Discrimination of malignant transformation from benign endometriosis using a near-infrared approach

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Received September 20, 2017; Accepted January 16, 2018

DOI: 10.3892/etm.2018.5779

Abstract. The aim of the present single-center retrospective study was to investigate the discrimination of malignant transformation from ovarian endometrioma (OE) using a near-infrared approach *ex vivo*. Cystic fluid samples were collected from patients with OE (n=34) and endometriosis-associated ovarian cancer (EAOC) (n=12). The light reflected from each sample of cystic fluid [change in luminance, Δl (cd/m^2) = background luminance-cystic fluid luminance at 800 nm] was spectrally measured by a near-infrared CCD camera with band-path filter (800 nm). The Δl in EAOC was significantly lower compared with that in OE. On regression analysis, a positive correlation was observed between the Δl and Hb level in the cystic fluid, and this association was exponential. The diagnostic sensitivity and specificity of Δl was 83.3 and 94.1% at the cutoff value of 21.5 cd/m^2, with an area under the ROC curve of 0.897. The present *ex vivo* study potentially provides a powerful near-infrared approach for quantitative discrimination between EAOC and benign OE, with high sensitivity and specificity, which may have clinical applications.

Introduction

Ovarian cancer represents one of the leading causes of cancer-related mortality, with an increasing prevalence in Japan (1). The pathogenesis of ovarian cancer is complex, and is affected by numerous epigenetic and genetic factors (2). Endometriosis is usually a benign disorder, but is associated with an increased risk for developing ovarian (3) and endometrial cancers (4). Endometrioid and clear cell carcinoma of the ovary (endometriosis-associated ovarian cancer, EAOC) originates from endometriosis.

Magnetic resonance (MR) imaging may be used as an adjunctive method to distinguish EAOC from benign ovarian endometrioma (OE). Specifically, we have recently demonstrated that the MR transverse relaxation rate provides a noninvasive predictive tool to discriminate between EAOC and OE (5). However, the implementation of MR imaging in the outpatient clinic is often difficult.

Recent progress in research on the pathogenesis of EAOC is based on key developments in two areas: i) New mechanistic concepts regarding the pathogenesis of EAOC revealed a key role of hemoglobin (Hb), heme and iron-induced oxidative stress in the OE cystic fluid, wherein an imbalance between the overproduction of iron-induced oxidative stress and defense mechanisms could trigger DNA damage and carcinogenesis; and ii) the establishment of novel approaches to identify stress biomarkers, such as Hb and iron, for the prediction of malignant transformation (2,6,7). A recent *ex vivo* study revealed that electronic absorption spectroscopy using visible light at 580 and 620 nm provides a measure of Hb species in endometriotic cystic fluid by monitoring the relative concentrations of oxyhemoglobin and methemoglobin, respectively (8). The 620/580 nm peak ratio of cystic fluid in EAOC patients was significantly lower compared with that measured in women with benign OE (8). Therefore, the cystic fluid Hb species may be used as biomarkers in the differential diagnosis between EAOC and OE. However, one limitation of this absorption-based method is that the camera must be placed opposite a halogen white light source, which is disadvantageous as light in the visible spectrum does not penetrate tissue.

Recent advances in optical technology have led to innovative quantitative monitoring tools, which include spatially-resolved reflectance, diffuse optical spectroscopy, diffuse optical tomography and diffuse correlation spectroscopy (9,10). Optical spectroscopy and tomography utilize the near-infrared spectral region to provide quantitative determination of several important biological chromophores, such as Hb (11) or cytochrome *c* oxidase (12). Furthermore, light in the near-infrared spectrum efficiently penetrates tissue, including bone and muscle (11). When near-infrared light is shone through cystic fluid, the influence of photon

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Key words: near infrared, endometriosis, malignant transformation, ovarian cancer

attenuation and scattering varies depending on Hb concentration. A backscattered photon or an on-axis luminance measurement can be detected by means of appropriate optical apparatus. We hypothesized that the changes in luminance across a fluid can typically be used to determine the Hb concentration of the cystic fluid using a near-infrared sensor camera. Thus, we developed a rapid and sensitive *ex vivo* assay based on the changes of dynamic light scattering or changes in luminance across the cystic fluid. Furthermore, we investigated the potential of the luminance measurement as an objective optical method to discriminate EAOC from benign OE.

