Promoter Methylation Down-regulates Osteoprotegerin Expression in Ovarian Carcinoma

JI-YE KIM^{1,2}, SE HOON KIM¹ and HYUN-SOO KIM³

¹Department of Pathology, Severance Hospital, Yonsei University College of Medicine, Seoul, Republic of Korea;

²Department of Pathology, Ilsan Paik Hospital, Inje University College of Medicine, Goyang, Republic of Korea;

³Department of Pathology and Translational Genomics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea

Abstract. *Background/Aim:* Previous studies have documented that osteoprotegerin (OPG) is involved in the development and progression of several human malignancies. However, OPG has also been shown to act as a tumor suppressor. The aim of this study was to examine the expression status of OPG in ovarian carcinoma cells and investigate the underlying mechanism responsible for alterations in OPG expression. *Materials and Methods:* The expression levels of OPG mRNA and protein were assessed in human ovarian carcinoma cell lines. The methylation status of the OPG promoter region was determined using the bisulfite pyrosequencing technique. The effects of demethylation on OPG expression were also analyzed. *Results:* The human ovarian carcinoma cell lines, SW 626, OVCAR-3, ES-2, TOV-112D, and TOV-21G, expressed significantly lower levels of OPG mRNA and protein than the normal human ovarian epithelial cell line, HS823.Tc. Moreover, three CpG sites in the OPG promoter region were highly methylated in the SW 626, OVCAR-3, ES-2, and TOV-112D ovarian carcinoma cell lines compared to normal control cells. Furthermore, in all the examined ovarian carcinoma cell lines, treatment with the demethylating agent, 5-aza-2-deoxycytidine, resulted in significantly increased expression levels of OPG mRNA and protein compared to the respective pre-treatment levels. *Conclusion:* OPG expression was down-regulated in the studied ovarian carcinoma cells compared to the normal control cells, while demethylation significantly restored OPG expression in the OPG-down-

regulated cell lines. Our results suggest that OPG down-regulation in ovarian carcinoma occurs, at least partly, through epigenetic repression, suggesting its involvement in ovarian carcinogenesis.

Ovarian carcinoma accounts for more than 90% of primary ovarian malignancies. It has the highest mortality rate of all gynecological tumors (1). Its high mortality is due to the lack of early detection methods and the high risk of recurrence. Most ovarian carcinoma cases are diagnosed at advanced stages, resulting in a five-year survival rate of approximately 47% (2, 3). Moreover, the existing treatment of patients with ovarian carcinoma is limited to aggressive debulking surgery and postoperative adjuvant chemotherapy (1). Thereby, a thorough understanding of the alterations in gene expression that occur during ovarian carcinogenesis may aid in efforts to improve its diagnosis and treatment. A diagnostic or prognostic biomarker for ovarian carcinoma is urgently needed to guide the treatment of these patients.

Osteoprotegerin (OPG) is a secreted protein that belongs to tumor necrosis factor (TNF) receptor superfamily. In human tissues, a transcript of similar size is detected at highest levels in the lung, heart, kidney, and placenta, although there are detectable levels in various hematopoietic and immune organs (4). OPG acts as a decoy receptor for receptor activator of nuclear factor- κ B ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL) (5). OPG has many biological functions; it was first described as a potent inhibitor of osteoclastic bone resorption (4). In addition to its role in bone metabolism, OPG is involved in the development, progression, and metastasis of several human malignancies (6-14). We have previously reported that OPG expression is down-regulated in primary colorectal carcinoma cells and tissues and that reduced OPG expression is significantly associated with aggressive oncogenic behavior and poor prognosis for colorectal carcinoma (11, 15). We have also demonstrated that the *OPG* gene promoter is highly methylated in colorectal carcinoma cell lines and that

Correspondence to: Dr. Hyun-Soo Kim, Department of Pathology and Translational Genomics, Samsung Medical Center, Sungkyunkwan University School of Medicine, 81, Irwon-ro, Gangnam-gu, Seoul 06351, Republic of Korea. Tel: +82 234101243, Fax: +82 234100025, e-mail: hyun-soo.kim@samsung.com

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treatment with a demethylating agent significantly elevates *OPG* mRNA and protein expression (11).

