行政院國家科學委員會專題研究計畫　成果報告

氧化性壓力引發子宮內膜異位症進行性之研究

計畫類別：個別型計畫
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計畫主持人：高淑慧

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附件一

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

氧化性壓力引發子宮內膜異位症進行性之研究

計畫類別：■ 個別型計畫　　□ 整合型計畫
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共同主持人：
計畫參與人員：趙湘台 副教授

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

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執行單位：**臺北醫學大學生物醫學技術研究所**

中　華　民　國　2003 年 10 月 31 日

中文摘要

根據資料顯示婦女罹患子宮內膜異位症的發生率愈來愈高，其發生率約佔婦女人口的15%-50%。依據研究顯示，子宮內膜異位症常為女性不孕的主要族群，大約 50%的不孕婦女患有此一疾病。所謂子宮內膜異位症是子宮內膜(endometrium)生長於腹腔內子宮以外的部位。此種異位性內膜上皮細胞具有可侵入性(invasion)及轉移性(metastasis)的特性。迄今，對於子宮異位性內膜上皮細胞侵入性及轉移性的機轉及成因尚未清楚。許多學者認為子宮內膜異位症與氧化壓力(oxidative stress)有關，在異位的組織受到發炎反應影響而有較高含量的氧化傷害(oxidative damage)，而使 oxidatively modified complexes 增加。此外，在免疫系統上, 經由 cytokine 及 chemokine 活化 macrophage 也會造成發炎反應及增加氧化性傷害。我們在先前的研究證明在子宮內膜異位症患者的病灶處的檢體中，檢測出高含量的氧化性傷害物質,如 8-OH-dG 及脂質過氧化產物(lipoperoxide)及粒線體基因突變的堆積(mitochondrial DNA mutation)。尤其於巧克力囊腫(chocolate cyst)檢測出遠高於正常值 5 倍的氧化性傷害如 8-OH-dG 及脂質過氧化產物。並且於病灶處標本中檢測出新的粒線體基因斷損突變（5755 bp 斷損)的堆積。根據實驗結果顯示，我們推測氧化性傷害於子宮異位性內膜上皮細胞引發(initiation)及進展(progression)扮演重要的角色。本計畫中我們將子宮內膜異位上皮細胞及基質細胞(stromal cell)加以分離，分別探討這兩者細胞於氧化性傷害(如 H_2O_2 and TCDD)處理後的分別變化。此外，針對體外初期培養的內膜細胞加以氧化劑處理(如 H_2O_2 and TCDD)，並且檢測內膜細胞上皮細胞侵入性增加 168%及 156%。由實驗結果得知 oxidative stress 確實使子宮內膜細胞上皮細胞侵入性增加，氧化傷害增加。未來尋找可降低侵入性及轉移性的因子及治療方法，使此疾病的治療有更進一步的進展。

關鍵字：子宮內膜異位症、侵入性、轉移性、粒線體 DNA 突變、氧化性傷害

Abstract

Endometriosis is a considerable threat to the physical, psychological and social integrity of women. More, up to 50% of infertile patients have this disease. The etiology and pathogenesis of this important disease is poorly understood, which is defined as the ectopic location of the endometrium-like glandular epithelium and stroma ouside the uterine cavity. Clinical observations and *in vitro* experiment imply that endometriotic cells are invasive and able to metastasize. To date, however, little is know about the mechanisms of invasion and metastasis in endometriosis. As a result of such stress, a sterile, inflammatory reaction with the secretion of growth factors, cytokines, and chemokines is generated, which is deleterious especially to successful reproduction. In our preliminary data, the significantly higher amounts of oxidative damages were detected in endometriotic lesions than in controlled normal endometrium such as the mitochondrial DNA rearrangement, 8-OH-deoxyguanosine (8-OH-dG), and lipoperoxide contents (TBA reacted compounds). There were approximately four to five folds of 8-OH-dG and lipoperoxides in the chocolate cyst. A novel 5755 bp deletion of mtDNA was identified in the endometriotic tissue. After DNA sequencing, the junction sites at nucleotide position (np) 8062-8064 or np 13819-13821 (5' to 3') on the heavy strand of mtDNA. According to these results, the central hypothesis proposes that oxidative damages might be anticipated in the

initiation or progression of endometriosis. In this study, we suggested such a pro-oxidant environment promotes growth of ectopic endometrium. In the study, we established the primary co-culture model of endometrial cells from the patients with endometriosis. Epithelial cells and stromal cells were separated and co-cultured. By using matrigel assay, the invasion rate of endometrial epithelial cells was increased to 168% and 156% by treating with oxidants (such as H_2O_2 and TCDD). We suggested the oxidative stress contributed in the pathogenesis of endometriosis. In the future, we will focus on the searching drugs of the therapeutic approaches.

Key words: Endometriosis, invasion, metastasis, mtDNA mutation, oxidative damage

研究計畫之背景及目的

子宮內膜異位症(Endometriosis)是一種具有細胞侵入性的良性婦科疾病。根據組織學上的特性，子宮內膜異位症被定義為是子宮內膜(endometrium)及內膜腺體和基質細胞生長於腹腔內子宮以外的部位。此種異位性內膜上皮細胞具有可侵入性(invasion)及轉移性(metastasis)的特性。根據資料顯示婦女罹患子宮內膜異位症的發生率愈來愈高，其發生率約佔婦女人口的15%-50%，並且影響婦女的生殖能力(1)。此種異位性內膜細胞與子宮內的內膜細胞相似，會隨著婦女生理週期而有週期性的增生及剝落，常造成異位處產生局部性的發炎反應。這些過程常引發患病婦女月經困難，性生活障礙(dyspareunia)，骨盆腔疼痛，月經性血尿(catamenial hematuria)，或其他受影響的器官所產生的症狀。此病症常為女性不孕的主要族群，約50%的不孕婦女患有此一疾病(2)。