Materials and methods

Study population. The research protocol was approved by the Nara Medical University Review Boards, and written informed consent was obtained from all subjects. A total of 46 patients with OE (n=34) or EAOC (n=12) were recruited between February 2013 and January 2015 at the Department of Gynecology, Nara Medical University Hospital (Kashihara, Japan). Histopathological examination confirmed the diagnoses of benign OE and EAOC. All cystic fluid samples were collected from the patients during surgery, and an aliquot of each sample was stored at -80˚C until testing.

Instrumentation and system design. Narrow-band optical filtering is required to achieve the highest signal-to-noise ratio (SNR) as an optical enhancement technology. During our preliminary study, the SNR was determined analytically using a band-path filter with varying wavelengths (750-1,000 nm), and the results were validated experimentally. We found a single optimum for the optical path length of the filter at 800 nm (data not shown). This was applied to in the current system.

The instrument was designed and set up. A diagram of the light path is illustrated in Fig. 1. This figure indicates the luminance (light reflected from the sample) measurement of the cystic fluid sample. Measurements were obtained by performing *ex vivo* phantom experiments. An aliquot of the cystic fluid sample (1 ml) was transferred to a disposable cuvette (10-mm wide x 10-mm thick). The light source was a halogen lamp with a 300 W quartz-tungsten-halogen bulb (EXR 82v; Eiko, Co., Ltd., Hitachinaka, Japan). The distance between the halogen light and the cystic fluid was 50 mm. Halogen light illuminated the sample and a near-infrared CCD camera with band-path filter (800 nm) recorded the light signal reflected from the sample. The change in luminance [Δl value (cd/m^2)] was calculated by subtracting a sample blank for each specimen (Δl = background luminance - cystic fluid luminance at 800 nm).

Hb assay. Cystic fluid total Hb concentrations were measured as described previously (6,8,13). From the correlation data, a formula was calculated to convert heme levels (mg/l) to hemoglobin (g/dl). We then investigated the correlation between Δl and Hb in cystic fluid.

Effects of an anatomical barrier on surface reflectance. Transvaginal ultrasound imaging is replacing radiological methods in the investigation of ovarian tumors. Due to the presence of an anatomical barrier (which may include an ovarian cyst wall or vaginal connective/muscle tissue) in this type of imaging, *ex vivo* experiments using appropriate modeling are important to establish a clinically relevant model. To investigate the effect of such an anatomical barrier, the surface of a disposable cuvette was covered with pieces of commercial Japanese chicken of different thicknesses (5 and 10 mm). The Δl value was measured by recording the light scattered (surface luminance) from the cystic fluid that was covered by these barriers. We generated three sets of experiments: Experiment 1 (0 mm; the surface of the cuvette was not covered); experiment 2 (5 mm; the surface of the cuvette was covered by a 5-mm-thick piece of chicken); and experiment 3 (10 mm; the surface of the cuvette was covered by a 10-mm-thick piece of chicken).

Statistical analysis. Statistical analysis was conducted using the SPSS 22.0 software package (IBM Corp., Armonk, NY, USA). Comparisons of non-parametric data (Δl and Hb levels) between the OE and EAOC groups were performed using the Mann-Whitney U test. Correlation analysis was performed using Pearson's correlation coefficient. The optimal cutoff value was defined according to analysis of the receiver operating characteristic (ROC) curve. The sensitivity and specificity of detection were calculated on the basis of cutoff value to differentiate EAOC from benign OE. The area under the ROC curve (AUC) was also calculated for each marker. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Δl and Hb of cystic fluid samples. The clinical characteristics, cystic fluid Δl levels and Hb concentrations of patients are summarized in Table I. Subjects in the EAOC group were older compared with the OE group ($P<0.001$). Fig. 2 shows box and whisker plots representing the median level and interquartile range (box) of Δl and Hb for each studied group. The EAOC patients showed significantly lower Δl values compared with the OE group ($P<0.001$) (Table I; Fig. 2A). The cystic fluid levels of Hb were also significantly lower in EAOC patients compared with OE patients (Table I; Fig. 2B). These results indicated that the OE and EAOC groups were clearly separated.

