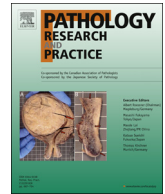




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Clinical significance of M2 macrophages expressing heme oxygenase-1 in malignant transformation of ovarian endometrioma

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ABSTRACT

Malignant transformation of endometriosis is a rare and still poorly understood event, but is associated with the distortion of the pro-oxidant and anti-oxidant balance. The aim of the present study was to quantify the numbers of macrophages polarized as M1 or M2 phenotypes and the expression of heme oxygenase (HO)-1 in tissue sections from patients with benign ovarian endometrioma (OE) and its malignant transformation (endometriosis-associated ovarian cancer, EAOC). We performed a retrospective study at the Department of Gynecology, Nara Medical University hospital from December 2012 to March 2015. This study included 53 patients with OE (n = 33) and EAOC (n = 20), and we evaluated polarized functional status of macrophages by immunohistochemical staining of CD68, CD11c, CD163 and HO-1. The number of the M1 phenotype (CD11c⁺, p = 0.001) and the M2 phenotype (CD163⁺, p = 0.009) was significantly lower in EAOC patients than in OE patients. Analyzing the correlations between the studied markers, the expression of CD68, CD11c, and CD163 proteins significantly correlated with each other (p < 0.001). The number of M2 phenotypes expressing HO-1 was significantly decreased in the EAOC group, compared with the OE group (P < 0.001), demonstrating sustained downregulation of an antioxidant marker, HO-1, in EAOC. In conclusion, reduced number of M2 macrophages expressing HO-1 may have an important role in promoting malignant transformation of OE.

1. Introduction

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterus, most often in the pelvic peritoneum and ovaries. This disorder affects an estimated 10% of women in the reproductive age group and is basically an estrogen-dependent benign gynecological disease. Repeated episodes of retrograde menstruation or ovarian hemorrhage occur in the peritoneal cavity or ovarian endometrioma (OE), respectively [1].

Endometriosis results in a local accumulation of hemoglobin, heme and iron species, which causes severe oxidative stress and antioxidants depletion, leading to distortion in the redox balance [2]. Altered homeostatic redox balance of the environment may support chronic inflammation, uncontrolled proliferation and then malignant transformation [2]. Actually, endometriosis increases the subsequent risk of developing endometriosis-associated ovarian cancer (EAOC) [3,4].

Endometriotic lesions are highly infiltrated with various leukocytes, including macrophages that secrete antioxidants and

immunosuppressive factors [1,5–7]. Endometriosis infiltrating macrophages might adapt to these stressful environmental conditions by secreting antioxidants that control excess oxidative stress [1].

Oxidative stress and inflammation in the surrounding environment contribute to several aspects of macrophage functions including recruitment, activation and the shift in cell polarity. Macrophage polarization may have a distinct role in the inflammatory, immune and neoplastic diseases [8,9]. Macrophages are classified into the pro-inflammatory, classically activated M1 macrophages that possess anti-tumor activity and the alternatively activated M2 macrophages that support tumor progression and malignancy [8,9]. For the phenotypical characterization of infiltrating macrophages, immunohistochemistry employing selected literature-based prototype-antibodies against CD11c, CD163 and CD68 was evaluated in this study. Although CD11c is a specific marker in macrophages and dendritic cells [10], CD11c-based immunohistochemistry is used as a M1 phenotype specific marker [11,12].

CD11c, a member of integrin family, induces tissue injury and the

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inflammatory response [13]. The protein expression of M2 phenotype specific marker CD163 is tested via immunohistochemistry. CD163 is a hemoglobin/haptoglobin scavenger receptor and acts to protect tissues from oxidative damage [5]. CD68 is specifically expressed by tissue macrophages and used as a pan-macrophages marker [14].

When the microenvironment is altered in endometriosis by an excess oxidative stress and inflammatory insult, knowledge on how macrophages respond to its changes is limited. An antioxidant enzyme, heme oxygenase-1 (HO-1), is a key mediator that allows the resolution of inflammatory processes [15,16]. HO-1 is responsible for the catabolism of heme to carbon monoxide (CO), biliverdin, and iron. HO-1 was poorly expressed in peritoneal mesothelium and macrophages, but highly expressed in endometriotic lesions [17].

