Evaluation of Serum Autoantibody Levels in the Diagnosis of Ovarian Endometrioma

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Objective: We analyzed autoantibodies against tumor-associated antigens (TAAs) in the serum of patients with endometrioma and healthy controls to determine whether autoantibodies can be accurate biomarkers for the diagnosis of ovarian endometrioma. **Methods:** Serum samples were obtained from 56 patients with endometriosis and 66 healthy women who served as normal controls. The titers of antibodies against a panel of eight TAAs were analyzed using enzyme-linked immunosorbent assay. **Results:** We found that the serum IGFII mRNA-binding protein 1 (IMP1) autoantibody and cyclin B1 autoantibody could discriminate between healthy controls and endometriosis patients (AUC–ROC 0.777; 95% confidence interval [CI] 0.694–0.860, $P < 0.0005$, and AUC–ROC 0.614; 95%

confidence interval [CI] 0.513–0.714, $P = 0.031$, respectively). Using 0.073 and 0.007 as the cutoff values for IMP1 and Cyclin B1 autoantibody, respectively, the sensitivity and specificity of IMP1 were 85.7 and 63.6%, respectively. When cyclin B1 was combined with IMP1, the specificity increased to 72.7% and the sensitivity slightly decreased to 83.9%. **Conclusions:** Our data suggest that IMP1 alone or combined with cyclin B1 seems to fulfill the requirements of sensitivity and specificity to become a useful clinical biomarker of endometrioma. However, further studies will be required to establish the predictive value and to support the clinical use of IMP1/cyclin B1 in the diagnosis and/or screening of endometriosis. *J. Clin. Lab. Anal.* 24:357–362, 2010. © 2010 Wiley-Liss, Inc.

Key words: endometriosis; autoantibody; IMP1

INTRODUCTION

Endometriosis is a common gynecologic disorder that presents with chronic pelvic pain or infertility and represents one of the most common admitting diagnoses in women of reproductive age. The “gold” standard for the diagnosis of endometriosis is laparoscopy. However, the invasiveness and associated morbidity of the laparoscopic procedure precludes its use for initial screening or monitoring recurrences and response to therapy. Less invasive tests, such as serologic testing for CA-125, have been widely used for detection of endometriosis and monitoring of progressive disease (1–4). However, a review of 23 articles showed a limited diagnostic performance of serum CA-125 in detecting endometriosis (5), so more effective biomarkers for endometriosis are needed.

Many studies demonstrated that sera from cancer patients contain autoantibodies which react with a

unique group of autologous cellular antigens generally known as tumor-associated antigens (TAAs) (6). Although the mechanisms underlying the production of such autoantibodies in cancer patients are not completely understood, emerging evidence indicates that many of the target antigens are cellular proteins whose dysregulation could have tumorigenic potential, such as mRNA binding protein p62 (7) and cell-cycle control protein cyclinB1 (8), or proteins whose aberrant regulation or overexpression could lead to tumorigenesis, such as p53 (9), HER-2/neu (10). The identification of novel autoantibodies is likely to be related to events

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associated with tumorigenesis. These autoantibodies can be used as reporters to identify aberrant cellular mechanisms in tumorigenesis, and can also serve as immunodiagnostic markers for cancer detection (6).

Although endometriosis is considered a benign disorder, it exhibits some characteristics similar to malignant lesions, such as cellular atypical hyperproliferation, invasion, metastasis, neoangiogenesis, and genomic instability (11), and current studies indicate some molecular aberrations commonly observed in cancer tissue are also present in endometriotic lesions (12,13). Besides, several studies have demonstrated that patients with endometriosis exhibit serum autoantibody response to cellular proteins (14–16). This finding led us to hypothesize that the sera of endometriosis patients contain antibodies which react with a unique group of TAAs, thus providing a new marker for endometriosis. Owing to the limited diagnostic performance of cancer antigen CA-125, the carbohydrate antigen currently used to detect endometriosis (5), alternative serum markers with higher sensitivity, specificity, and predictive value could help in the early detection and monitoring of disease progression as well as response to treatment (17). Thus, in this study, we evaluated whether the autoantibodies to TAAs can be accurate biomarkers for the diagnosis of ovarian endometrioma.

