Expression and clinical significance of methyl-CpG binding domain protein 2 in high-grade serous ovarian cancer

WANGANG GONG[1-4], MAOWEI NI[2-4], ZHONGBO CHEN[2-4] and ZHIGUO ZHENG[2-4]

[1]College of Life Sciences, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310053; [2]Institute of Cancer and Basic Medicine, Chinese Academy of Sciences; [3]Zhejiang Cancer Research Institute, Cancer Hospital of The University of Chinese Academy of Sciences; [4]Zhejiang Cancer Research Institute, Zhejiang Cancer Hospital, Hangzhou, Zhejiang 310022, P.R. China

Abstract. Platinum resistance is an important cause of clinical recurrence and mortality of patients with high-grade serous ovarian cancer (HGSOC). Methyl-CpG binding domain protein 2 (MBD2) serves an important role in tumor progression; however, its role in HGSOC remains unclear. The aim of the present study was to investigate the expression of MBD2 in HGSOC and its role in drug resistance and prognosis of HGSOC. MBD2 expression was analyzed by immunohistochemical staining and western blotting. The associations between MBD2 expression and clinical pathological features, platinum resistance and patient prognosis were analyzed using a χ^2 test, Kaplan-Meier analysis and Cox regression analysis. Positive MBD2 expression was detected in 73 (63.5%) of the HGSOC tissue samples, whereas it was undetectable in all 16 normal tissue samples (100%) analyzed, indicating a significantly higher expression level in tumor tissues compared with normal tissues (P<0.001). Additionally, MBD2 expression was significantly higher in platinum-resistant cases compared with that in platinum-sensitive cases (P<0.05). In addition, high expression of MBD2 was negatively associated with relapse-free survival (P<0.05). In conclusion, MBD2 was demonstrated to be a potential drug target and a biomarker for poor prognosis in HGSOC.

Introduction

Ovarian cancer is the deadliest type of malignant tumor in gynecology, and it seriously affects the lives of women (1). In 2018, 152,000 women died from ovarian cancer worldwide, and the overall 5-year survival rate was ~30% (2,3). High-grade serous ovarian cancer (HGSOC) is the most common form

of ovarian cancer, as well as the most deadly and invasive subtype (4). The standard treatment for HGSOC is cytoreductive surgery combined with platinum and paclitaxel-based chemotherapy (4). Although patients are initially sensitive to chemotherapy, the majority eventually develop drug resistance, which is associated with increased mortality (5). Thus, the identification of targets to treat HGSOC resistance and prognostic biomarkers is of great significance for the treatment of HGSOC.

Hypermethylation of CpG islands in the gene promoter region causes gene silencing and is an important characteristic of cancer cells (6). Methyl-CpG binding domain proteins (MBDs) silence gene expression by binding to methylated DNA and interacting with histone deacetylases (HDACs) and histone methyltransferases, thus affecting carcinogenesis (7,8). The central region of MBD2, a member of the MBD family, contains two binding domains, MBD and a transcriptional inhibition domain, which are responsible for methylation specific binding and transcriptional inhibition (9). MBD2 usually binds to methylated promoter CpG islands (clusters of high density CpG dinucleotides) and acts as a methylation-dependent transcriptional repressor (10). MBD2 can recruit the nucleosome remodeling and deacetylase/Mi-2 complex to silence genes through a methylation-related mechanism (11-14). However, one study also reported that MBD2 is a transcriptional activator of the cAMP response promoter (15). These controversial results suggest that MBD2 serves different roles according to the cell type. Increasing evidence indicates that MBD2 is associated with the occurrence and development of a variety of tumors, and its role in different tumors is also different. For example, MBD2 expression is higher in hepatocellular carcinoma (HCC), glioblastoma (GBM) and breast cancer tissues compared with normal tissues (16-18), whereas it is lower in gastric, cervical and colon cancer tissues compared with normal tissues (17,19,20). In addition, high expression of MBD2 is associated with poor prognosis of patients with HCC (21). A previous study demonstrated that stable short hairpin RNA (shRNA)-mediated downregulation of MBD2 inhibits the proliferation of SK-BR-3, MDA-MB-231 and MDA-MB-435 breast cancer cells cultured *in vitro* (22). However, Yuan *et al* reported that MBD2 downregulation combined with HDAC-1 promotes the growth and metastasis of colorectal cancer (23).

