Loss of OPCML expression and the correlation with CpG island methylation and LOH in ovarian serous carcinoma

H. Chen[1], *M.D.*; **F. Ye**[1], *M.D., Ph.D.*; **J. Zhang**[2], *M.D.*; **W. Lu**[1], *M.D.*; **Q. Cheng**[1]; **X. Xie**[2], *M.D.*

[1]*Women's Reproductive Health Laboratory of Zhejiang Province*
[2]*Department of Gynecologic Oncology, Women's Hospital, School of Medicine, Zhejiang University, Hangzhou (China)*

Summary

Purpose: To detect the expression of OPCML in ovarian serous carcinoma and investigate the correlation with CpG island methylation and LOH of OPCML. *Methods*: 20 normal tissues, 75 ovarian serous tumors, three cell lines, SKOV-3, CAOV3 and 3AO, were detected in OPCML expression by RT-PCR, CpG island methylation by methylation-sensitive restriction enzyme-PCR, and LOH analysis at four microsatellite marks (D11S4085, D11S1320, D11S874 and D11S969). *Results*: Loss of OPCML expression in ovarian serous carcinomas was significantly higher than in ovarian adenomas and normal tissues. OPCML expression was detectable in 3AO, but not in SKOV-3 and CAOV3. CpG island methylation was found in 53.4% of the carcinomas, while in none of the adenomas or normal tissues. Meanwhile, CpG island methylation was detectable in SKOV-3 and CAOV3, but not in 3AO. The correlation between CpG island methylation and loss of OPCML expression was found in carcinomas. The LOH rate at D11S4085 in carcinomas was significantly higher than that for adenomas and normal tissues. LOH at D11S4085 was also correlated with loss of OPCML expression. *Conclusions*: These results indicate that loss of OPCML expression occurs frequently in ovarian serous carcinoma. CpG island methylation and LOH are probably two mechanisms of OPCML inactivation.

Key words: Ovarian serous carcinoma; OPCML; CpG island methylation; LOH.

Introduction

The cellular adhesion molecule is an important part of costimulatory molecules. It has been shown that loss of those molecules assists in tumor cells escaping from attack of immune cells of the host, thereby promoting progression and metastasis of the tumors [1].

OPCML is a novel member of the IgLON family of immunoglobulin domain-containing glycosylphosphatidylinositol (GPI)-anchored cell adhesion molecules [2]. OPCML predominantly exists in the neural cells of the cerebral cortex and hippocampi [3]. In addition to neural cells, OPCML is also normally expressed in ovarian surface epithelium and weakly in the heart, placenta, liver, kidney, pancreas, and colon [4]. The effect of OPCML on neural cells suggests that it might function in the development and progression of some malignant tumors. Recently, Sellar and his colleagues found that OPCML was frequently somatically inactivated in epithelial ovarian carcinoma. They further verified that OPCML had functional characteristics consistent with tumor suppressor genes [5].

Epithelial ovarian carcinoma consists of different subtypes, including serous, mucinous, endometriod and others. Of those, serous carcinoma is the most common malignancy. A recent study indicated that carcinogenesis of serous carcinoma might also be different from that of others [6]. In order to narrow and verify the findings by Saller *et al.* in epithelial ovarian carcinoma and cell lines, we collected 75 ovarian serous tumors and three ovarian serous carcinoma cell lines, detected OPCML expression, and analyzed the correlation with CpG island methylation and loss of heterozygosity (LOH).

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Materials and Methods

Tissues

Twenty normal ovarian tissues, 17 benign ovarian serous adenomas and 58 ovarian serous carcinomas were collected from the Department of Gynecologic Oncology of the Women's Hospital, School of Medicine, Zhejiang University. Median age was 46.5 years (range, 38-58 years) in the normal group, 39.0 years (range, 21-70 years) in the adenoma group, and 51.5 years (range, 32-78 years) in the carcinoma group.

