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The Possible Role of Eukaryotic Translation Initiation Factor 3 Subunit e (eIF3e) in the Epithelial–Mesenchymal Transition in Adenomyosis

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Abstract

Epithelial–mesenchymal transition (EMT) has been reported to be involved in adenomyosis by promoting cell invasion and fibrogenesis. But few studies have identified critical factors that regulate EMT process during adenomyosis. The eukaryotic translation initiation factor 3 subunit e (eIF3e) protein is a component of the multisubunit eIF3 complex essential for cap-dependent translation initiation. The aim of this study was to investigate whether eIF3e is involved in EMT in adenomyosis. Ectopic endometrial tissue samples were collected from 40 premenopausal women with ultrasonographically diagnosed and histologically confirmed adenomyosis. As controls, endometrial samples were obtained from 40 cycling premenopausal women patients who underwent surgery for benign gynecologic disorders or cervical intraepithelial neoplasia but without endometriosis, adenomyosis, nor uterine fibroids. All tissue samples were subjected to immunohistochemistry analysis of eIF3e, transforming growth factor-β1 (TGF-β1), E-cadherin, vimentin, Snail, and proliferating cell nuclear antigen (PCNA). The epithelial component of ectopic endometrium showed significantly reduced immunoreactivity against eIF3e and E-cadherin but elevated immunoreactivity against TGF-β1, Snail, vimentin, and PCNA as compared with that of control endometrium (all *P* values <.05), and the difference was not affected by age, parity, or menstrual phase. The eIF3e staining levels correlated negatively with those of TGF-β1, vimentin, Snail, and PCNA (both *P* values <.05). These data suggest that decreased eIF3e expression may pave way for EMT in the development of adenomyosis through activating the TGF-β1 signaling pathway. Our study provided novel insights into the development and treatments of adenomyosis.

Keywords

eIF3e, adenomyosis, epithelial–mesenchymal transition, TGF-β1

Introduction

Adenomyosis is defined as the presence of heterotopic endometrial glands and stroma in the myometrium, with a poorly understood pathogenesis.[1,2] Patients with adenomyosis mostly suffered from dysmenorrhea, heavy menstrual bleeding, and subfertility.[3] Treatment of adenomyosis remains challenging. Progestogenic agents are not very effective, and the efficacy of gonadotropin-releasing hormone (GnRH) agonists is restricted by their short duration.[2] In addition, symptoms often recur after discontinuation of GnRH agonists therapy,[4] leaving the only perpetual effective treatment as hysterectomy.[2] Therefore, further research on pathogenesis and pathophysiology is urgently needed to relieve the suffering of patients.

The role of epithelial–mesenchymal transition (EMT) in the development of adenomyosis has received increasing attention.[5,6] Emerging evidence suggests that EMT may play a causal role in the development of adenomyosis by increasing the invasive propensity of adenomyotic epithelial cells.[7] Oh et al reported the elevated levels of nuclear β-catenin in adenomyosis and found that stabilized β-catenin expression in mouse led to altered expression of EMT markers, such as E-cadherin, Snail, and ZEB1, which was concomitant with increased incidence of adenomyosis.[8] It was further found that hepatocyte growth factor-induced EMT was a causal factor for the development of adenomyosis.[9] However, it remains largely

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unknown how EMT was promoted during the development of adenomyosis. It is desirable to identify key factors that strictly control the EMT processes in the development of adenomyosis.

Translational control plays an important role in the regulation of protein expression and occurs primarily at the initiation step.[10] Eukaryotic initiation factors (eIFs) are proteins involved in the initiation phase of eukaryotic translation. These proteins help stabilize the formation of functional ribosome around the start codon and also provide regulatory mechanisms in translation initiation. In mammals, eIF3 is the largest initiation factors, comprising 13 nonidentical subunits (3a-3m) that regulate translation initiation by orchestrating the formation of 43S-48S preinitiation complexes.[11] In particular, eIF3e, also known as Int6, is mainly involved in protein synthesis, due to its direct binding to the 40S ribosome and facilitating ribosome recruitment to messenger RNA (mRNA).[12,13] It has been reported that decreased eIF3e expression in both lung and breast epithelial cells leads to the overproduction of the transforming growth factor-β (TGF-β) cytokine and that inhibition of TGF-β signaling can reverse eIF3e-regulated EMT in lung epithelial cells, indicating that EMT in association with a decrease in eIF3e expression may be a general phenomenon in disease development and that it requires activation and maintenance of the TGF-β signaling pathway.[14] In addition, eIF3e silencing promotes angiogenesis and enhances wound healing, which further suggests that eIF3e expression may have critical roles in regulating cell migratory activities.[15]