There are a few previous studies that reported a possible association between *OPG* and apoptosis in ovarian carcinoma cells (5, 16). These studies documented that *OPG* attenuates TRAIL-induced apoptosis; however, its role in ovarian carcinogenesis is yet to be elucidated. Moreover, the expression of *OPG* and its precise regulatory mechanisms in ovarian carcinoma remain unclear. In this study, we investigated the expression levels of *OPG* mRNA and protein in ovarian carcinoma cells, as well as the methylation status of the *OPG* promoter region. Moreover, the effects of demethylation on *OPG* expression were analyzed. Our results indicated that promoter methylation is one of the mechanisms that contribute to the down-regulation of *OPG* expression in ovarian carcinoma.

Materials and Methods

Cell culture and treatment. The Hs823.Tc human normal ovarian cell line as well as the SW 626, OVCAR-3, ES-2, TOV-112D, and TOV-21G ovarian carcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium or Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) (all from Gibco, Life Technologies, Grand Island, NY, USA). All cell lines were treated as previously described (1, 11, 17-19). The cells were cultured at 37°C in a humidified atmosphere of 5% carbon dioxide. The demethylating agent 5-aza-2-deoxycytidine (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate-buffered saline to a concentration of 50 mg/ml as a stock solution and stored at -20°C until use. Immediately before use, stock solutions were diluted in Roswell Park Memorial Institute 1640 medium without FBS. The cells were seeded in 6-well plates at a density of 5×10⁵ cells/ml in the media and treated with 5-aza-2-deoxycytidine (final concentration, 1 µM). The 5-aza-2-deoxycytidine was replaced with freshly prepared solution every 24 h, and the cells were harvested 96 h after the initial treatment. Control cultures were treated under similar experimental conditions in the absence of 5-aza-2-deoxycytidine (phosphate-buffered saline only).

Complementary DNA synthesis. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. RNase-free DNase I (Thermo Fisher Scientific, Waltham, MA, USA) treatment was carried out to remove contaminating genomic DNA from the purified total RNA obtained from the cell lines. Isolated total RNA was diluted to 1 mg/ml with sterile diethylpyrocarbonate-treated water, and 2.5 ml was added to reactions containing 1× DNase I buffer and 1 U DNase I (final volume, 10 ml). After incubation at 37°C for 30 min, the reactions were stopped by incubation at 70°C for 10 min. DNase I-treated RNA was reverse-transcribed into first-strand cDNA using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). One microgram of DNase I-treated RNA and 250 ng of random primers were mixed in a 0.5-ml PCR tube and brought to 11 ml with sterile diethylpyrocarbonate-treated water, heated at

65°C for 5 min, and chilled quickly on ice. Other reagents were added to the 20-ml reaction volume at the following final concentrations: 1× First-Strand Buffer, 10 mM dithiothreitol, 0.5 mM each dNTP, and 200 U Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Reactions were incubated at 42°C for 1 h and heated to 70°C for 10 min, and the products were stored at -20°C. The amount of cDNA was determined using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). The cDNA was subjected to qRT-PCR analysis using the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). PCR was carried out in a 20-µl reaction containing 0.5 µM each primer, 1× Thunderbird SYBR qPCR Mix (Toyobo), and 2 µl of template DNA. PCR for *OPG* was performed on a C1000 Thermal Cycler (Bio-Rad Laboratories) using the following reaction protocol: polymerase activation at 95°C for 1 min, followed by 40 cycles at 95°C for 10 s for denaturation, 58°C for 10 s for annealing, and 72°C for 20 s for extension. Amplification patterns were analyzed and threshold cycle numbers (Ct) for each sample were determined using CFX Manager Software (Bio-Rad Laboratories). The primer sequences used for *OPG* were as follows: forward, 5'-TGC TGT TCC TAC AAA GTT TA-3', and reverse, 5'-CTT GAG TGC TTT AGT GCG T-3'. The $\Delta\Delta C_t$ method was used to calculate relative *OPG* expression after normalization to the expression of β -actin (20). Amplification of *OPG* was confirmed by melting-curve analysis, and target amplicon size (430 bp) by agarose gel electrophoresis (Thermo Fisher Scientific). Each sample was assayed in triplicate.

Enzyme-linked immunosorbent assay (ELISA). For analysis of *OPG* protein production, culture media were harvested and centrifuged to remove cellular debris. Media were concentrated by centrifugal filtration at 4,000 rpm for 20 min using the Amicon Ultra-10K concentrator (EMD Millipore, Burlington, MA, USA). A commercially available ELISA kit for Osteoprotegerin (Cloud-Clone Corporation, Katy, TX, USA) was used according to the protocols provided by the manufacturer to measure the concentrations of human *OPG*. Each sample was assayed in triplicate.

