Biomarkers, 2009; 14(8): 604–618

ORIGINAL ARTICLE

Cytokine array analysis of peritoneal fluid between women with endometriosis of different stages and those without endometriosis

Zhen Hou[1,2], Liang Sun[1], Liying Gao[1,2], Lianming Liao[1], Yundong Mao[1,2], and Jiayin Liu[1,2]

[1]*Center of Clinical Reproductive Medicine, Department of Obstetrics and Gynecology, First Affiliated Hospital of Nanjing Medical University, Nanjing, China, and* [2]*Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing, China*

Abstract

Cytokines are key mediators of intercellular communication and are likely to promote the development and progression of endometriosis. Previous studies provided evidence that endometriosis develops as a result of the pathogenetic factors in the peritoneal environment, especially the peritoneal fluid (PF). We determined different cytokine expression in peritoneal fluid between women with minimal/mild and moderate/severe endometriosis and those without endometriosis using the cytokine array. As a result, 78 cytokines were found to have a threefold change, including 74 increases and four decreases in endometriosis compared with the control group; 96 cytokines had a threefold change including 91 increases and five decreases in minimal and mild endometriosis compared with the control group; 83 cytokines had a threefold change including 14 increases and 69 decreases in moderate and severe endometriosis compared with minimal and mild endometriosis. The cytokine networks were produced by Pathway Studio software and revealed that most cytokines are involved in cell binding, interaction and protein synthesis and transportation regulation. Among them activin A, Smad7 and β-nerve growth factor are the most interesting as they may be involved in the pathogenesis of endometriosis. These results suggest that cytokines are very important factors in the development of endometriosis. The findings of differentially expressed cytokines improves our knowledge of the pathogenesis and development of endometriosis and these findings warrant further studies to develop potential targets for the diagnosis and treatment of endometriosis.

Keywords: *Endometriosis; cytokine network; pathogenesis*

Introduction

Endometriosis is a common gynaecological disorder associated with pelvic pain and infertility. It is estimated to affect at least 10% of women of reproductive age (Gruppo Italiano per lo studio dell'endometriosi 1994). It is defined as the presence of endometrial glands and stroma outside the uterus, mainly on the pelvic peritoneum and the ovaries.

The precise aetiology of endometriosis remains unknown despite years of research. The most widely accepted theory on the pathogenesis of endometriosis is Sampson's theory (Sampson 1927) suggesting that this disorder originates from retrograde menstruation of viable endometrial tissue through the Fallopian tubes into the peritoneal cavity where it implants on the peritoneal surface or pelvic organs. However, it cannot explain why the displaced cells survive in women with endometriosis and not in healthy women.

Several previous studies applied proteomic technology to the study of endometriosis (Poliness 2004). Since the 1990s, two-dimensional (2D) electrophoresis has been used to investigate molecules involved in the pathogenesis of endometriosis (Nothnick 1994). More recently, Fowler et al. (2007) investigated the effects of endometriosis on the proteome of human

Address for Correspondence: Jiayin Liu, Center of Clinical Reproductive Medicine, Department of Obstetrics and Gynecology, First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Gulou District, Nanjing 210029, China. Fax: +86-025-83674442. E-mail: jyliu@njmu.edu.cn

(Received 30 March 2009; revised 17 June 2009; accepted 14 July 2009)

ISSN 1354-750X print/ISSN 1366-5804 online © 2009 Informa UK Ltd
DOI: 10.3109/13547500903183970

eutopic endometrium by using 2D-polyacrylamide gel electrophoresis (PAGE) and mass spectrometry. Several dysregulated proteins were identified, including molecular chaperones, proteins involved in the cellular redox state, molecules involved in protein and DNA formation/breakdown and secreted proteins. In a similar study, Zhang et al. (2006) observed the abnormal expression of proteins involved in the cell cycle, signal transduction and immunological function. Recently, Protein-Chip technology has been applied to the study of protein expressed by the endometrium, endometriotic tissues and normal peritoneum obtained from women with and without endometriosis (Kyama et al. 2006).

An immunological or inflammatory aetiology has been suggested, as indicated by the increased concentrations of activated macrophages, cytokines, T cells and B cells in patients with endometriosis (D'Hooghe et al. 2003). Cytokines are key mediators of intercellular communication within the immune system, and exert proliferative, cytostatic, chemoattractant or differentiating effects on a variety of target cells (D'Hooghe et al. 2003) and are likely to promote the development and progression of endometriosis. Several studies have provided evidence that endometriosis develops as a result of the pathogenetic factors in the peritoneal environment, especially the peritoneal fluid (PF) (Ramey & Archer 1993, Koninckx 1998, 1999, Gazvani & Templeton 2002). PF is often seen in the pelvic cavity, particularly in the vesicouterine fold and in the cul-de-sac in proximity to the uterus, Fallopian tubes and ovaries.

Tabibzadeh et al. (2003) compared 2D-PAGE of PF in women with and without endometriosis. However, the gels exhibited a limited number of protein spots (approximately 73), and the identity of the majority of protein spots with abnormal expression in endometriosis was not determined by either immunoblotting or mass spectrometry. By using 2D-PAGE combined with semiquantitative computerized analysis and immunoblotting, Ferrero et al. demonstrated that the PF of women with endometriosis, when compared with that of control subjects, contains higher levels of a particular isoform of Hp β chain (Ferrero 2005a) and lower levels of an isoform of vitamin D binding protein (Ferrero 2005b). Then they used 2D-PAGE combined with liquid chromatography tandem mass spectrometry to search for other specific proteins present in the PF of women with endometriosis. In total, 98 protein spots were consistently (90% of the cases) present in the PF and 11 protein spots had significantly higher expression in the PF of women with endometriosis when compared with controls. These spots were identified as four isoforms of R2-Heremans Schmidt-glycoprotein, three isoforms of R1-antitrypsin, one isoform of protein S100-A8, one isoform of apolipoprotein A-1, one immunoglobulin kappa chain C region, and one serum albumin precursor. Two protein spots had significantly lower expression in the PF of women with endometriosis; they were identified as R-1B-glycoprotein and pantophysin.

In the present study we determined the expression of different cytokines in the PF from women with minimal/mild and moderate/severe endometriosis and those without endometriosis using the cytokine chip.

Materials and methods

Reagents

Biotin Label-based Human Antibody Array I was purchased from RayBiotech (Norcross, GA, USA) and used to detect the expression levels of 507 human proteins according to the manufacturer's instruction. Other reagents used included Cell & Tissue Protein Extraction Reagent and BCA Protein Assay Kit (KangChen, Shanghai, China).

Study population

Six women scheduled for laparoscopy for infertility or abdominal pain were recruited to participate in this study with written informed consent. The study was approved by the Ethics Committee on Human Research of the First Affiliated Hospital of Nanjing Medical University. All of the women were in the proliferative phase confirmed by the pathological examination of the endometrial biopsy (Noyes et al. 1975). None of the women included had active pelvic inflammatory disease, hydrosalpinges or any autoimmune disease. None of the patients had a history of endometrial hyperplasia or neoplasia, had received any anti-inflammatory or hormonal medications or had undergone hysterosalpingography in the previous 3 months. The diagnosis of endometriosis was confirmed by the presence of glands and stroma in the endometriotic lesions. Endometriosis was diagnosed and staged according to the revised American Fertility Society (rAFS) classification by visual inspection of the pelvis (American Society for Reproductive Medicine, 1996). Three women had minimal/mild disease (AFS stages I–II, aged 30 ± 2.65 years) with red endometriotic lesions, such as red vesicles, red flame-like lesions or gland-like lesions. Three women had moderate/severe disease (AFS stages III–IV, aged 29 ± 2.65 years).

Three women (aged 31.67 ± 2.31 years) who received a sterilization operation and were confirmed to have no endometriosis served as the control group. No lesions of endometriosis, active pelvic inflammatory disease and hydrosalpinges were observed via laparoscopy.

