Research article

Cyst fluid iron-related compounds as useful markers to distinguish malignant transformation from benign endometriotic cysts.

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Running title: Iron predicts malignant transformation of endometriosis

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Abstract

OBJECTIVE: The purpose of this study was to investigate cyst fluid levels of total iron, heme iron and free iron in benign endometriotic cysts and endometriosis-associated ovarian cancer (EAOC) and to demonstrate the significance of these biomarkers in differential diagnosis between EAOC and endometriotic cysts.

METHODS: Cyst fluid samples were obtained from eleven patients with EAOC and thirty-six women with benign endometriotic cysts at the time of surgery.

RESULTS: The median (± SD) total iron levels for endometriotic cysts and EAOC cysts were 244.4 ± 204.9 mg/L and 14.2 ± 36.6 mg/L, respectively. EAOC patients had much lower levels of iron-related compounds compared with endometriotic cyst samples ($p<0,001$). When the total iron results were analyzed using the receiver operating characteristics (ROC) curve method, the optimum diagnostic cut-off point was 64.8 mg/L, sensitivity was 90.9%, specificity was 100%, positive predictive value (PPV) was 100%, and negative predictive value (NPV) was 97.3%. Patient demographic characteristics such as tumor size, age at operation, parity and menopause were not correlated with cyst fluid iron levels.

CONCLUSIONS: We conclude for the first time that iron-related compounds are important biomarkers that can predict malignant transformation with high sensitivity and specificity for women with endometriosis.

Key words: Endometriosis; Malignant transformation; Iron; Cyst fluid.

Introduction

Endometriosis is the most common gynecological disease. A prospective cohort study in Japan supported the hypothesis that ovarian endometrioma increases the subsequent risk of developing ovarian cancer (8). Kobayashi et al. reported that the incidence of ovarian cancer in women with endometriotic cysts was 0.72% during follow-up of up to 17 years (8). Thus, this disorder is occasionally accompanied by malignant neoplasms (1). Women with long-standing endometriosis have an increased risk for developing EAOC, especially more than 10 years after the initial diagnosis of endometriosis (8,16).

It has been reported that an iron-rich environment in the endometriotic cysts may play a crucial role in carcinogenesis, indicating that the malignant transformation of endometriosis might be due to iron-induced oxidative stress derived from the repetition of hemorrhage (6,16). In the present study, iron-related compounds, including total iron, heme iron and free iron, were chosen based on previously reported studies (16). Hemoglobin is a heterotetramer protein composed of two alpha and two beta globin chains, which bind a heme group that provides the binding site for molecular oxygen. Heme is a ferrous iron (Fe^{2+})-protoporphyrin IX complex. Cell-free hemoglobin after hemolysis can oxidize oxyhemoglobin (oxyHb Fe^{2+}; ferrous) to methemoglobin (metHb Fe^{3+}; ferric) and free heme or iron, which can generate reactive oxygen species (ROS) and increasingly propagate oxidative damage to lipids, proteins and nucleic acids (2).

In this study, the diagnostic utility of the iron-related compounds was explored as cyst fluid biomarkers for the differentiation of benign and malignant endometriotic tumors.

Materials and methods

Patient material

The study was conducted under the guidelines that had been approved by the medical ethics committee of the Nara Medical University. A prospective cohort study of consecutive patients diagnosed with endometriotic cysts or their malignant transformation (endometriosis-associated ovarian cancer, EAOC) between December 2012 and July 2013 in Nara Medical University Hospital was enrolled in this study. After obtaining signed informed consent, cyst fluid samples were collected at the time of surgery. A histological diagnosis was confirmed by surgical pathology. Cyst fluid samples were obtained from Japanese patients with EAOC (n=11) and endometriotic cysts (n=36). Specimens were immediately aliquoted and frozen at −80° C within an

hour of collection.

Measurement of total iron concentration in cyst fluid

Each assay was performed in duplicate or triplicate. Cyst fluids were weighed and microwave digested with 50% HNO_3 and 5% H_2O_2. The final sample solution after digestion of each sample was diluted to 30 mL of 0.1 mol/L HNO_3. The amount of total iron was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista MPX, Varian, USA) with internal standard method (5).

