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Expression Pattern of Mitogen-inducible Gene 6 in Relation to the Extracellular Signal-regulated Kinase Pathway and Redox Status in Patients with Endometriosis

Nada Hashem Ayad[1*], Hanaa Hibishy Gaballah[1], Hesham Abd El-Aziz Salem[2], Sobhy Abd El-Hamid Hassan[1] and Safwat Mohamed Kasem[1]

[1]Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Tanta University, Egypt.
[2]Department of Gynaecology and Obstetrics, Faculty of Medicine, Tanta University, Egypt.

Authors' contributions

This work was carried out in collaboration among all authors. Author NHA performed experimental work, conducted the statistical analysis and wrote the manuscript. Authors HHG, HAEAS, SAEHH and SMK participated in designing the research protocol, supervised overall work execution and edited the manuscript. The final version of the manuscript was read and approved by all authors.

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ABSTRACT

Background: Endometriosis is a gynecological disorder characterized by the presence of ectopic endometrium outside the uterus. It is a multi-factorial disease, where different processes facilitate the implantation and survival of ectopic endometrial tissue.
The Aim: This study aims to evaluate the levels of the expression patterns of mitogen-inducible gene 6 (Mig-6) in eutopic and ectopic endometrial tissues of endometriosis patients and their relevance to the levels of phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) and redox status.

**Corresponding author: E-mail: nhashem274@yahoo.com, nada_85197_pg@med.tanta.edu.eg;*

Methods: Relative gene expression levels of Mig-6 and the levels of pERK1/2 were assessed in endometrial tissues of control group and in both eutopic and ectopic endometrial tissues of endometriosis patients .Serum levels of total protein thiols and total protein carbonyls were also assessed in endometriosis and control groups.
Results: Relative gene expression levels of Mig- 6 showed significant lower values in endometriosis group as compared to control group, and they were associated with significant increase in pERK 1/2 levels and altered redox status.
Conclusion: These data, signified a significant role of downregulated Mig-6 expression, and activated ERK 1/2 signaling in the pathogenesis of endometriosis. Moreover, it suggested that oxidative stress indicated by high protein carbonyls level contributes to the pathogenesis of endometriosis and that protein thiols have a defensive role against oxidative stress.

Keywords: Endometriosis; mitogen-inducible gene 6; phosphorylated extracellular signal-regulated kinase 1/2; protein thiols; protein carbonyls.

ABBREVIATIONS

Mig-6 : Mitogen-Inducible Gene 6;
pERK1/2 : Phosphorylated Extracellular Signal-Regulated Kinase ½;
Errfi1 : ErbB Receptor Feedback Inhibitor 1;
RALT : Receptor Associated Late Transducer;
EGFR : Epidermal Growth Factor Receptor;
PI3K/AKT : Phosphatidyl Inositol 3-Kinase/ Protein Kinase B Pathway;
MAPK : Mitogen-Activated Protein Kinases;
rASRM : Revised American Society of Reproductive Medicine;
DNPH : 2,4-Dinitrophenyl Hydrazine;
DTNB : 5, 5'- Dithiobis-(2-nitrobenzoic acid);
c-FOS : C-Finkel–BISKIS–Jinkins (FBJ) Murine Osteosarcoma Viral Oncogene Homolog;
BCL-2 : B-Cell Lymphoma 2;
ESR1 : Estrogen Receptor α;

1. INTRODUCTION

Endometriosis is a common estrogen-dependent inflammatory disease, characterized by the presence of endometrial-like tissue outside the uterus which can lead to ovarian cysts (endometriomas), fibrosis, and adhesions in the pelvis [1]. Endometriosis patients suffer from pain, and/or infertility [2]. Many theories have been proposed to clarify the pathogenesis of endometriosis [3]. However, its pathogenesis is still poorly understood [4].

Mitogen-inducible gene 6 (Mig-6) (also known as gene 33 [5], ErbB receptor feedback inhibitor 1 (Errfi1) [6], and receptor associated late transducer (RALT)) [7], is located on human chromosome 1p36.23 [8]. It encodes a cytosolic multiadaptor protein [9], that has an important tumor suppressor function [10] and it plays a role in modulating the response to steroid hormones in the uterus. Besides being required for normal response to progesterone in the uterus, [11] it mediates epithelial estrogen responses in the uterus, and thus Mig-6 is an important regulator of endometrial cell proliferation [12]. Mitogen-inducible gene 6 (Mig-6) is best known for its role as a negative feedback regulator of epidermal growth factor receptor (EGFR) induced signaling pathways [13], including phosphatidyl inositol 3-kinase /protein kinase B (PI3K/AKT) pathway [14,15]. PI3K/AKT pathway controls the cell cycle and metabolism [16].