Peritoneal fluid collection

PF samples were collected and processed as previously described (Ferrero 2005a, b). Briefly, all visible PF was aspirated from the cul-de-sac and the vesicouterine fold during laparoscopy. The PF was not used if contaminated by blood. PF samples collected from different sites in the pelvis were mixed before centrifuging at 2000*g* for 5 min. Supernatants were removed and stored in aliquots at -80°C until the cytokines were assayed.

Sample pooling

Appropriate sample pooling can provide equivalent power and improve efficiency and cost-effectiveness for microarray experiments with a modest increase in total number of subjects (Peng 2003). Pooling schemes in terms of replicates of subjects and arrays can be compared before experiments are conducted (pool sample). We pooled the three samples of each group with equal amounts of protein for the detection of cytokines.

Biotin labelling

PF protein quantification was performed by using a BCA Protein Assay Kit. Briefly, a standard curve was set up by plotting the 562 nm measurement for each BSA standard versus its concentration in μg ml^{-1}. The absorbance at 562 nm of the each PF sample was determined on a plate reader after the sample was prepared according to the manufacturer's instruction. Then the standard curve was used to determine the protein concentration of each sample. The PF samples were labelled with biotin according to the manufacturer's instructions.

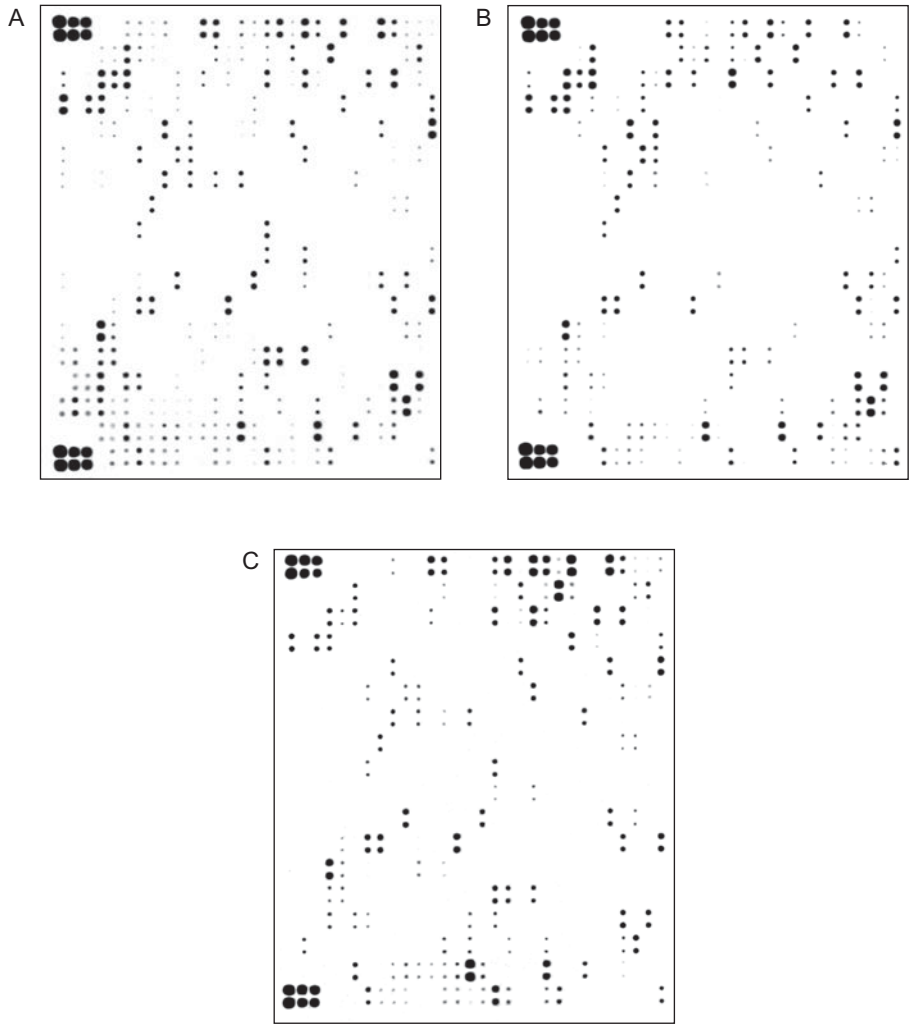

Figure 1. Cytokine array detection of peritoneal fluid in different groups. Biotin Label-based Human Antibody Array I purchased from RayBiotech was used to detect the expression levels of cytokines in peritoneal fluid. The signal was detected directly from the membrane using a chemiluminescence imaging system. (C) control; (A) endometriosis stage I/II; (B) endometriosis stage III/IV.

Table 1. Expression of cytokines in the peritoneal fluid from women in the endometriosis (EM) and control groups. In total 78 cytokines have a threefold change including 74 increases and four decreases in endometriosis compared with the control group.

Cytokine name	Control	EMs (I/II)	EMs (III/IV)	Fold expression
Activin A	0.00053	0.08800	0.00677	88.74
Activin B	0.00541	0.08468	0.00530	8.31
Activin C	0.00791	0.05350	0.00883	3.94
Activin RIB/ALK-4	0.00206	0.01599	0.00812	5.84
Activin RII A/B	0.00029	0.02548	0.01556	70.44
BCMA/TNFRSF17	0.00607	0.07222	0.01913	7.53
BD-1	0.00245	0.07391	0.04854	24.97
β-Catenin	0.00532	0.09098	0.04217	12.52
β-Defensin 2	0.00920	0.05896	0.01402	3.97
BMP-3	0.04568	0.29318	0.42959	7.91
BMP-3b/GDF-10	0.01075	0.05949	0.06019	5.56
BMP-4	0.03374	0.07752	0.39338	6.98
CCR4	0.01248	0.32246	0.08982	16.52
CCR6	0.00362	0.04967	0.01828	9.39
CCR8	0.07729	0.56392	0.63898	7.78
CD30 ligand/TNFSF8	0.00367	0.10469	0.29568	54.62
CD40/TNFRSF5	0.00345	0.01457	0.05325	9.84
CD40 ligand/TNFSF5/CD154	0.08712	0.30458	0.67308	5.61
Cerberus 1	0.01913	0.06702	0.49731	14.75
Chem R23	0.00495	0.00371	0.04321	4.74
CV-2/Crossveinless-2	0.00036	0.00889	0.00722	22.12
CXCR4 (fusin)	0.01299	0.11289	0.06370	6.80
DAN	0.01490	0.08358	0.37861	15.51
Dkk-1	0.00019	0.00599	0.00706	33.59
Dkk-4	0.01415	0.10935	0.04726	5.53
Erythropoietin	0.20691	0.67669	0.70349	3.34
Endothelin	0.01961	0.33334	0.60879	24.02
Fas ligand	0.00808	0.04820	0.00163	3.08
FGF-16	0.00767	0.11626	0.00864	8.14
FGF-18	0.00444	0.01832	0.01354	3.59
FGF-23	0.07530	0.52375	0.44470	6.43
Follistatin	0.07428	0.49690	0.74462	8.36
Follistatin-like 1	0.07486	0.47888	0.36075	5.61
GDF-15	0.01121	0.06878	0.01103	3.56
GDNF	0.00022	0.00333	0.00566	20.57
GFR α-2	0.00539	0.05345	0.01021	5.91
Glucagon	0.16926	0.56456	0.52719	3.23
Glut5	0.05139	0.45210	0.04156	4.80
Growth hormone (GH)	0.00039	0.00333	0.00369	9.04
HGF	0.00274	0.00867	0.01264	3.89
IL-1 ra	0.07581	0.48687	0.35237	5.54
IL-9	0.00558	0.01245	0.02470	3.33
IL-17B R	0.00083	0.04437	0.01347	35.04
IL-17C	0.05539	0.44597	0.02306	4.23
IL-17F	0.06976	0.45980	0.03348	3.54
IL-20 R β	0.02721	0.11077	0.48952	11.03
Inhibin B	0.00777	0.09205	0.01141	6.66
Kremen-2	0.05549	0.39180	0.38147	6.97
Lck	0.00257	0.00356	0.05105	10.61
MFRP	0.01032	0.05478	0.00999	3.14
MMP-8	0.00952	0.04955	0.01201	3.23
MMP-16/MT3-MMP	0.02454	0.09808	0.06524	3.33
MSP β-chain	0.01303	0.08337	0.06836	5.82

Table 1. continued on next page.