As adenomyosis has been characterized as loss of epithelial property and gain of mesenchymal propensity of cells, we hypothesized that eIF3e may play a role in EMT in adenomyosis. To this end, we evaluated the expression of eIF3e in ectopic and control endometrium using immunohistochemistry (ICH) analysis. We also evaluated the immunoreactivity of epithelial and mesenchymal markers to assess the association between eIF3e expression and EMT process. In addition, the immunostaining of TGF-β1, a prototypical inducer of EMT, was synchronously assessed in clinical tissues for mechanism studies.[16-18] Finally, coagulant parameters were also determined with supportive analysis of their relationship with eIF3e expression in light of the fact that platelets play an important role in endometriosis[19,20] and adenomyosis development.[6,21]

Methods and Materials

Patients and Tissue Samples

This study was approved by the ethics review board of Shanghai Obstetrics and Gynecology Hospital, Fudan University. Forty women with adenomyosis (28 with diffused adenomyosis, 12 with focal adenomyosis) visiting Shanghai Obstetrics and Gynecology Hospital, Fudan University, from 2015 to 2016, were recruited for this study. Their diagnoses were made by transvaginal ultrasound (28 in 40) before surgery and were all histologically confirmed postoperatively. Based on previous criteria, adenomyosis is histologically characterized by the presence of ectopic endometrial mucosa within the myometrium (invagination of endometrium in the myometrium at a depth of at least 2.5 mm below the basal layer of the endometrium) that leads to hypertrophy of the smooth muscle, which confirms the diagnosis.[22] The diagnosis of focal or diffuse adenomyosis was based on imaging appearances as previously clearly stated.[23] For example, in the diffuse form of adenomyosis, the uterus appears to be enlarged and globular. This is due to a globally thickened myometrium caused by the coexistence of glandular cysts and hypertrophy of the unevenly distributed smooth muscle cells. The ectopic endometrial tissue samples of all adenomyosis cases were collected during hysterectomy, after informed consent was obtained, and immediately fixed in 10% buffered formalin and then processed for paraffin embedding. For controls, we also collected, after informed consent, endometrial tissue samples through curettage from 40 women seen at the same hospital in 2013 and 2016. These control cases included teratoma (7, or 15.9%), cervical intraepithelial neoplasia-III (28, or 40.9%), stage Ia1 cervical cancer (3, or 6.8%), and cervical carcinoma in situ (2, or 4.5%), and were excluded from any clinical indication of adenomyosis or endometriosis. The selection of controls was based solely on menstrual phase besides disease status, and, to a lesser extent, patients' age. The characteristics of patients with adenomyosis and controls were summarized in Table 1.

Table 1. Characteristics of Patients Recruited With Adenomyosis and Controls.

Categories	Controls (n = 40)	Adenomyosis (n = 40)	*P* Values
Age (years)	36.9 ± 6.4	47.9 ± 5.3	.01
Menstrual phase			
Proliferative	26 (65.0%)	29 (72.5%)	.571
Secretory	14 (35.0%)	11 (27.5%)	
Parity			
0	6 (15.0%)	16 (40.0%)	.049
1	30 (75.0%)	21 (52.5%)	
2	4 (10.0%)	3 (7.5%)	
Severity of dysmenorrhea			
None	38 (95.0%)	19 (47.5%)	.02
Mild	2 (5.0%)	4 (10.0%)	
Moderate	0 (0.0%)	1 (2.5%)	
Severe	0 (0.0%)	16 (40.0%)	
Amount of menses			
Light	1 (2.5%)	6 (15.0%)	.450
Moderate	38 (95.0%)	17 (42.5%)	
Heavy	1 (2.5%)	17 (42.5%)	
Uterine myoma			
No	40 (100.0%)	24 (60.0%)	.01
Yes	0 (0.0%)	16 (40.0%)	

All women in both study and control groups were premenopausal and had no hormone therapy or intrauterine device use for ≥6 months prior to tissue collection. The menstrual phase was determined by histological endometrial dating. The amount of menses during menstruation was grouped into 3 classes: mild, moderate, and heavy, depending on whether

they changed their sanitary pads <3, between 3 and 6, or >6 times a day, respectively.[24]

For each patient with adenomyosis, the following information was collected through reading medical charts and interviewing: age at surgery, uterus size (calculated as $\pi D_1 D_2 D_3/6$, where D_1 = the distance from fundus to the internal os of the cervix, D_2 = transverse diameter at the level of the cornua, and D_3 = anteroposterior diameter at the level of cornua), complaint of dysmenorrhea, duration of dysmenorrhea, amount of menses (mild, moderate, or heavy), and parity. The severity of dysmenorrhea was quantified using a 10-cm visual analog scale, assessed before operation.