MATERIALS AND METHODS

Serum Samples and Antibodies

In this study, sera from 56 patients with endometriosis and normal human sera (NHS) from 66 healthy women were obtained from the Taichung Veterans General Hospital. All patient sera were collected when the patients were admitted for operation and had not yet received operation or other medical treatment. NHS were collected from adults who had no obvious evidence of malignancy, leiomyoma, adenomyosis, or endometriosis during their annual physical examination. Informed consent was obtained from all the women who were enrolled in this study. The research protocol was approved by the Ethics Committee for Human Research of Taichung Veterans General Hospital.

Expression and Purification of Tumor-Associated Antigens Fusion Protein

Eight TAAs were selected based on findings of previous reports (18). To generate TAA recombinant proteins, the cDNA of various TAAs were subcloned into a pET32a (Novagen) vector producing a fusion protein with NH-terminal 6x histidine and transformed into *Escherichia coli* strain BL21. After DNA sequences of expression constructs were obtained and confirmed,

the established bacterial clones were cultured and activated with IPTG in order to produce recombinant TAA protein antigens. Induction of expression of recombinant His-tagged recombinant protein was performed according to the protocol provided by Novagen. The (His)6-fusion protein was purified by using metal-affinity chromatography followed by gel filtration.

Enzyme-Linked Immunosorbent Assay

Purified recombinant proteins were diluted in coating buffer (50 mM NaHCO₃, 50 mM Na₂CO₃, pH 9.4) to a final concentration of 0.25 µg/µl for coating of the enzyme-linked immunosorbent assay (ELISA) plates (Costar EIA/RIA 96-well plate, Corning Inc., NY) overnight at 4°C. Wells were washed once with PBS containing 0.1% Tween 20 (PBST) and blocked using blocking buffer (2 mg/ml casein, 2 mg/ml bovine serum albumin (BSA) in PBS). Plates were washed again and the human serum samples were diluted at 1:200, incubated with the antigen-coated wells at 37°C for 90 min, followed by washing with PBS containing 0.05% Tween 20 (PBST). Then, the samples were incubated with horseradish peroxidase (HRP)-conjugated goat antihuman IgG (Caltag Laboratories, Burlingame, CA) as a secondary antibody diluted at 1:3000 for 90 min, followed by washing with PBST. The substrate 2, 20-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma, St. Louis, MO) was used as the detecting agent. The OD of each well was read at 405 nm. Each sample was then tested in duplicate. The detailed protocol of ELISA described by Rubin was used (19).

RESULTS

Frequency and Titers of Antibodies Against a Panel of Eight TAAs in Healthy Controls

One hundred and sixty women had peripheral venous blood drawn for autoantibody study, including 56 endometriosis patients and 66 healthy controls. The age of healthy controls ranged from 20 to 41 years with a mean of 33.1 years, and patients' age ranged from 18 to 45 years with a mean of 31.3 years. The absorbance readings of TAAs in the healthy controls are shown in Table 1. If mean+2SD was used as a cut-off value, the false-positive rate would be less than 5% for any TAAs; however, if a positive test for antibodies was taken as an absorbance reading above the mean+SD, a high false-positive rate would be observed in P53, Csk, P90, and P16. Based on the criteria of good discriminatory ability and a low false positive rate, IGFII mRNA-binding protein 1 (IMP1) and Cyclin B1 were chosen as the candidate biomarkers for further evaluation.

TABLE 1. Absorbance Reading of Eight TAAs in the Healthy Controls

	IMP1	P53	Cyclin-B1	Ras	Csk	Her-2/new	p90	P16
Absorbance reading (mean $\pm$ SD)	0.0639 $\pm$ 0.0359	0.1621 $\pm$ 0.0677	0.0338 $\pm$ 0.0431	0.0712 $\pm$ 0.0386	0.1877 $\pm$ 0.0565	0.0368 $\pm$ 0.0247	0.1636 $\pm$ 0.0578	0.2013 $\pm$ 0.0535
Number and percentage of autoantibody ^a	3(4.5)	2(3.0)	2(3.0)	2(3.0)	0(0)	1(1.5)	1(1.5)	1(1.5)
Number and percentage of autoantibody ^b	9(13.6)	60(90.9)	8(12.1)	9(13.6)	65(98.5)	11(16.7)	63(95.5)	66(100)

^aCut-off value: mean+2SD.^bCut-off value: mean+SD. **Fig. 1.** The absorbance reading of IMP1 and Cyclin B1 autoantibody in endometriosis patients and healthy controls.