Cell Lines

Ovarian serous carcinoma cell line SKOV-3 and CAOV3 were obtained from the American Type Culture Collection (USA), and 3AO from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Science, China. All the cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2.

Primer Design

The primers, including OPCML mRNA, promoter CpG island of OPCML and four microsatellites (D11S874, D11S969, D11S1320, D11S4085) were designed by Primer Designer 5.0 (Table 1), according to the sequence from the Genebank, and synthesized by Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd, China.

RT-PCR

Total mRNA was extracted using Trizol (Gibco BRL). cDNA was prepared by using oligo-$(dT)_{12\text{-}18}$ primer and MMLV retrotranscriptase. The PCR was 94°C, 40•sec; 57°C, 40 sec; 72°C, 40 sec with five cycles; 94°C, 40 sec; 55°C, 40 sec; 72°C, 40 sec with 33 cycles, and then followed by 10 min extention at 72°C. ß-actin was used as a positive control.

Table 1. — *Primers for PCR.*

Extended sequence	Product length	Primers
ß-actin mRNA	224 bp	5'-GAGCGGGAAATCGTGCGTGACATT-3'
		5'-GAAGGTAGTTTCGTGGATGCC-3'
OPCML mRNA	327 bp	5'-ACCGCAGCACCATCCTCTAC-3'
		5'-CCTGGCCTTCCTTGACTGAC-3'
Promoter CpG	559 bp	5' -CAGCTCCTGTATGTCAGAGA -3'
island of OPCML		5'-CAAGGAACAGCAGCCTGAGA-3'
		5'-GGTTTAAAAAGTCAGCCCTC-3'
D11S874	185 bp	5'-AATCATTTTCAAGCATAGGC-3'
		5'-TTGATTTGGAAGATTTTCAC-3'
D11S969	145 bp	5'-GGGGCAGAATGGGTAT-3'
		5'-AACATTACTAAAAGGTTAAATGAGC-3'
D11S1320	225-233 bp	5'-ATTAAGGCACCAAATGGG-3'
		5'-GCTACAATGCAATATCAATAGAAGG-3'
D11S4085	187-203 bp	5'-GGCCACAGGACTTTCAGAG-3'

Methylation-sensitive restriction enzyme-PCR (MSRE-PCR)

There were seven sites for the methylation-sensitive restriction enzyme (MSRE) in the OPCML sequence (Hap II,CCGG; Aat II,GACGTC; Acc II,CGCG; Cfr13 I,GGNCC). DNA (0.5 µg) was digested with 10 U methylation-sensitive restriction enzyme (TaKaRa) Hap II, Aat II, Acc II and Cfr13 I, or non-methylation-sensitive restriction enzyme MSP-I in 20 µl reaction volume for 10-12 h, and then digested for another 4-6 h after adding 0.5 µl enzymes into the systems to ensure complete digestion. The reaction was terminated by heating the samples at 70-80°C for 20 min. DNA was also methylated by SssI methyltransferase (New England) as a positive control. The PCR was 94°C, 1 min; 57°C, 1 min; 72°C, 1 min with five cycles; 94°C, 1 min; 55°C, 1 min, 72°C, 1 min with 33 cycles, and followed by 10 min extention at 72°C.

Cell Culture and 5'-aza-2'-deoxycytidine (AZA) treatment

AZA can demethylate CpG islands. The ovarian carcinoma cell line 3ao, SKOV-3 and CaoV3 were maintained in RPMI 1640. The medium was replaced after 24 h, and AZA (Sigma) was added to a final concentration of 20 µM. After four days, the cultured cells with and without AZA treatment were collected for RT-PCR and MSRE-PCR.