The extracellular signal-regulated kinase 1/2 (ERK 1/2) is a family member of the mitogen-activated protein kinases (MAPK) [17]. ERK1/2 is a serine threonine kinase with more than 200 known substrates, and with critical roles in multiple cellular processes [18]. It plays a central role in transducing signals controlling cell proliferation [19]. It also exerts an anti-apoptotic effect as the activation of ERK 1/2 cascade is connected with the inhibition of caspase-3 and caspase-9 [20].

Oxidative stress leads to the non-enzymatic modification of specific amino acid residues, where aldehyde or ketone functional groups are introduced, this is known as carbonylation [21]. Protein carbonylation can have deleterious effects on cell function, since it can lead to protein dysfunction and to the production of potentially harmful protein aggregates [22]. In addition, Protein carbonyl groups can be used as biomarkers for oxidative damage to proteins [23]. Novel 2,4-diphenylhydrazine (DPNH)-reactive carbonyl groups in proteins might be either produced by the direct metal-catalyzed oxidation of aminoacyl side chains (arginine, proline, lysine

and threonine) or introduced by stable adduct formation via the reaction of lysyl residues with reducing sugars and third-party reactive carbonyl species (RCS). The latter include the small reactive aldehydes glyoxal and methylglyoxal, deriving from free-radical attack to derivatives of both carbohydrate and lipid metabolism, and reactive aldehydes like acrolein, malonyldialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), which are lipoxidation products, formed upon free-radical attack to polyunsaturated fatty acids [24].

Thiols are organic compounds containing a sulfhydryl group (–SH) which act as physiological free radical scavengers [25]. The total plasma thiols include albumin thiols, other proteins containing free thiol groups and low-molecular-weight thiols, [26] such as cysteine and glutathione [27]. The oxidation of thiols participates in the defense against oxidative damage. In addition they also play critical role in enzymatic catalytic mechanisms and signal transduction processes [28].

2. SUBJECTS AND METHODS

2.1 Patients and Specimens

This study was approved by the Ethics and Research Committee of Tanta Faculty of Medicine with approval code 31137/09/16 and was in accordance with the principles of the Declaration of Helsinki II. Through an informed consent, patients were informed that the resected specimens may be used for scientific research with great concern for the privacy of participants. This study was carried out on 30 females who were admitted to the Department of Gynecology and Obstetrics at Tanta University Hospital. They were enrolled into 2 groups. This group comprised 12 laparoscopically confirmed endometriosis-free patients of reproductive age undergoing endometrial biopsy. These control subjects were infertile patients undergoing diagnostic laparoscopy and hysteroscopy during the work up of infertility. The endometriosis group comprised 18 patients who were diagnosed by laparoscopic visualization. Normal endometrium and putative lesions were confirmed by histopathological examination. All subjects were of reproductive age. During the laparoscopy procedure, the surgical team graded the stages of endometriosis in accordance with the revised American Society of Reproductive Medicine (rASRM) classification [29]. 5 ml venous blood samples were collected under strict aseptic technique from both groups. In order to get serum sample, blood was left for 20 min to allow proper clotting and then it was centrifuged for 10 min at 3000 rpm. Tissue samples were taken from both eutopic and ectopic endometrium of endometriosis patients and from eutopic endometrium of control group.

2.2 Quantitative Real-time PCR

RNA extraction: RNA was isolated from both eutopic and ectopic endometrium of endometriosis group and eutopic endometrium of control group using phenol chloroform based extraction followed by spin column based extraction according to the manufacturer's protocol. TRIzol Lysis Reagent (catalogue number 17063, INTRON Biotechnology), Chloroform (code number C/4966/17, Fischer Scientific, U.K), QIAamp RNA Blood Mini Kit (catalog no. 52304, QIAGEN, USA) were used for RNA extraction. The concentration and purity of the RNA samples were measured by a nanodrop (Scan Drop, analytikjena), and samples were used only if the ratio of the absorbance (A 260/280) was 2 before reverse transcription reaction.

The genomic DNA elimination and cDNA synthesis: It was performed using the Quanti Tect Reverse Transcription Kit (catalog no. 205310, QIAGEN, USA) according to the manufacturer's instructions. The genomic DNA elimination reaction was prepared by adding 2 µl of gDNA Wipeout Buffer to variable amounts of template RNA and RNase-free water to form total volume of 14 µl. The genomic DNA elimination reaction components were incubated for 2 min at 42ºC. The cDNA master mix was prepared according to the kit's protocol (14 µl of genomic DNA elimination reaction components were added to 1 µl of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript RT Buffer and 1 µl of RT Primer Mix) and was added to each sample, which was then incubated in the programmed thermal cycler for 15 min at 42ºC, followed by inactivation of enzymes at 95ºC for 3 min.The cDNA which was then stored at - 80ºC.