Table 1. Continued.

Cytokine name	Control	EMs (I/II)	EMs (III/IV)	Fold expression
NCAM-1/CD56	0.00240	0.08021	0.04743	26.56
NRG2	0.00675	0.02857	0.01546	3.26
NRG3	0.00517	0.04794	0.00476	5.10
Persephin	0.01466	0.07543	0.04291	4.04
PF4/CXCL4	0.10928	0.68141	0.23812	4.21
Prolactin	0.00248	0.01847	0.00954	5.66
Smad 7	0.03881	0.51743	0.05882	7.42
Smad 8	0.01211	0.06728	0.01089	3.23
SMDF/NRG1Isoform	0.01556	0.06804	0.04847	3.74
Sonic hedgehog (Shh N-terminal)	0.00985	0.05136	0.01037	3.13
TECK/CCL25	0.00068	0.00613	0.00237	6.25
TGF-β RI/ALK-5	0.05578	0.26100	0.08233	3.08
Thrombopoietin (TPO)	0.00876	0.06928	0.07272	8.10
Thrombospondin-1	0.06937	0.22478	0.46485	4.97
Thrombospondin-4	0.01585	0.05065	0.21411	8.35
Tie-2	0.00425	0.04991	0.01956	8.18
TNF-β	0.00061	0.00844	0.00741	13.06
TRAIL R3/TNFRSF10C	0.00153	0.00708	0.04546	17.18
TRAIL R4/TNFRSF10D	0.01850	0.05813	0.37707	11.76
TRANCE	0.05022	0.13230	0.35284	4.83
WISP-1/CCN4	0.01170	0.01367	0.06218	3.24
CCL14/HCC-1/HCC-3	0.04219	0.00979	0.01227	0.26
CCR1	0.12311	0.06436	0.01549	0.32
HB-EGF	0.40073	0.11125	0.15058	0.33
LIF	0.04100	0.01176	0.00923	0.26

Cytokine array

Human Antibody Array I was used to detect the expression levels of 507 human proteins according to the manufacturer's instruction. Briefly, the membrane was placed in the tray and incubated with blocking buffer at room temperature for 1 h. A PF sample was added to each array membrane and incubated at room temperature for 2 h. After thorough washing, HRP-conjugated streptavidin was added to each membrane and kept at room temperature for 2 h with gentle shaking. Detection Buffer C and D were sequentially added for chemiluminescence development. The signal was detected directly from the membrane using a chemiluminescence imaging system.

Data were revised by the background and standardized by ScanAlyze software (Stanford University, Stanford, CA, USA).

Statistical analysis

Concentration of the cytokines was compared between the different groups. Data were considered to be significant if the fold change was more than three times. Different cytokines were analysed by Pathway Studio software 5.0 (Ariadne, Rockville, MD, USA) and the cytokine network associated with the pathogenesis and development of endometriosis was established. A number of cytokines with a lot of connections with others were selected for the discussion.

Results

Cytokine array detection

Using the standard curve we determined the protein concentration of each sample. Protein concentrations in each PF sample were detected. Then we pooled the three samples of each group with equal amounts of protein for the detection of the cytokine (Figure 1).

Different cytokine expression between women with and without endometriosis

In total 78 cytokines had a threefold change including 74 increases and four decreases in the endometriosis group compared with the control group (Table 1). Different cytokine networks for the two groups were produced and are shown in Figure 2.

Different cytokine expressions of PF from the I/II endometriosis and control groups

In total 96 cytokines had a threefold change including 91 increases and five decreases in the minimal and mild

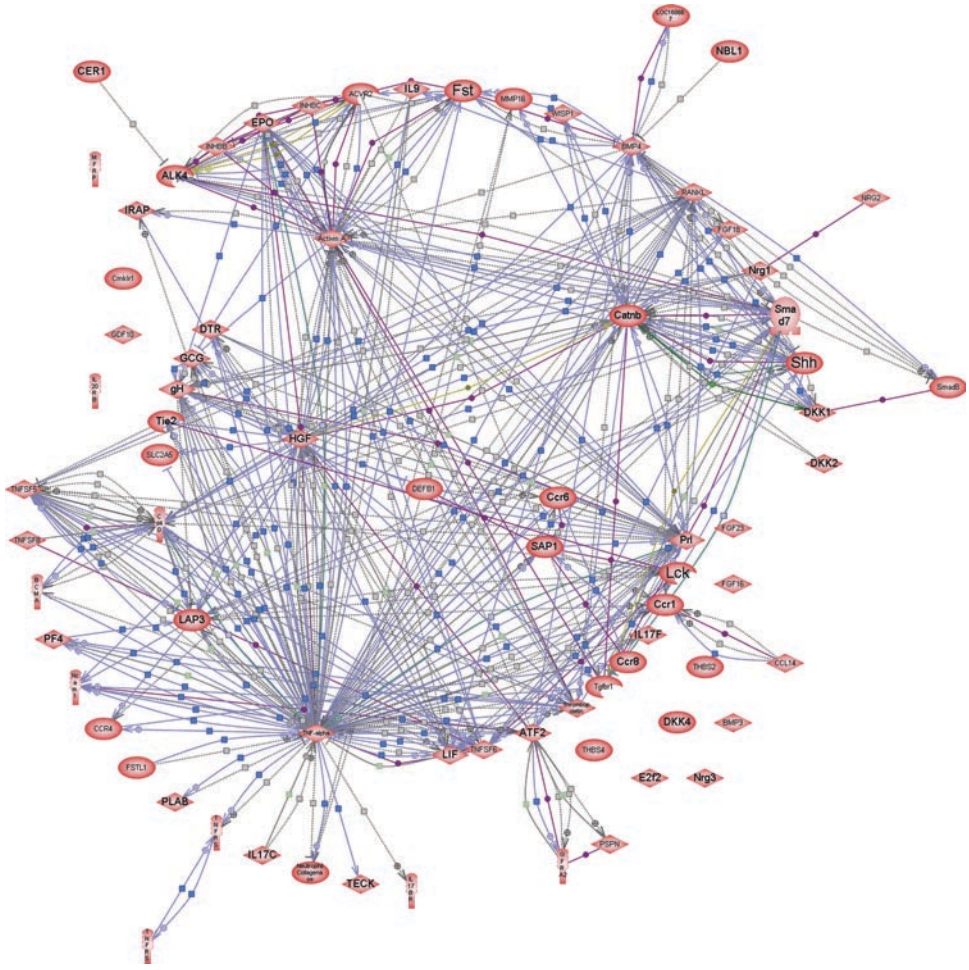

Figure 2. Cytokine network between the endometriosis and control groups. Different cytokines were analysed by Pathway Studio software 5.0 and the cytokine network between the endometriosis and the control group was established.

endometriosis group compared with the control group (Table 2). Among them activin A, activin receptor types A/B (activin RII/A/B), interleukin (IL)-17B receptor (IL-17BR) and β defensin (BD-1) were most significantly increased in the PF of women with endometriosis during stages I–II. These cytokines may be associated with the pathogenesis of endometriosis. The different cytokine networks from the two groups are shown in Figure 3.

