Opioid-binding protein/cell adhesion molecule-like (OPCML) gene and promoter methylation status in women with ovarian cancer

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Abstract

OBJECTIVE: The expression profile of the OPMCL gene was studied to find out if there was any evidence of a CpG island methylator phenotype and if there was an association of CpG island methylation with the gene downregulation in women with ovarian cancer. **MATERIAL AND METHODS:** Expression of OPCML in 43 ovarian cancer tumor samples and in 4 normal ovaries was determined by RT-PCR. Methylation status of OPCML promoter region was studied with methylation-specific PCR (MSP) method. Possible associations with selected clinicopathologic variables: FIGO stage, histological grade, patient's age and menopausal status were tested. **RESULTS:** In all normal ovarian samples OPCML mRNA was present, but it was not detectable in 24 of 43 ovarian cancer cases. We have not found the relationship between age and menopausal status with the presence of RTPCR product of OPCML. Hypermethylation of OPCML was not correlated to FIGO stage, however, in 80% of cases with methylated OPCML early clinical stage was also present. Tumor grading and histological type had no significant influence on the presence of hypermethylation of OPCML gene. In 20 of 43 cases of ovarian cancer methylated product of MSP amplification was present. In a group of OPCML*mRNA*-negative tumors there were 75% of cases with hypermethylated exon of OPCML and the correlation between these variables was statistically significant (χ^2 =17,7; p=0,00003). No promoter hypermethylation of the studied gene was found in normal ovaries. **CONCLUSIONS:** Reduced OPCMPL gene expression in ovarian cancer in comparison to normal ovaries could be related to the hypermethylation of promoter region. This epigenetic alteration may be the reason of gene silencing and the loss of suppressor function.

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Introduction

OPCML (opioid-binding protein/cell adhesion molecule-like) is a member of the IgLONs immunoglobulin subfamily of glycosylphosphatidylinositol-anchored cell adhesion molecules [4]. These proteins are present in neural tissue where OPCML is mainly expressed in the gray matter of the brain [10]. The possible functions of this protein are cell adhesion and cell-cell recognition processes [9]. Recent evidences suggested that OPCML could act as a tumor suppressor gene [11, 12]. It has been noted that *OPCML* expression was completely abrogated in the majority of primary ovarian tumors and in ovarian cancer cell lines compared with normal human ovary [12]. The loss of OPCML expresion was present in 83% of women with ovarian cancers. The mechanism underlying OPCML silencing was shown to be CpG island hypermethylation [12]. Aberrant methylation of multiple CpG islands is a frequent event in epithelial ovarian cancer compared with normal ovarian surface epithelium [1].

Altered methylation patterns are known to occur in the DNA of cancer cells [2]. Two patterns have been observed: wide areas of global hypomethylation along the genome and localized areas of hypermethylation at certain sites, the CpG islands, within the gene promoter regions [2,15]. The amplification of protooncogenes, or the silencing of tumor suppressor genes, disrupts the balance that normally controls cell proliferation and leads it through the succession of events leading to full malignant status. Thus, in theory, decreased methylation, and hence relief of transcriptional silencing, may allow the expression of previously quiescent protooncogenes to become active and induce the cell proliferation events. Alternatively, increased methylation at previously unmethylated sites, such as the promoter regions of a tumor suppressor gene, may result in their silencing through inhibition of transcription and their inability to suppress cell proliferation [2,13]. Recently, we have shown that hypomethylation of SNCG was very common in malignant tumors of the ovary and led to overexpression of synuclein-γ-neural protein, a possible important oncoprotein in ovarian cancer [3]. CpG island hypermethylation of genes such as p16, BRCA1, RASSF1A among others, is a relatively early event in ovarian tumorigenesis [1,14].

Since previous data suggested a role of changes in OPCML expression in ovarian carcinogenesis, we wanted to know if the abnormal methylation of CpG islands could be a possible mechanism for the alterations of OPCML expression. In order to determine if hypermethylation of the OPCML gene is a common molecular event in ovarian cancer, we examined the methylation status of promoter region of OPCML gene in women with ovarian cancer.

