

Levels of endoplasmic reticulum stress-related mRNA in peritoneal fluid of patients with endometriosis or gynaecological cancer

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Abstract

Objective: To compare the levels of endoplasmic reticulum (ER) stress-associated mRNAs and the clinical characteristics of patients with endometriosis or gynaecological cancer.

Methods: This prospective study obtained intraperitoneal fluid samples from female patients that underwent surgery. The levels of ER stress mRNAs in the peritoneal fluid, including C/EBP-homologous protein (CHOP), X-box binding protein 1 (sXBPI), activating transcription factor 6 (ATF6), immunoglobulin heavy chain-binding protein (BiP), inositol-requiring enzyme 1 α (IRE1 α) and protein kinase RNA-like endoplasmic reticulum kinase (PERK), were measured using real-time reverse transcription–polymerase chain reaction in patients with benign disease without endometriosis (control group), with endometriosis or with gynaecological cancer.

Results: This study enrolled 126 patients: 46 control patients; 47 with endometriosis; and 33 with cancer. The levels of CHOP and BiP mRNA were significantly higher in the control group compared with the cancer group. Levels of sXBPI and ATF6 mRNA were significantly higher in the cancer group than in the control and endometriosis groups. In the endometriosis group, ATF6 mRNA level was inversely correlated with age and positively correlated with serum cancer antigen 125 levels; and ATF6 and PERK mRNA levels were inversely correlated with parity.

Conclusion: The levels of ER stress-related mRNAs were related to the pathogenesis of endometriosis and gynaecological cancers.

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Keywords

Endoplasmic reticulum stress-related mRNA, endometriosis, gynaecological cancer

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Introduction

Endometriosis is a chronic inflammatory condition affecting approximately 5–15% of all women of reproductive age that is associated with chronic pelvic pain, dysmenorrhoea, dyspareunia, infertility and menstrual irregularities.¹ Although endometriosis is not malignant, its ability to infiltrate into and invade distant tissues is similar to that of the metastasis of malignant tumours.² Several hypotheses have been proposed to explain the aetiology of endometriosis such as retrograde menstrual reflux, entopic presence of endometrial stem cells and defects in the immune system.³ However, the exact pathogenesis of endometriosis has not yet been fully elucidated.

The endoplasmic reticulum (ER) is a multifunctional organelle critical for the proper folding and assembly of secreted and transmembrane proteins.⁴ In eukaryotic cells, secreted and membrane proteins fold and mature in the lumen of the ER, facilitated by a large spectrum of chaperones, and correctly folded proteins exit the ER and travel along the secretory pathway to their final destination.⁵ An accumulation of unfolded or misfolded proteins in the ER leads to stress conditions. To mitigate such circumstances, ER stress activates a homeostatic intracellular signalling network that is collectively called the unfolded protein response (UPR).⁶ Failure of the UPR to sustain ER proteostasis contributes to the development of several pathologies including metabolic, neurodegenerative and inflammatory diseases.⁵ Impaired ER

homeostasis by severe/prolonged ER stress-mediated UPR signalling pathways reportedly contributes to several reproductive tissue pathologies including endometriosis, cancers and recurrent pregnancy loss.⁷ Involvement of ER stress in the pathogenesis of endometriosis is thought to be related to angiogenesis of ectopic endometrial tissues, cell survival and tissue growth.⁸

This study investigated the levels of ER stress-related mRNA in the peritoneal fluid of patients with benign disease without endometriosis (control group), with endometriosis, or with gynaecological (ovarian, uterine and cervical) cancer and compared the levels between the three groups. The study also evaluated the correlations between the levels of ER stress-related mRNAs and clinical data and serum cancer antigen 125 (CA-125) level in the three groups.