Different cytokine expressions of I/II and III/IV endometriosis

In total 83 cytokines had a threefold change including 14 increases and 69 decreases in the moderate and severe endometriosis group compared with the minimal and mild endometriosis group (Table 3). Among them Lck, chemokine CCL23 (chemR23), tumour necrosis factor (TNF)-related apoptosis-inducing ligand receptor 3 (TrailR3), TrailR4 and CD30 were most significantly increased in the PF of women with endometriosis during stages III–IV. These cytokines may be associated with the development of endometriosis. The different cytokine networks from the two groups are shown in Figure 4.

Cytokines with different expression levels

We were interested in several cytokines that had a lot of connections with others, which included activin A, Smad 7, β-NGF, etc. Interestingly expressions of activin A, Smad 7 and β-NGF were increased more than three times in the minimal and mild endometriosis group compared with the control group while they were decreased more than three times in the moderate and severe endometriosis group (Figure 5).

Discussion

Protein arrays with antibodies are developing as a new tool for the rapid measurement of protein expression in various diseases (Belov et al. 2001, Lin et al. 2003). In

Table 2. Expression of cytokines in the peritoneal fluid from women with stage I/II endometriosis (EM) and control groups. In total 96 cytokines have a threefold change including 91 increases and five decreases in the minimal and mild endometriosis group compared with the control group.

Cytokine name	Control	EMs (I/II)	Fold expression
Activin A	0.00053	0.08800	164.80
Activin B	0.00541	0.08468	15.64
Activin C	0.00791	0.05350	6.76
Activin RIA/ALK-2	0.05622	0.17199	3.06
Activin RIB/ALK-4	0.00206	0.01599	7.75
Activin RII A/B	0.00029	0.02548	87.47
ALCAM	0.04753	0.17665	3.72
Angiogenin	0.01515	0.06141	4.05
BCMA/TNFRSF17	0.00607	0.07222	11.90
BD-1	0.00245	0.07391	30.15
β-Catenin	0.00532	0.09098	17.11
β-Defensin 2	0.00920	0.05896	6.41
β-NGF	0.01466	0.04461	3.04
BMP-3	0.04568	0.29318	6.42
BMP-3b/GDF-10	0.01075	0.05949	5.53
CCR3	0.00981	0.04660	4.75
CCR4	0.01248	0.32246	25.85
CCR6	0.00362	0.04967	13.73
CCR8	0.07729	0.56392	7.30
CD30 ligand/TNFSF8	0.00367	0.10469	28.56
CD40/TNFRSF5	0.00345	0.01457	4.23
CD40 ligand/TNFSF5 /CD154	0.08712	0.30458	3.50
Cerberus 1	0.01913	0.06702	3.50
CV-2/Crossveinless-2	0.00036	0.00889	24.41
CXCR4 (fusin)	0.01299	0.11289	8.69
DAN	0.01490	0.08358	5.61
Dkk-1	0.00019	0.00599	30.84
Dkk-4	0.01415	0.10935	7.73
Erythropoietin	0.20691	0.67669	3.27
Endothelin	0.01961	0.33334	17.00
Fas ligand	0.00808	0.04820	5.96
FGF-16	0.00767	0.11626	15.16
FGF-18	0.00444	0.01832	4.13
FGF-23	0.07530	0.52375	6.96
Follistatin	0.07428	0.49690	6.69
Follistatin-like 1	0.07486	0.47888	6.40
GDF-15	0.01121	0.06878	6.13
GDNF	0.00022	0.00333	15.23
GFR α-2	0.00539	0.05345	9.92
Glucagon	0.16926	0.56456	3.34
Glut2	0.11734	0.51681	4.40
Glut5	0.05139	0.45210	8.80
Glypican 5	0.14608	0.50833	3.48
Growth hormone (GH)	0.00039	0.00333	8.57
HGF	0.00274	0.00867	3.16
ICAM-5	0.18261	0.58521	3.20
IL-1 ra	0.07581	0.48687	6.42
IL-17B R	0.00083	0.04437	53.76
IL-17C	0.05539	0.44597	8.05
IL-17F	0.06976	0.45980	6.59
IL-20 R β	0.02721	0.11077	4.07
IL-22	0.01240	0.05364	4.32

Table 2. continued on next page.

Table 2. Continued.

Cytokine name	Control	EMs (I/II)	Fold expression
Inhibin B	0.00777	0.09205	11.85
Kremen-2	0.05549	0.39180	7.06
MFRP	0.01032	0.05478	5.31
MIF	0.01388	0.04480	3.23
MMP-8	0.00952	0.04955	5.21
MMP-10	0.01369	0.04765	3.48
MMP-16/MT3-MMP	0.02454	0.09808	4.00
MSP β-chain	0.01303	0.08337	6.40
NCAM-1/CD56	0.00240	0.08021	33.38
Neuritin	0.04015	0.17130	4.27
Neuropilin-2	0.08440	0.31910	3.78
Neurturin	0.07141	0.25551	3.58
NRG2	0.00675	0.02857	4.23
NRG3	0.00517	0.04794	9.27
Osteoprotegerin/TNFRSF11B	0.23166	0.71849	3.10
Pentraxin3/TSG-14	0.02537	0.09680	3.82
Persephin	0.01466	0.07543	5.15
PF4/CXCL4	0.10928	0.68141	6.24
PLUNC	0.08818	0.37120	4.21
Pref-1	0.06127	0.30285	4.94
Prolactin	0.00248	0.01847	7.46
RELT/TNFRSF19L	0.05546	0.21610	3.90
S100 A8/A9	0.09406	0.29470	3.13
Smad 4	0.15149	0.48863	3.23
Smad 7	0.03881	0.51743	13.33
Smad 8	0.01211	0.06728	5.55
SMDF/NRG1Isoform	0.01556	0.06804	4.37
Sonic hedgehog (Shh N-terminal)	0.00985	0.05136	5.21
SPARC	0.01418	0.05026	3.55
TECK/CCL25	0.00068	0.00613	9.02
TGF-β RI/ALK-5	0.05578	0.26100	4.68
Thrombopoietin (TPO)	0.00876	0.06928	7.91
Thrombospondin-1	0.06937	0.22478	3.24
Thrombospondin-4	0.01585	0.05065	3.20
Tie-1	0.01609	0.07120	4.42
Tie-2	0.00425	0.04991	11.75
TNF-β	0.00061	0.00844	13.90
TRAIL R3/TNFRSF10C	0.00153	0.00708	4.63
TRAIL R4/TNFRSF10D	0.01850	0.05813	3.14
CCL14/HCC-1/HCC-3	0.04219	0.00979	0.23
GDF8	0.04379	0.01338	0.31
HB-EGF	0.40073	0.11125	0.28
IL-11	0.02539	0.00703	0.28
LIF	0.04100	0.01176	0.29

this way it is possible to screen thousands of proteins that may be involved in disease development (Cahill 2001, Lal et al. 2002). The cytokine array is one of the protein arrays which will lead to a better understanding of diseases and the development of new treatments (Sellers & Yates 2003, Seliger & Keller 2002). In the present study the cytokine array was used in an effort to identify proteins that may be associated with or are potential biomarkers of endometriosis.