Genomic DNA extraction and bisulfite conversion. Genomic DNA was extracted from cultured cells using the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany) and quantified using a DropSense96 multichannel spectrophotometer (Trinean, Gentbrugge, Belgium). Bisulfite treatment was performed using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) on 2 µg of DNA according to the protocols provided by the manufacturer.

Pyrosequencing. DNA methylation of *OPG* was determined by bisulfite pyrosequencing. Primers were designed using Pyrosequencing Assay Design Software, Version 1.0.6 (Qiagen, Valencia, CA, USA). To ensure distinct pyrosequencing signals, the previously bisulfite-modified DNA (100 ng) was amplified by 45 cycles of PCR using the PyroMark PCR Kit (Qiagen) with a biotin-labeled primer according to the protocols provided by the manufacturer. The primer sequences used were as follows: forward, 5'-GGG TTT TGT AAT TTG AGG TTT TAG AA-3', and reverse, 5'-biotin-ACT TAT ATC TCC TCC ACC CTA AA-3'. PCR products were immobilized to Streptavidin Sepharose High-Performance (GE Healthcare, Chicago, IL, USA) beads *via* biotin affinity. PCR products were denatured to single-

Figure 1. Expression of osteoprotegerin (OPG) in ovarian carcinoma and normal ovarian cell lines and effect of demethylation on OPG. (A) OPG protein expression levels measured by enzyme-linked immunosorbent assay. * $p < 0.05$ versus Hs823.Tc cells (normal control). (B) Normalized OPG mRNA expression ratios measured by quantitative real-time reverse-transcriptase polymerase chain reaction. * $p < 0.05$ versus Hs823.Tc cells (normal control). (C) OPG promoter methylation status analyzed by bisulfite pyrosequencing. * $p < 0.05$ versus Hs823.Tc cells (normal control). (D) Effect of 5-aza-2-deoxycytidine treatment on OPG mRNA expression. * $p < 0.05$ versus pre-treatment level. (E) Effect of 5-aza-2-deoxycytidine treatment on OPG protein expression. * $p < 0.05$ versus pre-treatment level. Error bars represent the standard deviations.

stranded DNA and annealed with sequencing primers (5'-GAT AAA GGT TTG GGA TAT ATT-3'). Complexes of bead-bound single-stranded DNA and primer were applied to a PyroMark Q96 ID pyrosequencer (Qiagen) with PyroMark Gold Q96 Reagents (Qiagen).

Raw results were analyzed and visualized using Pyro Q-CpG Software, Version 1.0.0 (Qiagen). Pyrosequencing was performed for three independent bisulfite reactions, and the average methylation frequency for each CpG site was calculated.

Table I. *Osteoprotegerin (OPG) mRNA and protein expression levels in ovarian carcinoma and control normal ovarian cell lines.*

Cell line	Category	OPG mRNA expression		OPG protein level	
		Normalized ratio	p-Value	Mean±Standard deviation	p-Value
Hs823.Tc	Normal	1.000		66.510±0.008	
SW 626	Carcinoma	0.054	<0.001*	5.301±0.010	<0.001*
OVCAR-3	Carcinoma	0.012	<0.001*	0.932±0.029	<0.001*
ES-2	Carcinoma	0.002	<0.001*	0.283±0.014	<0.001*
TOV-112D	Carcinoma	0.003	<0.001*	0.230±0.008	<0.001*
TOV-21G	Carcinoma	0.020	<0.001*	0.472±0.003	<0.001*

*Statistically significant compared to control Hs823.Tc cells.

Table II. *Osteoprotegerin (OPG) promoter methylation status of three CpG sites in ovarian carcinoma and control normal ovarian cell lines.*

Cell line	Category	OPG promoter methylation status (%)					
		-186 CpG	p-Value	-182 CpG	p-Value	-166 CpG	p-Value
Hs823.Tc	Normal	1.44		1.72		1.11	
SW 626	Carcinoma	4.09	0.022*	4.10	0.029*	13.65	<0.001*
OVCAR-3	Carcinoma	40.83	<0.001*	51.61	<0.001*	47.40	<0.001*
ES-2	Carcinoma	4.86	0.017*	8.92	<0.001*	18.42	<0.001*
TOV-112D	Carcinoma	3.53	0.036*	5.95	<0.001*	7.37	<0.001*
TOV-21G	Carcinoma	1.59	0.211	3.54	0.077	3.95	0.045*

*Statistically significant compared to control Hs823.Tc cells.