Correspondence to: Dr Zhiguo Zheng, Zhejiang Cancer Research Institute, Zhejiang Cancer Hospital, 1 Banshan Road, Hangzhou, Zhejiang 310022, P.R. China
E-mail: zhengzg@zjcc.org.cn

To the best of our knowledge, the expression of MBD2 in HGSOC has not been studied to date; thus, the aim of the present study was to investigate the association between MBD2 expression and the prognosis of patients with HGSOC and platinum resistance.

Materials and methods

Bioinformatics analysis of MBD2 expression in human OC. The Human Protein Atlas (http://www.proteinatlas.org) is a database that provides immunohistochemical (IHC) staining data for common cancers, normal tissues and cell lines; it contains >10 million IHC images (24-27). The expression data of MBD2 in different normal tissues can be obtained by entering MBD2 in the Tissue Atlas module, and the results were displayed on the webpage.

Patient selection. The study was approved by the Ethics Committee of Zhejiang Cancer Hospital (approval no. IRB-2015-175) and written informed consent was obtained from all patients. In total, 131 female patients were enrolled onto the present study (age range, 39-74 years old), including 115 HGSOC (age range, 39-74 years old) and 16 normal patients (age range, 39-65 years old). The 131 frozen tissue samples used in this study were provided by the Biobank of Zhejiang Cancer Hospital (Hangzhou, China). All tissue samples were collected in the operating room of the Zhejiang Cancer Hospital (Hangzhou, China) from January 2008 to June 2014. The samples were fixed and paraffin embedded. Among all samples, there were 115 HGSOC tissue samples (including 1 case of stage I, 11 cases of stage II, 95 cases of stage III and 8 cases of stage IV) and 16 normal ovarian tissue samples. Normal ovarian tissues samples were obtained patients with other gynecological benign tumors. The diagnosis and stage of HGSOC are determined by two independent experienced pathologists in the Zhejiang Cancer Hospital (Hangzhou, China). Histological classification and tumor staging were performed according to World Health Organization histological classification criteria and the International Federation of Gynaecology and Obstetrics (FIGO) staging criteria (28).

All patients received standard chemotherapy and were divided into platinum-resistant and platinum-sensitive (including partially sensitive) groups according to the following criteria: i) Platinum-resistant group, patients displaying progression or recurrence <6 months after finishing platinum treatment; and ii) platinum-sensitive group, those displaying recurrence >6 months after platinum treatment or those who did not exhibit recurrence.

Immunohistochemical staining. The immunohistochemical staining kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. The tissue samples were fixed with 10% formalin at room temperature for 24 h. Paraffin-embedded tissue samples were cut into 4-μm-thick sections and placed at 72°C for 30 min, then dewaxed and hydrated by xylene and ethanol. The sections were dewaxed with xylene twice for 5 min each time, and the gradient ethanol rehydration (100% for 3 min twice, 95% for 3 min twice, 80% for 3 min) was rinsed with clean running water for 30 min. Antigens were retrieved by pressure cooker treatment for 90 sec
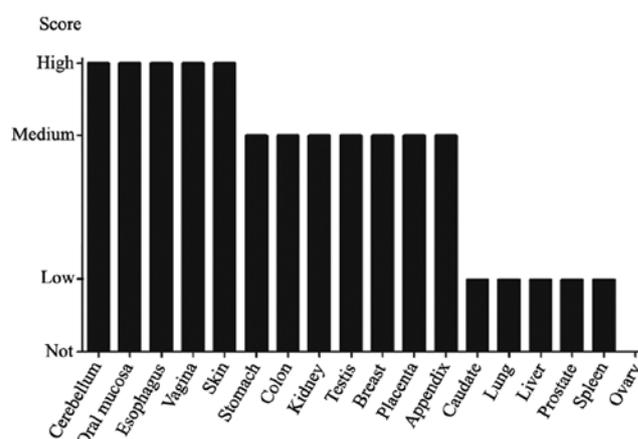