Real-time quantitative PCR: Quantitative real-time PCR was performed using the QuantiTect SYBR Green PCR Kit (catalog no. 204141, QIAGEN, USA) according to manufacturer's instructions.The primer sequences of Mig-6 were 5'-CTACTGGAGCAGTCGCAGTG-3'(forward) and 5'-CCTCT TCATGT GGTCC CAAG-3'(reverse) [30]. Glyceraldehyde-3-phosphate

dehydrogenase gene (GAPDH) was used as reference gene, primer sequences for GAPDH were:5'-CAACTACATGGTTTACATGTTC-3'(forward) and 5'-GCCAGTG GACTCC ACGAC -3' (reverse) [30]. 2.5 µl of cDNA were added to 12.5 µl of QuantiTect SYBR Green PCR Master Mix,1 µl of the specific primer pairs ,and 8 µl of RNase-free water. This cDNA was then amplified using Rotor - Gene Q cycler, as follows: Initial activation step at 95ºC for 15 min , followed by 45 cycles of denaturation at 95ºC for 15 s, annealing at 60ºC for 30 s and extension at 72ºC for 30 s. The cycle threshold(Ct) values were calculated for target genes and reference gene, then gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method [31].

2.3 Phosphorylated Extracellular Signal-regulated Kinase 1/2 Level Assay

Phosphorylated extracellular signal-regulated kinase 1/2 levels were determined using ELISA kit (catalogue number 201-12-6290, Sun Red Biological Technology Company, Shanghai, China) according to the manufacturer's instructions. Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer. The levels were expressed as pg/mg tissue protein.

2.4 Total Proteins Assay

Concentrations of total proteins in the tissue samples were determined according to Biuret method [32] using total protein kit, supplied by Diamond Diagnostics.

2.5 Assay of Total Protein Carbonyl Groups

Protein carbonyls levels were determined using 2,4-dinitrophenyl hydrazine (DNPH) protocol of Levine, et al. (1990) [33]. Where, 15 µl of the serum were mixed with 400 µl of 15 mM 2,4-dinitrophenyl hydrazine in 2 M HC1 and the blank was prepared by mixing 15 µl of the serum with 400 µl of 2 M HC1. After precipitation of protein with 1ml of 14% trichloroacetic acid, the pellets were washed with 1:1 ethanol-ethylacetate, and then redissolved in 900 µl of 6 M guanidine hydrochloride (dissolved in 2 M HCl, adjusted to pH 2.3 with diluted trifluoroacetic acid) at 37°C (using water path) for 15 min with vortex mixing. The absorbance was determined at 366 nm by spectrophotometer using the following equation [33], with a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$.

Total protein carbonyls level (µmol/l) = A 366 /22,000

2.6 Assay of Total Protein Free Thiol Groups

200 µl of serum were mixed with 600 µl of the Tris-EDTA buffer followed by adding 40 µl of 10 mM 5, 5'- dithiobis-(2-nitrobenzoic acid) (DTNB). To the sample blank only sample was added without DTNB. However, only DTNB was added to blank (B) .After 15 min of incubation at room temperature in the dark, 3.16 mL of methanol were added to all samples and they were centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant (A) was measured at 412 nm and subtracted from a DTNB blank (B) and a sample blank without DTNB. In agreement with Sedlak and Lindsay (1968) [34], a value of 0.03 for the sample blank was consistently obtained. Therefore, individual sample blanks were not critical and can be taken as 0.03.Total SH groups are calculated using an absorptivity of 13,600 cm^{-1} M^{-1} as follows: (A - B - 0.03) × (4.0/0.2)/13.6 = (A - B - 0.03) × 1.47 mM

There is normally a little difference between total thiol and protein thiol levels because of the low glutathione level in the blood. Therefore, protein thiol level was calculated by subtracting the glutathione level from the total thiol level [34]. The concentration of reduced glutatione (GSH) in serum was measured according to the method of Ellman (1959) [35].

2.7 Statistical Analysis

Statistical analysis of the results of the current study was conducted using the computer program Statistical Package for the Social Science (SPSS); version 23 for Microsoft Windows, and the data were presented as mean ± SD. The intergroup variations between two groups were measured using t-test, while Analysis of variance (ANOVA) followed by Tukey tests were used to evaluate the statistical significance between three experimental groups. Pearson's correlation test was performed to evaluate the relationship between different parameters. P value < 0.05 was considered significant.

