Occult microscopic endometriosis: undetectable by laparoscopy in normal peritoneum

Khaleque Newaz Khan1,*, Akira Fujishita2, Michio Kitajima1, Koichi Hiraki1, Masahiro Nakashima3, and Hideaki Masuzaki1

1Department of Obstetrics and Gynecology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan 2Saiseikai Nagasaki Hospital, Nagasaki, Japan 3Department of Tumor and Diagnostic Pathology, Atomic Bomb Disease Institute, Nagasaki, Japan

*Correspondence address: Tel: +81-95-819-7363; Fax: +81-95-819-7365; E-mail: nemokhan@nagasaki-u.ac.jp

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STUDY QUESTION: Is there any occurrence of hidden (occult) endometriotic lesions in normal peritoneum of women with and without visible endometriosis?

SUMMARY ANSWER: We detected a slightly higher occurrence of occult microscopic endometriosis (OME) in normal peritoneum of women with visible endometriosis than in control women.

WHAT IS KNOWN ALREADY: Based on a small number of cases, the concept of invisible microscopic endometriosis in visually normal peritoneum has been reported for more than a decade but there is controversy regarding their tissue activity and clinical significance.

STUDY DESIGN, SIZE, DURATION: This case-controlled research study was conducted with prospectively collected normal peritoneal samples from 151 women with and 62 women without visible endometriosis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Normal peritoneal biopsy specimens from different pelvic sites were collected during laparoscopy. A histological search of all peritoneal biopsy specimens for the detection of invisible endometriosis was done by immunoreaction to Ber-EP4 (epithelial cell marker), CD10 (stromal cell marker) and Calretinin (mesothelial cell marker). Tissue expression of estrogen/progesterone receptors (ER/PR) and cell proliferation marker, Ki-67, was performed by immunohistochemistry to identify tissue activity.

MAIN RESULTS AND THE ROLE OF CHANCE: Three different patterns of OME were detected based on (I) the presence of typical gland/stroma, (II) reactive hyperplastic change of endometrioid epithelial cells with surrounding stroma and (III) single-layered epithelium-lined cystic lesions with surrounding stroma. A higher tendency toward the occurrence of OME was found in women with visible endometriosis (15.2%, 23/151) compared with control women (6.4%, 4/62) ($P = 0.06$, $\chi^2$ test). The epithelial cells and/or stromal cells of OME lesions were immunoreactive to Ber-EP4 and CD10 but not reactive to Calretinin. ER and PR expression was observed in all patterns of OME lesions. Ki-67 index was significantly higher in pattern I/II OME lesions than in pattern III OME lesions ($P < 0.05$ for each).

LIMITATIONS, REASONS FOR CAUTION: Bias in the incidence rate of OME lesions in this study cannot be ignored, because we could not analyze biopsy specimens from the Pouch of Douglas of women with revised classification of the American Society of Reproductive Medicine Stage III–IV endometriosis due to the presence of adhesions in the pelvis.

WIDER IMPLICATIONS OF THE FINDINGS: We re-confirmed a decade long old concept of invisible (occult) endometriosis in visually normal peritoneum of women with visible endometriosis. The existence of a variable amount of tissue activity in these occult lesions may contribute to the recurrence/occurrence of endometriosis or persistence/recurrence of pain manifestation in women even after successful ablation or excision of visible lesions by laparoscopy.

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**Introduction**

Endometriosis is an estrogen-dependent chronic inflammatory disease mostly affecting women of reproductive age. Originally described over 300 years ago, endometriosis is classically defined by the presence of endometrial glands and stroma in extraterine locations (Burney and Giudice, 2012). There are some established hypotheses and regulatory factors supporting the development or maintenance of endometriosis (Attar and Bulun, 2006; Burney and Giudice, 2012). Recently, it has been demonstrated that besides hormonal regulation, both initial and secondary inflammatory mediators are known to be involved in the growth of endometriosis (Khan et al., 2008, 2009, 2010a,b). However, it is difficult to uniformly explain the pathogenesis of endometriosis by a single factor. Even after many years, most of the literature claims that the exact pathogenesis of endometriosis is unclear and that endometriosis is an enigmatic disease. The recurrence of pain and lesions is still occurring after effective medical or surgical therapies.

