Immunohistochemical expression of CD44v9 and 8-OHdG in ovarian endometrioma and the benign endometriotic lesions adjacent to clear cell carcinoma.

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Running title: CD44v9 and malignant transformation of benign OE

Abstract

Aim: Expression of CD44 variant isoforms (CD44v) promotes the synthesis of reduced glutathione and contributes to reactive oxygen species (ROS) defense through up-regulation of the intracellular antioxidant. The aim of the study was to investigate the expression of CD44v9 and oxidative DNA damage marker, 8-OHdG, in benign ovarian endometrioma (OE) and OE harboring clear cell carcinomas (CCC).

Methods: A retrospective study was performed at the Department of Gynecology, Nara Medical University hospital from January 2006 to December 2012. Patients with histologically confirmed benign OE (n = 27) and OE harboring areas of CCC (n = 8) were selected. Tissue samples were immunohistochemically analyzed for the presence of CD44v9 and 8-OHdG using avidin-biotin complex method.

Results: CD44v9 was located on the cell membrane of endometriotic epithelial cells and expressed in 88.9% (24/27) of benign OE tissues. Only 25.0% (2/8) of benign endometriotic lesions adjacent to CCC was found to stain weakly for CD44v9. Percentage of CD44v9 positive cells was 68.5 ± 20.2% (mean ± SD) of benign OE, 16.7 ± 16.5% of CCC endometriotic tissue ($p<0.001$). Compared to benign OE, CCC endometriotic tissue showed a significant increase in the proportion of 8-OHdG expression (77.3 ± 22.5% vs. 94.9 ± 3.0%, $p = 0.049$). A significant negative correlation was observed between CD44v9 status and 8-OHdG nuclear expression ($r = -0.458$, $p = 0.006$).

Conclusion: Alterations in CD44v9 and 8-OHdG may be associated with malignant transformation of benign OE.

Keywords: Endometriosis; Clear cell carcinoma of the ovary; CD44v9; 8-OHdG; Immunohistochemistry.

Introduction

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterus, most often in the pelvic peritoneum or ovaries [1]. This disorder is an estrogen-dependent benign gynecological disease of reproductive age. Endometriosis is linked to pelvic pain and infertility [2]. Ovarian endometrioma (OE) presents an increased risk of endometriosis-associated ovarian cancer (EAOC), predominantly in endometrioid and clear cell carcinomas [3,4]. Unopposed exposure to estrogen may be an important step toward understanding its potential implications for endometrioid carcinoma of the ovary [4]. Ovarian endometrioma also plays a role in the development of clear cell carcinoma (CCC) with time. In Japan, the majority (~80%) of EAOC had CCC with early stage tumors [5]. A pathological evaluation of specimens of EAOC demonstrated the transformation process from benign endometriosis through atypical endometriosis to ovarian cancer in a stepwise manner. Thus, benign OE is regarded as being potentially malignant and associated with increased risk of ovarian cancer [2,3,5,6]. The progressive accumulation of acquired epigenetic and genetic alterations was identified along the endometriosis-carcinoma sequence [7]. Tumor originates and progresses at the site of chronic inflammation and oxidative stress that create procarcinogenic and proangiogenic tumor microenvironment by inducing DNA damage and repair [6]. In recent years, evidence has emerged that, in endometriosis, hemoglobin, heme and iron overload resulting from repeated episodes of hemorrhage may create disruption of the homeostatic redox circuits and oxidative stress, which promotes DNA mutations or induces DNA damage and genome instability, possibly through autoxidation and Fenton chemistry [6,8]. The accumulation of DNA damage and (epi)genetic defects may emerge based on profound changes of microenvironment. Redox-active iron could induce oxidative stress, resulting in the development of CCC of the ovary [9].