The cytokine network is very complex in the pathogenesis of endometriosis. For example, Bedaiwy et al. (2002) reported that serum IL-6 and PF TNF-α could be used to discriminate between patients with and without endometriosis with a high degree of sensitivity and specificity. The study of cytokine expression with a cytokine array can provide more information than the traditional methods. The cytokine array allows the simultaneous observation of alterations

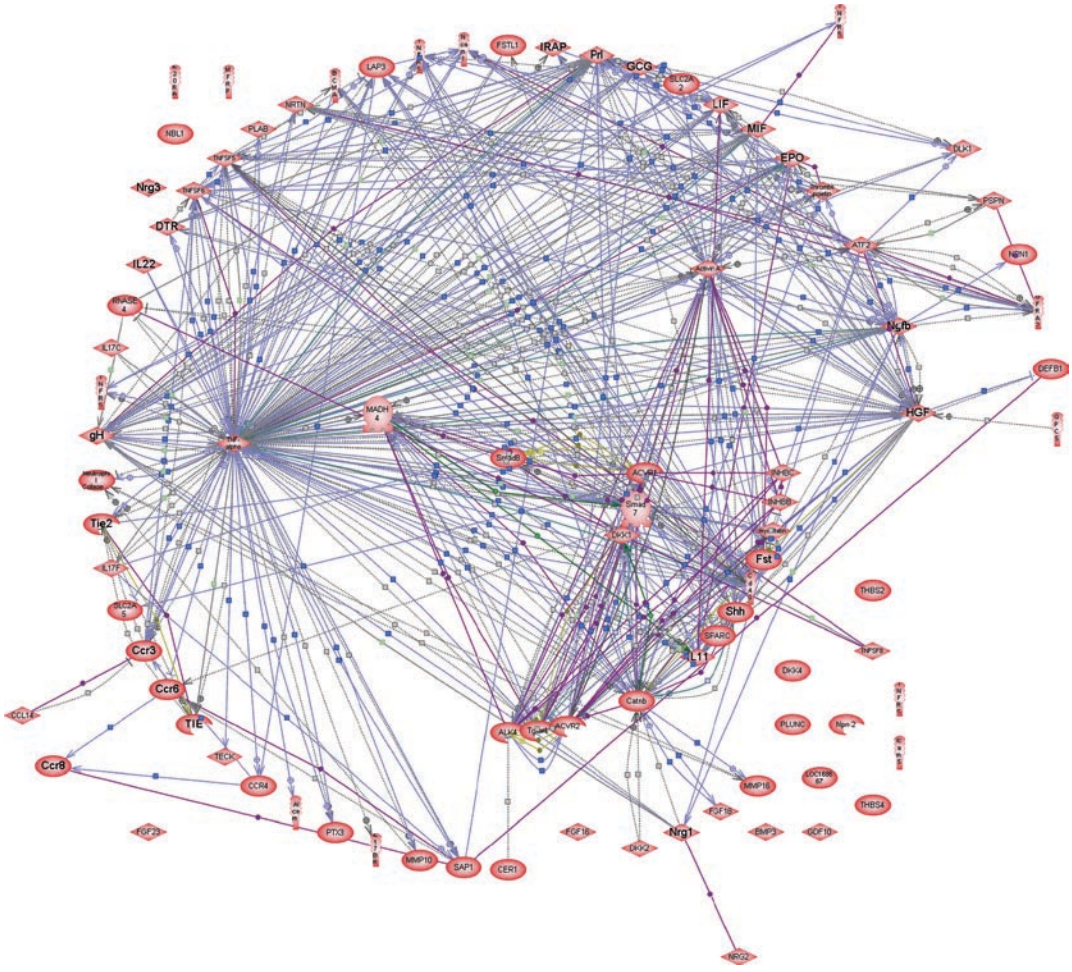

Figure 3. Cytokine network between endometriosis stage I/II group and control group. Different cytokines were analysed by Pathway Studio software 5.0 and the cytokine network between the stage I/II endometriosis group and the control group was established.

in cytokine expression which may be a precursor to disease development or a consequence of the disease (Petricoin 2002). Our study showed different cytokine expressions in the PF between women with minimal/mild and moderate/severe endometriosis and those without endometriosis. We also built the cytokine networks using the Pathway Studio software. Pathway Studio software revealed that most cytokines are involved in cell binding, interaction and protein synthesis and transportation regulation. Among them activin A, activin RII/A/B, IL-17BR and BD-1 are most significantly increased in PF of women with endometriosis during stages I and II. These cytokines may be associated with the pathogenesis of endometriosis. Lck, ChemR23, TrailR3, TrailR4, and CD30 are most significantly increased in PF of women with endometriosis during stages III and IV. These cytokines may be associated with the development of endometriosis.

Among the differently expressed cytokines, activin A, Smad7 and β-nerve growth factor (NGF) are the most interesting. Activin A is a growth factor expressed in increasing amounts throughout endometrial phases by both epithelial and stromal cells. In healthy women activin A concentrations in the PF were significantly higher than in serum at both phases of the menstrual cycle (Florio et al. 1998). Activin-A secretion by glandular and stromal endometrial cells was sevenfold and threefold higher, respectively, in women with endometriosis compared with women without endometriosis. The α- and β A-subunits of inhibin/activin were more abundant in eutopic glandular cells from patients with minimal to mild endometriosis compared with women without endometriosis, which was consistent with our results (Rombauts et al. 2006). In ovarian endometriosis activin A concentration in the cystic fluid was slightly higher than that in PF and significantly higher than that in serum. Immunoreactive α- and β A subunits were strongly expressed both in the epithelial and stromal components of ovarian endometrioma. The relative abundance of βA mRNA was significantly decreased in endometriotic cells compared with eutopic stromal cells. The results provide evidence for

Table 3. Expression of cytokines in the peritoneal fluid from women with stage I/II and III/IV endometriosis (EM). In total 83 cytokines have a threefold change including 14 increases and 69 decreases in the moderate and severe endometriosis group compared with minimal and mild endometriosis group.

Cytokine name	EMs (I/II)	EMs (III/IV)	Fold expression
BMP-4	0.07752	0.39338	5.07
CD40/TNFRSF5	0.01457	0.05325	3.66
Cerberus 1	0.06702	0.49731	7.42
Chem R23	0.00371	0.04321	11.66
DAN	0.08358	0.37861	4.53
GDF11	0.00770	0.05354	6.95
IL-11	0.00703	0.04527	6.44
IL-20 R β	0.11077	0.48952	4.42
Lck	0.00356	0.05105	14.32
Thrombospondin-4	0.05065	0.21411	4.23
TRAIL R3/TNFRSF10C	0.00708	0.04546	6.42
TRAIL R4/TNFRSF10D	0.05813	0.37707	6.49
WISP-1/CCN4	0.01367	0.06218	4.55
XEDAR	0.01062	0.05103	4.80
Activin A	0.08800	0.00677	0.08
Activin B	0.08468	0.00530	0.06
Activin C	0.05350	0.00883	0.17
Activin RIA/ALK-2	0.17199	0.00620	0.04
ALCAM	0.17665	0.04880	0.28
Angiopoietin-4	0.05233	0.01437	0.27
Angiopoietin-like 2	0.46564	0.13549	0.29
Artemin	0.36500	0.10599	0.29
Axl	0.05875	0.01542	0.26
B7-1 /CD80	0.05319	0.00665	0.13
BCMA/TNFRSF17	0.07222	0.01913	0.26
β-Defensin 2	0.05896	0.01402	0.24
β-NGF	0.04461	0.00999	0.22
CCR1	0.06436	0.01549	0.24
CCR2	0.55505	0.06803	0.12
CCR3	0.04660	0.00886	0.19
CCR4	0.32246	0.08982	0.28
CCR5	0.04349	0.01264	0.29
CLC	0.05190	0.01589	0.31
CTLA-4 /CD152	0.06814	0.01750	0.26
Fas ligand	0.04820	0.00163	0.03
FGF R4	0.58892	0.05962	0.10
FGF-16	0.11626	0.00864	0.07
FGF-17	0.01138	0.00320	0.28
GASP-1/WFIKKNRP	0.50536	0.06543	0.13
GDF-15	0.06878	0.01103	0.16
GFR α-2	0.05345	0.01021	0.19
Glut2	0.51681	0.08816	0.17
Glut5	0.45210	0.04156	0.09
Glypican 5	0.50833	0.04980	0.10
IL-4 R	0.65841	0.02375	0.04
IL-17B R	0.04437	0.01347	0.30
IL-17C	0.44597	0.02306	0.05
IL-17F	0.45980	0.03348	0.07
IL-22	0.05364	0.01286	0.24
IL-29	0.63438	0.06052	0.10
Inhibin B	0.09205	0.01141	0.12
MFRP	0.05478	0.00999	0.18

Table 3. continued on next page.

Table 3. Continued.