Patients and methods

Patient population

This prospective study obtained intraperitoneal fluid samples from female patients with benign disease without endometriosis (control group) and patients with endometriosis or gynaecological cancer that underwent surgery in the Department of Obstetrics and Gynaecology, St. Vincent's Hospital, The Catholic University of Korea, Suwon, Korea between July 2017 and July 2018. During laparoscopy, peritoneal fluid was collected aseptically from the Pouch of Douglas, taking care to avoid areas of

bleeding. Patients with inflammatory diseases, hormone producing conditions including pregnancy, or blood-contaminated peritoneal fluid and those in whom peritoneal fluid was not collected were excluded from this study. The samples were centrifuged at 1800 g for 10 min at 4°C using an Eppendorf® Centrifuge 5810R (Eppendorf, Hamburg, Germany) and the supernatants were stored at -80°C in 1.5 ml aliquots. Cell pellets were stored at -80°C in 1.5 ml aliquots after adding RNase inhibitor (Sigma-Aldrich, St Louis, MO, USA).

The study protocol was approved by the Institutional Review Board of St. Vincent's Hospital, The Catholic University of Korea, Suwon, Korea (no. VC16TISI0148) and written informed consent was obtained from each patient (VC16TISI0148).

RNA extraction and real-time RT-PCR

Total RNA was purified from the peritoneal fluid samples using TRIzol® reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed on a StepOnPlus real-time PCR system with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primer sequences are shown in Table 1. The relative amount of the target gene mRNA compared with β -actin was calculated using the formula $2^{-(\text{target gene}-\beta\text{-actin})}$.

Demographic characteristics and correlation with ER stress-related mRNA levels

The correlations between the levels of six ER stress-related mRNAs comprising those encoding C/EBP-homologous protein (CHOP), X-box binding protein 1 (sXBP1), activating transcription factor 6 (ATF6), immunoglobulin heavy chain-binding protein (BiP), inositol-requiring enzyme 1 α (IRE1 α), and protein kinase RNA-like endoplasmic reticulum kinase (PERK) and age, parity and CA-125 level in the three groups.

Table 1. Primers for real-time reverse transcription-polymerase chain reactions.

mRNA	Direction	Primer sequences	Length, base pairs
β -actin	Forward	5'-GCGAGAAGATGACCCAGATC-3'	77
	Reverse	5'-GGATAGCACAGCCTGGATAG-3'	
CHOP	Forward	5'-GTACCTATGTTTCACCTCCTGG-3'	150
	Reverse	5'-TGGAATCTGGAGAGTGAGGG-3'	
sXBP1	Forward	5'-TGGATTCTGGCGGTATTGAC-3'	146
	Reverse	5'-TCCTTCTGGGTAGACCTCTG-3'	
ATF6	Forward	5'-CCTGTCCCTACAAAGTACCATGAG-3'	148
	Reverse	5'-CCTTTAATCTCGCCTCTAACCC-3'	
BiP	Forward	5'-CCTGGGTGGCGGAACCTTCGATGTG-3'	358
	Reverse	5'-CTGGACGGGCTTCATAGTAGACCGG-3'	
IRE1 α	Forward	5'-GCGAACAGAATACACCATCAC-3'	147
	Reverse	5'-ACCAGCCCATCACCATTG-3'	
PERK	Forward	5'-GAACCAGACGATGAGACAGAG-3'	150
	Reverse	5'-GGATGACACCAAGGAACCG-3'	

CHOP, C/EBP-homologous protein; sXBP1, X-box binding protein 1; ATF6, activating transcription factor 6; BiP, immunoglobulin heavy chain-binding protein; IRE1 α , inositol-requiring enzyme 1 α ; PERK, protein kinase RNA-like endoplasmic reticulum kinase.

Evaluation of CA-125 levels

The levels of CA-125 were evaluated using an enzyme-linked immunosorbent assay kit (ELISA; MUCIN 16/CA125 Human ELISA Kit) according to the manufacturer's instructions (Invitrogen). The upper limit of detection was 1000 U/ml. The minimum detectable concentration of CA-125 was 1 U/ml. Intra-assay coefficients of variation for the ELSIA were 2.4% at the level of 43.5 U/ml and 3.2% at the level of 303.3 U/ml. Interassay coefficients variation for the ELISA were 3.9% at the level of 43.5 U/ml and 3.9% at the level of 303.3 U/ml.