迄今，對於子宮內膜異位症的病理形成機轉尚未清楚。當異位的子宮內膜細胞產生粘著後，於粘著處開始增生及逐漸地侵入粘著處附近組織如骨盆腔。有許多因子可以引發異位內膜細胞的著附生長處的血管形成，促使異位細胞可以繼續生長。這些因子如cytokines(7-9)及生長因子(growth factors(10)，如transforming growth factor、interleukin-8、interleukin-1、tumor necrosis factor、interferon-γ (11)以及 vascular growth factor(12))被認為與引發子宮內膜異位細胞的著附生長、細胞增生及血管新生成有關。此外，子宮內膜異位症中所引起的吞噬細胞(marcrophages)的選召(recruitment)及活化的增加, 這被認為與此疾病的發展有重要相關性(13)。子宮內膜異位症是屬於多因子性疾病並伴隨骨盆腔的發炎反應。許多學者認為子宮內膜異位症與氧化壓力(oxidative stress)有關(14)。並且氧化壓力可以增加異位內膜細胞的著附生長，及引發的局部細胞外間質(extracellular matrix)的破壞。有許多證據顯示氧化壓力確實與子宮內膜異位症有關，並提出許多假說來解釋氧化壓力引發子宮內膜異位症形成(如圖一所示)。如氧化損壞的紅血球(15)、凋亡的子宮內膜細胞和未分解完成的內膜組織(16)常可以黏附於骨盆腔，並產生訊息使吞噬細胞的產生選召及活化。但是於罹患子宮內膜異位症的婦女常見到其對於這些訊息刺激無法產生適當的macrophage scavenger receptor反應。而這些骨盆腔內的活化吞噬細胞可以產生氧化壓力，常造成脂質過氧化物、崩解的氧化產物、氧化的low-density lipoprotein (17)、apoprotein (18)以及其他相互作用而成的蛋白質形成。另一方面,由對抗氧化壓力所形成的自體抗體

(autoantibody)於罹患子宮內膜異位症的婦女中有顯著性增加的現象(19)。由此氧化壓力所形成的發炎及伴隨 growth factors、cytokines、chemokines的產生常危害女性的生殖能力。此外，暴露於環境毒物 (如戴奧辛等)及重金屬的環境中亦會引發高濃度的氧化性自由基的產生，並且破壞氧化劑和抗氧化劑的平衡。而高濃度氧化性自由基的產生常與子宮內膜異位症的形成有關，進而降低受孕的能力。本研究中，我們證實氧化性傷害於子宮內膜上皮細胞異位性引發(initiation)及進展(progression)扮演重要的角色。由結果顯示，我們在子宮內膜異位症患者的病灶處的檢體中，檢測出高含量的氧化性傷害物質,如粒線體基因重組的堆積(mitochondrial DNA rearrangement)、8-OH-dG 及脂質過氧化產物(lipoperoxide, TBA-reacted 化合物)。尤其於巧克力囊腫(chocolate cyst)檢測出遠高於正常值5倍的氧化性傷害如8-OH-dG及脂質過氧化產物。並且於病灶處標本中檢測出新的粒線體基因斷損突變（5755 bp斷損)的堆積。此外，我們針對病患的*GSTM1*的基因加以檢測，我們發現於null-type *GSTM1*的病患中有較高比例的子宮內膜異位症罹患率。並且於null-type *GSTM1*的病患中有較高含量的氧化性傷害物質堆積。本研究中，我們推測氧化性環境可以增進子宮異位性內膜細胞的生長。人類的子宮內膜是由子宮內膜上皮細胞及內膜腺體覆蓋於基質上所組成。子宮內膜上皮細胞及基質細胞的體外初期培養中發現，於體外初期培養的內膜細胞加以氧化劑處理(如H_2O_2及2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD))，其上皮細胞侵入性增加。未來尋找可降低細胞侵入性及轉移性的因子及治療方法。冀望經由對此疾病的治病機轉的了解以其對此疾病的診斷方法及治療有更進一步的進展。

Materials and Methods

1.Endometriotic Tissue and Preparation of Primary Endometriotic Cells

Endometriotic tissue samples are obtained from patients undergoing laparoscopy for unexplained infertility, known endometriosis, or lower abdominal pain. Biopsies are taken during the proliferative phase of the menstrual cycle. In the operation theater, biopsy material was transferred immediately after laparoscopy into phosphate-buffered saline (PBS) containing 0.25% collagenase A and 1.5U/ml dispase (Roche, Grenzach-Wyhlen, Germany) and digested at room temperature for 6 hours. Red blood cells and debris were removed by centrifugation on a 45% (v/v) Percoll cushion (Amersham-Pharmacia, Freiburg, Germany). Dissociated cells were plated onto the appropriate tissue culture vessels and maintained in Dulbecco's modified Eagle's medium containing antibiotics and 10% fetal calf serum (FCS). For growth of endometriotic cells different batches of FCS were screened: some batches of FCS did not support optimal growth of endometriotic cells.

2. Determination of the mtDNA mutations in human or mouse tissue

(1) Total DNA extraction

All the samples are then checked by microscopy for morphological changes and stored at -196℃until analysis. The All tissue samples are minced into small pieces and

incubated at 56℃ for 2 hr in 50 μl lysis buffer containing 2% SDS and 50 mM Tris-HCl (pH 8.3), and then followed by phenol-chloroform extraction method. All the DNA samples are finally conserved in 200 μl of 10 mM Tris-HCl, pH 8.3.

(2) Synthesis of oligonucleotide primers

Oligonucleotide primers used for the amplification of target sequences of mtDNA and genomic DNA are chemically synthesized by Roche Molecular System, Inc. (Branchburg, NJ). The nucleotide sequences and sizes of the PCR products obtained from these primer pairs are summarized in Tables 1.

(3) Detection of deletion and point mutations of mtDNA by PCR

The desired target sequence of mtDNA will be amplified from 15-20 ng of each DNA sample in a 50 μl reaction mixture containing 200 μM of each dNTP, 0.4 μM of each primer, 1 unit of Ampli-Taq® DNA polymerase (Perkin-Elmer/ Cetus, Roche Molecular System, Inc., Branchburg, NJ), 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 8.3. PCR is carried out for 35 cycles in a DNA thermal cycler (Perkin-Elmer/Cetus) using the thermal profile of denaturation at 94℃ for 40 sec, annealing at 55℃ for 40 sec, and primer extension at 72℃ for 40 sec. Long PCR will be proceed followed as the thermal profile of denaturation at 94℃ for 2 min, annealing at 68℃ for 1 min, and primer extension at 72℃ for 2 min.

