancer with 30 control samples. Using a 20% cut-off for methylation, we observed significantly higher methylation in genes *NTKR1, GATA4* and *WIF1* in the ovarian cancer group compared with the control group. These findings could potentially be used in screening of ovarian cancer, and may have implications for future chemotherapy based on epigenetic changes.

Introduction

Ovarian cancer is the 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, with a 5-year survival rate of over 90 % in women with stage I ovarian cancer (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 a good initial response. It has generally been accepted that diagnosis of ovarian cancer at an early stage may 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), *SLIT2* (Dong et al., 2012), *p53* (Chmelarova et al., 2013), among others, is a well known event in ovarian tumorigenesis. Moreover, aberrant methylation is one of the early markers of carcinogenesis, where cells of normal origin acquire methylation changes at least three years in advance of the first morphological changes (Zhuang et al., 2012). 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.

In the present study, we used an approach that allows simultaneous assessment of aberrant promoter methylation of a large set of genes which, in contrast to other high-throughput methods, requires only small quantities of short DNA fragments, making it very suitable for analysis of DNA isolated from formalin-fixed and paraffin-embedded (FFPE) tissue samples. Methylation-spe-

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Abbreviations: EDTA – ethylenediaminetetraacetic acid, EGF – epidermal growth factor, FFPE – formalin-fixed and paraffin-embedded, MS-MLPA – methylation-specific multiplex ligation-dependent probe amplification, *NTRK1* – neurotrophic tyrosine kinase receptor, type 1, gene, *WIF* – WNT inhibitory factor gene.

cific multiplex ligation-dependent probe amplification (MS-MLPA) has been applied to the multiplexed measurement of methylation of tumour suppressor genes in cancer in many studies, and has shown good correlation with other methylation detection techniques (Nygren et al., 2005; Bol et al., 2010; Moelans et al., 2011).

In this study we used MS-MLPA to compare the methylation status of 25 tumour suppressor genes between ovarian cancer tissue and normal tissue. In addition, the association between the methylation status of the analysed genes and clinicopathological characteristics was investigated.

Material and Methods

Formalin-fixed and paraffin-embedded tissue samples of ovarian adenocarcinomas and normal ovarian tissue were obtained from 74 women treated at the Department of Obstetrics and Gynaecology, University Hospital Hradec Králové, Czech Republic: 44 patients with ovarian cancer and 30 patients with normal ovarium. The samples of normal ovary were obtained from patients treated surgically for a non-malignant diagnosis (such as descent of uterus with adnexectomy, uterine leiomyomas, 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 (J. L.) and the carcinomas were 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 formalin-fixed, paraffin-embedded samples using a Qiagen (Hilden, Germany) DNA extraction kit.

Methylation-specific multiplex ligation-dependent probe amplification

The present study used the MS-MLPA probe set ME004 (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*I 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 up to 5 μl in TE-buffer (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0), denatured and subsequently cooled down to 25 °C. After addition of the probe mix, the probes were allowed to hybridize (overnight at 60 °C). Subsequently, the samples were divided into two: in one half, the samples

Table 1. Genes in the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) KIT ME004-A1 probemix (MRC-Holland)