8-hydroxy-2'-deoxyguanosine (8-OHdG) is a potential biomarker of DNA oxidative damage for various diseases, including atherosclerosis, diabetes, smoking and cancers [10]. 8-OHdG might have a high probability of harmful effects in some cells, but it also acts as a defense mechanism against oxidative stress in other cells. For example, the expression of nuclear 8-OHdG were downregulated in EAOC tumorous tissue compared with OE and EAOC endometriotic tissue [11]. However, limited information is still available about the expression of 8-OHdG protein between OE and benign endometriotic lesions adjacent to the clear cell carcinoma (CCC endometriotic tissues).

CD44 is the major cell surface receptor for hyaluronan and becomes a valid cancer stem cell marker. This molecule exerts potential protective effect against ROS-mediated DNA damage [12]. Expression of CD44, especially variant isoforms (CD44v), contributes to ROS defense through upregulation of the synthesis of reduced glutathione (GSH), the primary intracellular antioxidant [12]. CD44v directly binds to and stabilizes xCT, a subunit of a glutamate-cystine transporter, that results in accumulation of antioxidants and protection against oxidative stress-related pathologies [13]. Aberrant alternative splicing, CD44v, is very common in cancer and associated with malignant transformation, survival in the ROS-generated worse environment, aggressiveness and metastasis [13,14]. Among splice variant isoforms, CD44v9 exhibited

ovarian cancer-specific expression patterns, but not normal ovarian tissues [15]. These variant forms are also involved in the development of endometriotic lesion [16]. Mechanisms involved in oxidative stress together with CD44v9 expression in CCC endometriotic tissues have not been investigated so far.

Here we focused on the expression of the antioxidant marker, CD44v9, and the influence of oxidative DNA damage marker, 8-OHdG, levels in OE and the benign endometriotic lesions adjacent to CCC.

Methods

Tissue samples

The samples consisted of surgically resected tissues from hospitalized patients. No patients underwent hormonal therapy or chemotherapy for six months prior to the surgery. A total of 35 formalin-fixed, paraffin-embedded tissue specimens, including 27 cases of benign OE and 8 cases of endometriosis-associated CCC, were retrospectively collected from Department of Gynecology at Nara Medical University hospital, Japan, between January 2006 and December 2012. Endometriotic tissue compartment was targeted as relevant regions of interest for immunohistochemical analysis in relation to the endometriotic epithelial cells of benign OE and endometriotic lesions adjacent to CCC tumorous tissue (CCC endometriotic tissues).

CCC endometriotic tissues were selected from the resected ovary of patients with CCC, which includes endometriosis appearing epithelial cells adjacent to the primary tumor site.

Endometriosis was present adjacent to the CCC. The data of patient demographic features and clinicopathologic characteristics were collected from a database containing comprehensive medical records and pathology reports. The protocols were approved by The Ethics Committee of Nara Medical University (reference number: 2012-541). Written informed consent was obtained from all patients.

Immunohistochemistry

Three paraffin sections of serial 4 μm thickness were taken from each original block, one section was stained with hematoxylin and eosin for diagnostic confirmation and the other two sections were immunostained for CD44v9 and 8-OHdG by immunoenzyme polymer methods and conventional methods, respectively, using an avidin biotin complex immunoperoxidase technique. Slides were deparaffinized and rehydrated, incubate in 3% H_2O_2 in methanol to block endogenous peroxidase. Antigen retrieval was performed by autoclaving at 120°C for 10 min in 0.01M sodium citrate buffer (pH 6.0). They were blocked with serum-free protein block solution (Dako #X0909) for ten minutes and then probed with primary antibody CD44v9 (given by professor Dr. Saya H belonging to Division of Gene Regulation, Institute for Advanced Medical Research, School of Medicine, Keio University, Tokyo, Japan) at a dilution of 1:12500 for one hour, followed by the EnVision$^+$ Kits (DAKO #K400611-2) for 30 minutes, and the DAB chromogen (Dako, #K346711-2) for ten minutes. Furthermore, other slides were probed with primary

antibody 8-OHdG (abcam, ab48508) at a dilution of 1:200 for one hour, followed by the biotinylated rabbit anti-mouse IgG (Nichirei Corporation #424021-(2)) for 10 minutes, then by the peroxidase-labeled streptavidin (Nichirei Corporation #424021-(3)) for 7 minutes, and the DAB chromogen (Nichirei Corporation #415171) for ten minutes. The primary antibodies were replaced with PBS for negative control experiments. The slides were counterstained with hematoxylin, and mounted under coverslips with permount.