3. RESULTS

The clinical data of endometriosis group concerning symptoms and severity of the

disease according to rASRM classification are shown in Table 1. It also shows laterality of endometriomas and phases of menstrual cycle. In addition to the body mass index of the patients.

Table 2 shows the statistical comparison among all studied groups as regards relative gene expression levels of the mitogen-inducible gene 6 (Mig-6) and phosphorylated extracellular signal-regulated kinase 1/2 (pERK 1/2) level. There were significant differences in values of mRNA expression levels of Mig- 6 among all groups (f value = 916.6, P < 0.001*). By using the multiple comparisons test (Tukey's test), there were significant decrease in ectopic and eutopic endometrium of endometriosis patients as compared to control group (P < 0.001*). There were statistically significant differences in values of (pERK 1/2) level among all groups (f value was 150.58, P < 0.001*). By using the multiple comparisons test (Tukey's test), there

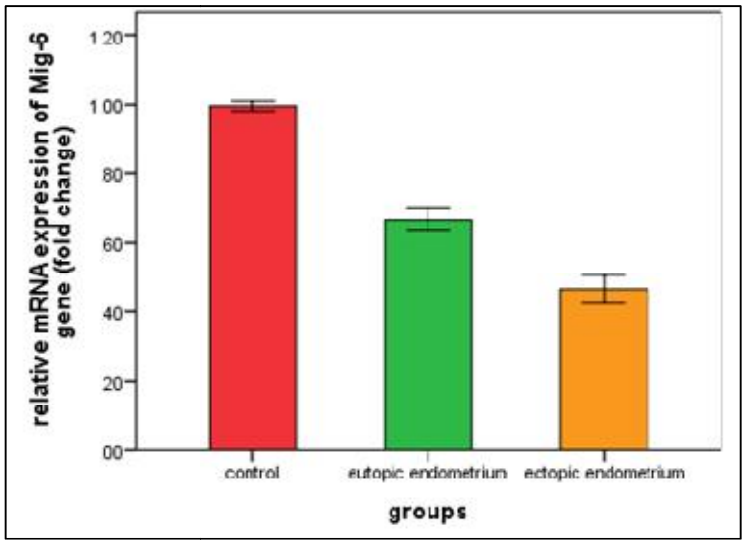

Fig. 1. Statistical comparison of mRNA relative gene expression levels of (Mig-6) among the studied groups using ANOVA test

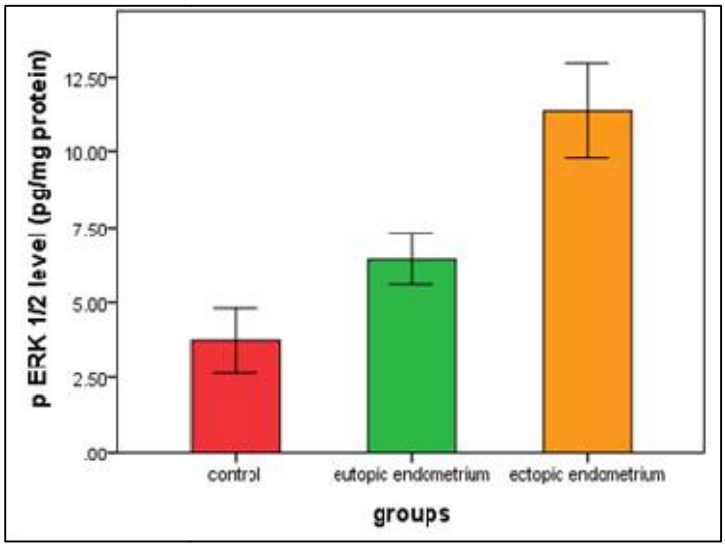

Fig. 2. Statistical comparison of (pERK 1/2) level (pg/mg protein) in the endometrial tissue using ANOVA test

Table 1. Summary of clinical data of endometriosis group

Variables	Endometriosis	
	Number (n)	%
Symptoms:		
• Pain		
a. Dysmenorrhoea	3	16.7%
b. Chronic pelvic pain	2	11.1%
c. Both dysmenorrhea and chronic pelvic pain	2	11.1%
• Infertility	11	61.1%
Phase of menstrual cycle:		
• Proliferative	15	83.3%
• Secretory	3	16.7%
Severity: (rASRM)		
• Stage I	5	27.7%
• Stage II	10	55.6%
• Stage III	3	16.7%
Body mass index (BMI):		
• 25>	1	5.6 %
• 30 –25	14	77.7 %
• > 30	3	16.7 %
Laterality:		
• Unilateral :	16	88.9%
• Bilateral :	2	11.1%

were statistically significantly higher values in eutopic and ectopic endometrium of endometriosis patients as compared to control group (P <0.001*). These data are illustrated in Figs. 1 and 2.