The detection and visible diagnosis of peritoneal endometriosis is usually performed by laparoscopy, a gold standard modality, and is microscopically confirmed by histopathology. Even with the careful eyes of expert surgeons, there is an obvious risk of missing or overlooking hidden (occult) lesions in visually normal peritoneum. For this reason, it is necessary to randomly collect visually normal peritoneum from different anatomical locations in the pelvis and to investigate the nature of any visually undetectable lesions of endometriosis. The concept of microscopic endometriosis in visually normal peritoneum was first reported by Murphy et al. (1986) and subsequently confirmed with an incidence rate of 6–13% (Nisolle et al., 1990; Balasch et al., 1996). However, with the elapse of more than one decade, further information on invisible or occult microscopic endometriosis (IME or OME) is lacking.

Therefore, we histologically examined biopsy specimens derived from the visually normal peritoneum of women with and without endometriosis to detect the possible occurrence of OME. The question remains, if hidden endometriosis lesions can be detected in normal peritoneum, are these invisible lesions really inactive as proposed in a previous report (Donnez and Langmondckt, 2004) or do they retain some tissue activity. If there is tissue activity in OME, this could be a clinically important issue. To address this question, we investigated the expression patterns of some tissue activity markers including ovarian steroid receptors and a cell proliferation marker in histologically confirmed OME lesions. With the belief in mind that all cells of epithelial origin are embryologically derived from coelomic epithelium, we further extended our experiment to examine the immunoreaction of CA125/MUC16, a marker of cells derived from coelomic epithelium and its derivatives, in visible peritoneal lesions and in OME lesions. Finally, we discussed the possible origin of OME.

**Materials and Methods**

**Subjects**

During the period between January 2005 and June 2010, peritoneal biopsy samples from different anatomical locations in the pelvis were randomly and prospectively collected from women with and without visible endometriosis during laparoscopy (Storz, Germany). With the concept of occult endometriosis in mind, we collected peritoneal biopsy samples together with visible peritoneal lesions. During this study period, we were able to collect a total of 895 peritoneal biopsy samples from 387 women with and without endometriosis.

The anatomical location in the pelvis and quality of each collected peritoneal biopsy specimens were retrospectively reviewed and confirmed by video image and tissue observation (K.N.K., A.F., M.K.). Visually normal peritoneum was examined from a distance of 3–4 cm and an effort was made to select normal peritoneum based on the following criteria: (i) smooth peritoneal surface with no text irregularity, (ii) no abnormal vascular pattern, (iii) transparent peritoneum, (iv) no sub-peritoneal cystic structures and (v) no superficial fibrosis. A sample of visually normal peritoneum is shown in Fig. 1. We used our laparoscopic technique where the laparoscope was 3–4 cm away from the peritoneal surface; the technique is very similar to that described by Nisolle et al. (1990). After careful observation and analysis, we could finally sort out 227 visually normal peritoneal samples from 151 women with visible endometriosis and 78 samples from 62 women without any visible peritoneal lesions (controls). We did not analyze remaining peritoneal samples from the remaining 174 women, because these samples did not satisfy the criteria of normal peritoneum.

**Figure 1** Photographs of visually normal peritoneum observed during laparoscopy (Storz, Germany) and taken from the area of Pouch of Douglas (A) and uterovesical space (B). The criteria of visually normal peritoneum are described in the Materials and Methods section.
confirmed by histology. The control group, between 19 and 48 years old, consisted of fertile women, without any evidence of visible endometriosis, who were operated on for benign ovarian cysts (dermoid cyst/serous cyst adenoma/mucinous cyst adenoma). The staging and the morphological distribution of the peritoneal lesions were based on the revised classification of the American Society of Reproductive Medicine (r-ASRM) (1997). All control women and women with endometriosis had regular menstrual cycles (28–35 days) except cases undergoing hormonal therapy. The phases of the menstrual cycle were determined by histological dating of eutopic endometrial samples taken simultaneously with the pathological lesions during laparoscopy.