Protein expression was evaluation by 2 independent observers (KS and CY). Five high powder fields were chosen for each sample and 1000 epithelial cells were evaluated. To determine the expression of the proteins in a section, the specimens were scored according to the staining intensity and the percentage of positive cells using a semi-quantitative scoring approach. Since most of the staining intensity were moderate brown particles, the intensity of staining was divided into no staining (a score of 0) and staining (a score of 1), respectively. In addition, the percentage of positive cells in each sample was defined as the ratio of positively stained endometriotic epithelial cells to all endometriotic epithelial cells. The percentage of positive cells was scored at four levels: 0 for <5%, 1 for 5–30%, 2 for 31–60%, and 3 for ≥61% [17]. The results were calculated in all the patients and were expressed as the mean ± standard deviation (SD). These two values (the staining intensity and percentage of positive cells) were combined to decide the expression of proteins in each group (the score <2 represents negative and ≥ 2 represents positive).

Statistical analysis

Numerical variables, including age, were presented as the mean ± SD. Variables that did not present normal distribution were expressed by median, minimum and maximum values. Differences between the groups of patients were estimated by Mann-Whitney U test. Categorical data were analyzed by the Chi-square test or the Fisher's 2-tailed test. The percentage of CD44v9 and 8-OHdG positive cells presented normal distribution, and correlation between CD44v9 and 8-OHdG was evaluated by Pearson correlation coefficient. Analyses were performed by SPSS version 21.0 (IBM SPSS, Armonk, NY, USA). Two-sided $P<0.05$ was considered to indicate a statistically significant difference.

Results

Study population

The study population consisted of 27 patients with benign OE and 8 patients with CCC endometriotic tissues. The clinicopathological characteristics of the two groups of patients are shown in **Table 1**. The patient age ($p = 0.001$), premenopausal status ($p = 0.011$) and a maximum diameter of the cyst ($p = 0.017$) differed significantly between patients with EC and CCC. Parity and serum CA125 levels did not differ between the two groups.

CD44v9 and 8-OHdG protein expression

Immunohistochemical analysis of CD44v9 and 8-OHdG expression was shown in **Figure 1**. CD44v9 was expressed on the cell membrane in endometriotic epithelial cells. Endometriotic stromal cells were negative for CD44v9 staining. 8-OHdG was restricted to the nucleus. 8-OHdG was identified in endometriotic epithelium and stroma, and its expression was greater in epithelial cells than in stromal cells. The staining was partially positive in endometriotic stromal cells. Therefore, only cell membrane and nuclear staining in endometriotic epithelial cells were evaluated.

The results of CD44v9 and 8-OHdG expression in two groups were summarized in **Table 2**. Of the 27 OE specimens, 24 had CD44v9 expression, with a positive rate of 88.9% (24/27), while the positive rate of CD44v9 was 25.0% (2/8) in CCC endometriotic tissue specimens. Cell membrane CD44v9 expression was markedly downregulated in CCC endometriotic tissues. The mean (± SD) percentage of CD44v9 positive cells in CCC endometriotic tissues was significantly lower than that in benign EC (68.5 ± 20.2% versus 16.7 ± 16.5%, $p < 0.001$).

8-OHdG immunohistochemistry was also available in 35 cases. Nuclear 8-OHdG positive staining was present in 26 EC patients (26/27, 96.3%) and 100% (8/8) of CCC endometriotic tissues ($P = 0.771$), which was usually moderate staining. However, the percentage of the 8-OHdG-positive endometriotic epithelial cells was significantly higher in the CCC group than in the OE group (94.9 ± 3.0% versus 77.3 ± 22.5%, $P = 0.049$).