Figure 1. Methyl-CpG binding domain protein 2 is expressed in normal tissues. Image was taken from The Human Protein Atlas (HPA), https://www.proteinatlas.org/ENSG00000134046-MBD2/tissue.

in 0.01 mmol/l citrate buffer. After three washes, the slides were placed in 3% hydrogen peroxide solution for 5-10 min, washed with running water twice and phosphate buffer saline (PBS) for 5 min, and incubated with rabbit anti-human MBD2 polyclonal primary antibody (1:100; cat. no. ab188474; Abcam) overnight at 4°C. After washing with PBS twice for 5 min, slides were incubated with horseradish peroxidase-conjugated rabbit secondary antibodies (cat. no. PV-6000, OriGene Technologies, Inc.) at room temperature for 20 min, followed by washing and 3,3'-diaminobenzidine staining at room temperature for 2 min. The sections were counterstained with haematoxylin for 1 min at room temperature and washed with running water three times. This was followed by incubation with 1% hydrochloric acid alcohol for 1 min and dehydration with a gradient of ethanol (80% for 10 sec, 95% for 10 sec, 100% for 5 min three times), and treated with xylene for 3 min at room temperature. Images were captured using a BX63 fluorescence microscope with a CCD camera (DP80; Olympus Corporation) at 200x magnification.

Evaluation of immunohistochemical staining. The expression of MBD2 detected by immunohistochemistry was evaluated by calculating the sum of staining intensity and the proportion of positively-stained cells, as previously described (29). Briefly, according to the staining intensity, there were four levels, including negative (0 point), weak positive (1 point), intermediate positive (2 points) and strong positive (3 points). The proportion of positively-stained cells was scored as follows: <5%, 0 point; 5-25%, 1 point; 26-50%, 2 points; 51-75%, 3 points; and >75%, 4 points. Finally, the sum of the percentage of positive cells score and the intensity score was calculated to provide the final score of MBD2 expression, which ranged between 0 and 7. A final score of 0 points indicated no expression, 1-4 points indicated low expression, and 5-7 points indicated high expression.

Western blotting. Western blotting was performed as previously described (17). Tissues were lysed in ice-cold cell lysis buffer (Beyotime Institute of Biotechnology) containing protease inhibitors (Thermo Fisher Scientific, Inc.), and the protein concentration in tissue extracts was quantified using
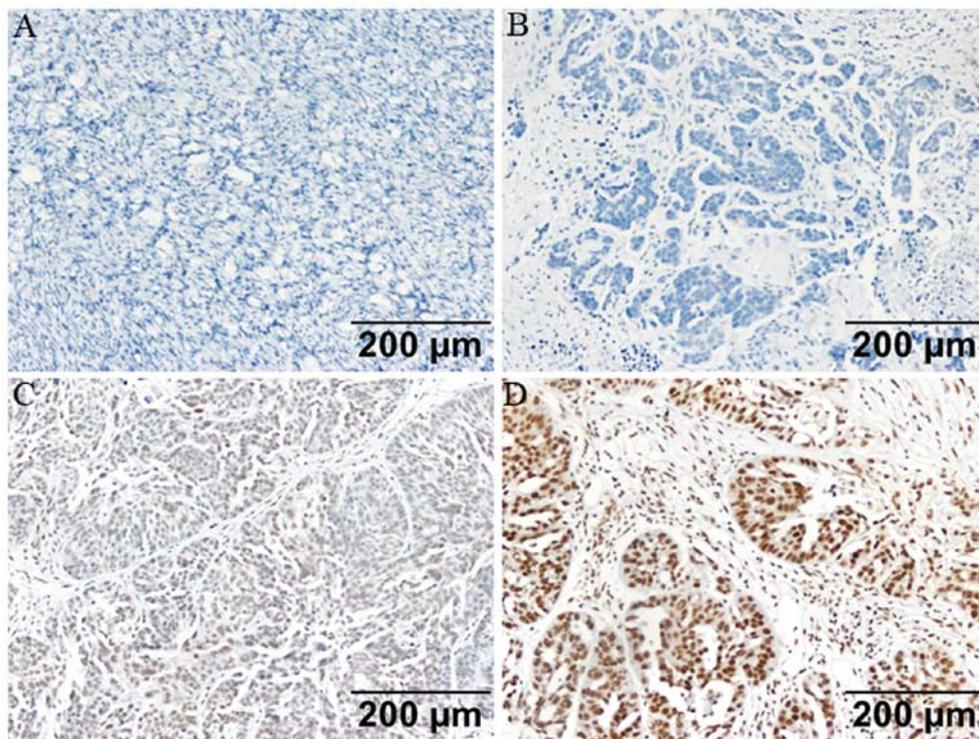
Figure 2. Detection of MBD2 by immunohistochemistry. Negative expression of MBD2 in (A) normal ovarian tissues and (B) HGSOC tissues. (C) Low expression of MBD2 in HGSOC tissues. (D) High expression of MBD2 in HGSOC tissues. MBD2, methyl-CpG binding domain protein 2; HGSOC, high-grade serous ovarian cancer.

the bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (25 μg/lane) from each extract were denatured and separated using a 10% polyacrylamide gel (Beyotime Institute of Biotechnology), and then transferred by electrophoresis onto a nitrocellulose membrane (Thermo Fisher Scientific, Inc.). The membranes were blocked with 5% non-fat dried milk in PBST for 1 h at room temperature and incubated with primary antibodies against MBD2 (1:1,000; cat. no. ab188474; Abcam) and β-actin (1:3,000; cat. no. EM21002; Hangzhou Huaan Biological Technology, Ltd.) at 4˚C overnight. Membranes were then incubated with horseradish peroxidase-conjugated anti-mouse (1:3,000; cat. no. 170-6516; Bio-Rad Laboratories, Inc.). or anti-rabbit secondary antibodies (1:3,000; cat. no. 170-6515; Bio-Rad Laboratories, Inc.). The membranes were developed using SuperSignal West Pico Substrate and CL-XPosure Film (Thermo Fisher Scientific, Inc.). Western blot results were analyzed using Image Lab 3.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. A Pearson Chi-square (χ^2) test or Fisher's exact test was used to analyze the associations between MBD2 expression and clinicopathological features, including age, FIGO stage, degree of differentiation, cancer antigen-125 (CA-125), lymph node metastasis and postoperative residual disease size. The associations between platinum resistance and MBD2 expression, as well as clinical features were analyzed using χ^2 test or Fisher's exact test and multiple logistic regression. The relapse date was defined as the time when new lesions were indentified by radiographic analysis in patients with HGSOC after the completion of surgery, so as to calculate the relapse-free survival (RFS) from the date of surgical

resection to the date of relapse. The Kaplan-Meier method was also used, and Cox proportional hazard regression, log-rank test and Breslow test were used for univariate and multivariate survival analyses of RFS. The unpaired Student's t-test or Fisher's exact test was used to compare the mean of two groups. Analyses were performed using SPSS version 18.0 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

MBD2 expression and clinicopathological characteristics of patients with HGSOC. A query from The Human Protein Atlas database demonstrated that MBD2 was not expressed in normal ovarian tissues (Fig. 1). The expression of MBD2 in 115 HGSOC tissue samples and 16 normal ovarian tissue samples from the patients with normal ovariectomy was investigated by immunohistochemistry (Fig. 2). The results demonstrated that MBD2 was expressed in 73 HGSOC samples (63.5%), but not expressed in 42 HGSOC samples (36.5%). No MBD2 expression was detected in all 16 normal ovarian tissue samples (100%). Fisher's exact test demonstrated that the expression of MBD2 in HGSOC tissues was significantly higher compared with that in normal ovarian tissues (P<0.001, Table I). In addition, among the 115 HGSOC samples, there was no significant association between MBD2 expression and age, FIGO stage, histological grade, CA-125, lymph node metastasis or postoperative residual tumor size (P>0.05), whereas MBD2 expression was significantly associated with platinum resistance (P=0.001; Table II). The expression of MBD2 was also detected by western blotting in eight HGSOC tissues and

Table I. Expression of methyl-CpG binding domain protein 2 in HGSOC and normal ovarian tissue samples.

	MBD2 expression		
Variables	Low	High	P-value
HGSOC	42	73	<0.001
Normal	16	0	

Statistical analysis was performed using a Fisher's exact test. HGSOC, high-grade serous ovarian cancer.