The distribution of biopsy specimens from visually normal peritoneum, based on anatomical location in the pelvis, is as follows. Women with visible endometriosis (n = 227) had samples from the Pouch of Douglas (n = 87), uterovesicle space (n = 104), right sacrouterine ligament (n = 20) and left sacrouterine ligament (n = 16), while control women (n = 78) had samples from the Pouch of Douglas (n = 35), uterovesicle space (n = 35), right sacrouterine ligament (n = 6) and left sacrouterine ligament (n = 5). We could not collect biopsy specimens from the Pouch of Douglas in women with r-ASRM Stage III–IV endometriosis due to the presence of adhesions. Instead, we collected peritoneal biopsy specimens from other anatomical locations in these women. After holding up the peritoneum with forceps, all peritoneal biopsy samples were collected from their respective sites by cutting with scissors a length of 1.0–2.5 cm at 1 cm depth. In our initial trial, we could not collect enough connective tissue just by punch biopsy from any of the anatomical sites.

All biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and were approved by the Institutional Review Board of Nagasaki University. Informed consent was obtained from all women.

**Antibodies**

We performed immunohistochemical studies to investigate immunoreactivity of target antigens in the serial sections of biopsy in the following antibodies: Ber-EP4 (epithelial cell marker, 1:200, M0804, mouse monoclonal, Dako, Denmark); CD10 (stromal cell marker, 1:40, 56C6, mouse monoclonal, Dako, Denmark); Calretinin (mesothelial cell marker, 1:50, SP13, rabbit monoclonal, Nichirei Bioscience, Japan); ER (estrogen receptor, 1:50, ER1DS, mouse monoclonal, Dako, Denmark); PR (progesterone receptor, 1:40, NCL-PGR, mouse monoclonal, Dako, Denmark); aromatase cytochrome P450 (aromatase metabolizing enzyme, 1:100, ab18995, rabbit polyclonal, abcam, Japan); Ki-67 (cell proliferation marker, 1:100, MIB-1, mouse monoclonal, Immunotech, Marseille, France); Toll-like receptor 4 (TLR4, a pattern recognition receptor, 1:50, ab22048, mouse monoclonal, abcam, Japan); D2-40 (lymphatic vessel marker, 1:100, M3619, mouse monoclonal, Dako, Denmark); von Willebrand factor (VWF, micro-vessel marker, 1:25, FB/86, M0616, mouse monoclonal, Dako, Denmark); CA125/MUC16 (marker of cells derived from coelomic epithelium, 1:20, M11, mouse monoclonal, Dako, Denmark). Non-immune mouse immunoglobulin (Ig) G1 antibody (1:50, Dako) was used as a negative control.

In an attempt to detect hidden lesions of endometriosis, all slides of biopsy specimens derived from visually normal peritoneum were histologically examined and any specimen with microscopic lesions suspected of being endometriosis was re-examined and confirmed by an expert histopathologist (M.N.).

**Immunohistochemistry**

The details of immunohistochemical staining were described elsewhere (Khan et al., 2003, 2004, 2005; Ishimaru et al., 2004). We used at least three slides per biopsy for immunohistochemical analysis. Briefly, 5-μm thick paraffin-embedded tissues were deparaffinized in xylene and rehydrated in phosphate-buffered saline. After immersion in 0.3% H2O2/methanol to block endogenous peroxidase activity, sections were pre-incubated with 10% normal goat serum to prevent non-specific binding and then incubated overnight at 4°C with respective antibodies. The slides were subsequently incubated with biotinylated second antibody for 10 min, followed by incubation with avidin-peroxidase for 10 min and visualized with diaminobenzidine. Finally, the tissue sections were counterstained with Mayer’s hematoxyline, dehydrated with serial alcohol rinses, cleared in xylene and mounted.