3. Analysis of oxidative damage in cellular molecules

(1) Determination of 8-OH-dG

Tissue samples are scraped by the cell lifter in 1.5 ml TE buffer and add 75 μl of 10% SDS, 30 μl of 200 mM butylated hydroxytoluene, and 15 μl of RNase A stock solution (10 mg/ml) then incubated the lysate at 37℃ for 1 hr. Proteinase K (100 μg/ml) and is incubated at 55℃ for 12 hr. The lysate is extracted by phenol/chloroform method. An aliquot of 100μg of DNA dissolved in 100 μl of 10 mM Tris-HCl (pH 7.4)/0.1 mM DFAM is digested by incubation with 1 μl of DNase I (20 units/μl) and 11 μl of 0.1 M $MgCl_2$ solution at 37℃ for 30 min. After adjusting the pH to 5.0 by adding 4.8 μl of 1 M sodium acetate (pH 5.3) and 1.2 μl of 0.1 M $ZnSO_4$, the DNA sample is digested with 5 μl of nuclease P1 (1 unit/3 μl in 20 mM sodium acetate, pH 5.3) at 65℃ for 10 min. The DNA molecules were hydrolyzed to the corresponding nucleosides by incubation with 5 μl of 1 U/μl alkaline phosphatase for 30 min at 37℃. Processed DNA samples are separated by C-18 column (particle size 5 μm, 200 × 4.6 mm, JT Baker, Inc.) on an HPLC system (Jasco, Japan) connected in series with an ECD detector (BAS, Inc.,) and a UV detector (at 254 nm). Elution was performed at the flow rate of 0.8 ml/min for 40 min with a mobile phase consisted of 12.5 mM citric acid, 25 mM sodium acetate, and 10 mM acetic acid containing 6 % of methanol (pH 5.8).

(2) *Determination of lipid peroxide*

In each analytical run, reagent blank, 1,1,3,3-tetraethoxypropane (TEP) standard working solutions and the sample are assayed in duplicate. An aliquot of 50 μl of each sample was pipetted into a test tube containing 0.6 ml of 0.44 M phosphoric acid. After

mixing, 0.2 ml of 42 mM thiobarbituric acid (TBA) solution is added to a final concentration of 7 mM and then placed in a 95℃dry bath for 1 hr. The samples were then cooled and neutralized with 1 N NaOH in methanol before HPLC analysis. An aliquot of 20 μl supernatant obtained above is injected into a narrow-pore C_{18} column (4.6 x 250 mm, particle size 5μm) using a Jasco PU-980 pump (Tokyo, Japan) with a solvent system made of methanol and 50 mM phosphate buffer (pH 6.8) (4:6, v/v) at the flow rate of 1 ml/min. The eluent is monitored with a fluorescence detector (excitation at 525 nm; emission at 550 nm).

(2) *Determination of carbonyl protein*

For the assessment of protein carbonyl content, plasma proteins are derivatized with DNP before SDS-polyacrylamide gel electrophoresis on 10% gradient gels, followed by Western transfer to nitrocellulose filters. Protein equivalent to 0.12 μL of plasma was loaded onto each lane for electrophoresis. DNP-derivatized tissue protein carbonyl groups are sequentially reacted with rabbit anti-DNP and goat anti-rabbit immunoglobulin G (IgG) antibodies (OxyBlot Oxidized Protein Detection Kit, Oncor, Gaithersburg, MD, now supplied by Intergen Company, Purchase, NY) followed by chemiluminescence detection with ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Baie d'Urfé, Canada). Total protein staining with amido black was done on duplicate nitrocellulose filters prepared at the same time from the same DNP-derivatized samples. Quantitation of densitometry scans from chemiluminescence detection and of total protein staining was done using a Univeral Software for analysis of 1D gels (American Applied Biotechnology, Fullerton, CA).

4. Matrigel-Invasion Assay

The ability of cells to migrate or invade through a Matrigel barrier was measured in Falcon BioCoat Matrigel invasion chambers (Becton Dickinson, Heidelberg, Germany) with 6.4-mm diameter and 8-μm pore size. Invasion chambers coated with Matrigel (for invasion measurement) or uncoated filters (for measurement of migration) are used according to standard protocols and the manufacturer's advice. Cells on the lower surface of the filter were stained with Hoechst dye no. 33258 (Sigma) and microscopically photographed with a 20x objective. Values for migration will be taken as the average number of migrated cells per photographic field over three independent fields per experiment and expressed as averages of triplicate experiments. Values for invasion were similarly recorded, but invasion values were expressed as the percentage of cells penetrating one field of a Matrigel-coated filter, compared to the numbers of the same cell type migrating in one field of a uncoated filter in a parallel control experiment.

Preliminary data

(1) Higher content of lipoperoxides in the endometriotic tissues

In this study, we detected the lipoperoxides (as malondialdehyde) in the endometrium, myoma, adenoma, ovary, and chocolate cyst from women with endometriosis or normal individuals. The content of lipoperoxides was 1.50±0.25 (n=10), 4.48±0.44 (n=10), 2.61±0.66 (n= 7), 1.85±0.11 (n= 6), 2.80±0.72 (n=12), 2.68±0.44 (n=10), 3.34(n= 1), 1.27 (n= 1), 2.61±0.43 (n=11), 19.84±2.50 (n=10) in the normal endometrium, chocolate cyst, endometrium, myometrium, myoma, ovary, peritoneal fluid, placenta, decidual tissue, and villi, respectively. The higher contents of lipoperoxides were detected in the tissues from the eutpotic and the ectopic endometriosis. There were approximately four to five folds of lipoperoxides in the chocolate cyst.