Table II. Associations between the expression level of MBD2 and clinicopathological characteristics.

	MBD2 expression		
Variables	Low	High	P-value
Age, years			0.157[a]
≤54	19	43	
>54	23	30	
FIGO stage			0.756[b]
I-II	5	7	
III-IV	37	66	
Histological grade			1.000[b]
G2	5	9	
G3	34	63	
Null	3	1	
CA-125, U/ml			0.078[a]
≤500	6	21	
>500	36	52	
Lymph node metastasis			0.884[a]
No	19	32	
Yes	23	41	
Residual tumor size, cm			0.547[a]
≤1	31	50	
>1	11	23	
Platinum resistance			0.001[b]
Present	2	22	
Absent	39	47	
Null	1	4	

Statistical analysis was performed with a [a]χ^2 test or [b]Fisher's exact test. The information of some individuals was missing or incomplete, therefore the total number is inconsistent. MBD2 methyl-CpG binding domain protein 2; FIGO, International Federation of Gynaecology and Obstetrics; CA-125, cancer antigen-125; Null, missing/unavailable data.

Table III. Associations between chemotherapy resistance and clinicopathological characteristics.

	Platinum resistance		
Variable	Present	Absent	P-value
Age, years			0.806[a]
≤54	13	49	
>54	11	37	
FIGO stage			0.065[b]
I-II	0	12	
III-IV	24	74	
Histological grade			0.498[b]
G2	4	10	
G3	19	73	
Null	1	3	
CA-125, U/ml			0.710[b]
≤ 500	5	21	
> 500	19	65	
Lymph node metastasis			0.106[a]
No	7	41	
Yes	17	45	
Residual tumor size			0.030[a]
≤1 cm	13	66	
>1 cm	11	20	
MBD2 expression			0.001[b]
Low	2	39	
High	22	47	

Statistical analysis was performed with a [a]χ^2 test or [b]Fisher's exact test. The information of some individuals was missing or incomplete, therefore the total number is inconsistent. FIGO, International Federation of Gynaecology and Obstetrics; MBD2 methyl-CpG binding domain protein 2; CA-125, cancer antigen-125; Null, missing/unavailable data.

Associations between platinum resistance and clinico-pathological characteristics of patients with HGSOC. It was demonstrated that postoperative residual tumor size (P=0.030) and MBD2 expression (P=0.001) were significantly associated with platinum resistance. However, platinum resistance was not associated with age, FIGO stage, histological grade, CA-125 or lymph node metastasis (P>0.05; Table III). Multivariate logistic regression analysis confirmed that >1 cm of residual disease and high expression of MBD2 were significantly associated with platinum resistance (P<0.05; Table IV).

Prognostic value of MBD2 expression in ovarian cancer. Kaplan-Meier univariate analysis demonstrated that MBD2 expression, FIGO stage, CA-125, lymph node metastasis and residual tumor size were significantly associated with relapse-free survival (RFS) in patients with HGSOC (P<0.05; Table V). Multivariate Cox regression analysis demonstrated that FIGO stage, CA-125, lymph node metastasis, residual tumor size and MBD2 expression were significantly associated with RFS (P<0.05; Table VI). Therefore, late FIGO, CA-125

eight normal ovarian tissues. The results demonstrated that the expression of MBD2 was significantly higher in HGSOC tissues compared with that in normal tissues (Fig. 3).

Table IV. Multivariate logistic analysis of the association between chemotherapy resistance and clinicopathological characteristics.

Variable	β	SE	Exp(β)	95% CI	P-value
Residual tumor (>1 cm)	-1.028	0.515	0.358	0.130-0.981	0.046[a]
MBD2 expression (High)	-2.212	0.777	0.109	0.024-0.502	0.004[b]