Materials and methods

All participants were informed of the nature of the study and gave their informed consent. The studied group included 43 women with malignant ovarian tumors. Tumor tissue was collected at surgery. Multiple representative samples were snap-frozen in liquid nitrogen until further processing. All collected specimens underwent histological examination in order to confirm the diagnosis of ovarian cancer. Histological types and gradings of the tumors were classified according to the criteria of World Health Organization (WHO). There were 23 serous, 14 mucinous, 2 endometrioid, 2 clear cell, 2 undifferentiated and 3 metastatic cancers. The proportion of malignant cells in all tumor tissues used in this study was more than 50%. The stage of each cancer was established according to International Federation of Gynecology and Obstetrics (FIGO) criteria. Additionally, four normal ovarian tissue samples were used as control for MSP and RTPCR methods

Detection of OPCML methylation by MSP(methylation specific PCR)

Genomic DNA from tumor tissue samples was extracted using the DNAeasy Qiamp Mini Kit (Qiagen, USA), according to manufacturer's protocol. Two μg of genomic DNA from each sample was modified by sodium bisulfite, which converts all of the unmethylated cytosines to uracils, whereas methylated cytosines remain unchanged. For DNA modification we use CpG Modification Kit (Chemicon, USA) according to manufacturer's instruction. The modified DNA was amplified according to the method described by Herman et al [6]. Detailed primer sequences and PCR conditions are available upon request. Briefly, the PCR mixture contained 2.5 μl of 10x PCR buffer (Qiagen,); 5μl 5xQ-Solution (Qiagen) 200 μM dNTPs; primers (0.5 μM each per reaction) and bisulfite-modified DNA ('30 ng) or unmodified DNA (30–50 ng) as a template and in a final volume of 25μl. This reaction mix was amplified with Qiagen Taq polymerase (Qiagen). Water blanks without added DNA were included as negative PCR controls in each assay. PCR products were analyzed on 2% agarose gels containing ethidium bromide

Detection of OPCML- mRNA expression by RTPCR RNA Isolation

Tissue samples were homogenized in 1 ml of Trizol Reagent (Life Technologies, USA); 1 ml/35–45 mg of tissue. The homogenized samples were then incubated for 5 minutes at room temperature. After the addition of 0.2 ml of chloroform, the samples were vigorously shaken for 15 seconds and incubated at room temperature for another 3 minutes. The samples were then centrifuged at 13500 rpm for 15 minutes at 4°C. After the centrifugation, the aqueous phase containing total RNA was transferred into a fresh tube, and the same volume of isopropyl alcohol was added. The samples were