However, HO-1 expression in the microenvironment of OE and its malignant transformation remain to be investigated.

The aim of the present study was to quantify the numbers of macrophages polarized as M1 or M2 and the expression of HO-1 in tissue sections from patients with OE and EAO.

2. Materials and methods

2.1. Tissue samples

The data of patient demographic features and clinicopathologic characteristics were collected from a database containing medical records and pathology reports. The samples consist of surgically resected tissues from hospitalized patients. A total of 53 formalin-fixed, paraffin-embedded tissue specimens, including 33 cases of pathologically confirmed benign OE and 20 cases of EAO, were retrospectively collected from Department of Gynecology at Nara Medical University hospital, Japan, between December 2012 to March 2015. In EAO cases, we confirmed morphologic documentation of the continuous transition from benign endometriotic epithelial cells to atypical endometriosis and finally to invasive carcinoma within the same specimen. No patients underwent hormonal therapy or chemotherapy prior to the surgery. The protocols were approved by the ethics committee of Nara Medical University (Approved number 1570/2017). This study is a retrospective observational study, carried out by the opt-out method of our hospital website.

2.2. Immunohistochemical staining for CD68, CD11, CD163, and HO-1

Paraffin sections of serial 2 μ m thickness were taken from each original block, one section was stained with hematoxylin and eosin for diagnostic confirmation and the other sections were immunostained for CD68, CD11c, CD163 and HO-1. Immunohistochemically staining for tissue sections was performed using anti-Human CD11c monoclonal antibody (1:100 dilution; Leica, Newcastle, UK), anti-Human CD68 monoclonal antibody (1:200 dilution; Dako, Denmark), anti-Human CD163 monoclonal antibody (1:200 dilution, Leica, Newcastle, UK), and anti-HO-1 monoclonal antibody (1:100 dilution; Abcam, Cambridge, UK). Immunohistochemistry was performed using the Leica Bond Max systems (Mitsubishi Chemical Medicine Co., Tokyo, Japan) following the manufacturer's instructions. The primary antibodies were replaced with PBS for negative control experiments. The slides were counterstained with hematoxylin, and mounted under coverslips.

Light microscopy was used to evaluate the intensity and localization of the staining. In each tissue section, round cells with a well-represented cytoplasm positive for CD68, CD11c, CD163, HO-1 were identified by screening the entire area in a low-power field (100 \times field) and five visual fields showing the highest infiltration rate of positive cells were selected (400 \times field) as described previously [14]. Results were expressed as the number of positive cell staining with brown in cytoplasm or cell membrane. Most of the staining intensity were moderate to strong brown particles. Cell counting results were obtained by 2 independent observers (TU and YY). If discrepant results were

obtained by two observers, these slides were re-evaluated using a dual-vision microscope, in order to achieve a consensus.

2.3. Statistical analysis

Analyses were performed by SPSS version 21.0 (IBM SPSS, Armonk, NY, USA). Numerical variables, including age, were presented as the mean \pm SD. Variables that did not present normal distribution were expressed by median, minimum and maximum values. Categorical data were analyzed by the Chi-square test or the Fisher's 2-tailed test. To determine the level of significance in differences in positive cell numbers between the two groups, Mann-Whitney U test and *t*-test were applied. The Pearson, Spearman's correlation coefficient analysis was implemented as descriptive statistics. Two-sided *P* < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Study population

The major clinical and pathological characteristics of the two groups of patients are listed in Table 1. There was a significant difference between the OE and EAO group in median values of variables such as age (*p* < 0.001), tumor size (*p* < 0.001), menopausal status (*p* < 0.001) and CA125 levels (*p* < 0.001). There were no statistical differences for parity status between the two study groups (*P* > 0.05).

3.2. The number and phenotypes of macrophages in the OE and EAO group

We identify and quantify the amounts of accumulated cells in tissue samples by immunostaining. Sample immunohistochemical images are shown in Fig. 1 CD68-, CD11c- and CD163-positive cell staining is mainly seen in the cytoplasm and/or membrane of macrophages. Generally, CD163⁺ cells and CD68⁺ cells were ampler than CD11c⁺ cells in both tissues. CD marker-positive cells revealed a randomly dispersed distribution, but "hot-spot" areas were identified in the OE and EAO tissue specimens. A greater number of CD68⁺ cells, CD11c⁺

Table 1
Demographic and clinical characteristics of the study population.