LOH

Forty-eight ovarian serous carcinoma samples collected both aberrant and normal paraffin-embedded tissues which were amplified by PCR labeled microsatellite marks in 11q25 (D11S874, D11S969, D11S1320 and D11S4085), in which the OPCML gene was located. D11S4085 was located in the second intron of OPCML, while D11S1320 and D11S874 belonged to HNT (neurotrimin), a paralog, and approximately 80 kb centromeric to OPCML in the opposite orientation. D11S969 was located in LOC253805, a hypothetical gene lying distal to OPCML; 20 ng of genomic DNA extracted from tissue was used for each PCR analysis. The products were separated by electrophoresis on the denaturing 8% polyacrylamide gel containing urea and the gel was dyed by fluorescence staining, showed by ImageQuant TL v2003.02. The allelic LOH rate was calculated as T1/T2/N1/N2 for the rate of area values of tumor (T) versus normal (N) alleles, using the software above. The LOH was defined as the allelic rate above 2.0 or below 0.5.

Statistical analysis

Fisher's exact probability in a 2x2 table was performed. Only p values < 0.05 were considered significant.

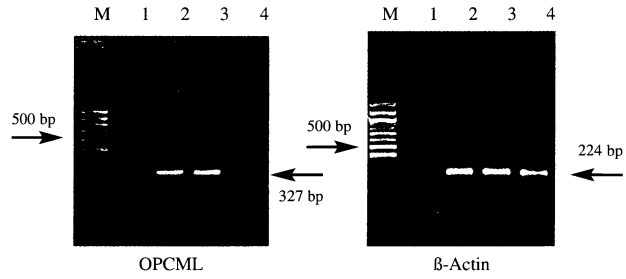


Figure 1. — Expression of OPCML mRNA in ovarian tissue.

Table 2. — *OPCML mRNA expression in ovarian tissues.*

Group	no.	Loss of expression (no.)	Rate of expression loss (%)
Normal	20	3	15.0
Adenoma	17	4	23.5$^{\Delta}$
Carcinoma	58	46	79.3$^{*\#}$

* compared with normal (x^2 = 26.334, p = 0.0000)
\# compared with adenoma (x^2 = 18.408, p = 0.000)
Δ Compared with normal (x^2 = 0.436, p = 0.680)

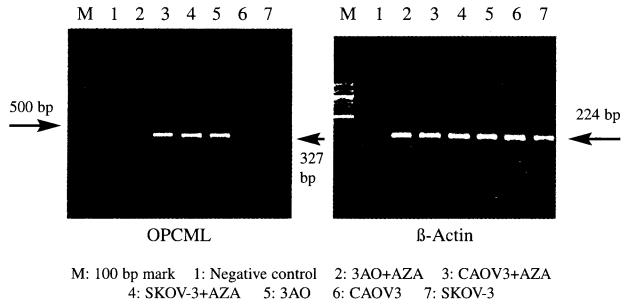


Figure 2. — OPCML mRNA expression in cell lines treated with or without AZA.

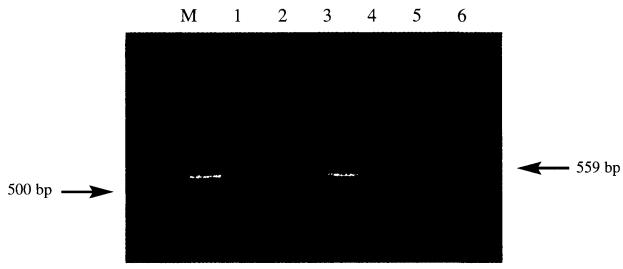
Results

Expression of OPCML mRNA

Carcinoma expression was significantly lower than for normal tissue and adenoma (Figure 1, Table 2). Furthermore, no differences in OPCML expression were found among different FIGO stages and cytological grades (Table 3). OPCML mRNA expression was detectable in 3ao, but not in SKOV-3 and CaoV3 (Figure 2).

CpG island methylation and the correlation with loss of OPCML mRNA expression

The CpG island methylation rate in the carcinoma group was significantly higher than that of the normal and adenoma groups (Figure 3, Table 4). CpG island methylation and loss of OPCML expression was significantly correlated (Table 5).