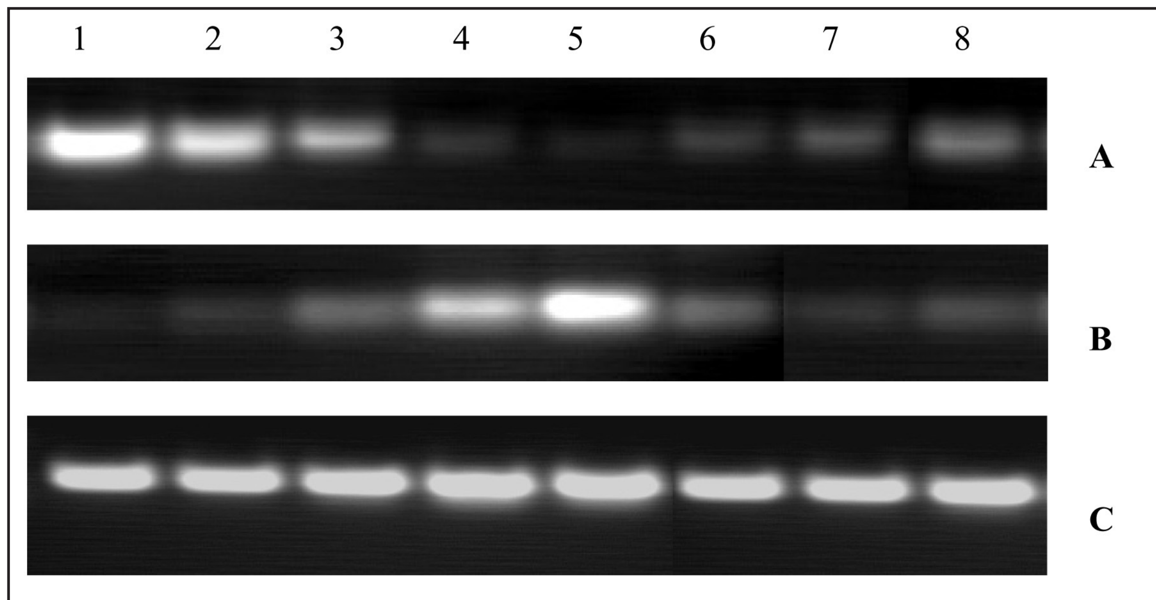

Figure 1:
A: Detection of OPCML mRNA by RTPCR methods
B: Detection of methylation of CpG island in OPCML gene by MSP methods.
C: Detection actin mRNA –control of integrity of isolated total RNA from samples.
Lane 1- normal control ovary, Lane 2-9 ovarian cancer samples

Table 1: Clinical information relating to patients.

Histologicaltype	Number of patiens	**Age (years)Mean (range)**	**Grading**			**Clinical stage**	
			G1	G2	G3	**I/II**	III/IV
serous	16	57 (21-72)	1	8	7	1	15
mucinous	4	64 (41-75)	0	3	1	0	4
endometroid	1	45 (43-47)	0	1	0	0	1
clear cell	3	65 (61-70)	0	2	1	2	1
undifferentiated	4	57 (53-60)	0	2	2	0	4
metastatic	6	58 (54-65)	0	2	6	0	8
other	7	49 (41-55)	1	0	5	2	4

incubated for 10 minutes at room temperature, and the RNA was precipitated by centrifugation. The RNA pellets were washed with 1 ml of 75% ethanol and dissolved in 40 ul of RNase-free water.

RT-PCR Analyses

RT-PCR analysis was performed by a standard RT-PCR with the primers specific for human OPCML and beta-actin. Primers were designed using Primer-3 v.0.2 software. Three µg of total RNAs were used for reverse transcription reaction using oligo(dT) primers. The samples were heated to 70°C for 10 minutes and then quickly chilled on ice. Then, 4 µl of 5X first strand buffer, 2 µl of 0.1 M DTT, 1 µl of 10 mM deoxynucleoside triphosphate, and 200 units of reverse transcriptase were added to the samples. The mixtures were incubated at 42 °C for 60 minutes, and one-fifth of this reaction was amplified by PCR using PCR kit. Each reaction consisted of 30 cycles in the GeneAmp PCR System 2400 (Perkin-Elmer). The parameters for PCR were as follows: denaturation at 95 °C for 30 seconds; annealing at 57°C for 30 seconds; and elongation at 72°C for 60 seconds. One-third of the PCR products were electrophoresed through 1% agarose gel. The integrity and the loading controls of the RNA samples were ascertained by β-actin expression with a set of primers for 314-bp beta-actin.

Statistical Analysis. The data were compared with the use of χ^2 test. In all calculations Statistica ver. 6.0. (Statsoft, USA) was used and $p>0.05$ was considered significant.

Results

Selected pathological characteristics of the tumor samples are listed in Table 1. Firstly, using RT-PCR assays, we examined OPCMLmRNA expression in malignant ovarian tumors Secondly, to determine whether the expression of OPCML in ovarian cancer is associated

Table 2: Detection of methylated CpG islands by MSP method and altered SNCGmRNA expression in ovarian cancer in relation to clinical parameters.

	Methylated OPCML	**Altered OPCML mRNA expression**
	Number of patients	
	20(46,5%)	24(55,8%)
	Menopausal status (MNP	
Before MNP	8(40%)	11(55%)
After MNP	12(52,7%)	13(56,5%)
	Histological Grading	
G1+G2	11(55%)	13(65%)
G3	9(39,1%)	11(47,8%)
	FIGO stage	
I and II	4(80%)	5(100%)
III and IV	16(42,1%)	19(50%)
	Histological type	
serous	8(53,3%)	8(53,3%)
nonserous	12(42,8%)	16(57,1%)

with hypermethylation, we analyzed the methylation status of DNA isolated from the samples of tumor tissues. Figure 1A shows a typical example of OPCML mRNA detection with the use of RTPCR. The OPCML mRNA was not detectable in 24 of 43 ovarian cancer cases (55,8%). Age and menopausal status of patients had no influence on the presence of OPCML mRNA (p=0,9). We have not found the relationship between histological type of the tumor and the presence of RTPCR product of OPCML (p=0,8). Also, the expression of OPCML gene was not correlated to the tumor grading (p=0,46). We have found that only FIGO clinical stage had a significant influence for absence of OPCMLmRNA (χ^2=4,47; p=0,03). Interestingly, all five tumor samples with FIGO stage I or II were OPCMLmRNA-negative.