We analyzed the correlation of the percentage (%) of the positive CD44v9 expression with that of the positive 8-OHdG expression in the two groups. A significant negative correlation between CD44v9 and 8-OHdG expression was identified, with a correlation coefficient of −0.458 ($P = 0.006$) (**Figure 2**)

Discussion

We performed immunohistochemical staining of CD44v9 and 8-OHdG in paraffin-embedded tissues of OE and the benign endometriotic lesions adjacent to CCC. A high level of 8-OHdG protein expression was found in endometriotic epithelial cells of two groups, while the CD44v9 immunoreactivity was significantly lower in CCC endometriotic tissues compared to benign OE. Our data suggest that the downregulation of CD44v9 and the upregulation of 8-OHdG in OE lesions may provide an excess oxidative stress and future risk of malignant step.

Oxidative stress is considered to be an important trigger for endometriosis carcinogenesis [18]. Repeated episodes of hemorrhage occur in endometriosis at the onset of menstruation. Extracellular hemoglobin, heme, and iron species in endometriotic cyst cause DNA damage and mutations, which create increased cellular susceptibility to oxidant-mediated cell killing or carcinogenesis [6,8,18,19]. Not only endometriosis but also genetic hemochromatosis, chronic viral hepatitis and asbestosis induce iron overload and have been associated with iron-induced carcinogenesis [20]. 8-OHdG is an established marker of

degree of DNA oxidative damage. Cyst fluid hemoglobin and iron overload may be associated with the upregulation of 8-OHdG in OE lesions [8,19]. Our data are consistent with previous studies reporting that oxidative stress is upregulated in endometriosis-associated CCC carcinogenesis [18]. The co-regulation and fine-tuning of oxidative stress and antioxidants may be more predictive of endometriosis-carcinoma sequence progression [19]. CD44v9 may play a critical role as a suppressor of CCC carcinogenesis in benign OE. Antioxidant strategy may be viewed as an approach to prevent malignant transformation of endometriosis.

This study has limitations. Firstly, the scope of the present study was limited to endometriotic epithelial cells in benign OE and benign endometriotic lesions next to clear cell carcinoma. In order to investigate the role of CD44v9 and 8-OHdG in CCC carcinogenesis, we focused our attention to the DNA oxidative damage and anti-oxidant properties in CCC endometriotic tissues. Actually, we attempted to compare the expression of these proteins among OE groups, CCC endometriotic tissues and CCC tumor tissues. And more prominent difference was observed between OE and CCC endometriotic tissues than OE and CCC tumor tissues (Table 3). Moreover, our data showed that the expression of 8-OHdG increased in EAOC endometriotic tissue than OE groups. This result is not contrary to the previous report that cyst fluid hemoglobin and iron overload are more severe in OE than EAOC [8,19].

Secondly, the number of samples was relatively small. In the 8 cases with CCC endometriotic tissues, a direct continuous transition was noted from clearly benign endometriosis through atypical endometriosis to CCC. The limitation can be addressed in the future with validation incorporating a larger sample size of CCC with morphologic documentation of the continuous transition. Finally, we found insufficient evidence to confirm whether results are more or less applicable for various subgroups of EAOC. The study enrolled patients with CCC; therefore, results may not be applicable to patients with endometriosis-associated endometrioid carcinoma. We must exemplify whether the CD44v9 protein expression has been downregulated, potentially due to its primary regulation by epigenetic or genetic mechanisms. Notwithstanding these limitations, reduced expression of CD44v9 may be associated with malignant transformation of benign OE.

In conclusion, present observations demonstrated that downregulation of CD44v9 and upregulation of 8-OHdG may be associated with tumor microenvironment of endometriosis-associated CCC carcinogenesis.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number JP16K11150, Takeda Science Foundation, Tohoku Bureau of Economy, Trade and Industry and Konica Minolta Science and Technology Foundation in 2017

Disclosure

None declared.