ROC curve in EAOC group vs. benign OE group. The sensitivity and specificity of cystic fluid Δl level for the diagnosis of malignant transformation were 83.3 and 94.1%, respectively, using a cutoff value of 21. The AUC was 0.897 (Fig. 3A; Table II, experiment 1). A Hb level of 1.99 g/dl was identified to detect EAOC with a sensitivity of 100% and a specificity of 91.7%, and an AUC of 0.988 (Fig. 3B). Since the patients with EAOC were significantly older than those with OE, correlations between age and each parameter were evaluated using Pearson's correlation coefficient. The age distribution of the subjects is shown in Fig. 4. There were no significant correlations between age and cystic fluid Δl (Fig. 4A; $r=-0.128$, $P=0.470$) or Hb level (Fig. 4B; $r=-0.159$, $P=0.370$) in the OE group. There were also no correlations between age

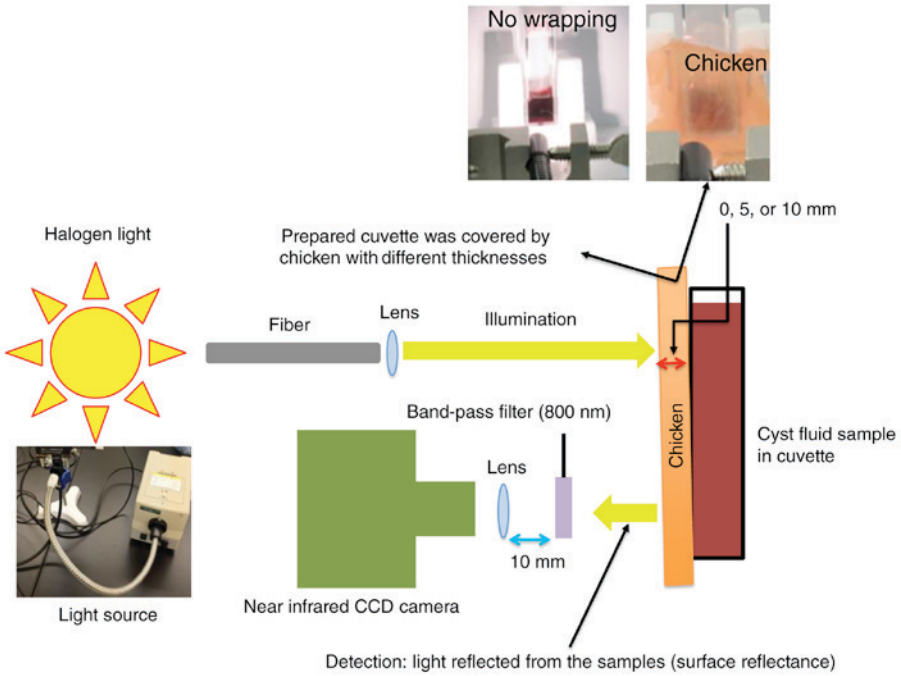

Figure 1. Schematic illustration of experimental setup to acquire a light reflected from the samples. Schematic diagram of the optical arrangement used for luminance measurements. The experimental arrangement of the halogen light illumination and photodetector are illustrated. The light source and CCD camera are precisely aligned to image the light signal. The prepared cuvette was covered by a commercially available chicken with a different thickness (0, 5 and 10 mm).

Table I. Patient demographics and tumor characteristics of two groups.

Patient and clinical characteristics	OE	EAOC	P-value
Number	34	12	
Age (years)			<0.001
Median (range)	39.0 (26-51)	49.5 (36-69)	
Mean ± SD	38±7	49±11	
Cyst size (cm)[a]			0.022
Median (range)	7.0 (2.7-19.3)	11.0 (4.2-22.5)	
Mean ± SD	7.7±3.2	12.1±5.7	
FIGO stage	-	Ia (n=5), Ib (n=1), Ic (n=6)	
Pathology	Endometriosis	Clear cell carcinoma (n=6) Endometrioid carcinoma (n=3) Mucinous carcinoma (n=1) Serous carcinoma (n=1) Seromucinous carcinoma (n=1)	
Δl (cd/m^2)			<0.001
Median (range)	29.0 (18.2-41.4)	16.2 (-5.5-32.3)	
Mean ± SD	29.6±5.0	14.4±10.8	
Hb (g/dl)			<0.001
Median (range)	6.1 (2.2-42.8)	0.77 (0.2-3.5)	
Mean ± SD	9.5±9.3	1.1±0.9	