Gene	Name	Probes	Chromosomal location
EPHB2	Ephrin receptor B2	07910-L07623	1p36.1
BCL2	B-cell CLL/lymphoma 2	10344-L10882	18q21.3
PTEN	Phosphatase and tensin homologue	10345-L11988	10q23.3
NF1 (a)	Neurofibromin 1	03845-L03318	17q11.2
RARRES1	Retinoic acid receptor responder (tazarotene induced) 1	03308-L13245	3q25
TERT	Telomerase reverse transcriptase	10346-L10884	5p15.3
THBS1	Thrombospondin 1	02739-L02166	15q15
SFRP1 (a)	Secreted frizzled-related protein 1	10564-L13246	8p11.2
IGF2R (a)	Insulin-like growth factor 2 receptor	02800-L02185	6q26
NF1 (b)	Neurofibromin 1	03844-L03317	17q11.2
TWIST1	Twist homologue 1	07015-L06626	7p21.2
APAF1 (a)	Apoptotic peptidase-activating factor 1	09165-L09458	12q23.1
PCNA	Proliferating cell nuclear antigen	03955-L03394	20p12.3
DNAJC15	DnaJ (Hsp40) homologue, subfamily C, member 15	03297-L02661	13q14.1
NTRK1	Neurotrophic tyrosine kinase, receptor, type 1	03970-L03357	1q23.1
PXMP4 (a)	Peroxisomal membrane protein 4	03303-L13247	20q11.2
MEN1 (a)	Multiple endocrine neoplasia I	09168-L09461	11q13.1
LMNA	Lamin A/C	12287-L13799	1q22
APAF1 (b)	Apoptotic peptidase-activating factor 1	09166-L09459	12q23.1
PCCA	Propionyl CoA carboxylase, α polypeptide	08676-L08688	13q32.3
PAX6	Paired box 6	03748-L03208	11p13
RBM14	RNA-binding motif protein 14	09429-L09678	11q13
MUS81 (a)	MUS81 endonuclease homologue	09157-L09315	11q13.1
IGF2R (b)	Insulin-like growth factor 2 receptor	02801-L02186	6q26
SFRP1 (b)	Secreted frizzled-related protein 1	10563-L11109	8p11.2
GATA4	GATA-binding protein 4	03754-L13255	8p23.1
PXMP4 (b)	Peroxisomal membrane protein 4	03304-L13248	20q11.2
MEN1 (b)	Multiple endocrine neoplasia 1	09167-L09460	11q13.1
MUS81 (b)	MUS81 endonuclease homologue	10574-L09314	11q13.1
WIF1	WNT inhibitory factor 1	10361-L10899	12q14.3
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	10364-L13254	16q22.1

were directly ligated, while for the other half ligation was combined with the *Hha*I digestion enzyme. 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 a 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 were separated and quantified by electrophoresis in an ABI 3130 capillary sequencer and analysed using GeneMapper4.0 (both Applied Biosystems). Peak identification and values corresponding to peak size in base pairs (bp), and peak areas were used for further data processing. The 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, a promoter was considered to be methylated if the dosage ratio was ≥ 0.20, which corresponds to 20 % of methylated DNA (Pavicic et al., 2011).

CpG universal methylated and unmethylated DNA (Chemicon International, Temecula, CA) were used 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 20% cut-off

In the present study we used the MS-MLPA probe set ME004 (MRC-Holland, Amsterdam, The Netherlands) to analyse samples from 44 patients with ovarian cancer and 30 control samples. Using a 20 % cut-off for methylation we observed statistically significant higher methylation in genes *NTRK1* (P = 0.008), *GATA4* (P < 0.001) and *WIF1* (P = 0.005) in ovarian cancer patients compared with the control group. For genes *APAF1a* and *DNAJC15* we observed high methylation (about over 80 %) in both ovarian cancer and control samples. For genes *EPHB2*, *NF1a*, *THBS1*, *PCNA*, *MEN1a*, *LMNA*, *RBM14*, *IGF2Rb*, *MEN1b*, *MUS81b* and *CDH1*, the methylation rate did not exceed the 20 % threshold; the other genes also showed relevant differences in methylation between ovarian cancer and control samples (Fig. 1).

Correlation with clinicipathological features

The mean (median) age of patients at the time of diagnosis was 53 years (range 21–79 years) in the carcinoma group and 56.5 years (range 40–84 years) in the control group. The methylation results from the ovarian cancer specimens were compared with clinicopathological characteristics including age, histological type, tumour stage and histological grade (Table 2).

Gene *GATA4* showed a significantly (P < 0.05) higher methylation in endometrioid type compared with the mucinous histological type of ovarian cancer (Table 2). There was also significantly higher methylation of the *WIF1* gene (P = 0.045) in the later stages than in stage I ovarian cancer. The *PTEN* gene showed significantly higher methylation (P < 0.05) in grade 2 than in other grades (1 and 3) of ovarian cancer.

