

Cyst fluid hemoglobin species in endometriosis and its malignant transformation: The role of metallobiology

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Abstract. The aim of the present study was to assess the relative concentrations of hemoglobin (Hb) species in endometriosis as a possible indicator of malignancy. Electronic absorption spectroscopy was employed to quantify the Hb species present in the cyst fluid collected from 8 patients with endometriosis-associated ovarian cancer (EAOC), and compared with those present in the cyst fluid of 35 patients with benign endometriotic cysts. The 620/580 nm ratio in the electronic absorption spectrum, which was used as a surrogate indicator of the methemoglobin (metHb)/(oxyhemoglobin+metHb) ratio, was measured in each cyst fluid by ultraviolet/visible grating spectrophotometric microplate reader. The optimal cutoff value was defined according to the analysis of receiver operating characteristic (ROC) curve. The sensitivity and specificity of detection were calculated on the basis of the cutoff value to differentiate EAOC from endometriosis. The 620/580 nm ratio of cyst fluid in EAOC patients was much lower than that measured in women with benign cysts (0.389 ± 0.266 vs. 0.666 ± 0.188 , $P=0.021$). ROC curve analysis performed using 0.35 as the optimal cutoff value indicated that the 620/580 nm ratio had a sensitivity, specificity, positive predictive value (PPV) and negative predictive value of 62.5, 100.0, 100.0 and 92.1%, respectively, in the diagnosis of EAOC. In conclusion, metHb is one of the most abundant Hb species in benign cysts, and the absorption 620/580 nm ratio of cyst fluid exhibits high specificity and PPV as a surveillance test for the early detection of malignant transformation of endometriosis. Thus, metallobiology highlights diverse features involved in Hb homeostasis and the pathogenesis of malignant transformation of endometriosis.

Introduction

Endometriosis is a hormone-dependent benign disease that affects ~10% of women during reproductive age (1). Retrograde flow of menstrual blood may lead to endometriosis (2). Individuals with endometriosis require a long follow-up, with particular attention paid not only to dysmenorrhea and infertility but also to potential malignant transformation (3). In Japan, <1% of women with endometriosis develop ovarian cancer (3-6). Substantial histological evidence indicates that endometriosis is associated with ovarian cancer, particularly endometrioid carcinoma and clear cell carcinoma (5). Although the pathogenesis of carcinogenesis remains unclear, it is assumed that malignant transformation may be derived from pathways associated with hyperestrogenism, chronic inflammation and oxidative stress (7). Inflammation and particularly oxidative stress, which is secondary to the influx of erythrocytes and subsequent hemolysis that occurs during retrograde menstruation and inner hemorrhage in endometriotic lesions, may be one of the causative factors in carcinogenesis (4). Hemoglobin (Hb), heme and iron may have a significant impact on oxidative stress in the development of endometriosis and malignant transformation (8). Therefore, metallobiology studies on Hb-mediated oxidative damage and antioxidant response are required.

The two most common redox states exhibited by iron are ferrous (Fe^{2+} , reduced) and ferric (Fe^{3+} , oxidized) (8). In methemoglobin (metHb), the iron in the heme moiety of Hb is oxidized from the ferrous (Fe^{2+}) to the ferric state (Fe^{3+}), while oxyhemoglobin (oxyHb) contains iron in the ferrous state (Fe^{2+}) (8). In Hb, iron is predominately in the ferrous state, and participates in redox chemistry producing free radicals (9). It has been previously reported that excess of Hb, heme and iron may be harmful to cells and may lead to cell death (10). Furthermore, sublethal oxidative stress may be linked with carcinogenic processes rather than cell death (11,12). However, no data are currently available regarding the concentrations of oxyHb and metHb present in the Hb species of endometriotic cyst fluid.

Therefore, the purpose of the present study was to investigate the proportion of metHb in benign cysts and endometriosis-associated ovarian cancer (EAOC), and to demonstrate the efficacy of this biomarker in the differential diagnosis of EAOC vs. benign cysts.