Measurement of heme iron concentration in cyst fluid

Cysts fluids were moved to 96 wells and alkalized with NaOH to adjust pH > 10. The resultant solution was subjected to heme measurement by Metalloassay LS Heme Assay Kit (Metallogenics, Japan) based on Triton-Methanol colorimetric assay (11). The optical density of 400 nm was determined by using a micro-titer plate reader (SH-1200, CORONA, Japan).

Measurement of free iron concentration in cyst fluid

Cyst fluids were extracted in 0.01M HCl and incubated at 4 °C for 30 min. The solution was centrifuged at 10,000 r.p.m. for 10 min. The collected supernatants were used as sample for chelate colorimetric assay. The sample was subjected to free-iron measurement by Metalloassay LS-MPR Kit (Metallogenics, Japan) based on chelate colorimetric method according to the manufacturer's protocol. The optical density of 750 nm was determined by using a micro-titer plate reader.

Statistical analysis

Differences between the groups of patients were estimated by Mann-Whitney U test. Analyses were performed by SPSS (version 21.0, IBM Corp., Armonk, NY, USA). Statistical significance was assumed at a two-sided P value lower than 0.05. Receiver Operative Curve (ROC) analysis was used to identify the best discriminating threshold of the cyst fluid iron levels for differential diagnosis between EAOC and endometriosis.

Results

Basal characteristics of the study subjects are shown in **Table 1**. EAOC subjects were significantly older than the endometriosis subjects (p=0.01). Tumor size were also significantly larger in patients with EAOC as compared to endometriosis controls

(p=0.003).

Cyst fluid iron levels

We measured three selected iron-related compounds, including total iron, heme iron and free iron. **Figure 1** shows box and whisker plots representing median levels and the interquartile range (box) of total iron (A), heme iron (B) and free iron (C) for each studied group. **Table 2** summarizes cyst iron values by each type of cyst. Total iron concentration in all samples ranged from 3.0-1046.3 mg/L. The concentrations were highly divergent between the patients, with a few having extremely high concentrations and some very low. Cyst fluid total iron levels were significantly lower in patients with EAOC than in endometriotic cysts (P<0.001, **Figure 1A**). EAOC patients had significantly lower heme iron levels than endometriotic cysts (P<0.001, **Figure 1B**). Compared to endometriotic cysts, free iron levels were also significantly lower in EAOC (P<0.001, **Figure 1C**). All of iron-related compounds showed significant difference between endometriotic cysts and EAOC.

Since the two groups of patients are not homogeneous, with a median age of 39.5 years old for the endometriotic cysts and of 46.0 years old for the EAOC group, we analyzed the correlation between age and cyst fluid iron levels. In analyses of data from all endometriosis subjects, no significant interaction between patient age and cyst fluid iron levels was observed ($y = -1.6446 x + 361.07$, $r = -0.064$, $p=0.709$; **Figure 2**, open circle). Also, cyst fluid total iron levels in EAOC were not correlated with age at surgery (y [total iron level] = 1.7476 x [age at surgery] - 47.279, $r = 0.274$, $p=0.416$; **Figure 2**, closed circle). Furthermore, we did not find any significant correlation between patient age and cyst fluid levels of heme iron (p=0.592) or free iron (p=0.463) (data not shown).

In addition, the cyst fluid levels of iron-related compounds were examined in 11 patients affected with EAOC (median age, 46.0 years old, range: 38–53 years) and in 24 age-matched endometriosis subjects (median age, 41.0 years old, range: 38–55 years). Compared to endometriosis, EAOC had significantly lower cyst fluid concentrations of total iron (14.2 ± 36.6 vs. 269.1 ± 172.6, $p<0.001$), heme iron (27.6 ± 53.4 vs. 271.5 ± 309.1, $p<0.001$), or free iron (3.9 ± 2.7 vs. 13.7 ± 10.1, $p<0.001$) (data not shown).