Immunohistochemistry

Serial 4-μm sections were obtained from each paraffin-embedded tissue block, with the first resultant slide being stained for Hematoxylin & eosin to confirm pathologic diagnosis, and the subsequent slides stained for ICH. Routine deparaffinization and rehydration procedures were performed.

The rabbit anti-human eIF3e (1:100; Abcam, Cambridge, United Kingdom), rabbit anti-human TGF-β1 (1:100; Abcam), E-cadherin (1:200; CST, Boston, MA, USA), rabbit anti-human vimentin (1:100; Abcam), mouse anti-human PCNA (1:100; Abcam), and goat anti-human Snail (1:100; Abcam) were used as primary antibodies. For antigen retrieval, the slides were heated at 98°C in a citrate buffer (pH 6.0) for a total of 30 minutes and cooled naturally at room temperature. Sections were then incubated overnight with the primary antibody at 4°C. After slides were rinsed, the biotinylated secondary antibody, Supervision Universal (anti-mouse/rabbit) detection reagent horse radish peroxidaase(HRP) (GK500705, Shanghai Gene Tech Company, Shanghai, China) was added for incubation at room temperature for 30 minutes. The bound antibody complexes were stained for 3 to 5 minutes or until appropriate for microscopic examination with diaminobenzidine and then counterstained with hematoxylin and mounted.

Immunoreactivity staining was characterized quantitatively by digital image analysis using the Image Pro-Plus 6.0 (Media Cybernetics, Inc, Rockville, Maryland) as reported previously[25] without prior knowledge of any of the clinicopathological information. Briefly, images were obtained with the microscope (Olympus BX51, Olympus, Tokyo, Japan) fitted with a digital camera (Olympus DP70, Olympus, Tokyo, Japan). A series of 10 random images on several sections were taken for each immunostained parameter to obtain a mean value. Staining was defined via color intensity, and a color mask was made. The mask was then applied equally to all images, and measurements were obtained. Immunohistochemical parameters assessed in the detected area included (a) integrated optical density (IOD), (b) total stained area (S), and (c) mean optical density (MOD), which is defined as MOD = IOD/S, equivalent to the intensity of stain in the positive cells.

The epithelial and stromal components were jointly evaluated under the circumstance of vimentin, TGF-β1, eIF3e, PCNA and Snail, which expressed positive staining in both epithelial and stroma cells. Epithelial cells were separately evaluated under the circumstance of E-cadherin, which exhibited positive staining exclusively in epithelial cells. To minimize potential bias, sections of control group were evaluated in the same way.

As positive controls for IHC analysis, mouse spleen and liver samples were used for the positive immunostaining of vimentin and TGF-β1, respectively, and women's breast cancer tissues were used for positive immunostaining of eIF3e, E-cadherin, and Snail. For negative controls, phosphate buffer saline (PBS) was used instead of primary antibodies before slides were exposed to secondary antibody. The negative controls did not show any nonspecific staining. The representative IHC results of positive and negative controls are shown in Figure S1 (Supplemental Material).

Statistical Analysis

For descriptive statistics, we used boxplot to graphically depict groups of immunoreactivity data. Comparison of means of continuous variables was made using the Wilcoxon test and Kruskal-Wallis test for 2 groups and ≥3 groups, respectively. Categorical variable comparisons were made using the chi-square or Fisher exact tests when necessary. The Spearman rank correlation was used to evaluate correlations between variables. The linear regression analysis was performed for assessed factors after immunoreactivity being square root transformed to improve normality. P values of less than .05 were considered statistically significant. All computations were made with R 3.3.1 (36) with the significance set of $p < 0.05$ (http://www.r-project.org).

Results

Clinicopathological Data

The characteristics of adenomyosis patients and the control subjects were summarized and compared. The adenomyosis group was comparable with the control group in menstrual phase ($P = .571$) and amount of menses ($P = .450$), but seemed to be older ($P = .01$) and had milder parity ($P = .049$), severer dysmenorrheal ($P = .02$), and higher incidence of uterine myoma ($P = .01$; Table 1).