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Materials and methods

Study population. The present study was approved by the Ethics Committee of Nara Medical University (Kashihara, Japan). A prospective single-center cohort study was performed in accordance with the Declaration of Helsinki (13). All patients signed written consent forms to participate in the present study. Patients were recruited from the Nara Medical University Hospital (Kashihara, Japan). From December 2012 to July 2013, 43 women underwent operation, 35 of whom received a diagnosis of benign endometriotic cysts, while 8 received a diagnosis of EAO. Cyst fluid samples were collected at the time of surgery. Specimens were immediately aliquoted and frozen at -80°C within 1 h of collection. Clinicopathologic data obtained from patients' medical records included age at the time of surgery, tumor size, stage and pathology. None of the patients had been consuming antioxidant vitamin supplements for ≥ 2 months.

Measurement of the electronic absorption spectra in cyst fluid. The electronic absorption spectra of the common and different species of Hb in cyst fluid were investigated in EAO and benign cysts. Frozen-thawed cyst fluid was diluted with phosphate-buffered saline (Takara Bio, Inc., Otsu, Japan) and added to a 96-well spectrophotometric plate (Cosmo Bio Co., Ltd., Tokyo, Japan). The electronic absorption spectra of the samples were measured with a grating spectrophotometric microplate reader (SH-1200; Corona Corporation, Sanjo, Japan). Previous studies demonstrated that a peak at 620 nm in the spectrum indicates specific absorption of metHb, whereas fairly sharp peaks located at 545 and 580 nm represent the absorption spectrum of a mixture of oxyHb and metHb (14-17).

There was marked light scattering in the samples, as evidenced by the non-zero absorbance at 700 nm. As a simple approach, the baseline was adopted using linear regression based on a linear function (Fig. 1). The absorption bands between wavelengths 605-655 nm and between wavelengths 565-605 nm were selected. The absorbance values at 620 and 580 nm were followed as surrogate markers of the relative concentrations of the Hb species present in the samples, and the ratio of the absorption measured at 620 nm divided by that measured at 580 nm was calculated as a predictive marker of the metHb/(oxyHb+metHb) ratio present in the cyst fluids.

Statistical analysis. Data are presented as the mean \pm standard deviation. Differences between the groups of patients were estimated by Mann-Whitney U test. 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 clinicopathological characteristics of the two groups of patients are shown in Table I. Since there were differences in age and tumor size, the clinical characteristics were not balanced. The median age in the benign cysts and EAO groups were 40.0 years (range, 21.0-55.0 years) and 49.5 years (range, 38.0-53.0 years), respectively ($P = 0.012$). The mean cyst size was 6.7 cm (range, 2.7-19.3 cm) in case