We next analyzed the correlation between cyst fluid iron levels and tumor size. The median cyst size was 6.7 cm (range, 2.7-19.3 cm) in case of endometriotic cysts and 10.2 cm (range, 4.2-17.6 cm) in case of EAOC, respectively (p=0.003). In endometriosis subjects, there was no statistically significant difference between cyst fluid total iron levels and tumor size (y [total iron level] = - 0.4183 x [maximum

diameter of tumors] + 326.4, r = - 0.064, p=0.711; **Figure 3**, open circle). Cyst fluid total iron levels demonstrated no association with tumor size in patients with EAOC (y = - 0.0827 x + 41.348, r = - 0.088, p=0.798; **Figure 3**, closed circle). Furthermore, no significant differences were seen between heme iron (p=0.797) and free iron levels (p=0.161) and tumor size (data not shown).

Evaluation of cyst fluid iron levels as potential biomarkers for differential diagnosis between EAOC and endometriosis

After quantitative measurement of iron-related compounds in 47 cyst fluid samples, we applied ROC curves to assess the potential utility of cyst fluid levels in diagnosing EAOC from endometriosis (**Figure 4**). Patients were divided into two groups based on cyst fluid total iron levels following a ROC analysis. A cut-off value of 64.8 mg/L to classify patients as benign endometriosis or having EAOC yielded 90.9% sensitivity, 100% specificity, 100% PPV and 97.3% NPV. ROC curves also showed an optimum cut-off point of 72.7 mg/L (heme iron) and 7.18 mg/L (free iron) to distinguish EAOC from benign endometriosis (data not shown). As shown in **Figure 1**, the dashed horizontal line represents the cut-off level for each marker. Sensitivity, specificity, PPV and NPV of heme iron < 72.7 mg/L were 90%, 100%, 100% and 97.2%; when the cut-off of free iron was < 7.18 mg/L they were, respectively, 90%, 91.4%, 75% and 97%. This analysis showed that iron-related compounds were significant discriminators for malignant transformation in endometriosis women.

Discussion

A single-center prospective study to evaluate the levels of iron-related compounds in ovarian cyst fluid from 47 patients with endometriotic cysts and EAOC was performed. A marked difference in iron values was observed between women with EAOC and those with endometriosis. To the best of our knowledge, this is the first study to reveal the significance of cyst fluid levels of iron-related compounds as predictors of malignant transformation of endometriosis.

In the present study, we focused on iron in endometriotic cysts, because iron-related compounds are derived from repeated hemorrhage. Extracellular hemoglobin after hemolysis can oxidize oxyhemoglobin (ferrous, Fe^{2+}) to methemoglobin (ferric, Fe^{3+}) and produce heme and iron, which can initiate or propagate oxidative damage to lipids, proteins and DNA. Endometriotic cyst leads to iron accumulation, which originates from intracystic hemorrhage over years.

Accumulating evidence suggests that iron overload has been associated with carcinogenesis, including renal cell carcinoma, malignant mesothelioma, and hepatocellular carcinoma (13). Iron-induced oxidative stress and frequent DNA mutations are involved in the progression of endometriosis and subsequent malignant transformation (7,16).

Yamaguchi et al. reported that the concentration of free iron in endometriotic cysts was higher than that in nonendometriotic cysts. They also showed that abundant free iron in the contents of endometriotic cysts could induced oxidative stress, DNA damage and subsequent cancer development (16). Although the findings might be accidental, the vast majority (~95%) of cyst fluid iron in endometriosis were heme-bound iron, but not free iron (~5%). Surprisingly, we discovered that significantly lower iron concentration was present in EAOC cysts than in benign endometriotic cysts. Our observations are inconsistent with the idea that excessive iron induces malignant transformation of endometriotic cells. At an inflammatory site in endometriosis, fine tuning of iron expression has proven to be of significant importance with respect to tumorigenesis and homeostatic apoptosis processes as the "double-edged sword" role of iron-induced ROS. This dichotomy might be differentiated at the level of amount and duration of iron that promotes the generation of highly reactive free radicals (e.g., $\cdot OH$ and $O_2.-$) through iron-induced Fenton chemistry and hemoglobin autoxidation (15). Excess ROS can induce oxidative stress, leading to cell damage that culminates in cell death, while sublethal ROS can increase the chance of a prolonged survival (3,9). A long-standing history of the exposure to iron accumulated in the ovarian endometriotic cysts during the reproductive period produces a powerful oxidant property that is a possible cause for the cell death rather than malignant change of the endometriotic cyst. Alternatively, chronic but moderate (sublethal) amount of oxidative stress can adapt to cell survival, suggesting that chronic exposure to oxidative stress sometimes increases tumorigenic potential of endometriotic cells. In this situation, ROS may act as a trigger for carcinogenesis via persistent DNA injuries and genetic instability. Each endometriotic lesion may display significant differences with regards to the level of responsiveness to ROS.