The immunoreactivities of ER/PR, aromatase cytochrome P450, TLR4 and CA125/MUC16 in biopsy specimens were quantified by a modified method of quantitative-histogram score (Q-H score) as described elsewhere (Khan et al., 2003, 2005; Ishimaru et al., 2004). The Q-H score was calculated using the following equation: Q-H score = ΣP/(i + 1), where i = 0, 1, 2 or 3 and P is the percentage of stained cells for each intensity. The staining intensity was graded as 0 = no, 1 = weak, 2 = moderate and 3 = strong. We calculated the mean Q-H scores of five different fields of one section by light microscopy at moderate magnification (×200).

The cell proliferation index (Ki-67 index) in each tissue section was calculated by measuring the mean percentage of Ki-67-positive nuclei among total cells in four different microscopic fields (×200) as we described previously (Khan et al., 2010b). The lymphatic vessel and micro-vessel density, as measured by total vessel number and as immunoreactive to D2-40 and VWF, respectively, were counted by light microscopy of those areas that contained the highest number of lymphatics, capillaries and venules, as described elsewhere (Khan et al., 2003). The total lymphatic vessel and micro-vessel counts were evaluated in the same specimen.

By the term ‘tissue activity’ in this study, we mean the capacity of a tissue or OME lesion to express different molecular markers and to have variable proliferative potentiality in the examined tissue or lesion.

**Statistical analysis**

All results are expressed as either mean ± SD or mean ± SEM. The clinical characteristics of the subjects were compared with one-way analysis of variance and the χ2 test was used for any difference between two groups. Mann–Whitney U-test or Student’s t-test was used to analyze any difference in protein expression between two groups. For comparisons among groups, the Kruskal–Wallis test was used. A box plot analysis of different protein expression was performed using the medians and inter-quartile range (IQR). A value of P < 0.05 was considered to be statistically significant.

**Results**

There were no significant differences in clinical characteristics between the 151 women with visible endometriosis and the 62 control women without endometriosis (Table I). We collected visually normal peritoneum from these two groups of women for our study purpose. All OME lesions were detected at a distance of 100–800 μm beneath the mesothelium of normal peritoneum.

We detected three patterns of OME: (I) the presence of typical gland/stroma, (II) reactive hyperplastic change of endometrioid epithelial cells with surrounding stroma and (III) single-layered epithelium-lined cystic lesions with surrounding stromal cells. We could detect variable patterns of OME in the peritoneum derived from 23 women with endometriosis (biopsy samples, n = 27) and 4 control women (biopsy samples, n = 4) without visible endometriosis. The detection rate of OME, by the number of patients and number of collected samples, respectively, was as follows: for women with endometriosis, 15.2% (23/151) and 11.8% (27/227); for control women, 6.4% (4/62) and 5.1% (4/78). A higher
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The concept of stromal endometriosis in mind, we found only three cases. We did not include them as a separate pattern (pattern IV) due to the small sample number. Each of these three cases with endometriotic stroma was immunoreactive to CD10 and non-reactive to Calretinin. The distribution of the three patterns of OME was as follows: women with endometriosis, pattern I (n = 18), pattern II (n = 9) and pattern III (n = 6); control women, pattern I (n = 3), pattern II (n = 0) and pattern III (n = 1). A total of 33 and 4 IME lesions were detected in women with visible endometriosis and control women, respectively. The detection pattern of OME lesions based on anatomical site of peritoneum was as follows: women with endometriosis (total sample, n = 27), Pouch of Douglas (n = 6), uterosacral space (n = 10), right sacrouterine ligament (n = 5) and left sacrouterine ligament (n = 6); control women (total sample, n = 4), Pouch of Douglas (n = 1), uterosacral space (n = 2), right sacrouterine ligament (n = 0) and left sacrouterine ligament (n = 1). A predominance of OME occurrence was observed in the Pouch of Douglas and uterosacral space.

Some normal peritoneal samples displayed more than one OME lesion. Therefore, a total of 33 OME lesions were detected in 27 peritoneal samples in women with visible endometriosis. In two cases, we found peritoneal pockets in the peritoneum derived from the rectovaginal pouch of women with visible endometriosis but these two cases lacked OME lesions anywhere in the pelvis including pockets.

The clinical profiles of patients with OME in the 23 women with visible endometriosis and 4 control women are shown in Table III.