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Figure 1

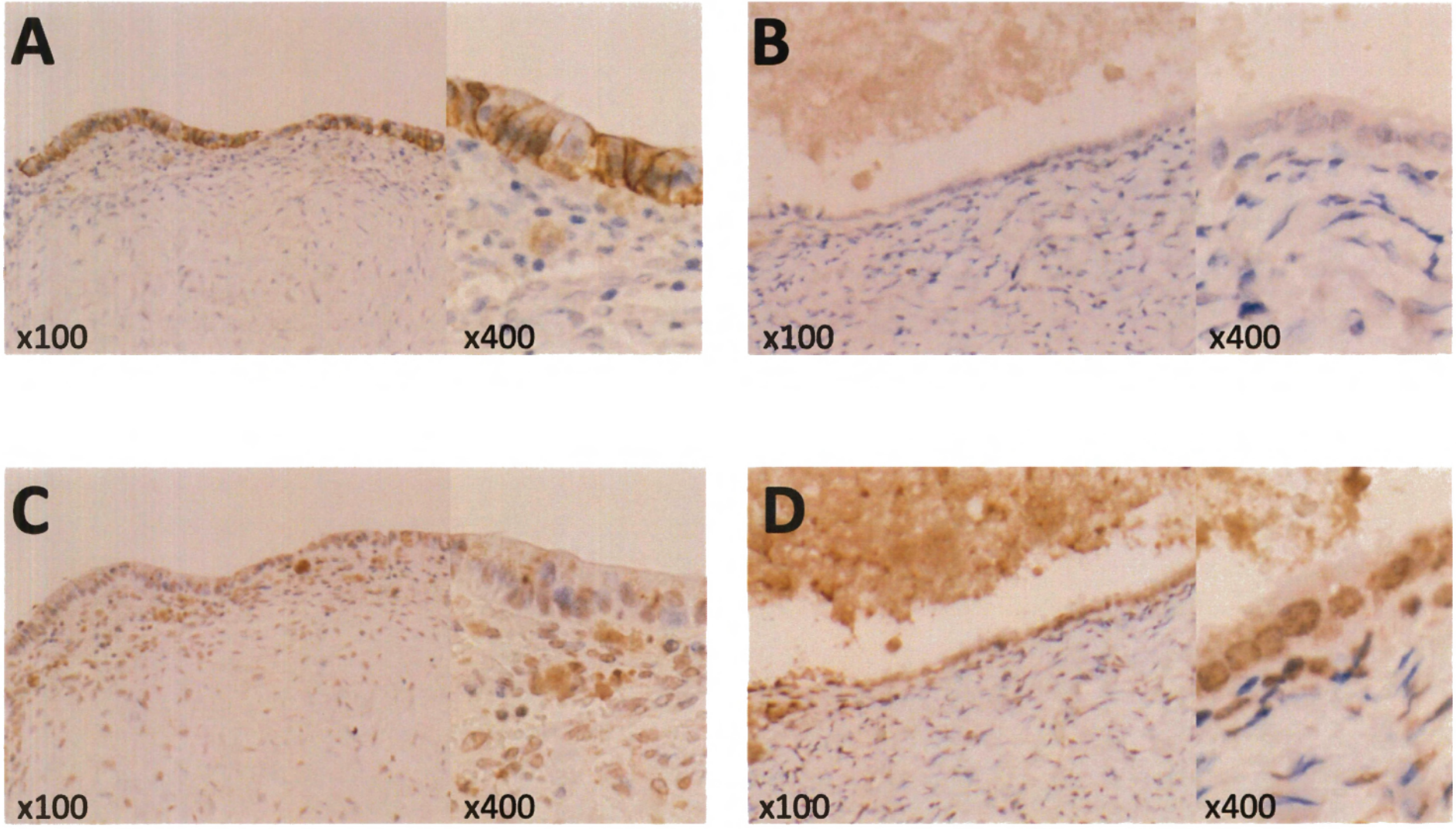
CD44v9 and 8-OHdG immunoreactions in benign OE and CCC endometriotic tissues. Original magnification × 100 and × 400. A and C, benign OE. B and D, CCC endometriotic tissue. A and B, CD44v9 immunostaining. C and D, 8-OHdG immunostaining.