[a]P<0.05, [b]P<0.01. MBD2 methyl-CpG binding domain protein 2; SE, standard error; CI, confidence interval.
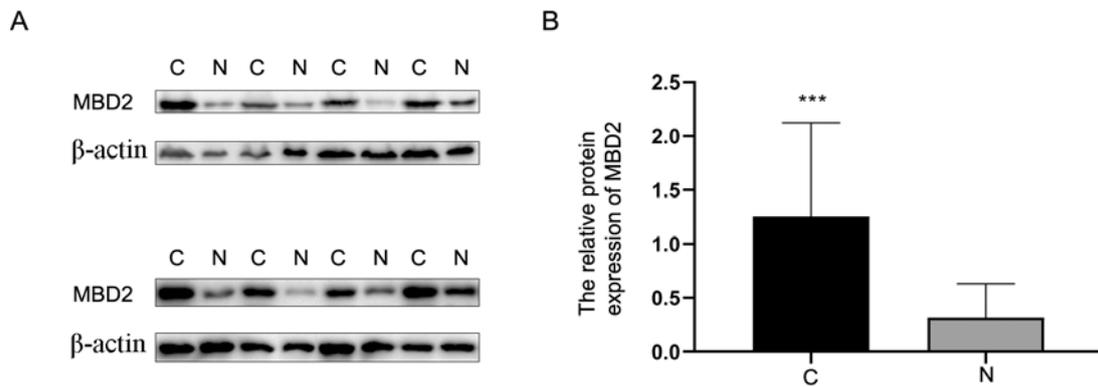

Figure 3. Detection of MBD2 using western blotting. (A) Representative western blots and (B) analysis demonstrating that MBD2 expression was higher in HGSOC tissues compared with that in normal tissues. ***P<0.001. MBD2, methyl-CpG binding domain protein 2; C, ovarian cancer tissue; N, normal ovarian tissue.

>500, lymph node metastasis, >1 cm of postoperative residual disease and high expression of MBD2 were identified as risk factors for recurrence of HGSOC.

Discussion

MBD2 is closely associated with the hypermethylation of CpG islands in some cancer types, such as breast and prostate cancer (9,30). And it has been reported to function as a tumor activator (31,32). Knockout of MBD2 upregulates the expression of tumor suppressor genes, such as p16 and p14 (33,34). MBD2 overexpression has been detected in a variety of solid tumors, including HCC, breast cancer, GBM and chronic myeloid leukemia (16-18,35). MBD2 promotes breast cancer progression by mediating tumor suppressor gene silencing (22,36). Zhu *et al* (16) reported that MBD2 overexpression leads to silencing of cerebral angiogenesis inhibitor 1 to promote the growth of GBM. Similar mechanisms have been reported in other cancers, such as HCC and gastrointestinal stromal tumors (17,37). However, MBD2 functions in a cell- and tissue-specific manner, and recent studies demonstrated that MBD2 has antitumor effects (21,38). MBD2 is downregulated in lung adenocarcinoma, and low expression of MBD2 is associated with poor prognosis (38). *In vitro* experiments further demonstrated that MBD2 inhibits metastasis (38). The results of the present study demonstrated that MBD2 was expressed at high levels in HGSOC, although the underlying mechanism remains unclear.

Approximately 20-30% of patients with ovarian cancer develop platinum resistance during chemotherapy (39). In the current study, 21.8% of patients developed platinum resistance, which is consistent with a recent study (39). Huang *et al* (40) performed a transcriptome analysis of platinum-sensitive and drug-resistant ovarian cancer cell lines and found differences in the expression of MBD2 between the two populations. Yu *et al* (41) used methyl-capture sequencing to precipitate methylated DNA from the recombinant methyl CpG binding domain of MBD2 for next generation sequencing. The results demonstrated that drug-resistant cells had lower levels of CpG methylation. The results of the present study revealed that MBD2 upregulation was related to platinum resistance in HGSOC, although the underlying mechanism needs to be further studied.

The results of the present study suggested that the recurrence of HGSOC was associated with numerous factors, such as late FIGO stage, lymph node metastasis and high expression of MBD2. The postoperative recurrence of epithelial ovarian cancer is related to clinical stage (42). The results of the present study demonstrated that postoperative recurrence of HGSOC was significantly correlated with advanced FIGO stage, which was consistent with a previous report (43). A higher tumor stage was associated with larger tumors, which may lead to local invasion. Surgical excision in these cases is difficult and may lead to injury, which increases the potential for cancer cells to invade blood vessels and lymphoid tissues, increases the depth of infiltration and promotes tumor metastasis (44,45). The present study also demonstrated that lymph node metastasis was a risk factor for recurrence of HGSOC, which was consistent with a previous study (46). One possible reason for this finding is that lymph node metastasis

Table V. Univariate survival analysis of relapse-free survival of patients with ovarian cancer.