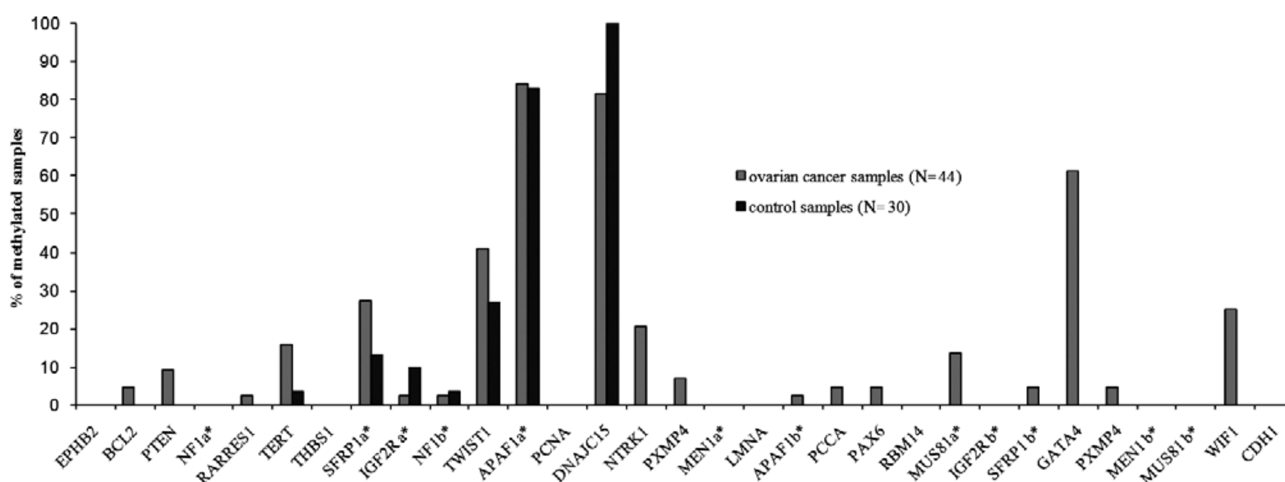

Fig. 1. Methylation of specific genes in ovarian cancer and control samples
Comparison of methylation frequencies (cut-off value 20 %) of the 25 analysed genes in ovarian cancer and control samples
* Two CpG loci (a and b) were analysed.

Table 2. Clinicopathological characteristics vs. methylation of specific genes

characteristic		number of samples	% of samples	% of methylated samples in specific genes					
				PTEN	***TERT***	***NTRK1***	***MUS81a***	***GATA4***	***WIF1***
age	**≤ 45 years**	10	22.7	10	10	20	10	70	50
	> 45 years	34	77.3	8.8	17.7	20.6	14.7	54.1	17.7
stage	**I + II**	16	36.4	6.25	6.25	31.25	12.50	62.50	12.50
	III + IV	28	63.6	10.7	21.4	14.2	14.2	60.7	32.1
histology	**serous**	31	70.5	6.5	16.1	16.1	9.7	58.1	29.0
	endometrioid	9	20.5	0	0	50	0	25*	0
	mucinous	4	9.0	22.2	22.2	22.2	33.3	88.9*	22.2
grade	**1**	13	29.5	0	7.7	7.7	7.7	61.5	15.4
	2	8	18.2	37.5*	25.0	37.5	37.5	62.5	37.5
	3	23	52.3	4.3*	17.4	21.7	8.7	60.9	26.1

* P value < 0.05

Discussion

The methylation of DNA is an enzymatic process that often occurs within CpG islands. It is now clear that *de novo* promoter hypermethylation is a common mechanism which can lead to down-regulation of tumour suppressor genes, resulting in their inactivation and reduced ability to suppress malignancies. The promoter methylation status has been reported in several genes in human neoplasms. The purpose of this study was to investigate promoter methylation of a set of common tumour suppressor genes in 44 ovarian cancer and 30 control samples using MS-MLPA.

The analysed loci of genes *EPHB2*, *NF1a*, *THBS1*, *PCNA*, *MEN1a*, *LMNA*, *RBM14*, *IGF2Rb*, *MEN1b*, *MUS81b* and *CDH1* did not show methylation above the threshold, suggesting that promoter methylation of the selected CpG loci of these tumour suppressor genes may not play an important role in ovarian carcinogenenesis. On the other hand, *BCL2*, *PTEN*, *RARRES1*, *TERT*, *SFRP1a*, *IGF2Ra*, *NF1b*, *TWIST1*, *APAF1a*, *DNAJC15*, *NTRK1*, *PXMP4*, *APAF1b*, *PCCA*, *PAX6*, *MUS81a*, *SFRP1b*, *GATA4*, *PXMP4* and *WIF* did show promoter methylation to a varying extent above the 20% threshold. *APAF1a* and *DNAJC15* methylation was very frequent in both ovarian cancer and normal ovarian tissue (Fig. 1).