There are several features of this study that limits the generalizability of these observed results. First, this is a single tertiary center analysis with a relatively small sample of cyst fluids. The impact of a small sample size, in particular the limited cases of malignant cysts, may include inadequate power to demonstrate a difference between malignant and benign cyst iron levels. This limitation can be addressed in the future with validation incorporating a larger sample size. Multi-center collaboration studies are

required to investigate whether iron levels have a diagnostic role in differentiation between endometriotic cysts and their malignant transformation.

Second, since this is not a serum-based test, non-invasive techniques for assessment of endometriotic cyst iron are clinically required. Recently, quantitative, safe and non-invasive tools using novel techniques for in vivo imaging of iron accumulations have been developed (10). MR imaging and optical imaging including MR spectrophotometry, based on proton transverse relaxation time (T2 and T2*) and its reciprocal relaxation rate (R2 and R2*), are unique among imaging modalities (12). A specific MR spectrophotometry algorithm yields brain and hepatic iron concentrations (4,14). These insights may lead to future therapeutic strategies targeting iron levels and MR spectrophotometry for early prediction of malignant transformation. This procedure may help in clinical decision making and proceed to surgical intervention instead of surveillance. We are now evaluating the in vivo iron levels by quantitative, non-invasive, non-destructive and rapid monitoring procedures using a specific spectrophotometry algorithm.

To date, there are no objective parameters for correct prediction of the malignant transformation of the endometriotic lesions. A biomarker that can accurately and reliably distinguish cancer among endometriosis remains an important clinical need. Some cases of unnecessary invasive interventions or missed opportunities to resect cancer may occur. Reduced cyst iron levels may help accurately identify the patient with cancer lesions that require immediate surgical attention. This result will facilitate validation in future studies.

In conclusion, we examined the diagnostic utility of iron-related compounds in endometriotic cyst fluid and observed significant difference in cyst iron concentrations between malignant and benign endometriotic cysts.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1. Patient demographics and tumor characteristics.

Patient and clinical characteristics	Endometriotic cysts	EAOC	p-value
Number	36	11	
Age (median, range)	39.5 (21-55)	46.0 (38-53)	0.01
tumor size(cm)* (median, range)	6.7 (2.7-19.3)	10.2 (4.2-17.6)	0.003
FIGO stage	-	Ia (n=4), Ib (n=1), Ic (n=6)	
Pathology	endometriosis	Clear cell adenocarcinoma (n=6) Endometrioid adenocarcinoma (n=3) Mucinous adenocarcinoma(n=1) Undifferentiated carcinoma (n=1)	

*, maximum diameter of tumors

Table 2. Cyst fluid iron values by each type of cyst.

	Endometriotic cysts	EAOC	p-value
total iron (mg/l)	244.4 ± 204.9* (65.3 - 1046.3)**	14.2 ± 36.6 (3.0 - 123.8)	<0.001
heme iron (mg/l)	303.9 ± 324.4 (82.9 - 1481.5)	27.6 ± 53.4 (7.5 – 187.6)	<0.001
free iron (mg/l)	13.5 ± 16.2 (6.2 - 92.9)	3.9 ± 2.7 (0.8 - 7.2)	<0.001

*, median ± SD

**, range

Figure 1. Cyst fluid iron levels in patients with endometriotic cysts and EAOC.
This figure shows the distribution of marker levels for each studied group. Cyst fluid iron levels were studied in patients with endometriotic cysts (n=36) and EAOC (n=11). A, total iron; B, heme iron; and C, free iron.