As indicated in Table 3, serum levels of total protein thiols showed statistically significant decrease in endometriosis group when compared to control group (P < 0.001*). However, serum levels of total protein carbonyls showed statistically significant increase in endometriosis group when compared to control group (P < 0.001*). These data are illustrated in Figs. 3 and 4.

Table 4 showed correlation matrix of Mig-6, pERK1/2, total protein thiols, and total protein carbonyls among all studied groups. There was significant negative correlation between pERK1/2 level and Mig-6 relative gene expression level among all studied groups (r = -0.86) (p <0.001*). There was also a significant negative correlation between level of serum total protein carbonyls and level of serum total protein thiols within all studied groups (r = - 0.672) (p <0.001*). These data are illustrated in Figs. 5 and 6.

4. DISCUSSION

The aetiology of endometriosis is complex and multifactorial, where several not fully confirmed theories describe its pathogenesis [36]. The current study revealed significant decrease in mRNA expression levels of Mig-6 in endometriosis groups as compared to control group, with significant differences in the expression level of the Mig-6 gene in the group of ectopic endometrium of endometriotic patients, compared to eutopic endometrium of endometriotic patient, and control groups.

In agreement with the current findings, Aznaurova, et al. [37] and Velarde et al. [38] reported that Mig-6 expression levels were down-regulated in endometrial tissue of endometriosis group compared to control group.This finding is biologically plausible as Mig-6 is an important mediator of progesterone action, which has anti proliferative effects [39,40]. Moreover, Yoo, et al. [39] reported that Mig-6 is pivotal in the suppression of epithelial proliferation through its inhibition of protein kinase B (AKT) activation. So, reduced expression of Mig-6 might enhance the proliferative changes of endometriosis [41].

The present study showed that the levels of pERK1/2 were significantly increased in endometriosis groups as compared to control group, with significant differences between its level in the groups of eutopic endometrium of endometriotic patients, ectopic endometrium of endometriotic patients, and endometrium of

control subjects. The attained finding is consistent with previous study by Erikson, et al. [42] reporting that pERK 1/2 level was significantly higher in stromal fibroblasts from women with severe endometriosis versus control subjects.

Several lines of evidence support this finding. Firstly, the endometriotic microenvironment may stimulate increased ERK activity in ectopic cells [43], as growth factors, and cytokines activate ERK1/2 signalling to enhance endometrial stromal cells migratory and invasive capacities in support of ectopic implantation [44].

Secondly, Extracellular signal - regulated kinase 1/2 (ERK 1/2) induces the expression of c- Finkel– Biskis– Jinkins (FBJ) murine

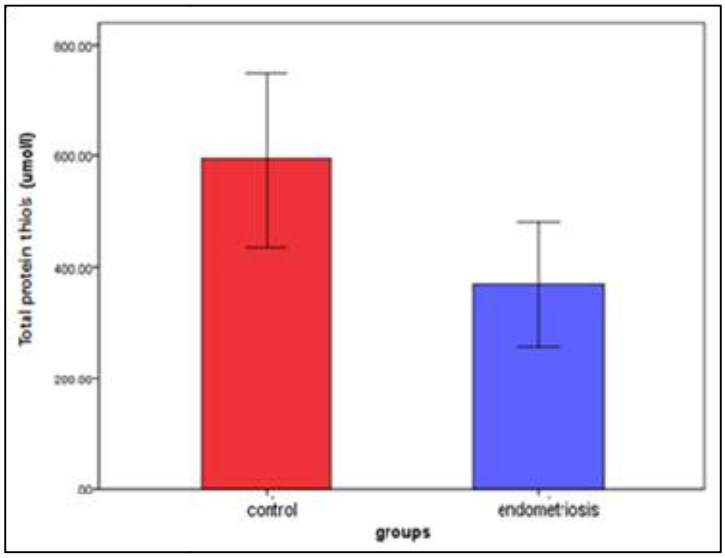

Fig. 3. Statistical comparison between the two studied groups as regards serum total protein thiols (µmol/l)