We found that methylated product of MSP amplification was present in 20 of 43 (46,5%) cases of ovarian cancer. In the group of OPCML*mRNA*-negative tumors there were 75% (18 of 24) cases with hypermethyletad exon of OPCML and the correlation between these variables was statistically significant (χ^2 =17,7; p=0,00003). Hypermethylation of OPCML was not correlated to FIGO stage (p=0,1), however, in 80% (4 of 5) of cases with methylated OPCML early clinical stage (FIGO I or FIGO II) was also present. Tumor grading and histological type had no significant influence for the presence of hypermethylation of OPCML gene (p=0,2 and p=0,5 respectively). Aberrant methylation of OPCML was more often found in postmenopausal women (52,7% vs 40%), however, the difference did not reach statistical significance (p=0,4). Figure 1B shows typical detection of MSP products from modified DNA samples.

Discussion

The onset of cancer is associated with the silencing of tumor suppressor genes and activation of protooncogenes. OPCML has been shown to exhibit functional characteristics of a tumor suppressor gene in an ovarian cancer cell line *in vitro* and also *in vivo* when xenografted into nude mice [12]. More than 50% of examined ovarian tumor samples in our study group presented with the lack of detectable expression of the OPCML gene. Our results are similat to those of Sellar et al [12] who found that abrogated expression was present in 83% of sporadic ovarian cancer samples. Zhang et al. [16] observed that among their ovarian epithelial cancer samples only 19.4% expressed OPCML mRNA in comparison to 85% of the normal ovarian tissue and 76.5% of benign ovarian tumors. The ratio of OPCML mRNA expression in ovarian epithelial cancer was significantly lower than those in normal and benign tumors. We observed altered *OPCML* expression in early-stage tumors which suggests that the dysregulation of *OPCML* translation may be an early event in ovarian tumorigenesis. Moreover, this process does not depend on the age of patients, tumor histology or grading. Sellar et al. [12] suggested that the loss of OPCML reduces intercellular adhesion and accelerates cell growth simultaneously, thereby promoting early steps of ovarian carcinogenesis.

Our results confirmed previous observations that the mechanism underlying OPCML silencing is related to CpG islands hypermethylation [7,12] . We found that 75% of samples with altered expression of OPCML also exhibited hypermethylation of OPCML. In another recent similar study by Zhang et al. [16] methylations of the gene's promoter region were detected in 44.4% samples of cancer tissue, while in no samples of normal

ovarian tissue and also in none of the benign ovarian tumors. The ratio of methylation of ovarian epithelial carcinoma was significantly higher than those of normal and benign tumors.

The mechanism underlying aberrant methylation of the promoter region of multiple suppressor genes in cancer remains uncertain. DNA methylation involves the enzymatic addition of a methyl group to the carbon-5 position of cytosine in DNA at CpG dinucleotides [5]. Altered expression of the DNMT enzymes is a possible mechanism for the dysregulation of DNA. Mei et al. [1] suggested that elevation of the RAS signaling pathway may play an important role in epigenetic inactivation of OPCML in human epithelial ovarian cancer. They found that oncogenic RAS activity is directly responsible for the observed OPCML promoter hypermethylation and epigenetic gene silencing of OPCML. The study suggests that the elevation of RAS signaling pathway may play an important role in epigenetic inactivation of OPCML in human epithelial ovarian cancer. In order to propose the role of OPCML and its relatives in ovarian carcinogenesis, more information is needed. Functions of these proteins in the normal ovary, as well as their localization within the ovarian environment and possible pathways that link them to ovarian cancer must be established.

Conclusion: Understanding the OPCML function and the possible use of surrogate molecules approach, with no requirement for restoration of gene function in every cell by gene therapy, may present a future potential therapeutic option.

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