We observed significantly higher methylation in genes *NTRK1*, *GATA4* and *WIF1* in the cancer group compared to the control group, suggesting that promoter methylation of these tumour suppressor genes could play an important role in ovarian carcinogenesis. These genes could be used in future screening for ovarian cancer because methylated DNA has also been detected in body fluids of ovarian cancer patients, e.g. in the plasma, and the levels correlated reasonably well with the methylation levels in tumour tissue (Ibanez de Caceres et al., 2004; Dong et al., 2012). 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).

The neurotrophic tyrosine kinase receptor, type 1 (*NTRK1*) gene encodes a member of the neurotrophic tyrosine kinase receptor (NTKR) family. This kinase is a membrane-bound receptor that, upon neurotrophin binding, phosphorylates itself and the members of the MAPK pathway. In the present study, we found a significantly higher level of methylation in this gene in ovarian cancer samples, suggesting the importance of this gene in ovarian carcinogenesis. Tapia et al. (2011) showed higher expression of *NTRK1* in ovarian cancer as a potential marker in epithelial ovarian cancer. This finding, in conjunction with our experiment, may suggest that the analysed CpG loci may be not only methylated, but hydroxymethylated, because hydroxymethylation has an opposite impact on DNA expression than methylation. However, we analysed only one CpG locus in the whole promoter region, which may not necessarily have an effect on gene expression.

The *GATA4* gene encodes a member of the GATA family of zinc-finger transcription factors. Members of this family recognize the GATA motif, which is present in the promoters of many genes. This protein is thought to regulate genes involved in embryogenesis and in myocardial differentiation and function. Methylation of the *GATA4* gene plays an important role in development of many types of cancer (Guo et al. 2004, 2006; Hellebrekers et al., 2009; Wen et al. 2010; Montavon et al., 2012). Wakana et al. (2006) found the importance of *GATA4* methylation in ovarian cancer cell lines, and Montavon et al. (2012) in a recent study found promoter methylation of the *GATA4* gene in 76.9 % of ovarian cancers and in 33.3 % of benign ovarian surface epithelium samples. In our study we confirmed the importance of *GATA4* promoter methylation in ovarian cancer, and we found new loci where promoter methylation was present in 61.4 % of ovarian cancer samples and in none of the control samples.

The protein encoded by the *WIF1* gene functions as an inhibitor of WNT proteins, which are extracellular

signalling molecules playing an important role in embryonic development. This protein contains a WNT inhibitory factor (WIF) domain and five epidermal growth factor (EGF)-like domains, and is thought to be involved in mesoderm segmentation. This gene functions as a tumour suppressor gene, and has been found to be epigenetically silenced in various cancers (Mazieres et al, 2004; Taniguchi et al., 2005; Deng et al., 2010). Our study indicates that *WIF1* methylation is also an important event in ovarian carcinogenesis, particularly in the advanced stages of the disease.

Methylation patterns are often associated with pathological features of ovarian cancer (Asadollahi et al., 2010). Montavon et al. (2012) showed high methylation in high-grade serous ovarian cancer. Abou-Zeid et al. (2011) showed higher methylation frequency of *CDKN2A* in poorly differentiated (grade 3) tumours. In our study the methylation levels of selected genes also correlated with the grade of ovarian cancer (Table 2). Grade 2 showed statistically higher methylation in the *PTEN* gene than other grades. However, we are unable to draw any conclusions because of the small number of samples in grade 2. A study with a larger number of samples will be necessary to determine whether *PTEN* methylation is associated with the grade of ovarian tumours.

In conclusion, our study showed that there are significant differences in promoter methylation in *NTRK1*, *GATA4* and *WIF1* genes between ovarian cancer and normal ovarian tissue, suggesting the importance of epigenetic changes in these genes in ovarian carcinogenesis. Such 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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