Cytokine name	EMs (I/II)	EMs (III/IV)	Fold expression
MIF	0.04480	0.00964	0.22
MMP-7	0.06281	0.01513	0.24
MMP-8	0.04955	0.01201	0.24
MMP-10	0.04765	0.00644	0.14
Neuritin	0.17130	0.05406	0.32
Neuropilin-2	0.31910	0.06758	0.21
Neurturin	0.25551	0.05304	0.21
NRG3	0.04794	0.00476	0.10
OSM	0.62269	0.18789	0.30
Osteoactivin/GPNMB	0.54274	0.17613	0.32
Osteoprotegerin/TNFRSF11B	0.71849	0.09341	0.13
PLUNC	0.37120	0.07279	0.20
Pref-1	0.30285	0.05335	0.18
RELT/TNFRSF19L	0.21610	0.00931	0.04
sgp130	0.04221	0.00862	0.20
Smad 4	0.48863	0.10511	0.22
Smad 7	0.51743	0.05882	0.11
Smad 8	0.06728	0.01089	0.16
Sonic hedgehog (Shh N-terminal)	0.05136	0.01037	0.20
SPARC	0.05026	0.01234	0.25
Spinesin	0.04696	0.01326	0.28
TACI/TNFRSF13B	0.04910	0.01115	0.23
TCCR/WSX-1	0.09409	0.01430	0.15
TGF-β 2	0.08271	0.00907	0.11
TGF-β RI/ALK-5	0.26100	0.08233	0.32
Tie-1	0.07120	0.01627	0.23
TLR3	0.05488	0.00355	0.06
TREM-1	0.09212	0.01151	0.12
TROY/TNFRSF19	0.07919	0.01781	0.22
Vasorin	0.05742	0.00933	0.16
VE-Cadherin	0.04482	0.01397	0.31

a local production and secretion of activin A in ovarian endometriotic cysts (Reis et al. 2001). A recent study revealed that activin A mRNA expression in eutopic endometrium of patients with endometriosis was significantly higher than in controls with a 10.2-fold and 7.3-fold increase in the proliferative and secretory phases, respectively. Activin RII mRNA expression were found to be similar in patients with and without endometriosis, while cripto (coreceptor) mRNA was markedly lower in eutopic and ectopic endometrium of women with endometriosis compared with healthy controls. So the altered endometrial expression of activin A and cripto support the involvement of the activin system in endometrial changes of women with endometriosis (Torres et al. 2009).

Smads are expressed in various cells and tissues whose altered expression has been associated with several abnormalities and cellular resistance to growth inhibitory action of transforming growth factor (TGF)-β (Massague &Wotton 2000, Moustakas et al. 2001, Kato et al. 2001). Smads comprise receptor-activated/pathway-specific Smad1, -2, -3, -5 and -8, common Smad (Smad4), and the inhibitory Smad (Smad6 and -7) (Moustakas et al. 2001). Activated TGF-β receptors induce recruitment of multiple intracellular signals, specifically Smads, whose activation and subsequent translocation into the nucleus results in gene expression in response to TGF-β (Moustakas et al. 2001, Kato et al. 2001, Chegini et al. 1992). The inhibitory Smad interacts with TGF-type I receptor and prevents R Smads phosphorylation, interrupting TGF-β induced signalling (Moustakas et al. 2001). Luo et al. (2003) showed that gonadotrophin releasing hormone (GnRH) increased the expression of inhibitory Smad7 mRNA in endometrial surface epithelial cells with a limited effect on endometrial stromal cells, while moderately increasing the common Smad7 protein levels in these cells. GnRH acts directly on the endometrial cells, altering the expression and activation of Smads, a mechanism that could lead to interruption of the TGF-β receptor signalling pathway in the endometrium. In our results concentrations of Samd4, 7, 8 and TGF-β RI were high in the minimal and mild endometriosis, but decreased more than three times in moderate and severe endometriosis, which suggests TGF-β and the Smad signal pathway have

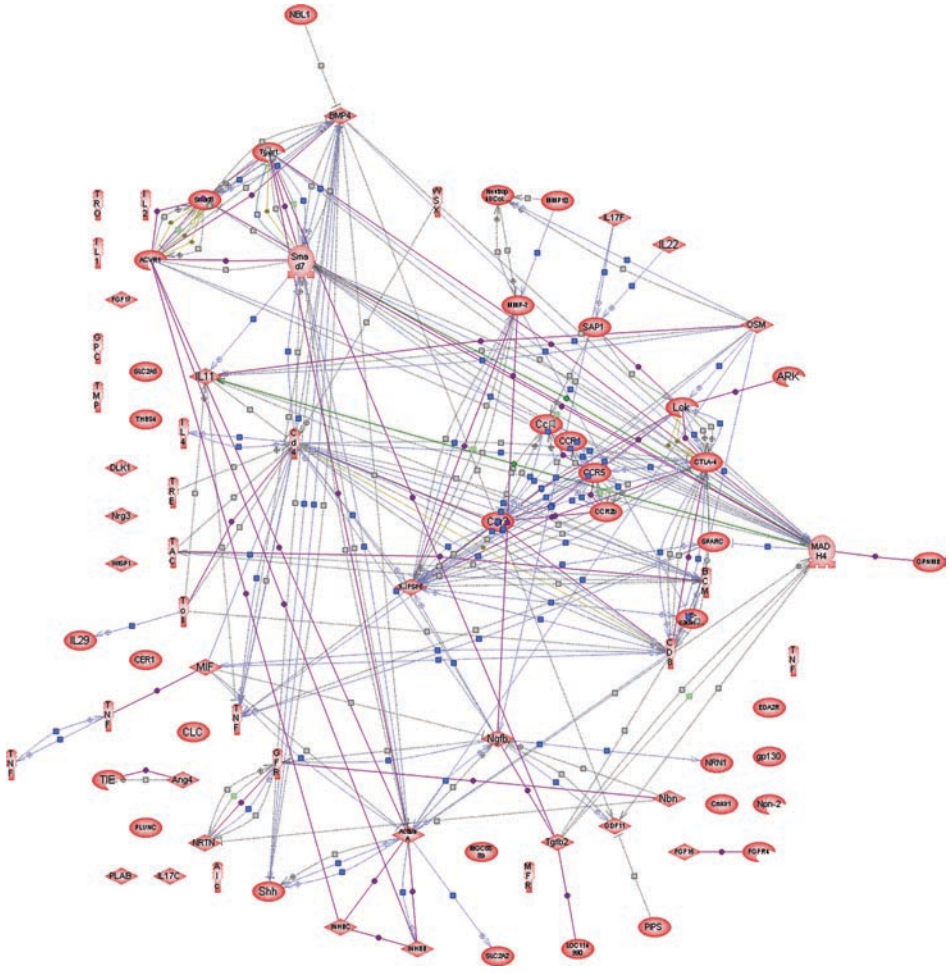

Figure 4. Cytokine network between stages I/II and III/IV endometriosis. Different cytokines were analysed by Pathway Studio software 5.0 and the cytokine network between stage I/II and III/IV endometriosis groups was established.

an important role in the pathogenesis of endometriosis and deserve further research.

Endometriosis is a gynaecological disease that can be associated with severe pelvic pain. However, the mechanisms by which pain is generated remain unknown. Increased β-NGF in the minimal and mild endometriosis may be related to the pain of the patient. Tokushige et al. (2006) revealed that there were significantly more nerve fibres identified in peritoneal endometriotic lesions than in normal peritoneum. There was an intense NGF immunoreactivity near endometriotic glands, and NGFRp75 immunoreactive nerve fibres were present near endometriotic glands and blood vessels in the peritoneal endometriotic lesions. Another study (Anaf et al. 2002) showed NGF expression was significantly stronger in deep adenomyotic nodules than in ovarian and peritoneal endometriosis, both in the proliferative phase in the glands and in the stroma, which suggests a role of β-NGF in endometriotic pain and hyperalgesia in deep adenomyotic nodules.