Evidence for Reduced eIF3e Expression, Activation of TGF-β1 Pathway, Occurrence of EMT, and Increased Proliferation in Ectopic Endometrium

The eIF3e immunoreactivity was investigated in ectopic and control endometrium. The micrographs of eIF3e immunoreactivity in ectopic and the control endometrium are shown in Figure 1. The eIF3e stained primarily in the epithelial component in both ectopic and control endometrium and did not vary significantly between proliferative and secretory phases in either control or ectopic endometrium. However, the eIF3e immunostaining levels in epithelium of both focal and diffused adenomyosis tissues significantly reduced compared with the

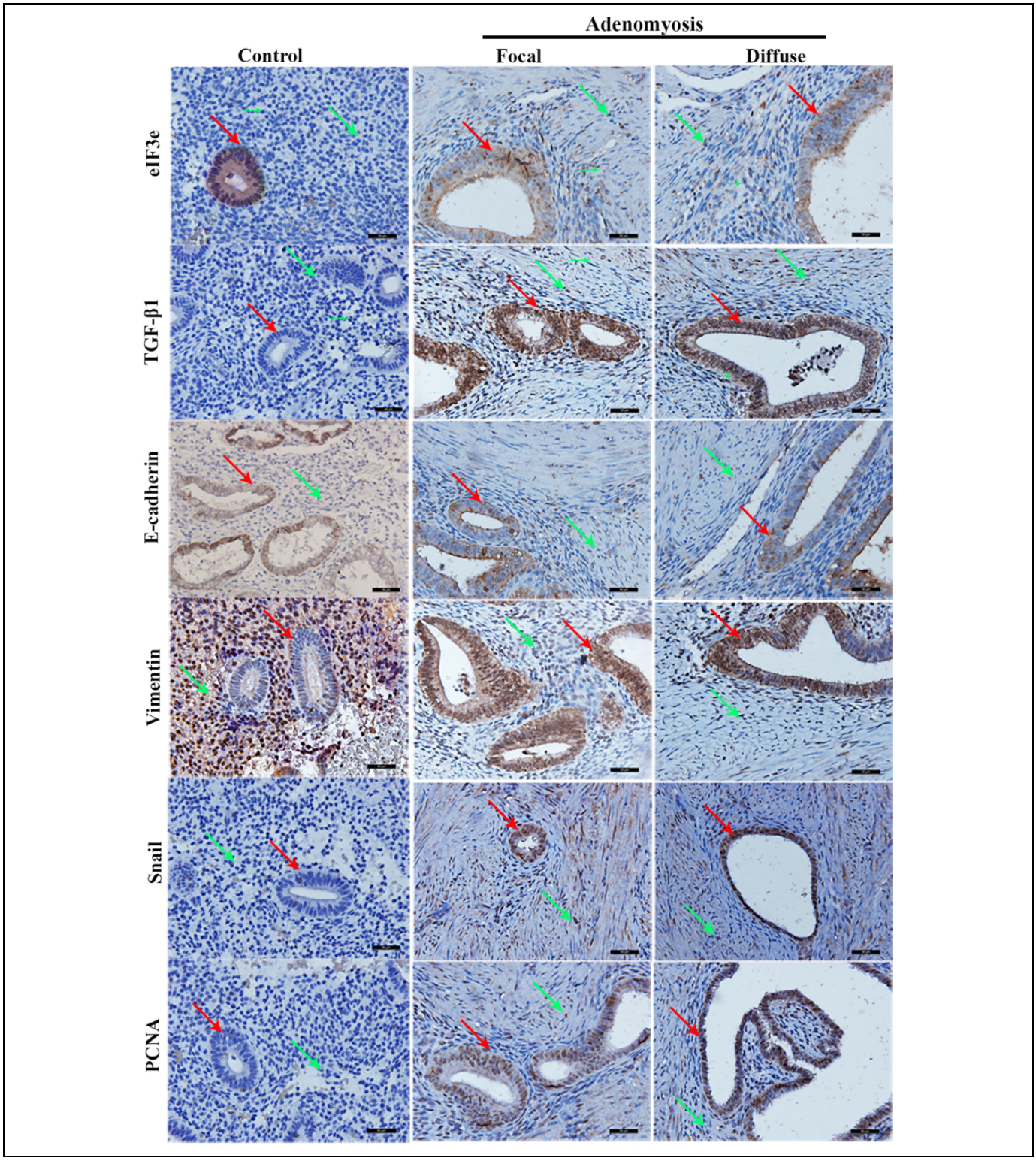

Figure 1. Immunohistochemistry staining in ectopic lesions and control endometrium. Representative immunoreactivity staining of eIF3e, TGF-β1, E-cadherin, vimentin, Snail, and PCNA in control (left column) and ectopic (right column) endometrium were shown. Red arrow indicates epithelial cells and green arrow indicates stromal cells. Scale bar = 50 μm. eIF3e indicates eukaryotic translation initiation factor 3 subunit e; TGF-β1, transforming growth factor-β1.