Baseline characteristics of the two groups	The OE group	The EAO group	p-value
Number	33	20	
FIGO stage	Benign ovarian endometrioma	Clear cell (n = 13) Endometrioid (n = 3) Serous (n = 1) Mucinous (n = 1) Other (n = 2)	
Age at diagnosis, mean \pm SD	37.2 \pm 8.5	51.1 \pm 11.7	< 0.001
median (range)	38 (26–65)	50 (36–69)	
Nulliparous n (%)	10 (30.3%)	10 (50.0%)	0.152
Pre-menopause n (%)	32 (97.0%)	11 (55.0%)	< 0.001
A maximum diameter of the cyst			
mean \pm SD	72.0 \pm 24.4	131.6 \pm 47.0	< 0.001
median (range)	70 (25–137)	120 (38–230)	
CA125			
mean \pm SD	73.9 \pm 47.1	687.7 \pm 1428.6	< 0.001
median (range)	61(17–235)	110(6–6272)	

OE ovarian endometrioma, EAO endometriosis-associated ovarian cancer, FIGO The International Federation of Gynecology and Obstetrics.

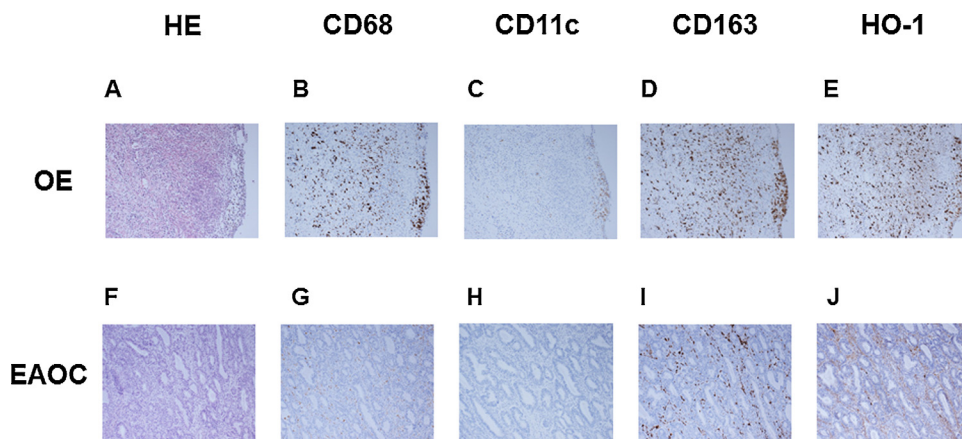


Fig. 1. Immunohistochemical analysis of CD68, CD11c, CD163 and HO-1 expression in OE and EAO tissues. To evaluate the changes in the macrophage phenotypes, consecutive tissue sections were immunohistochemically analyzed for the presence of CD68, CD11c, CD163 and HO-1 using avidin-biotin complex method. These slides show polarized macrophage infiltration. Representative images of hematoxylin and eosin stains (A and F), as well as CD68 (B and G), CD11c (C and H), CD163 (D and I) and HO-1 (E and J) immunohistochemical staining in OE (A, B, C, D and E) and EAO tissue (F, G, H, I and J) (original magnification $\times 100$). CD68, CD11c and CD163 were localized to the cytoplasm and cell membranes. HO-1 was predominantly expressed in the cytoplasm of macrophages. Hematoxylin was used to stain the nuclei.