(2) Higher amount of 8-OH-dG in the endometriotic tissues

The amounts of oxidized DNA base (e.g. 8-OH-dG) were detected by using HPLC-ECD in the endometrium, myoma, adenoma, ovary, and chocolate cyst from women with endometriosis or normal individuals. The content of 8-OH-dG was 0.17±0.07 ($x10^{-3}$%, n=5), 0.56±0.15 ($x10^{-3}$%, n=6), 1.21±0.10 ($x10^{-3}$%, n=10), 0.72±0.17 ($x10^{-3}$%, n=4), 0.55±0.18 ($x10^{-3}$%, n=5), 0.66±0.30 ($x10^{-3}$%, n=9), 0.62±0.05 ($x10^{-3}$%, n=9), 0.73 ($x10^{-3}$%, n=1), 0.31 ($x10^{-3}$%, n= 1), 0.65±0.19($x10^{-3}$%, n=9), 0.88±0.50($x10^{-3}$%, n=10) in the normal endometrium, adenomyoma, chocolate cyst, endometrium, myometrium, myoma, ovary, peritoneal fluid, lacenta, decidual tissue, and villi, respectively. The higher contents of 8-OH-dG were detected in the tissues from the eutpotic and the ectopic endometriosis. There were approximately four to five folds of lipoperoxides in the chocolate cyst.

(3) Accumulation of large scale deletion and DNA rearrangement of mtDNA in the endometriotic tissues

The accumulations of mtDNA rearrangements are shown in aged tissues, degenerated diseases, and several types of cancer in the humans. In our study, we detected mtDNA mutation in these tissues such as endometrium, myoma, adenoma, ovary, and chocolate cyst from women with endometriosis or normal individuals. Using primer-pair L8150-H14020, three types of PCR products were generated. The 5871 bp fragment was produced from the wild type mtDNA, the 894 bp fragment was from the 4977 bp deleted mtDNA, and the near 600 bp fragment was from 5270 bp mtDNA deletion. A scheme illustrating the strategy for the determination of multiple mtDNA deletion of various human tissues by the long-range PCR techniques (Fig.2) Lower panel, Lane 1 to 3 were examined from of ovaries, myometrium, and endometrium from 3 individuals with endometriosis, respectively. Lane M is the 100 bp DNA ladder and lane M' is 10 Kb DNA ladder in Fig 2.

(3) 4,977 bp deletion and a novel deletion of mtDNA in the endometriotic tissues

We applied primer-shift PCR to ensure the existence of 4,977 bp deletion and DNA sequencing to identify the novel 5,755 bp mtDNA deletion found in the endometriotic tissue. The primer-shift PCR products were amplified from the mtDNA with specific 4977 bp deletion in the ovaries with endometriosis. The lengths of the PCR products were according to the designed primer pairs. In Fig 3, Lane 1 to 3 indicate the PCR products of 894 bp (L8150-H14020), 793 bp (L8251-H14020), and 423 bp (L8251-H13650) amplified from 4977 bp deleted mtDNA. Furthermore, we sequenced the generated PCR products. In Fig. 4, a schematic illustration of the nucleotide sequence flanking the junction sites at the 5'-end of the novel 5755 bp deletion on the heavy strand of mtDNA in the endometriotic tissue. It revealed a 3-nucleotide indirect repeat (5'-CTT-3') located in the junction sites at nucleotide position (np) 8062-8064 or np 13819-13821 (5' to 3') on the heavy strand of mtDNA.

(4) *GST M1* genotyping in the endometriotic tissues

Glutathione S transferase M1(GSTM1) consists a super-family of Phase II enzymes that catalyze the conjugation of reduced glutathione with electrophilic compounds, including many environmental mutagens and carcinogens (26). The currently identified cytosolic GSTs are categorized into four main classes, α, μ, θ, and π, based on biochemical characteristics. The class μ of GST (GSTM1) have many substrates, including epoxides of polycyclic aromatic hydrocarbons such as benzo[a]pyrene, acrolein, and other unsaturated carbonyls generated by lipoperoxides and oxidative damage to DNA (27). *GSTM1* is deleted in about half of Caucasians (28). In this study, we examined the genotyping of *GST M1* in all the collected endometriotic tissues. Two major groups were classified into *GST M1* null type and non-null type. In Fig.5, by using different primer pairs, in lane 1, the 650 bp PCR products were amplified from *GSTM1*μ and 200 bp PCR products were amplified from *GSTM1*ψ. Lane 2 indicates the null type of *GSTM1* gene there was no PCR product was obtained. Lane 3, the 270 bp PCR products were amplified from GSTM1 by using different primer pair. In our study, the percentage of the null type were 49.2% and 50.8% were the non-null type. The high proportion of 4,977 bp deletion and 5755 bp deletion were found in the individuals with *GSTM1* null typing. In 18 cases which were identified with the 4,977 bp mtDNA deletion in the endometriotic tissues, 14 cases were detected in the individuals with *GSTM1* null typing and only 4 cases were found in the patients with GSTM1 non-null type(Table 2 and 3).

(4) Oxidative damages on the primary co-cultured cells derived from the endometriotic tissues

In order to underlying the effect(s) on the endometrial cells by oxidative stress, we established the primary co-cultured cells derived from the endometriotic cells *in vitro*. The epithelial cells and stromal cells were separated cultured or co-cultured. In the preliminary studies, we used two types of reagents, one was hydrogen peroxides and the other was TCDD (dioxin). Moreover, we traced the expression of DDH in the differentially treated cells. DDH was used as tracing marker of disease developing and progression. The ability of DDH

to generate ROS during the oxidation of PAH *trans*-dihydrodiols (proximate carcinogens) may have important implications for tumor initiation and promotion. In our preliminary data, the differential expression of DDH was detected in the different types of endometriotic tissues. The highly expressed *DDH* mRNA was detected in the endometrial cell line and differentially expressed in adenomyosis, not in normal endometrium (Fig. 7). The 961 bp was generated from the cDNA of *DDH* and the 475 bp was from *β-actin*. Differential expression of DDH was detected in the different types of endometriotic tissues. The highly expressed DDH was detected in the endometrial cell line (lane 1) and differentially expressed in adenomyosis (lane 2, 3, 4,5, and 9). In addition, the culture cells were treated with H_2O_2 and TCDD. The dose-dependent mRNA expression was shown in the treated culture cells. (A) The different dose of H_2O_2 from 0, 50, 100, 250 and 500 μM (Panel A, lane 1 to lane 5) was applied in treating cells. (B) Dose and time dependence expression of DDH was also detected in the treated cells with TCDD from 0 nM for 1 hr, 100 nM for 1 hr, 500nm for 1 hr, 100 for 3 hr, 500nm for 5 hr (Panel B, lane 1 to lane 5) of the treated cells.