Relationship Between TAAs and Endometriosis

The absorbance readings for IMP1 and Cyclin B1 autoantibodies in endometriosis patients and healthy controls are shown in Figure 1. The IMP1 and Cyclin B autoantibodies in the women with endometriosis were higher than in healthy controls, and the difference was significant ($P = 0.039$) for IMP1 autoantibodies.

Analysis of Diagnostic Accuracy of Serum Autoantibody Levels

As shown in Figure 2, the AUC-ROC curve was determined by plotting the ROC curve. Serum IMP1 autoantibodies could discriminate between healthy controls and endometriosis patients (AUC-ROC 0.777; 95% confidence interval [CI] 0.694–0.860, $P < 0.0005$). Similarly, serum Cyclin B1 autoantibodies could discriminate between healthy controls and endometriosis patients (AUC-ROC 0.614; 95% CI 0.513–0.714, $P = 0.031$).

Evaluation of Diagnostic Values of Serum Autoantibodies in Endometriosis

To further test if the IMP1 and Cyclin B1 autoantibodies can serve as endometriosis biomarkers, cutoff values were obtained by plotting a line parallel to the diagonal line on the ROC curve. The cutoff values for IMP1 and Cyclin B1 autoantibodies were correspondent to 0.073 and 0.007, respectively, when the line was tangential with the ROC curve. The sensitivity and specificity using these cutoff values are shown in Table 2. The sensitivity and specificity of IMP1 were 85.7 and 63.6%, respectively. With the addition of Cyclin B1, the specificity increased to 72.7% and the sensitivity slightly decreased to 83.9%. The Youden's index also increased from 0.493 to 0.566.

DISCUSSION

IMP1 is a member of the IMP family that determines the cytoplasmic fate of specific mRNAs. The IMP protein family directs mRNA localization, translation,

Fig. 2. Receiver operating characteristic (ROC) curves of MP1 and Cyclin B1 autoantibody for the diagnosis of ovarian endometrioma.

TABLE 2. Sensitivity, Specificity of TAAs

	Frequency (%)					
	Endometriosis (n = 56)	NHS (n = 66)	P value	Sensitivity	Specificity	Youden's index
<i>TAA</i>						
IMP1	48/56	24/66	0.00	85.7	63.6	0.493
Cyclin B1	53/56	42/66	0.00	94.6	36.4	0.310
IMP1 or cyclin B1	54/56	48/66	0.00	96.4	27.3	0.237
IMP1 and cyclin B1	47/56	18/66	0.00	83.9	72.7	0.566

or mRNA stability of their target transcripts. It has been shown that all IMP proteins are primarily expressed during embryogenesis and mid-gestation in mice with almost no detectable expression in the postnatal phase (20). Evidence indicates that IMP1 is essential for the regulation of c-myc, bTrCP1, and CD44 mRNA turnover, and also facilitates translational control of the IGF-II and b-actin mRNAs (7,21). Although the regulation of IGF-II and c-myc expression IMP1 is thought to affect cell proliferation, growth, and survival (22), the regulation of CD44 or b-actin expression via IMP1 is suggested to modulate cell adhesion, invadopodia [singular form—invadopodium] formation and actin dynamics (23).

In human cancers, de novo synthesis of IMPs has been identified in different tumors, such as colorectal carcinomas, bone and soft tissue sarcomas, breast carcinomas, and nonsmall cell lung carcinomas (22,24,25), and is associated with advanced clinical stage and poor prognosis (24,26,27). In primary ovarian carcinoma, overexpression of IMP mRNA (26) and protein (28) was also identified. In vitro studies on ovarian carcinoma-derived ES-2 cells demonstrated that

IMP1 knock-down reduced cell proliferation and survival associated with decreased c-myc mRNA half-life, suggesting IMP1 acts as an oncogenic factor that is involved in promoting elevated proliferation by stabilizing the c-myc mRNA in ovarian cells (28).