[a]Maximum diameter of tumor cysts. OE, ovarian endometrioma; EAOC, endometriosis-associated ovarian cancer; FIGO, International Federation of Gynecology and Obstetrics; SD, standard deviation.

and cystic fluid Δl (Fig. 4A; r=0.518, P=0.084) or Hb level (Fig. 4B; r=0.532, P=0.075) in the EAOC group. Fig. 5 shows a scatter plot of correlation between Δl and cystic fluid Hb level for the OE and the EAOC groups. When the interaction

Table II. Effects of an anatomical barrier against surface reflectance.

Patients	OE (n=34)	EAOC (n=12)	AUC	95% CI	P-value	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Experiment 1 (Samples not-covered by a chicken)										
Δl (cd/m^2)	29.6±5.0 (18.2-41.4)	14.4±10.8 (-5.5-32.3)	0.897	0.772-1.000	<0.001	21.5	83.3	94.1	83.3	94.1
Experiment 2 (Samples covered by a 5 mm-thick chicken)										
Δl (cd/m^2)	2.1±10.2 (-16.6-49.1)	-9.7±7.6 (-19.8-2.7)	0.859	0.733-0.985	<0.001	-5.0	75.0	91.2	75.0	91.2
Experiment 3 (Samples covered by a 10 mm-thick chicken)										
Δl (cd/m^2)	-9.9±5.1 (-20.1-2.0)	-17.6±7.6 (-26.3-6.3)	0.778	0.609-0.948	0.005	-15.5	66.7	85.3	61.5	87.9

Upper, experiment 1; middle, experiment 2; and lower, experiment 3 panels represent the Δl values and the discriminative value of each parameter in the OE and EAOC groups. OE, ovarian endometrioma; EAOC, endometriosis-associated ovarian cancer; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

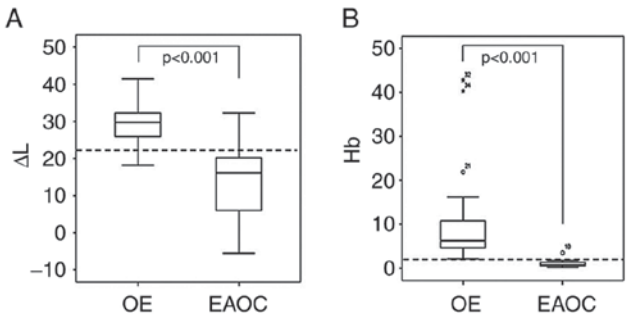

Figure 2. (A) Cystic fluid Δl and (B) Hb levels in patients with EAOC and OE. This image shows the distribution of marker levels for each studied group. Cystic fluid levels were studied in patients with EAOC (n=12) and OE (n=34). (A) The EAOC patients showed significantly lower Δl values compared with the OE group (P<0.001). (B) The EAOC patients showed significantly lower Hg values compared with the OE group (P<0.001).

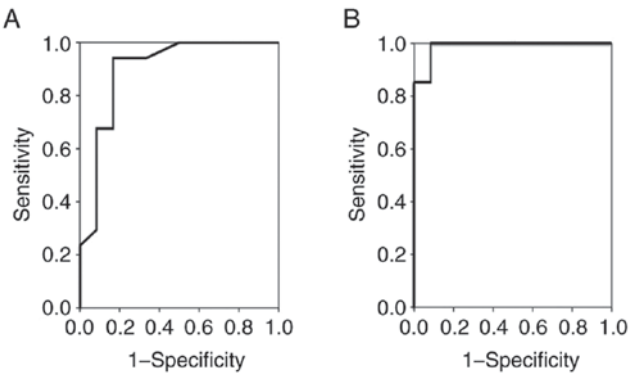

Figure 3. ROC curve comparing (A) Δl and (B) Hb levels in patients with EAOC group vs. patients with benign OE group. Receiver-operating characteristic (ROC) analysis shows a higher area under the curve (AUC) for ratio of the pixel density value of Δl and Hb. (A) The proper cut off for the Δl level which was determined for the diagnosis of malignant transformation in our study was 21.5. The area under the ROC curve of the Δl level was 0.897. The ROC curve exhibited 83.3% sensitivity and 94.1% specificity. (B) The best cut-off point for cystic fluid Hb level was 1.99 mg/l. The area under the ROC curve was 0.988. Sensitivity and specificity for detecting EAOC from benign OE were 100 and 91.7%, respectively.