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA). The Kolmogorov–Smirnov test was used to assess normality and Leven's test was used to assess the equality of variance between the groups. Between-group differences in levels of mRNA were determined using independent *t*-test and correlations were assessed using Pearson's correlation analysis. A *P*-value <0.05 was considered statistically significant.

Results

This prospective study enrolled 126 patients that underwent surgery and collected peritoneal fluid from them. Of these patients, 46 control patients had benign non-endometriotic disease, 47 patients had endometriosis and 33 patients had gynaecological cancer. The demographic and clinical characteristics of the three groups of patients are shown in Table 2. The concentration of serum CA-125 was significantly higher in the cancer group than in the other two groups and significantly higher in the endometriosis group than in the control group (*P* < 0.05 for all comparisons). There were no other significant differences between the three groups in terms of demographic and clinical characteristics.

The levels of ER stress-related mRNAs in the three groups are compared in Figure 1. The levels of CHOP and BiP mRNA were significantly lower in the cancer group than in the control and endometriosis groups (*P* < 0.05 for all comparisons). The levels of sXBP1 mRNA were significantly higher in the cancer group than in the control and endometriosis groups (*P* < 0.05 for all comparisons). The levels of ATF6 mRNA were significantly different among the three groups; being significantly higher in the endometriosis group

Table 2. Demographic and clinical characteristics of the patients (*n* = 126) included in a study to investigate the levels of endoplasmic reticulum stress-related mRNA in peritoneal fluid stratified according to their disease diagnosis.

Characteristic	Control group <i>n</i> = 46	Endometriosis group <i>n</i> = 47	Cancer group <i>n</i> = 33
Age, years	40.5 ± 12.6	38.2 ± 13.5	49.2 ± 14.1
Diabetes mellitus	1 (2.2%)	0 (0.0%)	2 (6.1%)
Hypertension	3 (6.5%)	3 (6.4%)	6 (18.2%)
Menopause	2 (4.3%)	1 (2.1%)	6 (18.2%)
Nulligravida	17 (37.0%)	18 (38.3%)	9 (27.3%)
Multigravida	29 (63.0%)	29 (61.7%)	24 (72.7%)
CA-125, U/ml*	25.35 ± 23.8	58.44 ± 42.9	125.8 ± 214.1

Data presented as mean ± SD or *n* of patients (%).

**P* < 0.05 for between-group comparisons; independent *t*-test.

CA-125, cancer antigen 125.