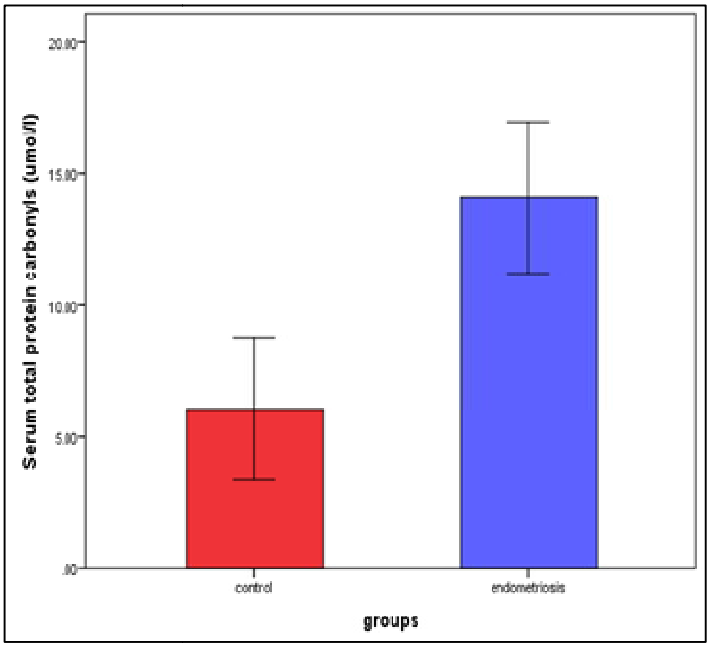

Fig. 4. Statistical comparison between the two studied groups as regards serum levels of total protein carbonyls (µmol/l)

Table 2. Statistical comparison of relative gene expression levels of the mitogen-inducible gene 6 (Mig - 6) and phosphorylated extracellular signal-regulated kinase 1/2 (pERK 1/2) level (pg/mg protein) among the studied groups using ANOVA test

Variables	Groups			ANOVA Test	
	Control n = 12	Eutopic endometrium n = 18	Ectopic endomerium n = 18		
		Mean ± SD		F	P-value
Tissue homogenate Mig-6 gene expression level	0.99±0.01	0.66 ± 0.03 [a]	0.04±0.46 [b,c]	916.6	< 0.001*
Tissue homogenate pERK ½ (pg/mg protein)	3.72 ±1.09	6.45 ± 0.87 [a]	11.37± 1.58 [b,c]	50.58	<0.001*

**p-value < .05 significant; a :significant eutopic endometrium group versus control group; b: significant ectopic endometrium group versus control group; c: significant eutopic endometrium group versus ectopic endometrium group*

Table 3. Statistical comparison between the two studied groups as regards serum levels of total protein thiols (µmol/l) and total protein carbonyls (µmol/l) using un-paired t-test

Variables	Groups		t-Test	
	Control n = 12	Endometriosis n = 18	t	P-value
	Mean ± SD			
Serum total protein thiols	594.24±156.93	368.81±112.43	4.59	<0.001*
Serum total protein carbonyls	7.07±3.35	20.83±3.206	-11.33	<0.001*

** p-value < .05 significant*

Table 4. Correlation matrix of Mig-6, pERK1/2, total protein thiols, and total protein carbonyls among all studied groups

Correlations		Mig-6 in endometrial tissue	pERK1/2 in endo-metrial tissue	total protein thiols in serum
pERK1/2 in endometrial tissue	r	- 0.86		
	P-value	<0.001*		
Total protein thiols in serum	r	- 0.049	- 0.254	
	P-value	0.879	0.425	
Total protein carbonyls in serum	r	- 0.147	0.305	- 0.672
	P-value	0.648	0.336	<0.001*