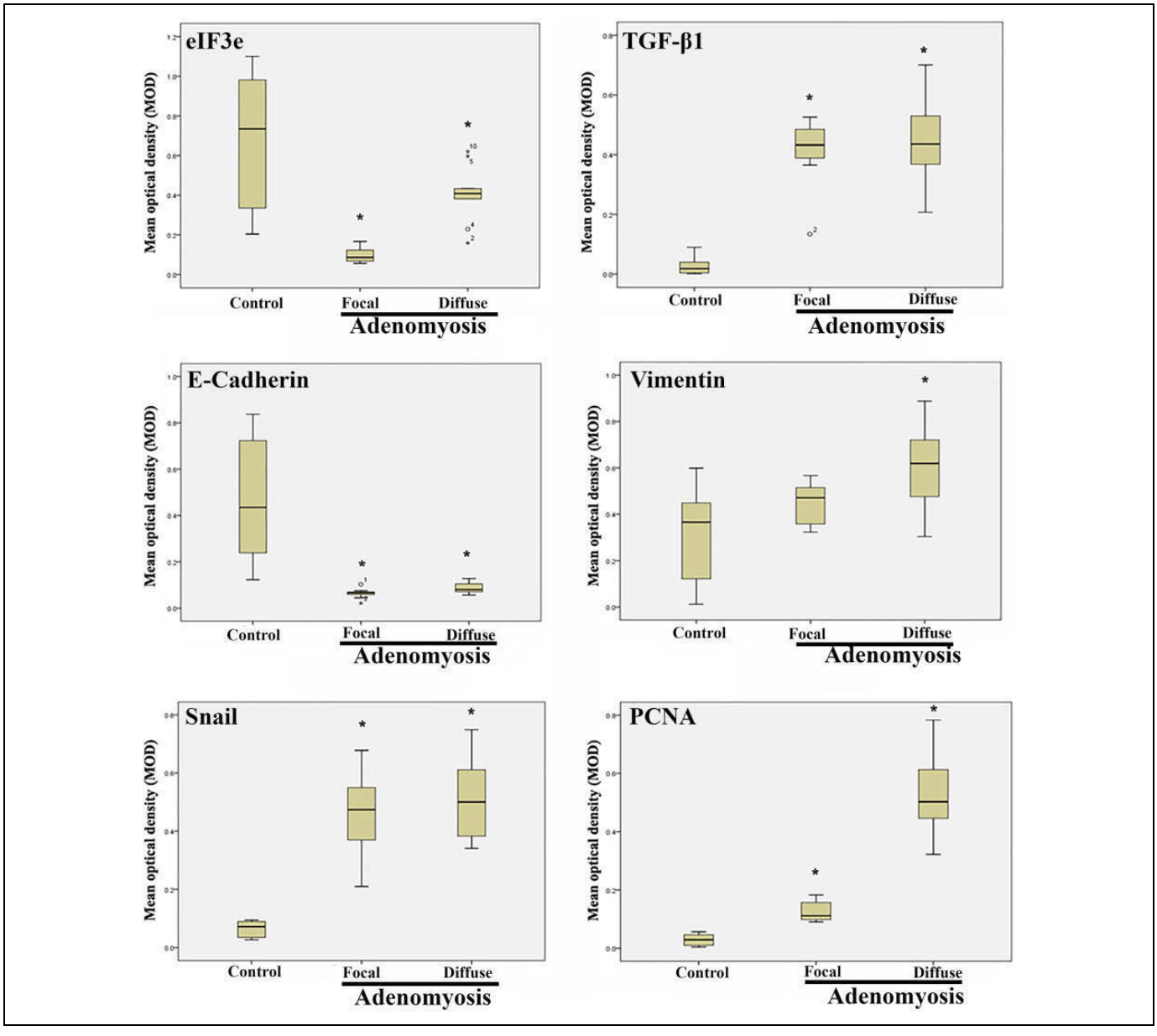

Figure 2. Summary of the differential immunoreactivity between control and ectopic endometrium. Boxplots showed the differential immunoreactivity of eIF3e, TGF-β1, E-cadherin, vimentin, Snail, and PCNA in Control (n = 40) and ectopic endometrium (n = 12 and 28 for focal and diffuse adenomyosis, respectively). *P < .05 versus control. eIF3e indicates eukaryotic translation initiation factor 3 subunit e; TGF-β1, transforming growth factor-β1.

control endometrium (Figure 2). The lesional eIF3e staining levels had no relationship with age, menstrual phase, parity, severity of dysmenorrhea, nor co-occurrence of uterine myoma (data not shown).