M: 100 bp mark 1: Carcinoma without enzyme cut 2: Negative control
3: Carcinoma with MSP-I cut after methylated by Sss I methylatransferase
4: Carcinoma with Hap II cut after methylated by Sss I methylatransferase
5: Carcinoma with MSP-I cut 6: Carcinoma with HAP-II cut

Figure 3. — CpG island methylation in ovarian serous carcinoma (Hap II site).

Table 3. — *OPCML Expression in ovarian carcinoma with different clinical and pathologic states.*

Group	no.	OPCML mRNA(n) +	OPCML mRNA(n) −	Rate of expression loss (%)	*p* value
FIGO stage					0.721*
I	1	0	1	100	
II	11	3	8	72.7	
III	43	9	34	79.1	
IV	3	0	3	100	
Differentiation					0.448$^{\Delta}$
Grade 1	6	1	5	81.0	
Grade 2	8	3	5	62.5	
Grade 3	44	8	36	81.8*	

* $x^2 = 1.336$, $\Delta x^2 = 1.606$.

Table 4. — *CpG ialand methylation in ovarian tissues.*

Group	no.	Methylation	Rate of methylation (%)
Normal	20	0	0
Adenoma	17	0	0
Carcinoma	58	31	53.4*#

* compared with normal ($x^2 = 17.740$, p = 0.0000)
# compared with adenoma ($x^2 = 15.488$, p = 0.000)

Table 5. — *Correlation between CpG island methylation and loss of OPCML expression in ovarian serous carcinoma.*

Group		CpG island methylation +	CpG island methylation −	Rate of methylation (%)
OPCML expression	+	0	12	0
	−	31	15	67.4
Rate of expression loss (%)		100	55.6	

* r = 22.043, p = 0.000.

Methylation at Hap II, Aat II, Acc II, Cfr 13 I site and the correlation with OPCML mRNA expression

The methylation rate at the Hap II site in the carcinoma group was significantly higher than that in the normal ($x^2 = 7.099$, $p = 0.005$) and adenoma ($x^2 = 6.091$, $p = 0.010$) groups. At Aat II, Acc II and Cfr13 I sites, no differences were found among the normal, adenoma and carcinoma groups. CpG island methylation at Hap II site was significantly correlated with loss of OPCML expression (Table 6).

Table 6. — *Correlation between CpG island methylation at HapII, Aat II, Acc II, and Cfr 13 I sites and loss of OPCML expression in ovarian serous carcinoma.*

		Hap II +	Hap II −	Aat II +	Aat II −	Acc II +	Acc II −	Cfr13 I +	Cfr13 I −
OPCML expression	+	0	12	0	12	0	12	0	12
	−	21	25	6	40	2	44	8	38
r		12.512		2.957		0.946		4.031	
p		0.0021		0.328		1.000		0.185	

Table 7. — *LOH of microsatellite marks.*

	D11SS4085 +	D11SS4085 −	D11S1320 +	D11S1320 −	D11S874 +	D11S874 −	D11S969 +	D11S969 −
Normal	0	20	0	20	0	20	0	20
Adenoma	1	16	0	17	0	17	0	17
Carcinoma	18	30	5	43	10	38	6	42

CpG island methylation and the corelation with loss of OPCML mRNA expreesion in cell lines

CpG island methylations at Hap II, Aat II, Cfr13 I sites were found in SKOV-3, and at the Hap II site in CAOV3, but no methylation was found in 3AO. Treated with AZA, SKOV-3 and CAOV3 reexpressed OPCML mRNA (Figure 3).

LOH of OPCML

The rate of OPCML LOH at D11S4085 in the carcinoma group was 37.5%, which was higher than that in the normal ($x^2 = 10.200$, $p = 0.001$) and adenoma ($x^2 = 6.067$, $p = 0.014$) groups. The LOH rate at D11S874 in carcinoma group was 20.8%, higher than that in normal ($x^2 = 4.885$, $p = 0.028$), but not in adenoma ($x^2 = 4.186$, $p = 0.052$). No differences at D11S1320 and D11S969 were found between carcinoma and adenoma. The correlation between LOH at D11S4085 and loss of OPCML expression was significantly correlated (Table 8).