** p-value < .05 significant*

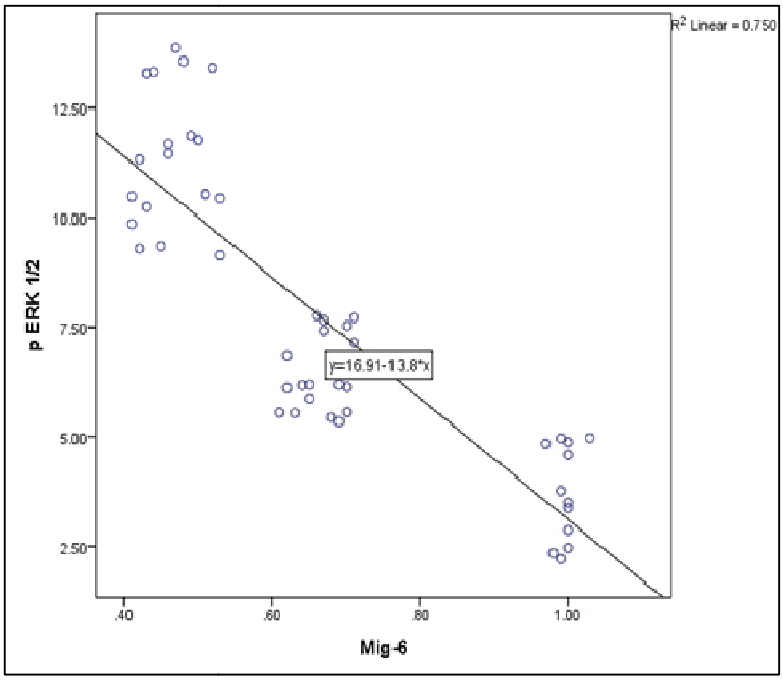

Fig. 5. Correlation of pERK1/2 level and Mig-6 relative gene expression level in endometrial tissue of studied groups

osteosarcoma viral oncogene homolog (c-FOS) [45], which, in turn, is responsible for cellular proliferation [46]. Therefore, the enhanced proliferation and survival of endometrial cells from patients with endometriosis compared with healthy women could be attributed to abnormal activation of ERK1/2 [47]. Concomitantly, Matsuzaki, et al. [48] suggested that the AKT and ERK signaling pathways may co-operate to support growth of deep endometriotic lesions by enhancing endometriotic stromal cell proliferation.

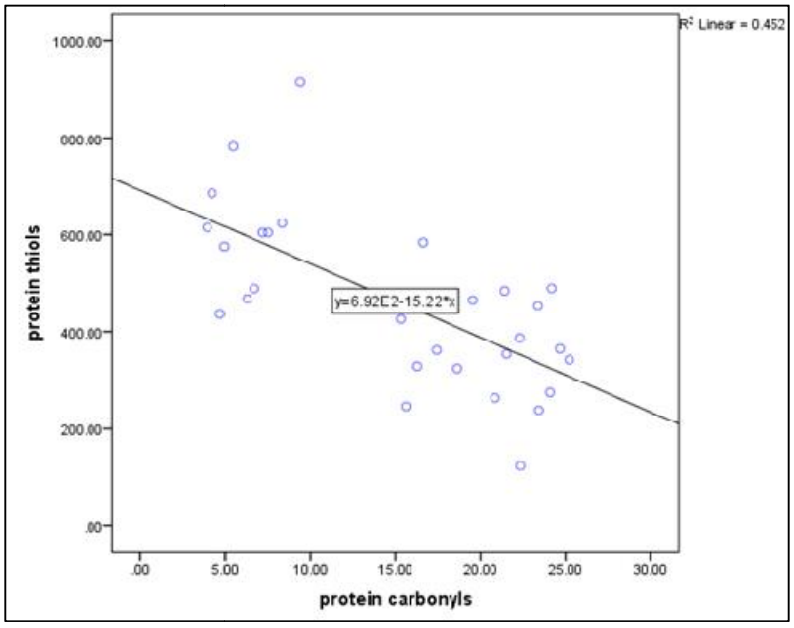

Fig. 6. Correlation of serum level of total protein carbonyls and serum level of total protein thiols in studied groups