Variable	n	Mean ± SE	95% CI	P-value
Age, years				0.978[b]
≤54	62	21.760±3.049	15.783-27.737	
>54	48	24.375±3.607	17.306-31.444	
FIGO stage				<0.001[a]
I-II	12	67.917±9.288	49.712-86.121	
III-IV	98	17.991±1.859	14.347-21.635	
Histological grade				0.488[a]
G2	14	20.643±5.808	9.260-32.026	
G3	92	24.140±2.725	18.799-29.480	
Null	4			
CA-125, U/ml				0.022[a]
500	26	32.138±5.746	20.877-43.400	
>500	84	19.698±2.306	15.179-24.217	
Lymph node metastasis				<0.001[a]
No	48	33.089±4.547	24.177-42.001	
Yes	62	15.414±1.888	11.713-19.115	
Residual tumor size, cm				0.002[a]
≤1	79	27.309±3.163	21.109-33.508	
>1	31	13.645±2.664	8.423-18.867	
MBD2 expression				0.011[b]
Low	41	28.786±4.221	20.514-37.059	
High	69	18.662±2.408	13.942-23.381	

Analysis was performed with a [a]log-rank test or [b]Breslow test. The information of some individuals was missing or incomplete, therefore the total number is inconsistent. MBD2 methyl-CpG binding domain protein 2; CA-125, cancer antigen-125; SE, standard error; CI, confidence interval; Null, missing/unavailable data.

Table VI. Multivariate survival analysis of relapse-free survival of patients with ovarian cancer.

Variable	β	SE	HR	95% CI	P-value[a]
FIGO stage (III-IV)	1.786	0.618	5.965	1.776-20.041	0.004
CA-125 (>500)	0.678	0.281	1.971	1.135-3.421	0.016
Lymph node metastasis (Yes)	0.450	0.227	1.568	1.004-2.448	0.048
Residual tumor size (>1 cm)	0.477	0.234	1.610	1.017-2.549	0.042
MBD2 expression (High)	0.525	0.222	1.691	1.093-2.615	0.018

[a]According to Cox regression analysis. FIGO, International Federation of Gynaecology and Obstetrics; MBD2 methyl-CpG binding domain protein 2; CA-125, cancer antigen-125; SE, standard error; CI, confidence interval; HR, hazard ratio.

is the most common type of ovarian cancer metastasis, which also increases the risk of recurrence (47,48). The results of the present study demonstrated that a postoperative residual tumor size ≤1 cm prevented postoperative recurrence, which was consistent with previous reports (49,50). Therefore, maximum radical resection of the tumor and reduction of residual cancer tissue are essential to improve the prognosis of HGSOC. In addition, the expression level of MBD2 in HCC is higher compared with that in normal tissues (51). MBD2 is an independent prognostic factor that affects the overall survival

and disease-free survival of patients and is considered to be a potential clinical prognostic marker (17). Notably, the current study also identified MBD2 as an independent prognostic factor for relapse-free survival of HGSOC.

To the best of our knowledge, the present study is the first to demonstrate that high expression levels of MBD2 are associated with platinum resistance and poor prognosis in patients with HGSOC. The results suggested that MBD2 is a promising target for cancer treatment. Sequence-specific anti-sense MBD2 inhibitors suppress the anchorage-independent

growth of human tumor cell lines *in vitro* and the growth of human tumor xenografts *in vivo* (52). The newly discovered MBD2 inhibitor KCC-08 combined with the retinoic acid receptor agonist isoretinoic acid can significantly reduce the growth and survival of cancer cells (53). However, the present study had some limitations, such as a small sample size and the nature of the study as a single-center study. In conclusion, patients with HGSOC with high MBD2 expression levels are more likely to develop platinum resistance and have a poor prognosis; thus MBD2 is a potential biomarker for HGSOC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WG wrote the paper and performed the experiments. MN and ZC participated in the data collection and analysis. ZZ conceived and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Zhejiang Cancer Hospital (approval no. IRB-2015-175) and written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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