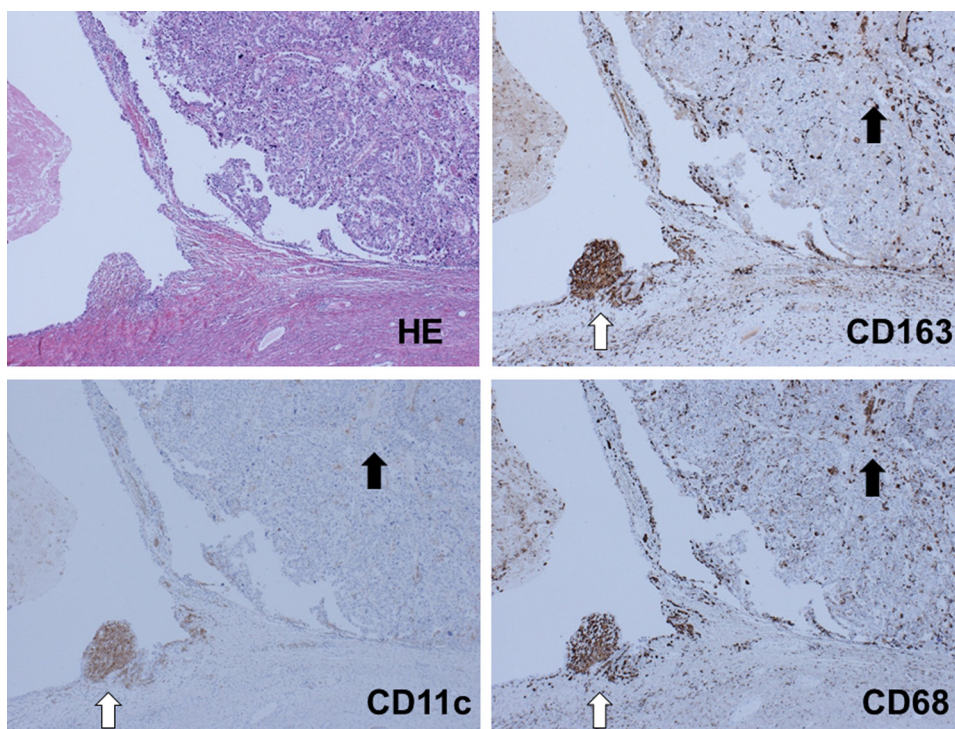


Fig. 2. CD68, CD11c and CD163 expression in EAO tissues and matched adjacent nontumor tissue samples. Our immunohistochemical results showed that CD11c, CD163, and CD68 expression was downregulated in the majority of EAO samples (black arrow) compared with corresponding adjacent noncancerous endometriotic tissues (white arrow).

cells, or CD163⁺ cells was observed in the stroma of both tissues. The number of CD68⁺ cells (Fig. 1B and G), CD11c⁺ cells (Fig. 1C and H) and CD163⁺ cells (Fig. 1D and I) were lower in the EAO group, compared with the OE group. Our immunohistochemical results also showed that CD68, CD11c and CD163 expression was downregulated in the majority of EAO samples compared with corresponding adjacent noncancerous endometriotic tissues (Fig.2).

The number of macrophage infiltration according to CD68, CD11c and CD163 counts are summarized in Table 2. The mean CD68⁺, CD11c⁺ and CD163⁺ counts in the EAO group were significantly lower than those in the OE group. We further analyzed M1-like cells/M2-like cell ratio (M1/M2) based on cell staining with CD11c⁺ cells and CD163⁺ cells in the consecutive sections. The M1/M2 ratio was significantly lower in the EAO group, compared with the OE group ($p < 0.001$).

Table 3 shows comparison of the correlations between the studied markers, including CD68, CD11c, and CD163. Analyzing the correlations between the studied markers, the expression of CD68, CD11c, and CD163 proteins significantly correlated with each other ($p < 0.001$)

Table 2

Distribution of cells expressing CD11c, CD68, CD163 and HO-1 in OE and EAO tissues. OE ovariana endometrioma, EAO endometriosis-associated ovarian cancer, HO-1 heme oxygenase-1.

The results of CD11c, CD68, CD163 and HO-1 expression Number	The OE group	The EAO group	p-value
CD11c expression median (range)	34.0 (0.6-73.8)	4.1 (0.8-21.8)	0.001
CD68 expression mean \pm SD	100.9 \pm 40.0	68.6 \pm 18.3	0.007
CD163 expression mean \pm SD	94.5 \pm 28.6	72.8 \pm 27.4	0.009
CD11c/CD163 (M1/M2) median (range)	0.321 (0.007-1.0)	0.065 (0.011-0.213)	<0.001
HO-1 expression mean \pm SD	60.0 \pm 23.4	17.4 \pm 10.9	<0.001

OE ovariana endometrioma, EAO endometriosis-associated ovarian cancer, HO-1 heme oxygenase-1.