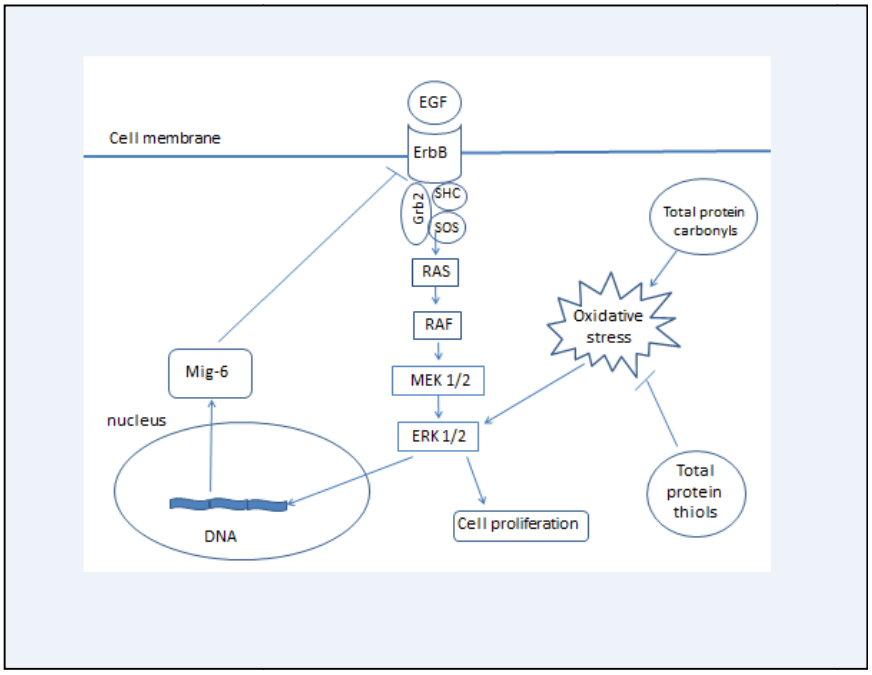

Fig. 7. Flow chart

Well in line, Velarde, et al. [38] suggested that increased activation of ERK1/2 in endometrial cells from women with endometriosis may be responsible for persistent proliferative changes in secretory-phase endometrium .Alternatively, Wang, et al. [49] demonstrated reduced cell apoptosis through CD 147-ERK-B-cell lymphoma 2 (BCL-2) intrinsic apoptosis signaling axis suggesting that this may contribute to the progression of human endometriosis.

The current study showed a significant negative correlation between pERK1/2 levels and Mig-6 relative gene expression levels in eutopic and ectopic endometrium of endometriosis patients and endometrium of control subjects. In agreement with this finding, Li, et al. [50] reported that down-regulation of Mig-6 activates the ERK signaling pathway. This is in accord with previous study by Kim, et al. [51] reporting an inverse correlation between Mig-6 and pERK1/2 in human endometrial cancer.

This finding might be indebted to the negative feedback regulation of ERK signaling pathway by Mig-6. As, Mig-6 binds to epidermal growth factor receptor (EGFR) family tyrosine kinases via its EGFR-binding domain ,leading to inhibition of EGFR autophosphorylation and inhibition of EGFR/ERK pathway [52,53]. Moreover, Mig-6 mediates progesterone action to inhibit ERK1/2 signaling [51], so the downregulation of Mig-6 relieves its inhibitory effect on ERK 1/2.

The current study showed altered redox status in endometriosis patients as evident by decreased serum total protein thiols levels and increased total protein carbonyls levels in endometriosis group when compared to control group. This finding extend previous observation by Turkyilmaz, et al. [54] and Rosa e Silva, et al. [55] revealing that total serum thiols were significantly lower in patients with endometriosis as compared to healthy controls .In addition, Carvalho, et al. [56] found that the protein carbonyls levels in the peritoneal fluid were significantly higher in patients with endometriosis compared to control group.

On the contrary, Santulli, et al. [11] showed that the concentrations of peritoneal protein carbonyls were not different between the endometriosis and control groups. This apparent discrepancy between results might be explained by the fact that Santulli, et al. study has limitations as the control group involved women who had undergone surgery for benign gynecological conditions such as fibroids, ovarian cysts or tubal infertility, possibly associated with altered peritoneal protein oxidative stress markers.

One hypothesis to explain the attained finding might be based on the fact that protein thiols have a significant role in the cell by minimizing the toxic effects of reactive oxygen species [57]. So in high oxidative stress, indicated by high protein carbonyls levels, the concentration of protein thiols decline to neutralize reactive oxygen species [27].

5. CONCLUSION

Collectively, this study strongly nominated Mig-6, pERK 1/2 and oxidative modifications of the proteome as key players in the multifactorial pathogenic mechanism of endometriosis**.** Mig-6 might integrate signal transduction through combinations of its functional domains via different signaling pathways to prevent endometriosis through its anti-proliferative actions. However, pERK1/2 promotes cell proliferation thus induces endometriosis. Indeed, detection of elevated levels of protein carbonyls is generally a sign not only of oxidative stress but also of protein dysfunction. Moreover, protein thiols have a defensive action against reactive oxygen species (ROS).

It is important that future research focuses on the pathogenesis of different symptoms of the disease as pain and infertility and to correlate the severity of the disease with the changes in differnet biochemical parameters. A specific ERK1/2 dependency for endometriotic lesions, through an activating genetic mutation is yet to be confirmed. Identifying the kinase dependency for endometriosis will, be a key for creating a new effective kinase inhibiting treatments of endometriosis. In addition, further elucidation of how ERK1/2 executes its function through downstream factors will help us understand completely ERK1/2 function.

CONSENT AND ETHICAL APPROVAL

This study was approved by the Ethics and Research Committee of Tanta Faculty of Medicine with approval code 31137/09/16 and was in accordance with the principles of the Declaration of Helsinki II. Through an informed consent, patients were informed that the resected specimens may be used for scientific research with great concern for the privacy of participants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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