Statistical analysis. Data are expressed as mean±standard deviation of three independent experiments, each performed in triplicate, and are presented relative to control. Wilcoxon rank sum test was used to compare the promoter methylation frequency and the expression levels of *OPG* mRNA and protein between the normal ovarian cell line and ovarian carcinoma cell lines. We used the Wilcoxon signed-rank test to compare the expression levels of *OPG* mRNA and protein before and after 5-aza-2-deoxycytidine treatment in each ovarian carcinoma cell line examined. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 20 (IBM Corporation, Armonk, NY, USA). A *p*-value <0.05 was considered statistically significant.

Results

***OPG* expression in ovarian carcinoma cell lines.** The ELISA results revealed that *OPG* protein expression was significantly reduced in SW 626 (5.301±0.010 ng/ml; *p*<0.001), OVCAR-3 (0.932±0.029 ng/ml; *p*<0.001), ES-2 (0.283±0.014 ng/ml; *p*<0.001), TOV-112D (0.230±0.008 ng/ml; *p*<0.001), and TOV-21G (0.472±0.003 ng/ml; *p*<0.001) cells compared to Hs823.Tc cells (66.510±0.008 ng/ml; Figure 1A). Consistent with these results, *OPG* mRNA expression was significantly lower in SW 626 (normalized mRNA expression ratio, 0.054; *p*<0.001), OVCAR-3 (0.012; *p*<0.001), ES-2 (0.002; *p*<0.001), TOV-112D (0.003; *p*<0.001), and TOV-21G (0.020;

p<0.001) cells compared to the control Hs823.Tc cells (1.000; Figure 1B). Table I summarizes the expression levels of *OPG* mRNA and protein in the examined ovarian carcinoma cells.

Mechanisms contributing to the down-regulation of OPG expression in ovarian carcinoma cell lines. We have previously observed that down-regulation of *OPG* expression is associated with promoter hypermethylation in colorectal carcinoma cells (11). Based on this observation, we hypothesized that promoter hypermethylation might explain the reduced *OPG* expression in ovarian carcinoma cells. We analyzed *OPG* promoter methylation status at three CpG sites (-186 CpG, -182 CpG, and -166 CpG) in the *OPG* promoter region using a pyrosequencing technique. When compared to that in Hs823.Tc cells, *OPG* promoter methylation was significantly increased in SW 62, OVCAR-3, ES-2, and TOV-112D cells (Figure 1C), in all the examined CpG sites, indicating that promoter hypermethylation may be a possible mechanism for the reduced *OPG* expression in these cell lines. Table II summarizes the *OPG* promoter methylation status and its differences between normal and carcinoma cell lines.

To further investigate the role of DNA methylation in the regulation of *OPG* expression, the ovarian carcinoma cell lines were treated with the demethylating agent 5-aza-2-

deoxycytidine. Treatment resulted in significant restorations of *OPG* mRNA expression, with a 13.419-fold increase in SW 626 cells ($p<0.001$), a 4.609-fold increase in OVCAR-3 cells ($p<0.001$), a 3.187-fold increase in ES-2 cells ($p=0.002$), and a 9.980-fold increase in TOV-112D cells ($p<0.001$) compared to the respective pre-treatment levels (Figure 1D). The restorative effect of 5-aza-2-deoxycytidine on *OPG* expression in these cell lines was also confirmed by ELISA (Figure 1E). When compared to the respective pre-treatment *OPG* protein levels, the post-treatment levels were significantly elevated in SW 626 (6.183 ± 0.096 ng/ml to 9.921 ± 0.033 ng/ml; $p<0.001$), OVCAR-3 (0.570 ± 0.001 ng/ml to 0.982 ± 0.002 ng/ml; $p<0.001$), ES-2 (0.184 ± 0.016 ng/ml to 0.348 ± 0.006 ng/ml; $p<0.001$), and TOV-112D (0.101 ± 0.032 ng/ml to 0.252 ± 0.029 ng/ml; $p=0.003$) cells (Figure 1E). These findings suggest that promoter methylation affects *OPG* expression in ovarian carcinoma cell lines.

Discussion

In this study, we analyzed the expression of *OPG* mRNA and protein in human ovarian carcinoma cells. We observed that all the examined ovarian carcinoma cell lines exhibited significantly lower *OPG* mRNA expression levels than the normal ovarian cell line. Consistent with this result, *OPG* protein expression was significantly reduced in ovarian carcinoma cell lines. Our observations indicate that *OPG* expression is down-regulated in ovarian carcinoma, suggesting that it may serve as a potential diagnostic biomarker for ovarian carcinoma.