between Δl and Hb was analyzed using a linear model, the best model fitted an exponential function. Therefore, data were linearized by log transformation. The association of Δl with Hb became steeper with lower Hb levels (<2 g/dl). Δl was strongly correlated with the cystic fluid Hb concentration (r=0.558, P<0.001).

Effects of an anatomical barrier on surface reflectance. The results of the *ex vivo* approach are summarized in Table II, which shows the comparison of measurements obtained from each experiment. The AUC for diagnosing EAOC from OE was 0.897 (experiment 1: Sensitivity, 83.3%; specificity, 94.1%), 0.859 (experiment 2: Sensitivity, 75.0%; specificity, 91.2%) and 0.778 (experiment 3: Sensitivity, 66.7%; specificity, 85.3%).

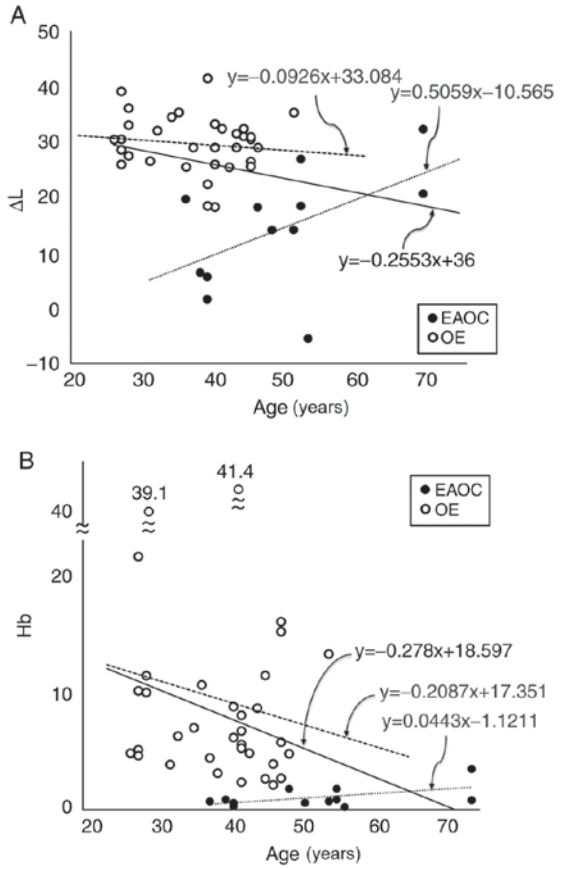

Figure 4. Cystic fluid (A) Δl and (B) Hb levels per age for subjects in the OE and EAOC samples. Open circle represents an individual value of OE subject. Closed circle represents an individual value of EAOC subject. (A) Correlation between cystic fluid Δl levels and age at surgery in women with OE (y = -0.0926x + 33.084, r=-0.128, P=0.470) and in patient with EAOC (y = 0.5059x - 10.565, r=0.518, P=0.084,). x, age at surgery; y, cystic fluid Δl level (cd/m^2). (B) Correlation between cystic fluid Hb levels and age at surgery in women with OE (y = -0.2087x + 17.351, r=-0.159, P=0.370) and in patient with EAOC (y = 0.0443x - 1.1211, r=0.532, P=0.075,). x, age at surgery; y, cystic fluid Hb level (mg/l).