The other cytokines, IL-17BR, BD-1, TrailR3, TrailR4, etc., that were most significantly increased in the PF of women with endometriosis during stages I–II or stages III–IV are also very important.

IL-17 is a representative cytokine secreted from T-helper (Th) 17 cells. The patients with minimal/mild endometriosis had a significantly higher level of IL-17 in the PF compared with those with moderate/severe endometriosis or without endometriosis (Zhang et al. 2005). We obtained the same result. Another study revealed IL-17 enhanced IL-8 secretion from Endometrial Stromal Cells(ESCs) in a dose-dependent manner. The IL-17-induced secretion of IL-8 from ESCs was suppressed by anti-IL-17R antibodies. Addition of TNF-α synergistically increased IL-17-induced IL-8 secretion from endometriotic stromal cells. IL-17 also enhanced the expression of cyclooxygensase-2 and proliferation of endometriotic stromal cells. So IL-17 may play a role in the development of endometriosis by stimulating inflammatory responses and proliferation of endometriotic stromal cells (Hirata et al. 2008).

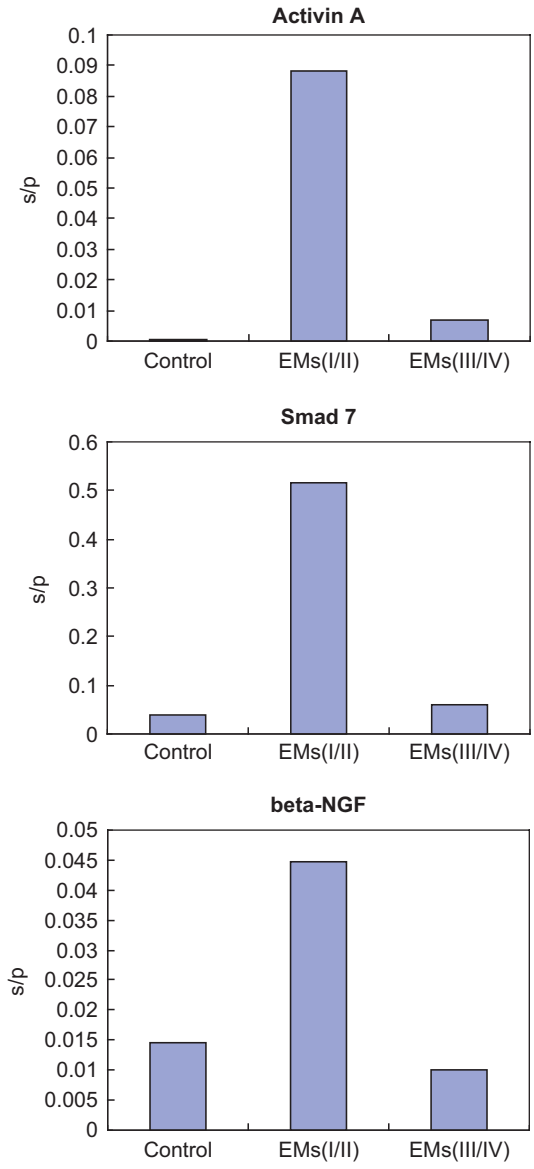

Figure 5. Different expressions of activin A, Smad 7, β-nerve growth factor (NGF) in patients and controls. Expressions of activin A, Smad 7 and β-NGF were increased more than three times in the minimal and mild endometriosis stage group compared with the control group while they were decreased more than three times in the moderate and severe endometriosis stage group. s/p, the relative results standardized by positive sample.

Glandular epithelium is the main source of BD-1 expression in the human endometrium. BD-1 was the only β-defensin detected by immunochemical staining in the mid-luteal phase endometrium. The intensity of staining was significantly different in the endometrial stroma, luminal and glandular epithelia. BD-1 expression is not significantly higher in female factor infertility (Das et al. 2007). However the function of increased expression of BD-1 in endometriosis is unknown.

Trail is a member of the TNF superfamily of cytokines that induces apoptosis upon binding to its death domain-containing transmembrane receptors, TrailR1 and TrailR2. Failure of apoptosis of refluxed endometrial cells within the peritoneal cavity is a possible aetiological factor for development of endometriosis. The effect of tunicamycin, a possible apoptosis enhancer, on Trail-induced apoptosis in endometriotic stromal cell was studied. Tunicamycin increases both TrailR2 mRNA and Trail-induced apoptosis in endometriotic stromal cell. The combined treatment with tunicamycin and Trail may have therapeutic potential in the treatment of endometriosis (Hasegawa et al. 2009). Osteoprotegerin is a survival factor that exerts its effect by binding to Trail, thus preventing Trail from binding to the apoptosis receptors TrailR1 and TrailR2. With respect to the stages of the disease, the concentrations of osteoprotegerin in women with stage III/IV endometriosis were significantly higher than in those without endometriosis and those with stage I/II endometriosis. These findings suggest that the Trail/osteoprotegerin system is involved in the pathophysiology of endometriosis, possibly affecting the apoptosis of endometriotic cells (Harada et al. 2004). The other two types of Trail receptor (TrailR3, TrailR4) deserve further study.

However there are many cytokines, such as TNF-α, IL-1, -6, -8, -10, -12, etc., that have been widely documented in the PF of women with endometriosis (Bergqvist et al. 2001, Lebovic et al. 2001). These cytokines were detected in our experiments, but the results revealed no significant difference between women with endometriosis of different stages and those without endometriosis. This may be because of the small sample size and high standard level (threefold) used as the threshold level for significance. A partial comparison of endometrial gene microarray data from the five published studies has been presented. Strikingly only one gene was consistently upregulated. There is lacking a large list of consensus genes in the five microarray studies. It is very common that no significant difference can be found in DNA or protein array analysis data while the significant differences were widely documented in the other studies by the methods of Western blot or enzyme-linked immunosorbent assay (Horcajadas et al. 2007).

Our results provide further insights into the mechanism of immunological modulation of the cytokine network during the process of endometriosis, suggesting that cytokine network analysis may be a new strategy for the development of a novel approach for diagnosis and treatment of endometriosis. However, these findings also indicate that the pathogenesis of endometriosis is very complex and involves many cytokines, which warrant further study.

Acknowledgements

This work was supported by National 973 and 863 programmes of China (no. 2006CB944005,

2006AA02Z4A4, IRT0631) and programmes of Education Bureau of Jiangsu Province for innovative project of graduated students (No. cx07B-217Z) and the 135 Grants from the Health Bureau of Jiangsu Province for Clinical Reproductive Medicine. Gratitude is extended to the patients who participated in the study and to Dr Guo Xuejiang for excellent technical assistance.

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

References

American Society for Reproductive Medicine. (1996). Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril* 67:817–21.

Anaf V, Simon PE, Nakadi I, Fayt I, Simonart T, Buxant F, Noel JC. (2002). Hyperalgesia, nerve infiltration and nerve growth factor expression in deep adenomyotic nodules, peritoneal and ovarian endometriosis. *Hum Reprod* 17:1895–900.

Bedaiwy MA, Falcone T, Sharma RK, Goldberg JM, Attaran M, Nelson DR, Agarwal A. (2002). Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial. *Hum Reprod* 17:426–31.

Belov L, de la Vega O, dos Remedios CG, Mulligan SP, Christopherson RI. (2001). Immunophenotyping of leukemias using a cluster of differentiation antibody microarray. *Cancer Res* 61:4483–9.

Bergqvist A, Bruse C, Carlberg M, Carlström K. (2001). Interleukin 1β, interleukin-6, and tumor necrosis factor-alpha in endometriotic tissue and in endometrium. *Fertil Steril* 75:489–95.

Cahill DJ. (2001). Protein and antibody arrays and their medical applications.*J Immunol Methods* 250:81–91.

Chegini N, Rossi MJ, Masterson BJ. (1992). Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and EGF and PDGF β-receptors in human endometrial tissue: localization and *in vitro* action. *Endocrinology* 130:2373–85.