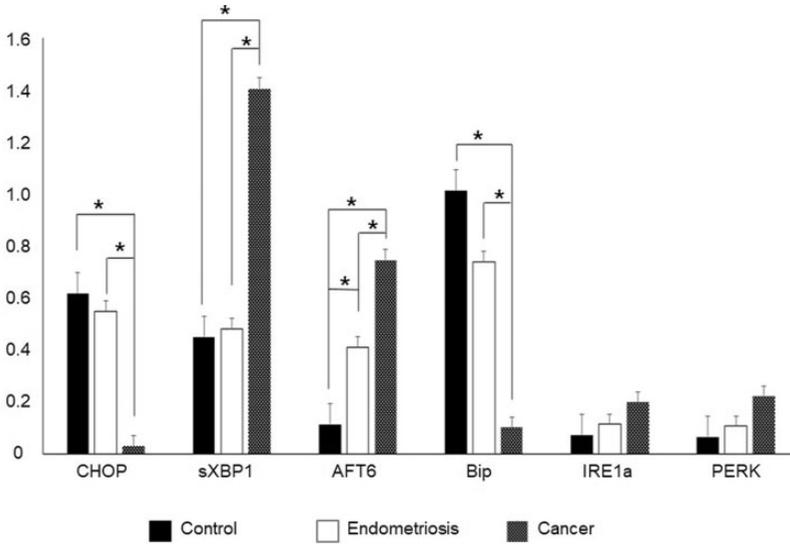


Figure 1. Relative levels of endoplasmic reticulum stress-related mRNAs in the peritoneal fluid of patients ($n = 126$) that were grouped based on their disease diagnosis as follows: benign disease without endometriosis (control group); endometriosis; or gynaecological (ovarian, uterine and cervical) cancer. Data presented as mean \pm SD. * $P < 0.05$ for between-group comparisons; independent t -test. CHOP, C/EBP-homologous protein; sXBP1, X-box binding protein 1; ATF6, activating transcription factor 6; Bip, immunoglobulin heavy chain-binding protein; IRE1 α , inositol-requiring enzyme 1 α ; PERK, protein kinase RNA-like endoplasmic reticulum kinase.

than in the control group and significantly higher in the cancer group than in the endometriosis and control groups ($P < 0.05$ for all comparisons). The levels of IRE1 α and PERK mRNAs were low in all three groups and there were no significant differences among the three groups.

In the endometriosis group, ATF6 mRNA levels were inversely correlated with age and positively correlated with serum CA-125 level; and ATF6 and PERK mRNA levels were inversely correlated with parity ($P < 0.05$ for all correlations) (Table 3). There were no significant correlations between the ER stress-related mRNAs and age, parity or CA-125 level in either the control or cancer groups.

Discussion

Impaired ER homeostasis induced by severe/prolonged ER stress-mediated UPR

signalling pathways is known to be related to several reproductive tissue pathologies including endometriosis, cancer, recurrent pregnancy loss and pregnancy complications.⁸ ER stress responses occur via three major ER transmembrane receptors: PERK, ATF6 and IRE1.⁹ Accumulated unfolded proteins in the ER bind to and recruit heat shock 70kDa protein 5 (HSPA5) away from those complexes, thereby activating the transmembrane receptors.¹⁰ PERK activates ATF4 transcription factor and the chaperone BiP/GRP78 through phosphorylation of the translation initiation factor (eIF2 α) and reduces the global synthesis of proteins and cell growth.¹¹ IRE1 can act as an endoribonuclease and induce splicing of XBP1 mRNA to generate sXBP1.¹² sXBP1 is involved in the quality control of proteins in the ER, ER expansion and export and

Table 3. Correlations between levels of endoplasmic reticulum stress-related mRNAs in peritoneal fluid and clinical characteristics in patients ($n = 126$) that were grouped based on their disease diagnosis.

Characteristic	mRNA	Control group		Endometriosis group		Cancer group	
		Pearson's coefficient	P-value	Pearson's coefficient	P-value	Pearson's coefficient	P-value
Age	CHOP	-0.156	NS	0.196	NS	0.031	NS
	sXBP1	-0.049	NS	-0.075	NS	-0.166	NS
	ATF6	0.059	NS	-0.408	$P = 0.005$	-0.014	NS
	BiP	0.061	NS	0.082	NS	-0.132	NS
	IRE1 α	-0.098	NS	-0.060	NS	0.099	NS
	PERK	-0.038	NS	-0.210	NS	-0.143	NS
Parity	CHOP	-0.158	NS	0.166	NS	0.043	NS
	sXBP1	0.080	NS	-0.132	NS	0.001	NS
	ATF6	-0.014	NS	-0.316	$P = 0.034$	-0.013	NS
	BiP	-0.054	NS	0.243	NS	-0.142	NS
	IRE1 α	-0.079	NS	-0.109	NS	-0.042	NS
	PERK	0.001	NS	-0.332	$P = 0.026$	-0.085	NS
CA-125	CHOP	-0.215	NS	-0.162	NS	-0.202	NS
	sXBP1	-0.065	NS	0.178	NS	-0.194	NS
	ATF6	0.171	NS	0.414	$P = 0.005$	-0.227	NS
	BiP	-0.022	NS	-0.092	NS	-0.228	NS
	IRE1 α	0.054	NS	0.125	NS	-0.214	NS
	PERK	-0.037	NS	0.227	NS	-0.208	NS