CD44v9; CD44 variant 9, 8-OHdG; 8-hydroxy-2'-deoxyguanosine, OE; ovarian endometrioma, CCC; clear cell carcinoma.

Figure 2

A significant negative correlation between CD44v9 expression and 8-OHdG expressions ($r=-0.458$, $P=0.006$). $y = -0.331x + 99.997$. x, the positivity of CD44v9 expression; y, the positivity of 8-OHdG expression. Open circle represents an individual value of benign OE. Closed circle represents an individual value of CCC endometriotic epithelial cells.

CD44v9; CD44 variant 9, 8-OHdG; 8-hydroxy-2'-deoxyguanosine, OE; ovarian endometrioma, CCC; clear cell carcinoma.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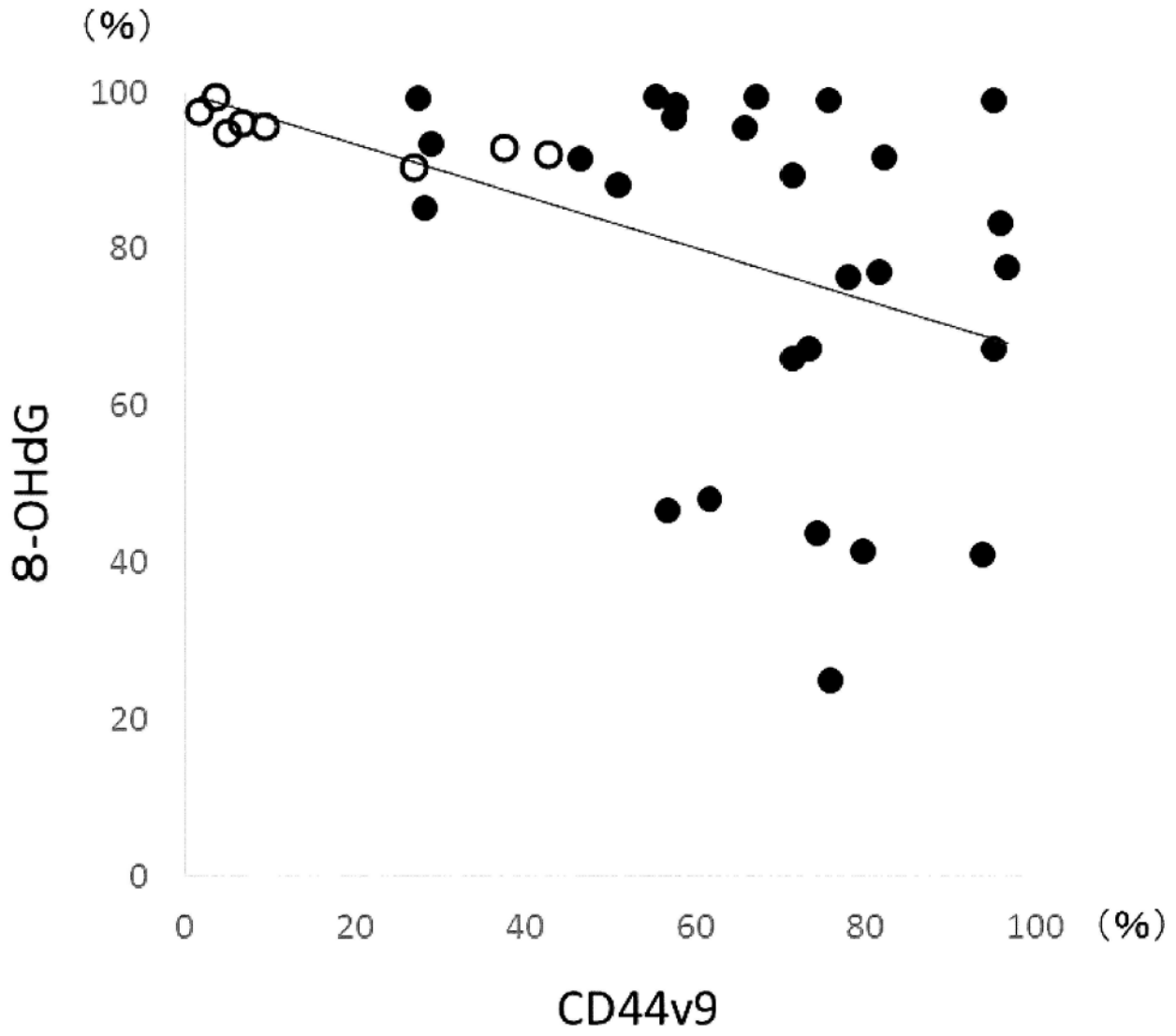
(%)
100
80
60
40
20
0
8-OHdG
0
20
40
60
80
100
(%)
CD44v9