(5) Increased invasion of epithelial and stomal cell co-culture

The ability of epithelial cells to migrate or invade through a Matrigel barrier was measured in Falcon BioCoat Matrigel invasion chambers. The invasion rate was apparently increased in the H2O2 (168%) or TCDD (156%) treated cells.

Table 1 Various tissues collected from 46 women with or without endometriosis. MtDNA mutation with 4977 bp deletion or 5755 bp deletion and GSTM1 genotype were examined in each sample.

Type of tisssue	Endometriosis	4977 bp mtDNA deletion	5755 bp mtDNA deletion	GST M1 gene Null type
Adenomyoma	8/8	1/8	2/8	2/8
Chocolate cyst	12/12	3/12	2/12	3/12
Endometrium	4/8	2/8	1/8	4/8
Myometrium	3/8	0/8	0/8	3/8
Myoma	11/17	1/17	1/17	8/17
Ovary	2/3	0/3	0/3	3/3
Peritoneal fluid	1/1	0/1	0/1	1/1
Uterus	1/1	1/1	1/1	0/1
Placenta	0/1	1/1	0/1	1/1
Decidual tissue	1/17	2/17	2/17	9/17
Villi	10/19	1/19	5/19	13/19

Table 2 The biomarkers of oxidative damage were examined in this study.

Type of tisssue	8-OH-dG/dG ($x10^{-3}$%)	Lipoperoxide content (pmole/μg protein)
Normal	0.17±0.07 (n= 5)	1.50±0.25 (n=10)
Adenomyoma	0.56±0.15 (n= 6)	N.D.
Chocolate cyst	1.21±0.10 (n=10)	4.48±0.44 (n=10)
Endometrium	0.72±0.17 (n= 4)	2.61±0.66 (n= 7)
Myometrium	0.55±0.18 (n= 5)	1.85±0.11 (n= 6)
Myoma	0.66±0.30 (n= 9)	2.80±0.72 (n=12)
Ovary	0.62±0.05 (n= 9)	2.68±0.44 (n=10)
Peritoneal fluid	0.73 (n= 1)	3.34 (n= 1)
Placenta	0.31 (n= 1)	1.27 (n= 1)
Decidual tissue	0.65±0.19 (n= 9)	2.61±0.43 (n=11)
Villi	0.88±0.50 (n=10)	19.84±2.50 (n=10)

Table 3 The various tissues collected from 46 women with or without endometriosis. Each sample with specific 4977 bp mtDNA deletion was compared with the GSTM1genotype in each sample.

Type of tisssue	4977 bp mtDNA deletion	GST M1 gene Null type	Endometriosis History
Adenomyoma (n=1)	+	Null type	+
Chocolate cyst (n=3)	+	Null type	+
	+	Null type	+
	+	Wild type	+
Endometrium (n=2)	+	Null type	-
	+	Wild type	+
Myometrium (n=2)	+	Null type	-
	+	Wild type	+
Myoma (n=1)	+	Null type	+
Ovary (n=3)	+	Null type	+
	+	Null type	+
	+	Null type	+
Peritoneal fluid (n=1)	+	Null type	+
Uterus (n=1)	+	Null type	+
Placenta (n=1)	+	Wild type	-
Decidual tissue (n=2)	+	Null type	-
	+	Null type	-
Villi	+	Null type	+

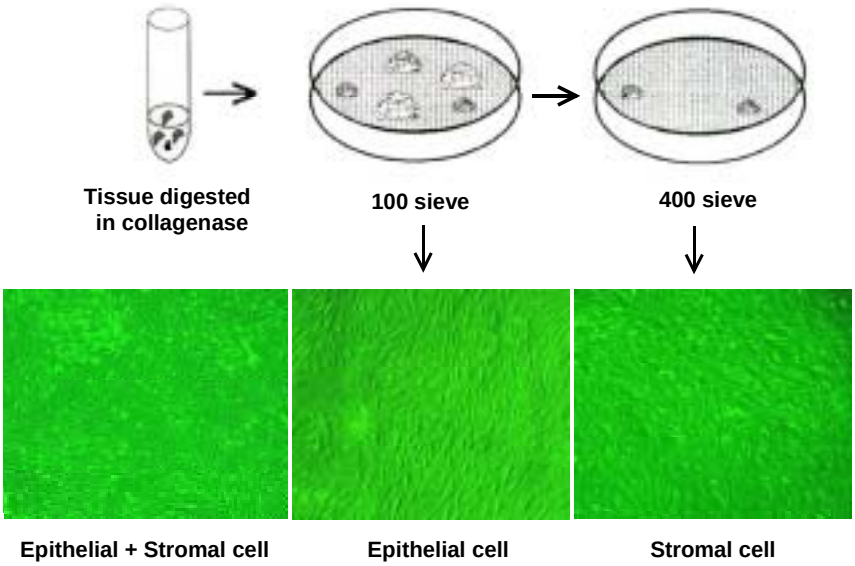

Fig.1 Schematic showing the strategy for isolation of endometrial stromal and epithelial components from primary tissues. This technique of using collagenase followed by sequential sieves to isolate glandular epithelium and stroma is based on the work of Satyaswaroop and collagues.