### Immunostaining pattern of Ber-EP4/CD10/Calretinin in OME

We found that the glandular epithelial cells/rim-like epithelial cells and surrounding stromal cells of three patterns of OME lesions were immunoreactive to Ber-EP4 and CD10, respectively, but not reactive to Calretinin, which is a marker of mesothelial cells (Fig. 2). Only three OME lesions of pattern II or III showed negative immunoreaction Ber-EP4, CD10 and Calretinin. Flat mesothelial cells derived from normal peritoneum were immunoreactive to Calretinin, not reactive to CD10 and partially reactive to Ber-EP4. We used biopsy specimens from mesothelium as a positive control and found that these cells were strongly immunoreactive to Calretinin (inset, Fig. 2).

### Immunostaining pattern of ER and PR in OME

A variable pattern of ER and PR immunoreexpression was observed in all OME lesions detected in women with visible endometriosis and control women (Fig. 3A and B). The immunoreactivity of PR as measured by Q-H score appeared to be higher in all patterns of OME lesions.
detected in women with visible endometriosis when compared with ER expression (Fig. 3C). In contrast to OME lesions, ER/PR expression did not show any apparent difference in the corresponding eutopic endometria of these two groups of women across the phases of the menstrual cycle (data not shown).

**Immunostaining pattern of aromatase in OME**
In an attempt to examine the possibility of local estrogen production by OME lesions, we found that all of the OME lesions expressed a variable amount of aromatase P450. An apparent immunoreaction to aromatase was found in visible peritoneal lesions and in OME lesions derived from women with visible endometriosis or control women (Supplementary data, Fig. S1).

**Immunostaining pattern of TLR4 in OME**
As a pattern recognition receptor, immunoreexpression of TLR4 was found in red and black lesions, and in all patterns of OME from women with visible endometriosis and control women (Fig. 4A–C). In addition to visible and occult lesions of endometriosis, TLR4 immunoreactivity was also found in micro-vessels as observed in different anatomical sites of peritoneum derived from women with and without endometriosis (Fig. 4D).

**Immunostaining pattern of D2-40 and VWF in OME**
As a marker of lymphatic vessels and vascular micro-vessels, we examined immunoreaction of D2-40 and VWF, respectively, in serial sections derived from visually normal peritoneum. A variable immunoreexpression of D2-40 and VWF was found in lymphatic cells and micro-vessels of all examined biopsies derived from women with visible endometriosis and control women. The number of D2-40/VWF immunoreactive lymphatic/vascular channels appeared to be higher in women with visible endometriosis when compared with ER expression (Fig. 3C). In contrast to OME lesions, ER/PR expression did not show any apparent difference in the corresponding eutopic endometria of these two groups of women across the phases of the menstrual cycle (data not shown).

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endometriosis than in controls in the peritoneum adjacent to OME lesions. In contrast, lymphatic/vascular numbers appeared to be slightly higher in controls than in women with endometriosis in the peritoneum distant from OME lesions. There was no significant difference in the lymphatic or micro-vessel counts between these two groups of women in either area (Supplementary data, Figs S2 and S3).

Immunostaining pattern of CA125/MUC16 in OME

A variable immunoexpression of CA125/MUC16 was found in red/black lesions, pattern I/II/III OME lesions detected in women with visible endometriosis and in pattern I/III OME lesions detected in control women (Fig. 6A–C). It was interesting to observe that all three OME lesions (pattern II/III) that were not reactive to Ber-EP, CD10 or Calretinin were still immunoreactive to CA125/MUC16 (Fig. 6D). We did not find any difference in the immunoreactivity of CA125/MUC16 among these visible and occult lesions as measured by Q-H scores (data not shown). Normal peritoneal mesothelium was also found to be immunoreactive to CA125/MUC16 (inset, Fig. 6D).