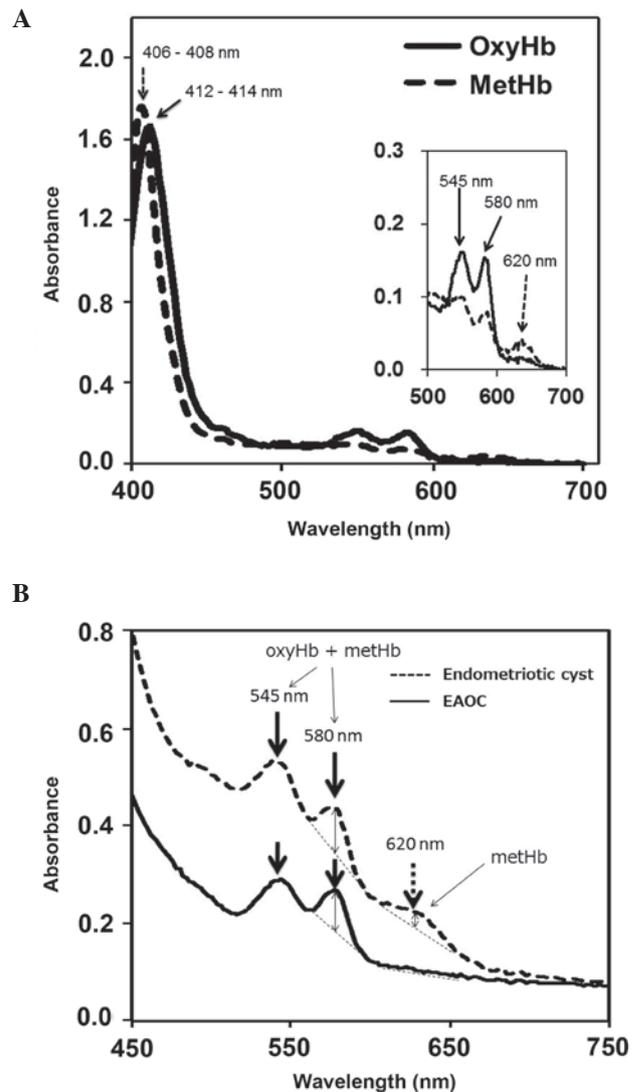


Figure 1. Electronic absorption spectra. (A) Electronic absorption spectra of purified oxyHb and metHb in phosphate-buffered saline, which were used as standards. Bold line, 30 mg/ml oxyHb; dotted line, 30 mg/ml metHb. The electronic absorption spectra of the different Hb species were represented as a function of the wavelength at the visible/near-infrared region of the electromagnetic spectrum, between 400 and 700 nm. The insert shows the region of the higher power image on the electromagnetic spectrum between 500-700 nm. (B) Electronic absorption spectra of the common and variable species of Hb present in the cyst fluid of patients with endometriotic cysts and EAO. The spectrum shown is a representative image of the independent experiments conducted to evaluate the absorption spectra of cyst fluids. Bold line, cyst fluid sample obtained from EAO patients; dotted line, cyst fluid sample obtained from patients with endometriotic cysts. The electronic absorption spectra of the different Hb species were represented as a function of the wavelength at the visible/near-infrared region of the electromagnetic spectrum, between 450 and 750 nm. MetHb exhibits absorption peaks at ~ 620 , 545 and 580 nm, while oxyHb exhibits absorption peaks at ~ 545 and 580 nm. oxyHb, oxyhemoglobin; metHb, methemoglobin; EAO, endometriosis-associated ovarian cancer; Hb, hemoglobin.

of benign cysts and 10.6 cm (range, 4.4-14.3 cm) in case of EAO; thus, the size of the primary tumor was significantly larger in the EAO than in the benign cyst group ($P = 0.003$).

Absorption spectra of hemoglobin species in cyst fluids derived from endometriotic cysts and EAO. As represented in Fig. 1A, purified oxyHb solution exhibited double

Table I. Demographic and clinical characteristics of the study population.

Characteristics	Endometriotic cysts	EAOC	P-value
Number	35	8	-
Median age (range), years	40.0 (21.0-55.0)	49.5 (38.0-53.0)	0.012
Mean tumor size (range), cm	6.7 (2.7-19.3)	10.6 (4.4-14.3)	0.003
FIGO stage			
Ia	-	1	-
Ib	-	1	-
Ic	-	6	-
Pathology			
Endometriosis	35		
Clear cell adenocarcinoma	-	5	-
Endometrioid adenocarcinoma	-	2	-
Mucinous adenocarcinoma	-	1	-

EAOC endometriosis-associated ovarian cancer, FIGO, Federation of Gynecology and Obstetrics (18).

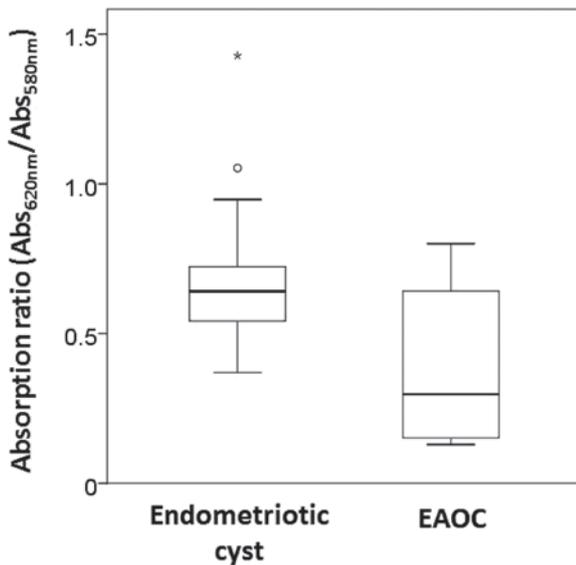


Figure 2. Comparison of the absorption ratio at 620 vs. 580 nm of cyst fluids derived from 35 patients with benign endometriotic cysts and 8 patients with endometriosis-associated ovarian cancer. *P=0.021. EAOC, endometriosis-associated ovarian cancer; Abs, absorption.