Table 3

Comparison of the correlations between the studied markers. Correlations between the distribution of CD68⁺, CD11c⁺, and CD163⁺ cells of each case are shown.

	CD11c	CD163
CD68	$y = 0.484x - 19.370$ $r_s^{**} = 0.524, p < 0.001$	$y = 0.610 + 33.011$ $r^* = 0.552, p < 0.001$
CD163	$y = 0.423x - 14.328$ $r_s^{**} = 0.270, p < 0.001$	

* Pearson's product moment correlation coefficient.

** Spearman's rank correlation coefficient.

3.3. The HO-1 expression in OE and EAOC tissues

Next, the HO-1 expression was evaluated using immunohistochemistry in the OE and EAOC group (Fig. 1E and J). The ectopic endometrial cells and epithelial cancer cells were generally unstained. HO-1 staining was strongly observed in the stroma. The number of HO-1-positive cells were significantly lower in the EAOC tissues, compared with the OE tissues ($P < 0.001$) (Table 2). Since HO-1 acts as a target for M2 macrophages, we further investigated the co-expression pattern of HO-1 and CD163 in OE and EAOC tissues. Indeed, examination of consecutive sections of OE tissue revealed that HO-1 is expressed in the majority of the cells expressing CD163, suggesting that HO-1 is co-expressed in a major subset of M2 macrophages (Fig. 1D and E). This co-expression was observed in all specimens, regardless of age (data not shown).

Fig. 3 shows comparison of the association between the numbers of CD163⁺ cells and HO-1⁺ cells. We observed statistically significant correlations between CD163 and HO-1 ($r = 0.201, p = 0.003$).

4. Discussion

To our knowledge, this is the first study that investigates the number and phenotypes of macrophages in malignant transformation of endometriosis. Here we evaluated the immunohistochemical expression of CD68 (pan-macrophage marker), CD11c (M1 marker), CD163 (M2 marker) and the influence of HO-1 in a benign OE and its malignant transformation, EAOC. A higher number of macrophages were observed in patients with OE compared to those with EAOC, and most had the M2 phenotype expressing HO-1. This study demonstrates sustained down-regulation of an antioxidant marker, HO-1 in EAOC, suggesting that reduced number of M2 macrophages expressing HO-1 may have an important role in promoting malignant transformation of OE.

First, we found that CD163⁺ M2-like cells were outnumbered by

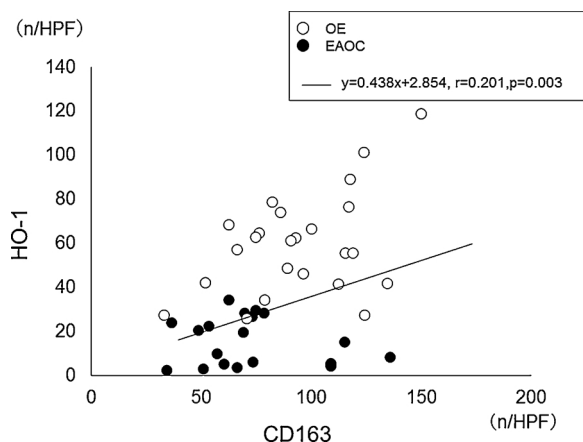


Fig. 3. Comparison of the association between CD163 and HO-1. The Pearson's correlation coefficient analysis for the correlation of CD163⁺ cells with HO-1⁺ cells. Plot of the number of HO-1⁺ cells against that of CD163⁺ cells.

CD11c⁺ M1-like cells in the two groups. M1 macrophages secrete inflammatory cytokines and contribute to the adaptive immune response through Th1 responses [8,18]. In contrast, anti-inflammatory M2 macrophages induce Th 2 responses and tissue repair [8,18]. Environmental stimuli shape macrophage plasticity in the OE and EAOC groups, and modify macrophages phenotypes from an anti-tumor M1 type to a pro-tumor M2 type [8,18–20]. It has been established that CD163 is classified into the marker of alternatively activated M2 phenotype and acts as a hemoglobin scavenger receptor on macrophage [5]. The possible role of macrophages in carcinogenesis has not been directly examined between the OE and its malignant transformation. However, our data support the finding that endometriosis environment itself has a propensity to develop into ovarian cancer.