The role of IMP1 in the pathogenesis of endometriosis is still uncertain, but studies revealed that some genetic alterations and molecular aberration of ovarian cancer are also present in endometriotic lesions (11). Previous studies demonstrated that c-myc is overexpressed in endometriosis (12,29) and the deregulation of the MYC genes may be responsible for the loss of cellular homeostasis in endometriotic lesions (13). A potential role of IMP1 in endometriosis may be hypothesized because IMP1 is involved in promoting elevated proliferation by stabilizing the c-myc mRNA in ovarian cells (28).

Cyclin B1 is a key component of the cell cycle machinery. Cyclin B1 binds to cdc2 at the beginning of the G2 phase, forming an activated Cyclin B1-cdc2 complex (maturation-promoting factor) and then phosphorylates a number of important substrates to control the G2 to M transition. Previous study has revealed the

expression of Cyclin B1 in endometriosis, suggesting it may play an important role in the pathogenesis of endometriosis by mediating ectopic endometrial cell proliferation under regulation of ovarian hormones (30).

Whatever the putative role of IMP1 and Cyclin B1 in endometriosis is, the clinical significance of the higher serum IMP1 and Cyclin B1 autoantibody levels in women with ovarian endometrioma warrants consideration. To our knowledge, this is the first study to measure serum IMP1/Cyclin B autoantibody concentration in patients with ovarian endometrioma. These autoantibodies against TAAs were reported as immunodiagnostic markers for cancer detection (6), and initial studies focused on combined different TAAs autoantibodies panel to increase the sensitivity of cancer detection and claimed that the reactivity of NHS was low, ranging from 0 to 2.4% to any individual TAA (31). As we know, most current available tumor markers show limited specificity and sensitivity, and levels also increase under benign conditions. The increased serum autoantibodies against TAAs in ovarian endometrioma than healthy controls as described in our study, is a direct evidence that the TAAs autoantibody also increase in benign conditions, and interpretation of these biomarkers in cancer screening should be cautioned.

Besides demonstrating the possible limitation of IMP1 and Cyclin B1 autoantibody in the application in cancer screening, the statistically significant higher serum IMP1 and Cyclin B1 autoantibody levels in women with endometriomas suggest they can be a candidate biomarker of ovarian endometrioma. Ultrasonography and CA 125 measurements are the usual methods of noninvasive diagnosis of endometrioma, but they do not achieve optimal sensitivity and specificity. According to one study, CA 125 detects only half the cases of endometriosis stage III/IV with appropriate specificity (5). Other noninvasive methods, such as transvaginal ultrasonography and pelvic examination, achieve high sensitivity for ovarian endometriosis, but their false positive rate is still a reason for concern. On the other hand, our data suggest that IMP1 alone or combined with Cyclin B1 seems to fulfill the requirements of high sensitivity and reasonable specificity to become a useful clinical biomarker of endometrioma. However, our results are based on women with ovarian endometriomas, and in that condition, transvaginal ultrasound can achieve high sensitivity, but nevertheless, incorporate the biomarker with other serum test in health examination to eliminate multiple examination method as an attractive utility. Though IMP1 and Cyclin B1 that can be used as biomarker in ovarian endometrioma has been evaluated in our study; whether IMP1/Cyclin B1 also detects nonovarian endometriosis, as well as low-stage disease, are conditions for which a serum marker is even more

necessary, owing to the poor performance of noninvasive methods that are not evaluated in this preliminary study, and another utility of tumor markers: to predict the clinical outcome and response to treatment cannot be answered at present. However, our data revealed IMP1/Cyclin B1 autoantibody are candidate of endometriosis biomarker, but more time and further studies are needed to support the clinical use of IMP1/Cyclin B1 in the diagnosis and/or screening of endometriosis.

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