We also investigated the regulatory mechanisms for *OPG* expression. A possible mechanism by which *OPG* is down-regulated in ovarian carcinoma cells is inhibition of gene expression by promoter hypermethylation. First, increased promoter methylation status was confirmed in SW626, OVCAR-3, ES-2, and TOV-112D cells *via* *OPG* promoter region (CpG site) bisulfite pyrosequencing. Then, treatment with a demethylating agent significantly restored *OPG* mRNA and protein expression in all examined ovarian carcinoma cell lines, which had very low pre-treatment mRNA and protein levels. These findings suggest that promoter hypermethylation plays a critical role in *OPG* down-regulation in ovarian carcinoma cells. Our results are in agreement with our previous *in vitro* studies on colorectal carcinoma (11), in which *OPG* mRNA and protein levels were significantly reduced among colorectal carcinoma cells compared to normal colonic epithelial cells. *OPG* down-regulation in colorectal carcinoma was found to be a result of hypermethylation in the promoter region. Similar to our findings, Lu *et al.* also demonstrated that nasopharyngeal (NPC-TW04) and colorectal (HCT 116) carcinoma cell lines were highly methylated at the upstream or downstream of

OPG transcription start site compared to normal nasal epithelial cells (12). Moreover, they observed a uniform reduction in *OPG* expression levels across a wide spectrum of human malignancies such as carcinomas of the oral cavity, nasopharynx, cervix, lungs, breast, pancreas, kidneys, prostate, and liver (12). Furthermore, Delgado *et al.* revealed that in the HEK-293 human embryonic kidney cell line, CpG islands of the *OPG* promoter were hypermethylated, resulting in a significant decrease in *OPG* mRNA expression levels (21). They also documented that treatment with a demethylating agent promoted a 20-fold induction of *OPG* mRNA expression in HEK-293 cells. Taken together, these results support our findings that promoter hypermethylation is a likely mechanism by which *OPG* is down-regulated.

However, our observations of *OPG* down-regulation conflict with previous reports in other malignancies showing that *OPG* exerts tumor-promoting effects. Analysis of the Cancer Genome Atlas (<https://cancergenome.nih.gov/>) dataset for lung carcinoma revealed a significant increase in *OPG* expression in lung carcinoma tissues compared to that in normal lung tissues (22). Prostate carcinoma cells were found to produce *OPG* *in vitro* and protect themselves against TRAIL-induced apoptosis (10). In the same context, an *in vivo* study demonstrated that *OPG* administration significantly decreased the growth of prostate carcinoma xenografts in nude mice (23). Furthermore, in osteosarcoma and lung carcinoma cell lines *OPG* overexpression was associated with aggressive oncogenic behavior such as increased cellular proliferation, migration, and invasion (22, 24). In contrast, studies on breast carcinoma using publicly available microarray data showed that *OPG* expression was associated with a better prognosis for patients with estrogen receptor-positive breast carcinoma (25). We have previously demonstrated that reduced *OPG* expression is significantly associated with aggressive oncogenic behavior in primary colorectal carcinoma and that *OPG* expression is an independent predictor of recurrent hepatic metastasis and independent prognostic factor for worse survival rates (11, 15). These conflicting data regarding the role of *OPG* may be related to the differences in organs and cell types, as well as the inadequacy of using a single model to explain the complex process of carcinogenesis.

In conclusion, we demonstrated that *OPG* expression was significantly down-regulated in ovarian carcinoma cells compared to normal ovarian cells, suggesting that it may serve as a potential diagnostic biomarker for ovarian carcinoma. Moreover, the three studied CpG sites of the *OPG* promoter were highly methylated, and treatment with a demethylating agent significantly restored *OPG* expression in all the examined ovarian carcinoma cell lines. Our data strongly suggest that methylation-dependent

mechanisms influence the transcription of the *OPG* gene. This study is the first to explore the expression status of OPG in ovarian carcinoma cell lines and to show that promoter hypermethylation is one of the mechanisms involved in the regulation of OPG expression. Further studies on the role of OPG in ovarian carcinoma could reveal important evidence regarding its potential use as a therapeutic target.

Authors' Contributions

All Authors were responsible for substantial contributions to the conception and design of the study, acquisition, analysis, and interpretation of the data, as well as drafting the manuscript, revising the manuscript critically for important intellectual content, and providing final approval of the version to be published.

Conflicts of Interest

None of the Authors have any conflicts of interest to declare regarding this study.

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