Figure 2. Correlation between cyst fluid total iron concentrations and patient age at surgery.
Open circle represents an individual value of endometriosis subject. Closed circle represents an individual value of EAOC subject. Correlation between cyst fluid total iron levels and age at surgery in women with endometriosis ($y = -1.6446 x + 361.07$, $r = -0.064$, $p=0.709$) and in patient with EAOC ($y = 1.7476 x - 47.279$, $r = 0.274$, $p=0.416$). x, age at surgery; y, total iron levels (mg/L).

Figure 3. Correlation between cyst fluid total iron concentrations and tumor size.
Open circle represents an individual value of endometriosis subject. Closed circle represents an individual value of EAOC subject. Correlation between cyst fluid total iron levels and tumor size at surgery in women with endometriosis ($y = - 0.4183 x + 326.4$, $r = - 0.064$, $p=0.711$) and in patient with EAOC ($y = - 0.0827 x + 41.348$, $r = - 0.088$, $p=0.798$). Cyst fluid total iron levels were not correlated with tumor size. x, tumor diameter (mm); y, total iron levels (mg/L).

Figure 4. Receiver-operating characteristic analysis
Receiver-operating characteristic analysis shows a higher area under the curve for ratio of the pixel density value of total iron. At a cut-off level of < 64.8 mg/L, the ROC curve exhibited 90.9% sensitivity and 100% specificity for detecting EAOC from benign endometriosis.