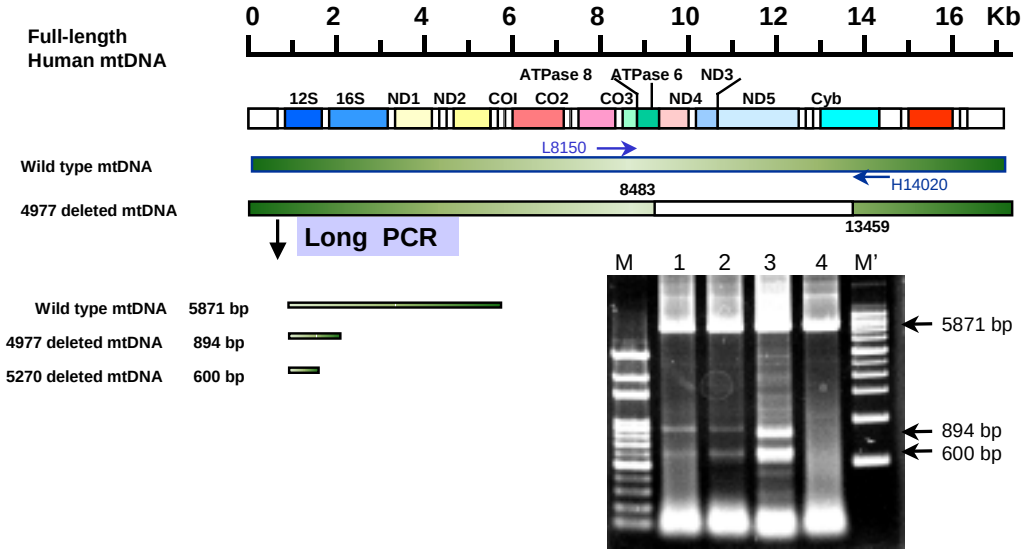

Fig2 Upper panel. A scheme illustrating the strategy for the determination of multiple mtDNA deletion of various human tissues by the long-range PCR techniques. Using primer-pair L8150-H14020, three types of PCR products were generated. The 5871 bp fragment was produced from the wild type mtDNA, the 894 bp fragment was from the 4977 bp deleted mtDNA, and the near 600 bp fragment was from 5270 bp mtDNA deletion. Lower panel, electrophorectogram of the PCR products amplified from mtDNA with specific deletions in women tissues with or without endometriosis. Lane 1 to 3 were examined from of ovaries, myometrium, and endometrium from 3 individuals with endometriosis, respectively. Lane M is the 100 bp DNA ladder and lane M' is 10 Kb DNA ladder.

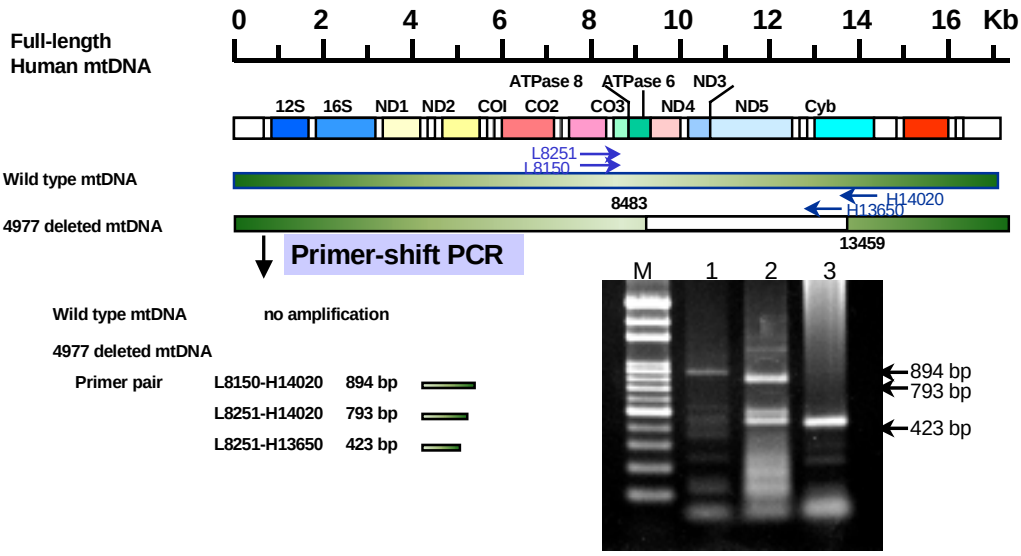

Fig. 3 Primer shift and electrophorectogram of the PCR products amplified from the mtDNA with specific 4977 bp deletion in the ovaries with endometriosis. Upper panel, by using different primer pairs, the PCR products were amplified from the 4977 bp deleted mtDNA. Lane 1 to 3 indicate the PCR products of 894 bp (L8150-H14020), 793 bp (L8251-H14020), and 423 bp (L8251-H13650) amplified from 4977 bp deleted mtDNA. Lane M is the 100 bp DNA ladder size marker.

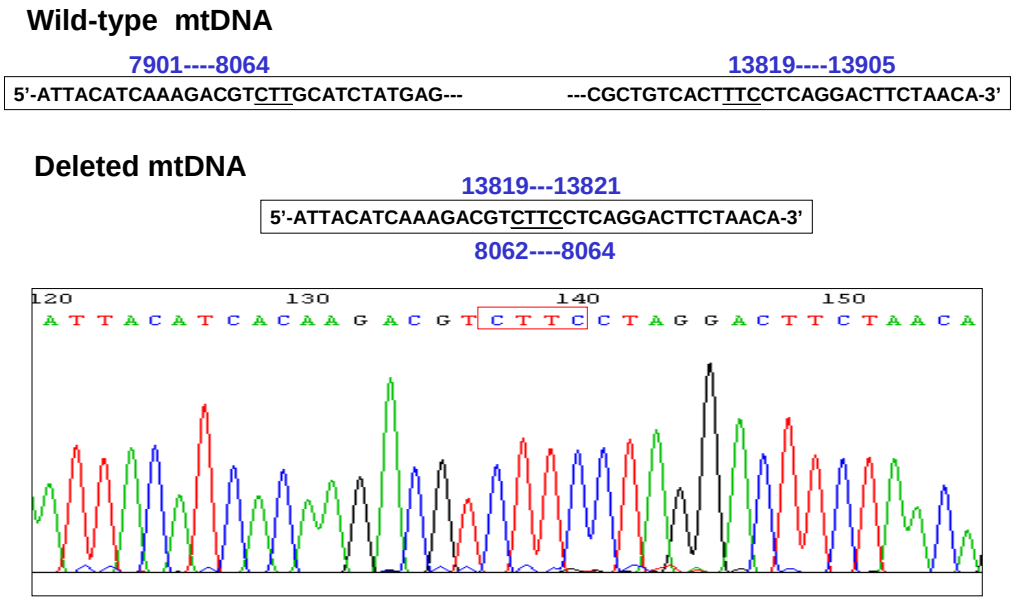

Fig 4 A schematic illustration of the nucleotide sequence flanking the junction sites at the 5'-end of the novel 5755 bp deletion on the heavy strand of mtDNA in human sperm. It revealed a 3-nucleotide indirect repeat (5'-CTT-3') located in the junction sites at nucleotide position (np) 8062-8064 or np 13819-13821 (5' to 3') on the heavy strand of mtDNA.