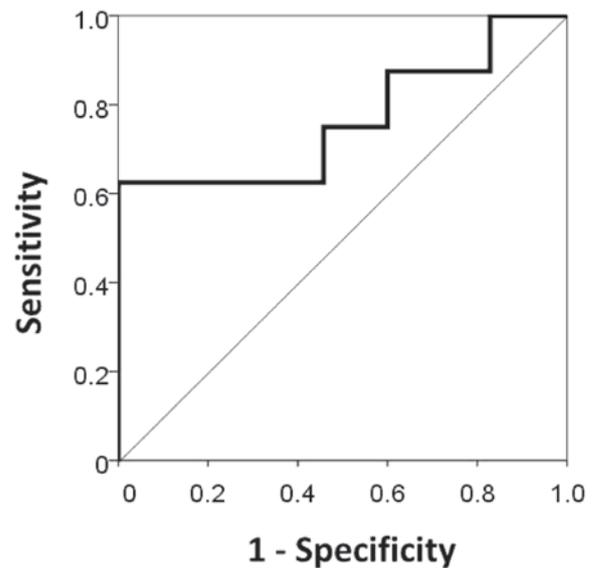


Figure 3. Receiver operating characteristic curve analysis of the absorption ratio at 620/580 nm of cyst fluid samples derived from individuals with benign endometriosis and patients with endometriosis-associated ovarian cancer.

sharp peaks at 545 and 580 nm in the electronic absorption spectrum, while purified metHb produced three absorption spectral peaks at 545, 580 and 620 nm, respectively. The intense peak at 620 nm appears to be composed of metHb, while the peaks at 545 and 580 nm are probably the result of a mixture of oxyHb and metHb. Fig. 1B represents the typical absorption spectra of Hb species in cyst fluids from endometriotic cysts and EAOC in the spectral range of 450-750 nm. The experimental data demonstrated good reproducibility, with the electronic absorption spectra exhibiting prominent peaks at ~545, 580 and 620 nm in benign cysts, and 545 and 580 nm in EAOC. At 545 and 580 nm, the absorption spectra were not obviously different between benign cysts and EAOC. Notably, the present results revealed a consistent difference

in the region of 620 nm between the absorption spectrum of benign cysts and that of EAOC. These differences in the absorption spectra may have been caused by differences in the degree of metHb formation between benign cysts and EAOC.

The relative concentrations of Hb species in cyst fluid was assessed as a possible indicator of malignancy in endometriosis. For that purpose, quantification of the 620/580 nm ratio in the absorption spectra was used as a surrogate marker of the metHb/(oxyHb+metHb) ratio present in the sample. Fig 2 contains box and whisker plots representing the median levels and interquartile range (box) of the absorption at 620 nm/absorption at 580 nm ratio for each of the groups studied. The 620/580 nm absorption ratio was significantly

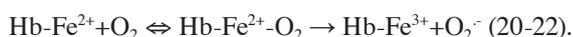
lower in the cyst fluid of EAOc individuals than in that of patients with benign cysts (0.389 ± 0.266 vs. 0.666 ± 0.188 , $P=0.021$).

Receiver operating characteristic (ROC) curve analysis for prediction of optimal cutoff values. The results of ROC curve analysis based on the detection of 8 EAOc patients and 35 women with benign endometriosis are shown in Fig. 3. The optimal cutoff value was determined by analyzing the ROC curve among EAOc and endometriosis. When the cutoff value was set to 0.35, the sensitivity, specificity, positive predictive value and negative predictive value of the 620/580 nm absorption ratio in the diagnosis of EAOc vs. benign endometriosis were 62.5, 100.0, 100.0 and 92.1%, respectively.

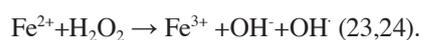
Discussion

In the present study, the absorption ratio at 620/580 nm was assessed to quantify the relative concentrations of Hb species present in cyst fluid collected from patients with EAOc vs. those present in cyst fluid collected from benign cysts, as a possible indicator of malignancy in endometriosis. To the best of our knowledge, the present study reports for the first time that the 620/580 nm absorption ratio was altered in cyst fluid of individuals with EAOc, compared with those with benign cysts. The relative amount of oxyHb and metHb released into the cyst fluid was spectrophotometrically measured. Patients with endometriosis exhibited higher levels of metHb in their cyst fluid, compared with EAOc patients. This indicated that the 620/580 nm ratio was a significant discriminator for malignant transformation in women with endometriosis, and the optimal cutoff point to predict malignant transformation was 0.35.