To further study the possible role of eIF3e in EMT and cellular proliferation, we performed immunostaining of ectopic and control endometrium using antibodies against TGF-β1, E-cadherin, vimentin, Snail, and PCNA (a cell proliferation marker). It was observed that TGF-β1 showed a positive staining in the cytoplasm of ectopic epithelium, in contrast to its negative staining in control group. Transforming growth factor-β1 was also positively stained in the cytoplasm of ectopic stroma, but its intensity was weaker in stroma than in the epithelium (Figure 1). Compared with control endometrium, TGF-β1 expression in adenomyosis endometrium was significantly elevated (Figure 2). As a marker of epithelial cells, E-cadherin expressed in membrane of epithelial cells of both groups (Figure 1), but had a significant decrease in adenomyosis ones (Figures 1 and 2). The immunoreactivity of vimentin was observed in the cytoplasm of stromal cells in control and adenomyosis group but had a significant increase in the cytoplasm of ectopic epithelial cells (Figure 1). The immunoreactivity of vimentin was mildly increased in adenomyosis tissues (Figure 2). Snail showed a positive staining in the nuclei of

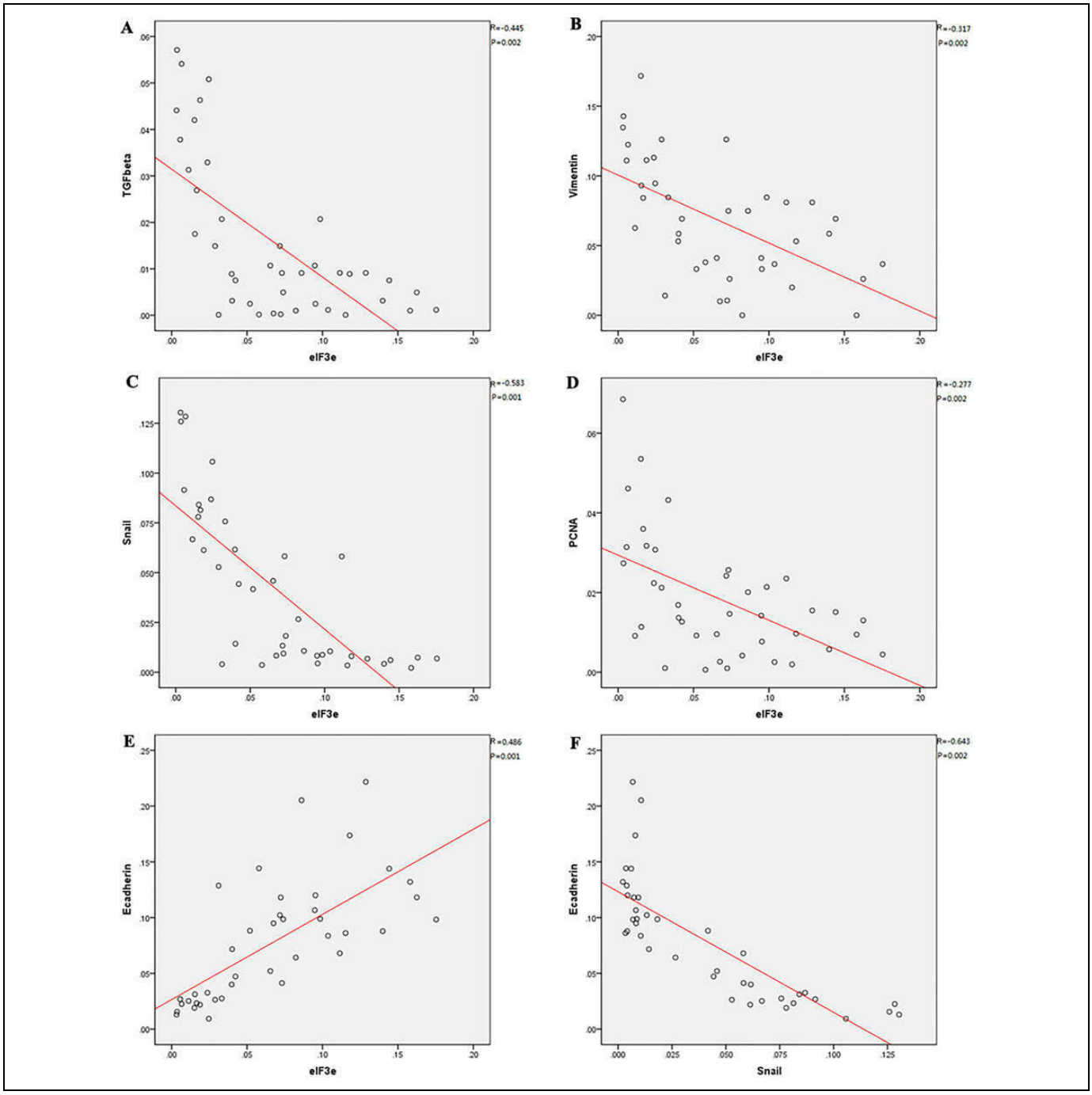

Figure 3. Correlation of eIF3e level with those of TGF-β1 and EMT markers in adenomyosis endometrium epithelial cells. Liner regression analysis was performed to show the staining levels of TGF-β1 (A), vimentin (B), Snail (C), PCNA (D), and E-cadherin (E) versus that of eIF3e staining. The relationship between Snail and E-cadherin staining was also analyzed (F). The correlation coefficient (*R* value) and significance (*P* value) were as indicated. n = 40 for each panel. eIF3e indicates eukaryotic translation initiation factor 3 subunit e; EMT, epithelial–mesenchymal transition; TGF-β1, transforming growth factor-β1.

epithelial and stromal cells in the ectopic lesions in adenomyosis groups (Figure 1) and had a significant increase in expression levels as compared with the control groups (Figure 2). PCNA expressed positively mostly in the nuclei of epithelial cells and in a few stromal cells of ectopic tissue (Figure 1) and was significantly increased as compared with the control ones (Figure 2). All these observations suggested the overexpression of TGF-β1, Snail, vimentin, and PCNA and the downregulation of E-cadherin in the epithelial component of ectopic endometrium.

Furthermore, we observed eIF3e staining levels correlated well with other EMT markers in the endometrium epithelial