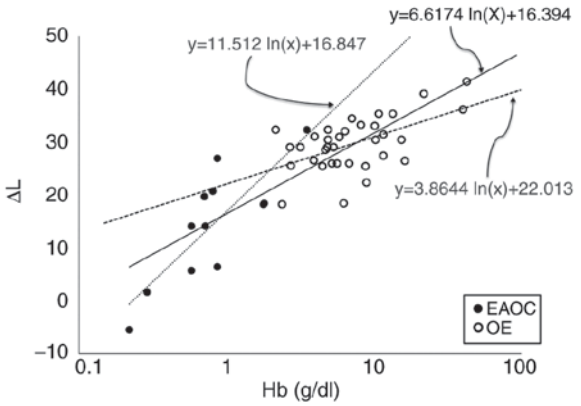

Figure 5. Relation between Δl and Hb in the OE group and the EAOC group. The relationship between Δl and Hb level was better described by an exponential function (y = 6.6174ln(x) + 16.394, r=0.558, P<0.001). Correlation between cystic fluid Δl levels and cystic fluid Hb level in women with OE (y = 3.8644ln(x) + 22.013, r=0.585, P<0.001) and in patient with EAOC (y = 11.512ln(x) + 16.847, r=0.704, P=0.011). x, cystic fluid Hb level (mg/l); y, cystic fluid Δl level (cd/m^2).

Discussion

To the best of our knowledge, this is the first *ex vivo* study of cystic fluid measurements via optical properties at 800 nm, which can discriminate malignant transformation from benign OE. The Δl of cystic fluid from the EAOC group was significantly lower compared with that of the OE group (P<0.001). Δl level could serve as a simple, rapid and accurate method to discriminate EAOC from benign OE, with high sensitivity (83.3%) and specificity (94.1%). In our *ex vivo* experiments, the samples were covered by 5- or 10 mm-thick pieces of chicken. Our measurements showed that the 10 mm-thick sample attenuated the power of ΔL to discriminate between benign and malignant specimens, with relatively lower sensitivity (66.7%) and specificity (85.3%).

Furthermore, the cystic fluid Hb concentrations were reduced in patients with EAOC (6-8). The Δl values and total Hb concentrations in 46 samples exhibited an exponential correlation (r=0.558), suggesting that the Δl value may reflect the Hb concentration. The present results were in agreement with those of Yoshimoto *et al* (6), who reported the cystic fluid concentration of total iron, heme iron, free iron and Hb species (6,7). Previous studies of Hb species have reported differences between OE and EAOC samples (8). Transvaginal ultrasound-guided luminance measurements using near-infrared approaches may advance medical imaging technology as a tool for discriminating malignant transformation in endometriosis.

Despite the advantages discussed above, there are several limitations in the present study. Firstly, an exponential curve of the Hb levels was a better-fitting model compared with the linear model. However, whether and how the Δl level reflects absolute Hb concentration has not yet been studied. We could not exclude the possibility of cross-contamination of other factors, such as heme iron and free iron, in these data acquired at an 800-nm wavelength. Secondly, a major limitation is the lack of large-scale evaluation. Finally, the complexity of reproductive organ anatomy poses several challenges for *in vivo* luminance imaging. Non-invasive imaging in deep tissue requires a near-infrared CCD camera with strong sensitivity and high spatial resolution. By adding detectors at multiple distances from the emitted light source, specific algorithms can subtract superficial light absorption from deep absorption to provide qualitative information of the cystic fluid Hb level (11). Despite these limitations, there is a great need to develop a clinically useful, noninvasive and reliable tool that accurately predicts the malignant transformation of endometriosis.

In conclusion, the luminance value obtained from *ex vivo* cystic fluid samples at an 800-nm wavelength may discriminate EAOC from benign OE patients. Transvaginal near-infrared approaches may provide a non-invasive assessment of malignant transformation of OE, and may have further clinical applications in an outpatient setting.

The aim of this study was to investigate the discrimination of malignant transformation from OE using a near-infrared approach *ex vivo*. The diagnostic sensitivity and specificity for Δl (change in luminance, cd/m^2) were 83.3 and 94.1%, respectively, at the cutoff value of 21.5 cd/m^2, with an AUC of 0.897. This *ex vivo* study potentially provides a powerful near-infrared approach for discrimination between

EAOC and benign OE, with high sensitivity and specificity. This study provides a basis for developing future clinical approaches.

Acknowledgements

This study was supported by grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (H.K.).

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