Das S, Vince GS, Lewis-Jones I, Bates MD, Gazvani R. (2007). The expression of human alpha and β defensin in the endometrium and their effect on implantation. *J Assist Reprod Genet* 24:533–9.

D'Hooghe TM, Debrock S, Hill JA, Meuleman C. (2003). Endometriosis and subfertility: is the relationship resolved? *Semin Reprod Med* 21:243–54.

Ferrero S, Gillott DJ, Remorgida V, Anserini P. (2005a). Haptoglobin β chain isoforms in the plasma and peritoneal fluid of women with endometriosis. *Fertil Steril* 83:1536–43.

Ferrero S, Gillott DJ, Anserini P, Remorgida V. (2005b). Vitamin D binding protein in endometriosis.*J Soc Gynecol Invest* 12:272–7.

Florio P, Luisi S, Viganò P, Busacca M, Fadalti M, Genazzani AR, Petraglia F. (1998). Healthy women and patients with endometriosis show high concentrations of inhibin A, inhibin B, and activin A in peritoneal fluid throughout the menstrual cycle. *Hum Reprod* 13:2606–11.

Fowler PA, Tattum J, Bhattacharya S, Klonisch T. (2007). An investigation of the effects of endometriosis on the proteome of human eutopic endometrium: a heterogeneous tissue with a complex disease. *Proteomics* 7:130–42.

Gazvani R, Templeton A. (2002). Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis. *Reproduction* 123:217–26.

Gruppo Italiano per lo studio dell' endometriosi. (1994). Prevalence and anatomical distribution of endometriosis in women with selected gynaecological conditions: results from a multicentric Italian study. *Hum Reprod* 9:1158–62.

Harada M, Osuga Y, Hirata T, Hirota Y, Koga K, Yoshino O, Morimoto C, Fujiwara T, Momoeda M, Yano T, Tsutsumi O, Taketani Y. (2004). Concentration of osteoprotegerin (OPG) in peritoneal fluid is increased in women with endometriosis. *Hum Reprod* 19:2188–91.

Hasegawa A, Osuga Y, Hirota Y, Hamasaki K, Kodama A, Harada M, Tajima T, Takemura Y, Hirata T, Yoshino O, Koga K, Yano T, Taketani Y. (2009). Tunicamycin enhances the apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand in endometriotic stromal cells. *Hum Reprod* 24:408–14.

Hirata T, Osuga Y, Hamasaki K, Yoshino O, Ito M, Hasegawa A, Takemura Y, Hirota Y, Nose E, Morimoto C, Harada M, Koga K, Tajima T, Saito S, Yano T, Taketani Y. (2008). Interleukin (IL)-17A stimulates IL-8 secretion, cyclooxygensase-2 expression, and cell proliferation of endometriotic stromal cells. *Endocrinology* 149:1260–7.

Horcajadas JA, Pellicer A, Simón C. (2007). Wide genomic analysis of human endometrial receptivity: new times, new opportunities. *Hum Reprod Update* 13:77–86.

Kato S, Ueda S, Tamaki K, Fuji M, Miyazono K, ten Dijke P, Morimatsu M, Okuda S. (2001). Ectopic expression of Smad7 inhibits transforming growth factor-β responses in vascular smooth muscle cells. *Life Sci* 69:2641–52.

Koninckx PR, Kennedy SH, Barlow DH. (1998). Endometriotic disease: the role of peritoneal fluid. *Hum Reprod Update* 4:741–51.

Koninckx PR, Kennedy SH, Barlow DH. (1999). Pathogenesis of endometriosis: the role of peritoneal fluid. *Gynecol Obstet Invest* 47:23–33.

Kyama CM, T'Jampens D, Mihalyi A, Simsa P. (2006). ProteinChip technology is a useful method in the pathogenesis and diagnosis of endometriosis: a preliminary study. *Fertil Steril* 86:203–9.

Lal SP, Christopherson RI, dos Remedios CG. (2002). Antibody arrays: an embryonic but rapidly growing technology. *Drug Discov Today* 7:143–9.

Lebovic DI, Mueller MD, Taylor RN. (2001). Immunobiology of endometriosis. *Fertil Steril* 75:1–10.

Lin Y, Huang R, Chen L, Lisoukov H. (2003). Profiling of cytokine expression by biotin-labeled-based protein arrays. *Proteomics* 3:1750–7.

Luo X, Xu J, Chegini N. (2003). Gonadotropin releasing hormone analogue (GnRHa) alters the expression and activation of Smad in human endometrial epithelial and stromal cells. *Reprod Biol Endocrinol* 16:125.

Massague J, Wotton D. (2000). Transcriptional control by the TGF-β/Smad signaling system. *EMBO* 19:1745–54.

Moustakas A, Souchelnytskyi S, Heldin CH. (2001). Smad regulation in TGF-β signal transduction. *J Cell Sci* 114:4359–69.

Nothnick WB, Curry TE, Muse KN, London SN, Vernon MW. (1994). Detection of a unique 32-kd protein in the peritoneal fluid of women with endometriosis. *Fertil Steril* 61:288–93.

Noyes RW, Hertig AT, Rock J. (1975). Dating the endometrial biopsy. *Am J Obstet Gynecol* 122:262–3.

Peng X, Wood CL, Blalock EM, Chen KC, Landfield PW, Stromberg AJ. (2003). Statistical implications of pooling RNA samples for microarray experiments. *BMC Bioinformatics* 4:26.

Petricoin EF, Zoon KC, Kohn EC, Barrett JC, Liotta LA. (2002). Clinical proteomics: translating benchside promise into bedside reality. *Nat Rev Drug Discov* 1:683–95.

Poliness AE, Healey MG, Brennecke SP, Moses EK. (2004). Proteomic approaches in endometriosis research. *Proteomics* 4:1897–902.

Ramey JW, Archer DF. (1993). Peritoneal fluid: its relevance to the development of endometriosis. *Fertil Steril* 60:1–14.

Reis FM, Di Blasio AM, Florio P, Ambrosini G, Di Loreto C, Petraglia F. (2001). Evidence for local production of inhibin A and activin A in patients with ovarian endometriosis. *Fertil Steril* 75:367–73.

Rombauts L, Donoghue J, Cann L, Jones RL, Healy DL. (2006). Activin-A secretion is increased in the eutopic endometrium from women with endometriosis. *Aust NZ J Obstet Gynaecol* 46:148–53.

Sampson JA. (1927). Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol* 14:442–69.

Seliger B, Keller R. (2002). Design of proteome-based studies in combination with serology for the identification of biomarkers and novel targets. *Proteomics* 2:1641–51.

Sellers TA, Yates JR. (2003). Review of proteomics with applications to genetic epidemiology. *Genet Epidemiol* 24:83–98.

Tabibzadeh S, Becker JL, Parsons AK. (2003). Endometriosis is associated with alterations in the relative abundance of proteins and IL-10 in the peritoneal fluid. *Front Biosci* 8:70–8.

Tokushige N, Markham R, Russell P, Fraser IS. (2006). Nerve fibres in peritoneal endometriosis. *Hum Reprod* 21:3001–7.

Torres P, Florio P, Galleri L, Reis FM, Borges L, Petraglia F. (2009). Activin A, activin receptor type II, nodal, and cripto mRNA are expressed by eutopic and ectopic endometrium in women with ovarian endometriosis. *Reprod Sci* 16:727–33.

Zhang H, Niu Y, Feng J, Guo H, Ye X, Cui H. (2006). Use of proteomic analysis of endometriosis to identify different protein expression in patients with endometriosis versus normal controls. *Fertil Steril* 86:274–82.

Zhang X, Xu H, Lin J, Qian Y, Deng L. (2005). Peritoneal fluid concentrations of interleukin-17 correlate with the severity of endometriosis and infertility of this disorder. *BJOG* 112:1153–5.