Discussion

We have re-established a more than decade old concept of IME (Redwine, 1988a,b, 1990; Balasch et al., 1996) using a large number of visually normal peritoneal samples. In order to avoid confusion in the use of the term ‘invisible’ or ‘non-visible’ microscopic endometriosis among laparoscopists, here we used the term ‘occult’ microscopic endometriosis instead of IME to indicate any hidden lesion in visually normal peritoneum. We demonstrated that the occurrence of OME in visually normal peritoneum was higher in women with visible peritoneal endometriosis than in control women. Our detection rate of OME in women with visible endometriosis (15.2%) and in control women (6.4%) is in consistent with the published incidence rate of 6–13% (Nisollle et al., 1990) and of 6–11% as reported by Balasch et al. (1996). Although rejected previously by Redwine (2003), we have re-ignited the controversial debate that the concept of IME or OME indeed exists. Our findings coincide with the findings of Nisollle et al. (1990), Balasch et al. (1996) and Walter et al. (2001) and have further reinforced the concept of OME, the so-called IME.

We support the opinion of Donnez and Langendonckt (2004), and oppose the proposal made by Redwine (2003) that OME is a rare and
clinically unimportant entity. We followed some criteria of visually normal peritoneum and collected a large number of samples over a period of 5 years to re-confirm the previous concept of IME. Unlike previous studies (Balasch et al., 1996; Donnez and Langeronckt, 2004), we confirmed our findings by random collection of peritoneal biopsy specimens from different anatomical locations and not from a single site. In fact, we found a higher incidence rate of OME in dependent part of pelvis and not in sacrouterine ligaments as reported previously.

The classical histological diagnosis of endometriosis differs between gynecologists and histopathologists. All clinical gynecologists accept diagnosis of endometriosis based on the findings of typical endometrial glands with a peripheral rim of stromal cells. But the majority of histopathologists disagree with this definition and define endometriosis just by the presence of stromal cells around cells of coelomic epithelium such as gland cells, endometrioid epithelial cells or mesothelial cells. Since this is a histopathology-based study, we defined endometriosis according to the opinion of an expert pathologist (M.N.).

In line with the opinion of our pathologist (M.N.), Redwine and Yokom (1990) reported that any glandular structure within peritoneal tissue cannot be accepted as true endometriosis without the presence of stroma around glands. With this knowledge in mind, we classified OME lesions into three categories based on the presence of stromal cells in and around glandular structure (pattern I), hyperplastic endometrioid epithelial cells (pattern II) and single-layered epithelium-lined cystic lesions (pattern III). All these three patterns of OME lesions were immunoreactive to Ber-EP4/CD10 but not reactive to Calretinin. The immunoreaction of Ber-EP4 in pattern II and III lesions may dictate the usefulness of Ber-EP4 staining in differentiating the rim of epithelial cells that might be confused with mesothelial-like cells by hematoxylin and eosin stain. In fact, the antibody against Ber-EP4 has been reported to distinguish epithelia from mesothelia (Latza et al., 1990). Only three OME lesions of pattern II/III showed negative immunoreaction to Ber-EP4 and CD10 as well as Calretinin. A lack of cellular differentiation or improper histogenesis of cells may explain this negative immunoreaction. But we cannot exclude the possibility of endosalpingiosis for these three lesions. Further in depth investigation is needed to clarify this issue.

In our study, laparoscopic tip differed from that used in previous studies by (Redwine, 2003; Nezhat et al., 1991), such that the distance between the tip and the peritoneal surface was ≏4 cm instead of 2 cm or less. Thus, it is quite possible that some OME lesions were detected under microscope rather than by the visible power of laparoscopic tip or the sharp visual acuity of surgeon’s eyes. We presume, however, that it is not always possible to identify a lesion located at 100–800 μm beneath the mesothelium of normal peritoneum even with the laparoscopic lens in close proximity to the peritoneal surface. In fact, our findings clearly support the findings as depicted in Fig. 2 of Redwine’s review article (2003). It is clear from the findings of Redwine (2003) that with a distance of 3–4 cm from the peritoneal surface, the incidence of IME (here OME) ranges from 5 to 10%, which coincides with our findings and the findings.