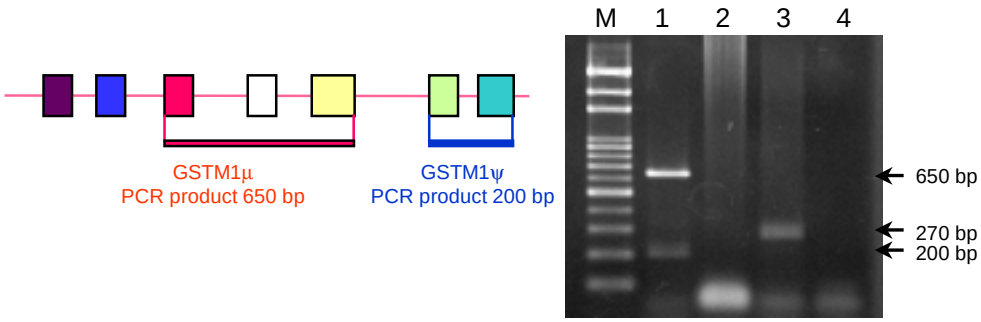

Fig. 5 Electrophorectogram of the PCR products amplified from the genomic DNA of glutathione S transferase M1(GSTM1) in the ovaries with endometriosis. Left panel, by using different primer pairs, in lane 1, the 650 bp PCR products were amplified from GSTM1μ and 200 bp PCR products were amplified from GSTM1ψ . Lane 2 indicates the null type of GSTM1 which no PCR product was obtained from GSTM1μ. Lane 3, the 270 bp PCR products were amplified from GSTM1μ by using different primer pair. Lane M is the 100 bp DNA ladder.

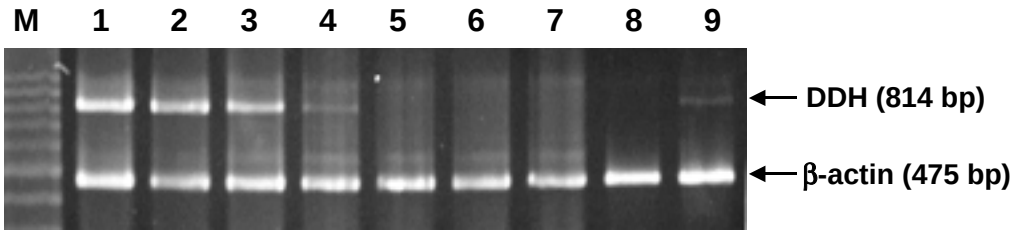

Fig 6 Electrophorectogram of the RT-PCR products amplified from the dihydrodiol dehydrogenase (DDH) mRNA in the endometriotic tissues. The 961 bp was generated from the cDNA of DDH and the 475 bp was from β-actin. Differential expression of DDH was detected in the different types of endometriotic tissues. The highly expressed DDH was detected in the endometrial cell line (lane 1) and differentially expressed in adenomyosis (lane 2, 3, 4,5, and 9).

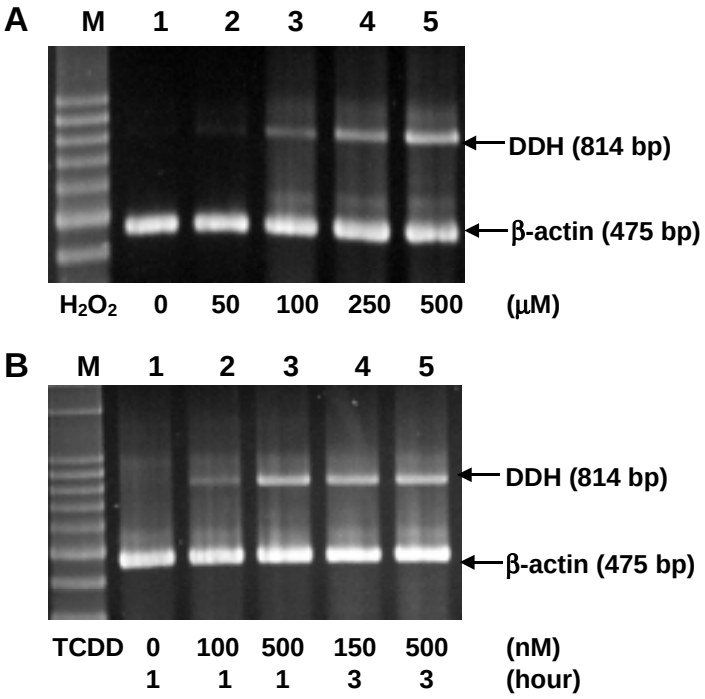

Fig 8 Electrophorectogram of the RT-PCR products amplified from the dihydrodiol dehydrogenase (DDH) mRNA in the endometriotic culture cells from two individuals with adenomyosis. The primary cell culture of endometriotic epithelium cells from the individual with adenomyosis was established. The culture cells were treated with H_2O_2 and TCDD. The dose-dependent mRNA expression was shown in the treated culture cells. (A) The different dose of H_2O_2 from 0, 50, 100, 250 and 500 μM (Panel A, lane 1 to lane 5) was applied in treating cells. (B) Dose and time dependence expression of DDH was also detected in the treated cells with TCDD from 0 nM for 1 hr, 100 for 1 hr, 500nm for 1 hr, 100 for 3 hr, 500nm for 5 hr (Panel B, lane 1 to lane 5) of the treated cells.