Table 8. — *Correlation between LOH at D11S4085 and loss of OPCML expression.*

		LOH +	LOH −	Rate of LOH (%)
OPCML expression	+	0	7	0
	−	18	23	43.9
Rate of loss of expression (%)			100	76.7

* r = 7.283, p = 0.036.

Discussion

OPCML inactivation and its clinical significance in ovarian serous carcinoma

Sellar *et al.* detected OPCML expression in 18 primary ovarian tumors, and found that OPCML expression was completely abrogated in 15 (83%) ovarian tumors [5]. Considering the heterogeneity of epithelial ovarian carcinoma, we determined OPCML expression in 75 ovarian serous tumors, and found that loss of OPCML expression existed in 79.3% of serous carcinomas, while only in 23.5% of ovarian benign tumors and in 15% of normal ovarian tissues. Similarly, two of three ovarian serous carcinoma cell lines did not express OPCML. Our findings strongly suggest that

loss of OPCML expression is very frequent in ovarian serous carcinomas, but not in benign ovarian serous tumors and normal ovarian tissues. Additionally, we found that no differences of OPCML expression existed between different cytological grades and between different FIGO stages. Our findings further suggest that OPCML inactivation might occur early in ovarian carcinogenesis.

It has been shown that adhesion molecules play an important role in cytotoxic T-cell recognition in a MHC-I restricted fashion. Decreased expression of adhesion molecules will reduce T-cell mediated attacks [7, 8]. Thus we suppose that loss of OPCML expression not only assists in tumor cells escaping from host immune attack, but also probably reduces intercellular adhesion and accelerates tumor invasion in ovarian serous carcinoma.

Mechanism of OPCML inactivation in ovarian serous carcinoma

There are three pathways to tumor suppressor gene inactivation, including gene mutation, CpG island methylation and loss of heterozygosity. Sellar *et al.* reported that the OPCML CpG island was methylated in 82% (14 of 17) of ovarian cancers, and further found that a significant correlation existed between OPCML CpG island methylation and loss of OPCML expression [5]. In our results, the rate of OPCML CpG island methylation was 53.4% (31 of 58) in ovarian serous carcinoma, while no methylation was found in 20 normal tissues and 17 benign tumors. CpG island methylation was correlated with loss of OPCML expression. Furthermore, we found that CpG island methylation at the Hap-II site was predominant compared with other sites, and also correlated with loss of OPCML expression. Meanwhile, we found that two ovarian serous carcinoma cell lines, SKOV3 and CAOV3, in which the CpG island was methylated, did not express OPCML. In contrast, cell line 3AO, in which the CpG island was unmethylated, expressed OPCML. To confirm the effect of CpG island methylation, we exposed SKOV3 and CAOV3 to AZA, and found reexpression of OPCML in both cell lines. Our findings suggest that CpG island methylation is an important epigenetic mechanism of OPCML inactivation in ovarian serous carcinoma.

LOH studies have been widely used to define regions of chromosomal loss in sporadic cancers. If the lost region corresponds to the location of a gene related to tumor, the cells may experience a dysregulated division, eventually leading to malignant transformation[9]. We analyzed LOH in the region associated with the gene encoding OPCML, and found a peak LOH rate of 37.5% at D11S4085 in ovarian serous carcinoma, significantly higher than that in benign tumors and normal tissues. All the ovarian serous carcinomas with LOH did not express OPCML. Our findings indicate that the LOH of OPCML is a second mechanism of OPCML inactivation in ovarian serous carcinoma. Our results have strongly shown that CpG island methylation and LOH of OPCML are frequent mechanisms of OPCML inactivation in ovarian serous carcinoma.

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Address reprint requests to:
X. XIE, M.D.
Department of Gynecologic Oncology,
Women's Hospital, School of Medicine,
Zhejiang University
Hangzhou (China)