Table 1 Demographic and clinical characteristics of the study population.

Baseline characteristics of two groups	The OE group	The CCC group	P-value
Number	27	8	
FIGO stage	-	I (n=5), II (n=1), III (n=1), IV (n=1)	
Age at diagnosis,			
mean ± SD	40.6±10.3	57.0±10.1	0.001
median (range)	42(20 - 68)	55.5(47 - 75)	
Nulliparous			
n (%)	15 (55.6%)	2 (25.0%)	0.112
Premenopause			
n (%)	21 (77.8%)	2 (25.0%)	0.011
A maximum diameter of the cyst (cm)			
mean ± SD	6.9 ± 3.9	12.7 ± 5.5	0.017
median (range)	6.3 (1.2 – 21.0)	13.0 (4.7 – 20.0)	
CA125(U/ml)			
mean ± SD	112.4 ± 161.4	113.4 ± 156.5	0.983
median (range)	40 (6 - 577)	36.0 (7 - 465)	

The patient age ($p = 0.001$), premenopausal status ($p = 0.011$) and a maximum diameter of the cyst ($p = 0.017$) differed significantly between patients with EC and CCC.
FIGO, International Federation of Gynecology and Obstetrics; EC, endometrial cyst; CCC, clear cell carcinoma.

Table 2 The results of CD44v9 and 8-OHdG expression in two groups.

The results of CD44v9 and 8-OHdG expression	The OE group	The CCC group	P-value
Number	27	8	
The percentage of CD44v9 expression	68.5 ± 20.2%	16.7 ± 16.5%	<0.001
CD44v9 expression			
Positive	24	2	0.001
Negative	3	6	
The percentage of 8-OHdG expression	77.3 ± 22.5%	94.9 ± 3.0%	0.049
8-OHdG expression			
Positive	26	8	0.771
Negative	1	0	

Cell membrane CD44v9 expression was markedly downregulated in CCC endometriotic tissues. The mean (± SD) percentage of CD44v9 positive cells in CCC endometriotic tissues was significantly lower than that in benign EC (68.5 ± 20.2% versus 16.7 ± 16.5%, $p < 0.001$).
CD44v9, CD44 variant 9; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; CCC, clear cell carcinoma; EC, endometrial cyst.

Table 3 The relation of CD44v9 and 8-OHdG expression in three groups.

The results of CD44v9 and 8-OHdG expression	The OE group	The CCC		p-value
		Endometriotic tissues	Tumor tissues	
Number	27	8	8	
The percentage of CD44v9 expression	68.5 ± 20.2%	16.7 ± 16.5%	56.9 ± 32.8%	<0.001
CD44v9 expression				
Positive	24	2	6	
Negative	3	6	2	
The percentage of 8-OHdG expression	77.3 ± 22.5%	94.9 ± 3.0%	88.9 ± 14.6%	0.103
8-OHdG expression				
Positive	26	8	8	
Negative	1	0	0	

Remarkable difference in CD44v9 expression was observed among OE groups, CCC endometriotic tissues and CCC tumor tissues. These data are shown in mean (± SD).
CD44v9, CD44 variant 9; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; CCC, clear cell carcinoma; EC, endometrial cyst.