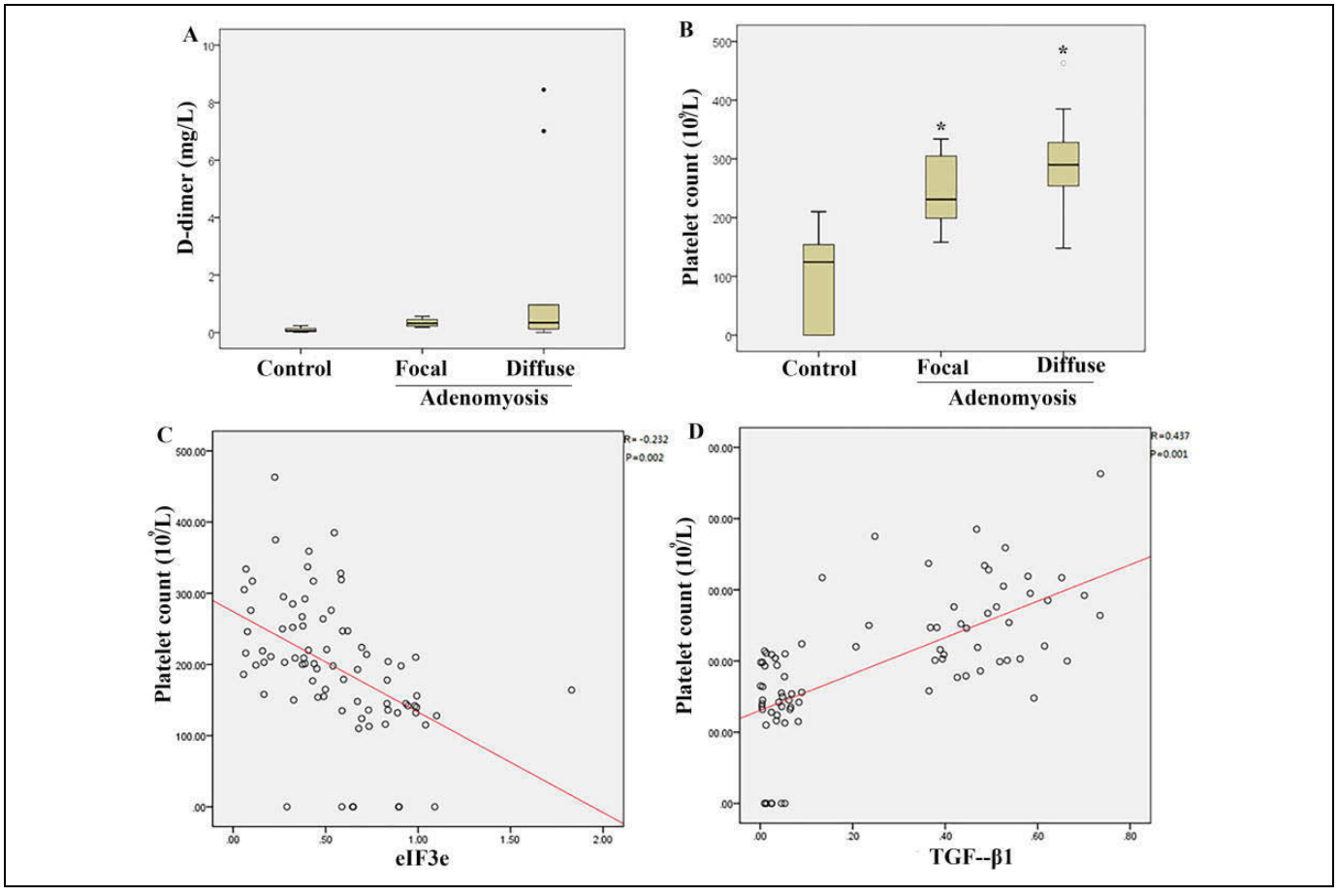

Figure 4. Relationship between coagulation parameters and eIF3e and TGF-β1 staining levels. (A, B) the D-dimer level and platelet count were determined in control cases (n = 40), focal adenomyosis (n = 12), and diffused adenomyosis (n = 28) cases. *$P < .05$ versus control. (C, D) Correlation of platelet count with eIF3e and TGFβ1 staining levels. The correlation coefficient (R value) and significance (P value) were as indicated. eIF3e, eukaryotic translation initiation factor 3 subunit e; TGF-β1, transforming growth factor-β1.

cells from the 28 cases with diffuse adenomyosis (Figure S2). The focal and diffuse adenomyosis cases were then pooled together to perform linear regression analysis. Consistently, our results showed that the eIF3e staining levels negatively correlated with those of TGF-β1 ($R = -.445$, $P = .002$), vimentin ($R = -.317$, $P = .002$), Snail ($R = -.583$, $P = .001$), and PCNA ($R = -.277$, $P = .002$) but positively with that of E-cadherin ($R = .486$, $P = .001$) (Figure 3A-E). As expected, the Snail staining levels correlated negatively with that of E-cadherin in the 40 cases with adenomyosis ($R = -.513$, $P = .001$; Figure 3F).

Relationship Between Coagulation Parameters and eIF3e and TGF-β1 Staining Levels

To test the hypothesis of platelet-induced EMT in adenomyosis, we further identified the coagulation parameters that associated with adenomyosis, and based on this parameter, we performed correlation studies. Interestingly, it was found that the platelet counts, but not the fibrinogen levels nor D-dimer, in women with adenomyosis were significantly higher than that in controls (Figure 4A and B). Using age, menstrual phase, parity, severity of dysmenorrhea, the amount of menses, and group