Second, although HO-1 expression in the stromal macrophages has been seen in the other cancer tissues [21], the impact of HO-1 expression on EAOC progression has not been explored. We have found that the CD163⁺ M2-like cells were HO-1⁺ in OE, but malignant transformation may be associated with, and at least partly due to, reduced number of M2 phenotypes expressing HO-1. Endometriotic cyst fluid contains much higher levels of iron-related compounds, such as hemoglobin species, heme and free iron, compared with EAOC sample [22]. Repeated episodes of hemorrhage in OE induce excess oxidative stress and trigger DNA damage, mutations and genome instability, demonstrating the dichotomy between cytotoxicity and proliferation in endometriotic cells [1,2,19,23]. Thus, stimuli in environment create increased cellular susceptibility to oxidant-mediated cell killing or carcinogenesis. In benign OE, autoxidation and Fenton reaction of hemoglobin from the ferrous Fe²⁺ (oxyhemoglobin) state to the ferric Fe³⁺ (methemoglobin) state lead to production of excess reactive oxygen species (ROS), such as O₂⁻ and [•]OH, that are major sources of DNA damage and mutation [24]. HO-1 is strongly and rapidly induced in response to oxidative cytotoxic conditions, such as excessive production of ROS. The ferric Fe³⁺ rich in OE can induce a high expression of HO-1 [25], supporting the finding that the number of HO-1⁺ cells was significantly higher in the OE group than in the EAOC group. In contrast, EAOC showed lower oxidant activity than OE, due to the decreased expression of ferric Fe³⁺ (methemoglobin) and increased expression of ferrous Fe²⁺ (oxyhemoglobin) [2]. EAOC appears to be associated with an antioxidant environment and decreased response to oxidative stress. Our findings support that M2 phenotypes no longer produce HO-1 in the EAOC microenvironment. EAOC macrophages demonstrate sustained downregulation of an antioxidant marker, HO-1, possibly due to decreased oxidative stress. Taken together, the pattern of redox balance supports that reduced oxidative stress may be involved in the pathogenesis of malignant transformation.

Third, the expression of HO-1 in macrophages was decreased in some cancers such as lung cancer [26]. Theoretically, HO-1 down-regulation leads to the increase of ROS and DNA damage in cells, which may promote the initiation of carcinogenesis [27]. Conversely, HO-1 has been detected in tumor-infiltrating macrophages and shows the impact on cancer progression, aggressiveness, invasion, metastasis, and poor prognosis [27,28]. At late phase of tumorigenesis, HO-1 over-expression may promote cancer progression through inducing the expression of angiogenic factors, such as vascular endothelial growth factor (VEGF) [29]. Several lines of evidence have highlighted the role of HO-1 in cancer progression through modulating tumor microenvironment [27]. Thus, the function of HO-1 in the pathogenesis of cancer progression remains controversial.

In conclusion, the aberrant microenvironment in endometriotic milieu can induce alterations in macrophage recruitment and its polarization phenotype, which significantly induces the shift to M2 phenotype expressing HO-1. Reduced number of M2 macrophages expressing HO-1 may have an important role in promoting malignant transformation of endometriosis. Although the exact reasons for EAOC carcinogenesis are unclear at the present time, this study supports the homeostatic redox balance hypothesis that there are at least two phases

of EAO tumorigenesis: the initial wave of the reduced infiltration of M2 macrophages would be followed by the second wave of subsequent increase of ROS and DNA damage in endometriotic cells, and then the final big wave of EAO carcinogenesis. The specific molecular mechanisms by which reduced HO-1 expression favors the promotion of malignant transformation of endometriosis still require further investigation.

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This study was not funded.

Conflict of interest

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.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The protocols were approved by the ethics committee of Nara Medical University (Approved number 1570/2017). This study is a retrospective observational study, carried out by the opt-out method of our hospital website.

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