Hb may be converted to metHb in the endometriotic cyst fluid solution. When erythrocytes are lysed in endometriotic cysts, Hb, heme and iron are released from lysed red blood cells into the cyst fluid space (19). Harmful reactive oxygen species (ROS) such as O_2^- are produced during Hb autoxidation of oxyHb-Fe²⁺ to metHb-Fe³⁺, as follows:



Furthermore, generation of free oxygen radicals (OH) responsible for cellular destruction by the ferrous state of iron has been documented in Haber-Weiss-Fenton chemistry:



Therefore, in endometriotic cyst fluid, a burst of ROS (including O_2^- and OH) would be generated via Hb autoxidation and Haber-Weiss-Fenton reaction. These data suggest that the high levels of metHb observed in patients with endometriosis may be due to excess free radical formation. Excess ROS may oxidize lipids, proteins and DNA, and subsequently induce apoptosis and cell death (25,26).

Notably, significantly lower levels of metHb were present in the cyst fluid of EAOc patients, compared with those present in the cyst fluid of patients with benign cysts. This finding suggests the presence of potential endogenous antioxidant compounds, with a reduction of the total oxidant

status. Reductase systems may catalyze the reduction of ferric iron Fe³⁺ to the ferrous Fe²⁺ state (22), suggesting a role for antioxidant response in the pathogenesis of EAOc. Antioxidant activity may be upregulated to address the redox imbalance in a subset of endometriotic cells, which may be exposed to sublethal levels of ROS and able to adapt to cell survival (27-29). The present data support the idea that excess free radicals may lead to cell damage that culminates in cell death, whereas persistent exposure to sublethal ROS with an increased antioxidant state increases the tumorigenic potential of endometriotic cells (24,30).

Limitations of the present study include the lack of a significant number of patients and the generalizability of the observed results. In addition, the pH may affect the features of the metHb spectra (31), but this was not evaluated in the present study. Furthermore, we hypothesize that other absorbing species are likely to be present in cyst fluid, including hemichromes, and possibly ferrylhemoglobin, sulfhemoglobin or deoxyhemoglobin (deoxyHb), although the levels of each of these species may be relatively low. Also, the absorbance of the samples at each wavelength is the result of the sum of the products of the extinction coefficients of each of the species present in the sample, multiplied by the concentration of those species (8). Therefore, the ratio of the absorption measured at 620 nm divided by that measured at 580 nm does not accurately report the metHb/(oxyHb+metHb) ratio. A more accurate method to determine the concentration of Hb species using absorption spectroscopy would be to collect data using an integrating sphere detector, so that scattered light is collected and true absorbance could be obtained. Thus, the present findings warrant further studies on the utilization of an integrating sphere detector. Finally, the method reported in the present study is not a serum-based test; thus, it may not exhibit the characteristics of the reliable, fast, low-cost, efficient bedside tests and non-invasive techniques that are clinically required for the assessment of the metHb/(oxyHb+metHb) ratio in cyst fluid. A number of studies have used broadband diffuse optical spectroscopy and frequency-domain near-infrared spectroscopy (NIRS) to assess tissue concentrations of metHb, oxyHb and deoxyHb (32,33). Lee *et al.* (33) reported noninvasive, quantitative *in vivo* measurements of metHb formation using broadband diffuse optical spectroscopy (33). NIRS may provide new avenues for future determination of the metHb/(oxyHb+metHb) ratio through transvaginal application of this modality. We hypothesize that these techniques would offer the potential for bedside measurement of Hb and hemodynamics.

Despite the aforementioned limitations, the results of the present metallobiology study are discussed in association with the current understanding of the pathogenesis of malignant transformation of endometriosis. Thus, if the value of the 620/580 nm ratio in the absorption spectrum of cyst fluid derived from patients with endometriosis is <0.35, it may be considered a possible marker to accurately identify patients with endometriosis at greatest risk of malignant transformation. A reduced 620/580 nm absorption ratio may aid to identify patients who require immediate clinical evaluation for cancer surveillance and surgical attention.

In conclusion, the present study examined the diagnostic utility of the absorption ratio at 620/580 nm of cyst fluid in

endometriosis and its malignant transformation. Further studies with larger patient groups are required to identify an optimal cutoff value of this ratio for accurate diagnosis of EAOC.

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