Figure 1A

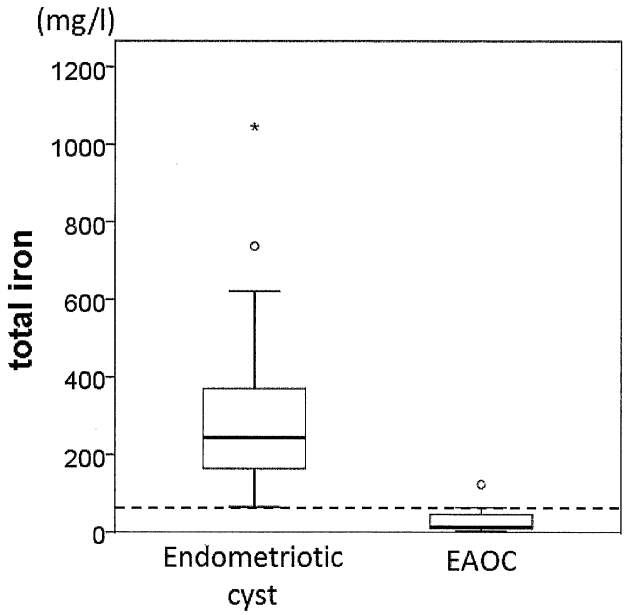

Figure 1B

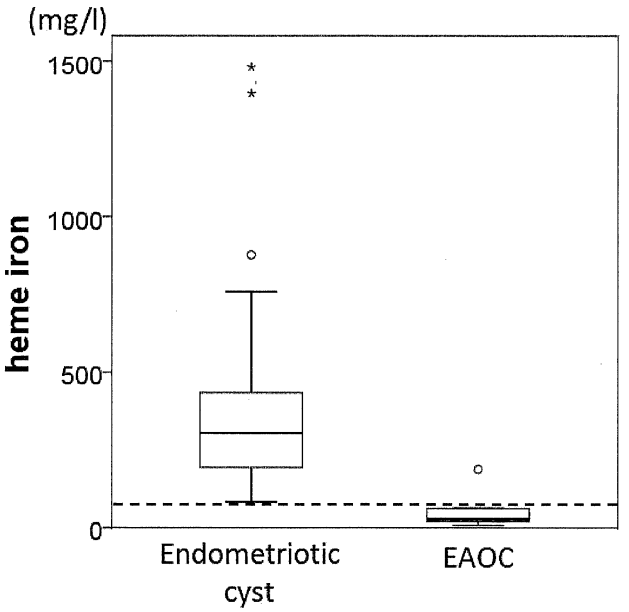

Figure 1C

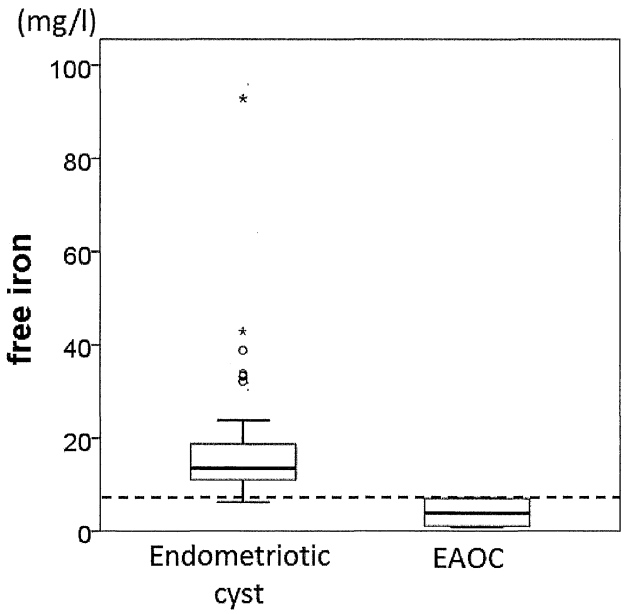

Figure 2

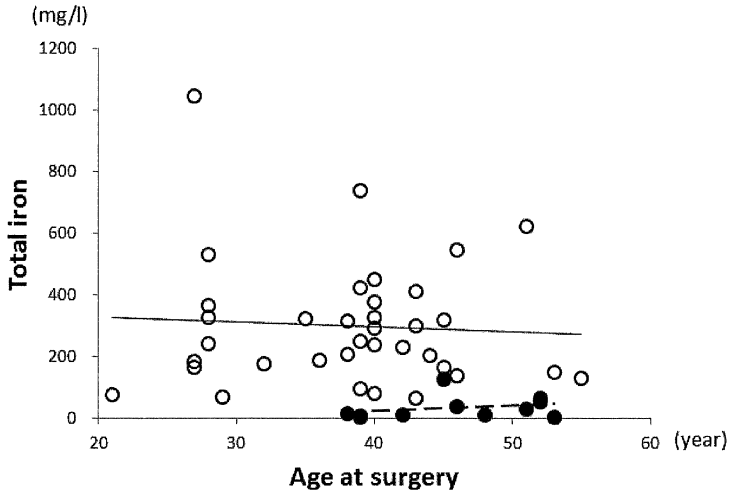
(mg/l)
1200
1000
800
600
400
200
0
Total iron
20
30
40
50
60
(year)
Age at surgery

Figure 3

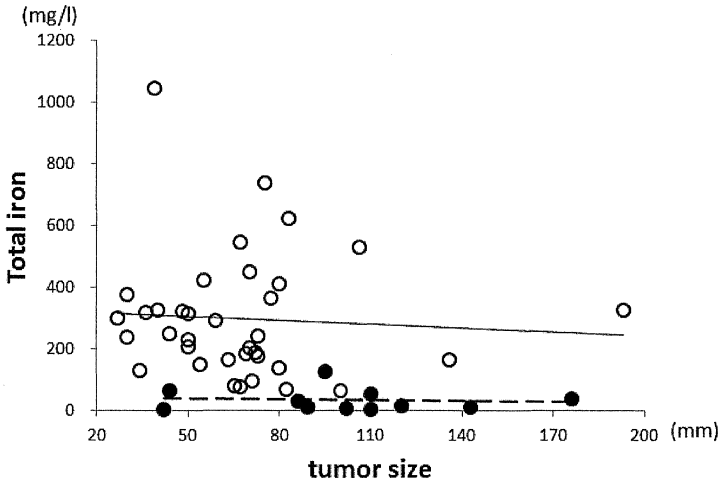
(mg/l)
1200
1000
800
600
400
200
0
Total iron
20
50
80
110
140
170
200
(mm)
tumor size

Figure 4