CHOP, C/EBP-homologous protein; sXBP1, X-box binding protein 1; ATF6, activating transcription factor 6; BiP, immunoglobulin heavy chain-binding protein; IRE1 α , inositol-requiring enzyme 1 α ; PERK, protein kinase RNA-like endoplasmic reticulum kinase; CA-125, cancer antigen 125.

NS, no significant correlation ($P \geq 0.05$).

degradation of misfolded proteins.¹³ ATF6, activated by unfolded proteins, exits the ER and migrates to integrate the Golgi apparatus membrane where it is cleaved.¹⁴ Cleaved ATF6 enters the nucleus and acts as a transcription factor targeting genes including *BiP/GRP78*, *XBP1* and *CHOP*.¹⁵ This current study found lower levels of CHOP and BiP mRNA in the cancer group than in the control group. Although the function of CHOP in oncogenesis has not yet been clearly determined, a number of studies demonstrated that CHOP induction in response to prolonged ER stress causes apoptosis of pre-malignant cells, thus preventing tumour progression.¹⁶ BiP, an indicator of ATF6 inhibition, is also involved in the stress signalling pathway by activating apoptosis thereby inducing suppression of

endometriosis progression.¹⁷ Thus, the reduced levels of CHOP and BiP mRNAs in the cancer group appears to promote cell survival pathways and tumour progression.

The levels of sXBP1 and ATF6 mRNAs were significantly elevated in the cancer group in the current study, suggesting that the ATF6 pathway might be associated with the pathogenesis of gynaecological cancers. The levels of IRE1 and PERK mRNA were slightly elevated in the cancer group, but there was no significant difference among the three groups in the current study. The ATF6 pathway is specialized in regulating the transcription of ER quality control proteins.¹⁸ In contrast, ATF6 can trigger cellular senescence through activation of the ER stress-ATF6 α axis.^{4,19} The role of XBP1 in tumorigenesis is quite

controversial. While some studies highlighted the importance of the IRE1/XBP1 axis for cell survival in an hypoxic environment and tumour growth,^{20,21} others reported decreased levels of XBP1 in the cancer group,²² the role of XBP1 in suppressing tumour formation²³ and the pro-apoptotic effect of the IRE1/XBP1 signalling pathway.²⁴ However, in this current study, the levels of sXBP1 mRNA were significantly increased in the cancer group compared with the control and endometriosis groups, supporting the role of sXBP1 in tumorigenesis. This result was consistent with that of a previous study reporting the crucial role of XBP1 in the oestrogen-induced growth of endometrial cancer.²⁵ Another study demonstrated that it is not activation of a single UPR signalling pathway, but the relative timing of IRE1/XBP1 and PERK/ATF4 signalling that determines the shift from cell survival to apoptosis.²⁶ In other words, sXBP1 may play a role in both cell survival and apoptosis depending on interactions with other ER stress-induced signalling pathways. IRE1 induces the splicing of XBP1 to generate sXBP1. In Figure 1, the level of sXBP1 mRNA was significantly elevated in the cancer group compared with the control and endometriosis groups, although the level of IRE1 mRNA was not. Activated IRE1, in turn, activates the transcription factor XBP-1 by splicing its mRNA, suggesting that the expression of IRE1 mRNA is related to the expression of sXBP1 mRNA. However, the levels of the two mRNAs do not appear to be directly proportional to each other. The level of sXBP1 mRNA was higher in the endometriosis group than in the control group, with the level being even higher in the cancer group in the current study. Moreover, although the differences were not statistically significant, the level of IRE1 mRNA was higher in the endometriosis group than in the control group and was even higher in

the cancer group in the current study, similar to the levels sXBP1 mRNA.²⁷ That is, the results showed the same trend. In addition, the level of sXBP1 mRNA is affected not only by the levels of IRE1 mRNA, but by ATF6.²⁸ Similar to the expression of sXBP1 mRNA, this current study found that the level of ATF6 was higher in the endometriosis group than in the control group and was higher in the cancer group than in the endometriosis group.²⁹ It should be noted that this current study measured mRNA levels not protein levels. Therefore, future studies are required to measure protein levels.