Figure 4  Hematoxylin and eosin stain (upper column) and immunohistochemical staining of TLR4 (lower column) in peritoneal endometriosis (A), different patterns of OME detected in women with visible endometriosis (B) and in control women (C). TLR4 immunoreaction was also observed in microvessels of normal peritoneum derived from the pouch of Douglas (D, upper column) and uterovesicle space (D, lower column) of women with endometriosis and control women. Magnification of (A–D) (×200).
of Nisolle et al. (1990) and Balasch et al. (1996). However, due to our large sample size, our samples were likely to contain some retroperitoneal glandular inclusions. Therefore, further study with a contact laparoscope closer to the peritoneal surface is necessary to carefully differentiate normal from abnormal peritoneal samples and determine the incidence of OME.

We found a variable amount of tissue expression of ER, PR, aromatase and TLR4 in OME lesions and these were detected in samples from both women with visible endometriosis and control women. Although, PR expression appeared to be higher than ER expression in OME lesions, there was no obvious difference in the expression of steroid receptors in the corresponding endometria across the phases of menstrual cycle. Higher PR expression and lower ER expression in OME lesions could be due to the difference in the binding affinities of the antibodies to their respective antigens. The higher PR expression may be involved in a proper decidual reaction and slow progression of these OME lesions once they are established. The immunoexpression of aromatase in all OME lesions is another interesting finding. With the influence of both systemic and local estrogen, these OME lesions, even though minute in size, may in time increase in size to be recognized by histology.

We previously reported from our laboratory (Khan et al., 2010a) that a small amount of lipopolysaccharide (LPS) is available in the pelvis across the phases of the menstrual cycle. This LPS derived from higher colony formation of Escherichia coli in menstrual blood (Khan et al., 2010a) may promote the growth of OME lesions after its binding with TLR4. In fact, we found moderate expression of TLR4 in all OME lesions. This growth promoting effect was clearly supported by the significantly higher index of Ki-67, a cell proliferation marker, in pattern I and pattern II OME lesions diagnosed in women with visible endometriosis than in pattern III OME lesions or lesions from control women. We are against the argument by Donnez and Langendonckt (2004) that IME (or OME) lesions are quiescent and inactive and that these lesions are clinically irrelevant. From our findings, we can at least argue that OME lesions are indeed active and retain variable growth potentiality in response to cyclic estrogen and/or various inflammatory mediators in pelvis, even if their concentration is minimal. They could be responsible for the subsequent recurrence or occurrence of endometriotic lesions even after successful excision or ablation of visible peritoneal lesions by laparoscopy. From a logical point of view, it is difficult to trace these growing lesions on the peritoneal surface by repeated surgical

Figure 5  Hematoxylin and eosin stain (upper column) and immunohistochemical staining of Ki-67 (lower column), a cell proliferation marker, in lesions derived from visible peritoneal endometriosis (A) and different patterns of OME detected in women with endometriosis (B) and in control women (C). The Ki-67 index (percentage of Ki-67 immunoreactive cells among total cells) in the respective lesions is shown in the lower right panel (D). Ki-67 index was significantly higher in red lesions (white bar) than in black lesions (hatching bar, *P < 0.05). Ki-67 index was also significantly higher in pattern I or II OME lesions (white/hatching bar) than in pattern III lesions (gray bar, P < 0.05 for each) detected in women with visible endometriosis. Boxes represent the distance (IQR) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values. Magnification of (A–C) (×200).
procedures in human. Therefore, the consistency between Fig. 2 of Redwine’s article (2003) and the existence of variable biological activity of OME lesions in our study can be considered as something new and important in both biological and clinical science. We can at least speculate from our current findings that OME lesions are not clinically irrelevant rather more or less clinically significant, because these subtle lesions of OME displayed some degree of biological activity.

The questions may arise now: ‘How can we decide the origin of OME lesions?’ or ‘Is Sampson’s theory enough to explain OME lesions?’ (Sampson, 1927a,b). There is no definite answer at this moment. But we argue that we can link each theory supporting the origin of visible endometriosis (Burney and Giudice, 2012) to the pathogenesis of OME lesions. If Sampson’s theory does not directly support the origin of OME lesions, they could be indirectly explained by the lymphatic or hematogenous spread of menstrual debris and its subsequent localization within lymphatic/vascular channels deep in the peritoneum as already proposed (Sampson, 1927a,b; Javert, 1952; Hey-Cunningham et al., 2011). However, our current findings did not support this phenomenon for OME. We failed to locate any OME lesion within lymphatic channels or vasculatures. Instead, we found an increased angiogenic and lymphangiogenic response in and around OME lesions. This could be due to local inflammatory reaction in the pelvis of women with visible endometriosis or to the activity of OME lesions.