References

1. Starzinski-Powitz, A., Handrow-Metzmacher, H., and Kotzian, S. (1998) The putative role of cell adhesion molecules in endometriosis: can we learn from tumor metastasis? *Mol. Med. Today* 5, 304-309.
2. Battista, G. (1991) Mild endometriosis and infertility: A clinical review of epidemiological data, diagnostic pitfalls, and classification limits. *Obstet. Gynecol. Surv.* 46, 374-379.
3. Koks, C.A.M., Demir-Weusten, A.Y., Groothuis, P.G., Dunselman, G.A.J., de Goeij, A.F.P.M., Evers, J.L.H. (2000) Menstruum induces changes in mesothelial cell morphology. Gynecol. Obstet. Invest., 50, 13-18.
4. Beliard, A., Donnez, J., Nilsolle, M., Foidart, J.M. (1997) Localization of laminin, fibronectin, E-cadherin and integrins in endometrium and endometriosis. Fertil. Steril. 67, 266-272.
5. Kokorine, I., Nisolle, M., Donnez, J., Eeckhout, Y., Courtoy, P.J., Marbaix, E. (1997) Expression of interstitial collagenase (matrix metalloproteinase-1) is related to the activity of human endometriotic lesions. Fertil. Steril. 68, 246-251.
6. Sillem, M., Prifti, S., Neher, M., Runnebaum, B. (1992) Extracellular matrix remodeling in the endometrium and its possible relevance to the pathogenesis of endometriosis. Human Reprod. Update, 4, 730-735.
7. Murphy, A.A., Santanam, N., Morales, A.J., and Parthasarathy, S. (1998) Lysophos phatidyl choline, a chemotactic factor for monocytes/T-lymphocytes is elevated in endometriosis. *J Clin Endocrinol Metab* 83, 2110-2113.
8. Sawastri, S., Desai, N., Rock, J.A., and Sidell, N. (2000) Retinoic acid suppresses interleukin-6 production in human endometrial cells. *Fertil Steril* 73, 1012-1019.
9. Bedaiwy, M.A., Falcone, T. Sharma, R.K., Goldberg, J.M., Attaran, M., Nelson, D.R., and Agarwal, A. (2002) Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial. *Hum. Reprod.* 17, 426-431.
10. Giudice, L.C., Dsupin, B.A., Gargosky, S.E., Rosenfeld, R.G., and Irwin, J.C. (1994) The insulin-like growth factor system in human peritoneal fluid: its effects on endometrial stromal cells and its potential relevance to endometriosis. *J Clin Endocrinol Metab* 79, 1284-1293.
11. Viantier, D., Cosson, M., Dufour, P. (2000) Is endometriosis an endometrial disease? Eur. J. Obstet. Gynecol. Reprod. Biol. 91, 113-125.
12. Donnez, J., Smoes, P., Gillerot, S., Casanas-Roux, F., Nisolle, M. (1998) Vascular endothelial growth factor (VEGF) in endometriosis. Hum. Reprod. 13, 1686-1690.
13. Halme, J., Becker, S., Wing, R. (1984) Accentuated cyclic activation of peritoneal macrophages in patients with endometriosis. Am. J. Obstet. Gynecol., 148, 85-90.
14. Langendonckt, A.V., Casanas-Roux, F., and Donnez, J. (2002) Oxidative stress and peritoneal endometriosis. Fertil. Steril., 77, 861-870.
15. Arumugam, K., Yip, Y.C. (1995) De novo formation of adhesion in endometriosis. The role of iron and free radical reactions. Fertil. Steril. 64, 62-4.
16. Murphy, A.A., Santanam, N., Parthasarathy, S. (1998) Endometriosis: a disease of oxidative

stress? Semin. Reprod. Endocrinol. 16, 263-273.
17. Ota, H., Igarashi, S., Hatazawa, J., and Tanaka, T. (1999) Endometriosis and free radicals. Gynecol. Obstet., Invest., 48, 29-35.
18. Langedonckt, A.V., Casanas-Roux, F., and Donnez, J. (2002) Oxidative stress and peritoneal endometriosis. Fertil. Steril. 77, 861-870.
19. Shanti, A., Santanam, N., Morales, A.J., Parthasarathy, S., and Murphy, A.A. (1999) Autoantibodies to markers of oxidative stress are elevated in women with endometriosis. *Fertil Steril* 71, 1115-8.
20. Rier, S.E. (2002) The potential role of exposure to enviromentsl toxicants in the pathophysiology of endometriosis. Ann.N.Y. Acad. Sci. 955, 201-212.
21. Zeitvogel, A., Baumann, R., and Starzinski-Powitz, A. (2001) Identification of an invasive, N-Cadherin-expressing epithelial cell type in endometriosis using a new cell culture model. *Am J Pathol* 159, 1839-1852.
22. Penning, T.M.,Tsuyoshi Ohnishi, T.S., Ohnishi, T., and Harvey, R.G. (1996) Generation of reactive oxygen species during the enzymatic oxidation of polycyclic aromatic hydrocarbon *trans*-dihydrodiols catalyzed by dihydrodiol dehydrogenase. Chem. Res. Toxicol., *9,* 84-92.
23. Burczynski, M.E., Lin, H.K., and Penning, T. M. (1999) Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogensase. Cancer Res., 59, 607-614.
24. Burczynski, M.E., and Penning, T. M. (2000) Genotoxic polycyclic aromatic hydrocarbons *ortho*-quinones generated by aldo-reductases induce CYP1A1 via nuclear translocation of the aryl hydrocarbon receptor. Cancer Res., 60, 908-915.
25. Rier, S., Foster, W.G. (2002) Environmental dioxins and endometriosis. Toxicol. Sci., 70, 161-70
26. Helzlsouer, K., Selmin, O., Huang, H., Strickland, P., Hoffman, S., Alberg, A., Watson, M., Comstock, G., and Bell, D. (1998) Association between glutathione S-transferase M1, P1, and T1 genetic polymorphisms and development of breast cancer. J. Natl. Cancer Inst., 90, 512-518.
27. Hayes, J.D., and Pulford, D.J. (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isozymes to cancer chemoprotection and drug resistance. Crit. Rev. Biochem. Mol. Biol., 30,445-600
28. Perera, F.P., Moony, LaV. A., Stampfer, M., Phillips, D.H., Bell, D.A., Rundle, A., Cho, S., Tsai, W.Y., Ma,J., Blackwood, A., and Tang, D. (2002) Associations between carcinogen-DNA damage, glutathione S-transferase genotype, and risk of lung cancer in the prospective Physicians' Health Cohort Study. Carcinogenesis, 23, 1641-1646.