Both ATF6 and IRE1-XBP1 pathways are activated under medium to high levels of ER stress, whereas only the ATF6 pathway is activated by low levels of ER stress.¹³ Therefore, the elevated levels of both ATF6 and sXBP1 mRNAs in the cancer group in the current study suggests that there are high levels of ER stress in gynaecological cancer. A previous study also demonstrated that *ATF6* gene expression was significantly elevated in patients with endometrioid cancer compared with healthy controls, while the expression of the *CHOP* gene was decreased in the cancer group compared with that in the healthy group,²² similar to the findings of the current study. ATF6 induces XBP1 mRNA, which is spliced by IRE1, and this sXBP1 mRNA produces a highly active transcription factor in response to ER stress.¹³ ATF6 and sXBP1 upregulate ER stress-mediated prion protein gene expression and promote cell proliferation, invasion and metastasis in many cancers including breast cancer.³⁰ On the other hand, CHOP is a stress-inducible nuclear protein that is crucial for the development of programmed cell death and regeneration.³¹ The *CHOP* gene and its mRNA induce apoptosis via several ER stress-mediated transcription factors in cancer cell lines and suppress cell growth.³²⁻³⁴

Therefore, the elevated levels of ATF6 and sXBP1 mRNA and reduced levels of CHOP and BiP mRNA in the cancer group indicate that the UPR signalling pathway was unregulated toward cell survival and tumour growth and inhibited apoptosis.

This current study found that the level of ATF6 mRNA was positively correlated with serum CA-125 level in the endometriosis group; therefore, ATF6 mRNA levels could serve as another useful biomarker for endometriosis. Whether increased ATF6 mRNA levels and CA-125 are causative factors or a consequence of the ER stress response is unclear.

Several studies have examined the relationship between oestrogen/progesterone and the ER stress response.^{7,25,34} Oestrogen seems to cross-talk with the UPR cascade by interacting with HSPA5, a molecular chaperone within the ER.⁷ Oestrogen-induced transcription of XBP1 plays a crucial role in oestrogen-induced growth of specific breast and endometrial cancer cells.²⁵ A previous study reported that progesterone increased expression of the *GRP78*, *CHOP* and *TRIB3* genes, which are involved in the pro-apoptotic pathway and decreased cellular invasiveness.³⁴ An abnormal ER stress response to progesterone increased endometriotic stromal cell invasiveness.³⁴ Considering these reports and the current results, ER stress-mediated mRNAs interact with oestrogen/progesterone and might be related to the pathogenesis and regulation of hormone-induced endometriotic cell growth and invasion.⁸

This current study had several limitations. First, it was not possible to obtain peritoneal fluid from healthy subjects due to ethical reasons. Instead, the study used peritoneal fluid from patients with benign tumours without endometriosis as a control group. Secondly, the study did not classify the levels of mRNA of ER stress markers by menstrual phase. Thirdly, the levels of ER stress-related mRNAs in peritoneal fluid

were measured rather than the levels in lesions. Fourthly, this current study measured mRNA levels not protein levels. Fifthly, the study only measured the levels of some ER stress-related mRNAs in these samples.

In conclusion, the levels of ER stress-mediated mRNAs were related to the pathogenesis of endometriosis and gynaecological cancers. These current findings suggest that elevated levels of ATF6 and sXBP1 mRNAs are associated with an increased likelihood of cancer. Furthermore, elevated levels of CHOP and BiP mRNAs might be associated with an increased likelihood of benign lesions.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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