We cannot exclude the possibility of genetic factors or metaplastic transformation of peritoneal mesothelial cells in response to inflammation or environmental factors (Burney and Giudice, 2012). Despite the possible origin of OME as a result of epithelial–mesenchymal transition/mesenchymal–epithelial transition or from stem cells (Gaetje et al., 1997; Sasson and Taylor, 2008; Maruyama and Yoshimura, 2012), embryonic development (Mülleriosis) within peritoneum may be another possible mechanism to explain the origin of IME, as described by Redwine (1988a,b).

In addition to recognizing Müllerian tissue and epithelial ovarian cancer cells, CA125/MUC16 can be used as a marker to identify cells derived from coelomic epithelium (i.e. embryonic origin) and its derivatives. With this concept in mind, we extended our experiments to immunolocalize cells of embryonic origin by immunoreaction to CA125/MUC16 in OME lesions. CA125 is a high molecular weight mucin-type glycoprotein encoded by MUC16 gene. CA125/MUC16 is expressed in epithelial ovarian cancer cells and in the apical surface of coelomic epithelia and its derivatives such as endometrium, mesothelial cells lining body cavities.

Figure 6 Hematoxylin and eosin stain (upper column) and immunohistochemical staining of CA125/MUC16, a marker of cells derived from coelomic epithelium (lower column) in visible peritoneal endometriosis (red/black lesions A), different patterns of OME detected in women with visible endometriosis (B) and in control women (C). Pattern II and III OME lesions that were non-reactive to Ber-EP4/CD10/Calretinin were still immunoreactive to CA125/MUC16 (D). Normal peritoneal mesothelium appeared to be immunoreactive to CA125/MUC16 (inset, D). Magnification of (A–D) (×200 and ×400).
and Fallopian tube (Cheon et al. 2009). As a marker of coelomic epithelial cells, we found immunoreaction of CA125/MUC16 in visible endometriosis and in all patterns of OME lesions. Three cases with OME lesions, which were not reactive to Ber-EP4, CD10 or calretinin, were still immunoreactive to CA125/MUC16. Our findings were further supported by the immunoreaction of CA125/MUC16 to normal peritoneal mesothelium. These findings may support the notion that cells of visible and occult endometriosis could be of embryonic origin (Mülleriosis) and CA125/MUC16 can be expressed irrespective of the presence of peritoneal pockets and anatomical sites of OME lesions. We speculate that the transformation of peritoneal mesothelial cells (metaplasia theory) or the time-dependent activation of coelomic epithelium (induction theory) within the peritoneum may explain the origin of OME lesions in our study.

Although the theory of Mülleriosis cannot be ignored, as proposed by Batt and Yeh (2013), as an additional factor in the origin of OME lesions from Müllerian tissue associated with peritoneal pockets, our findings did not support this theory. Even though we could detect two cases with peritoneal pockets derived from rectovaginal pouch in women with visible endometriosis, both of these cases lacked OME lesions anywhere in the pelvis, including the peritoneal pockets.

Finally, we conclude that existence of OME, previously described as IME, in visually normal peritoneum is true and acceptable. We refute the previous argument, and confirm here, by serial experiments, that OME lesions are indeed biologically active and retain their growth potential. This may be involved in the persistence and/or recurrence of pain, and recurrence of endometriosis after successful surgery or in the possible development of time-dependent overt endometriosis. Further studies are needed to strengthen our current findings.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

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**Authors’ roles**

K.N.K. was involved in the concept, study design, experiments, data analysis and manuscript writing; A.F., M.K. and K.H. contributed equally to sample collection and experimental assistance; M.N. was involved in histopathological reading and experimental advice and H.M. was involved in reading the draft manuscript.

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**Conflict of interest**

The authors declare that there is no conflict of interest related to this article.

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