identity (control vs adenomyosis) as covariables, the multiple linear regression analysis identified that the group identity was the only covariable that was significantly associated with the platelet counts, suggesting that platelet count was a contributing factor to the genesis of adenomyosis. Based on this finding, it was further found that the platelet count was negatively correlated with the staining levels of eIF3e ($R = -.232$, $P = .002$, Figure 4C), but positively with that of TGF-β1 ($R = .437$, $P = .001$; Figure 4D).

Discussion

Epithelial-to-mesenchymal transition (EMT) has been widely observed in the development of adenomyosis.[26-29] In adenomyosis, it could be detected that the epithelial marker E-cadherin decreases and the mesenchymal marker vimentin increases.[9,30] Snail, as a repressor of E-cadherin, also increased and led to EMT during the development of adenomyosis.[18] All these studies have emphasized that EMT is a causal process resulting in adenomyosis. However, limited cytokines and transcription factors have been reported to mediate EMT in

adenomyosis so far, making the occurrence of EMT remain mysterious in adenomyosis.

A recent global transcriptome analysis of eutopic endometrium in women with adenomyosis found the involvement of eIF2 signaling pathway.[31] As a member of eIFs family, as well as the largest initiation factor in mammals, eIF3e is expected to be an important factor involved in adenomyosis.[31] Therefore, the present study investigated the potential of eIF3e as an upstream factor that regulate the EMT occurrence in adenomyosis and discussed the possible mechanisms.

We found that eIF3e immunoreactivity was significantly reduced in ectopic endometrium as compared with control endometrium. On contrast, the immunoreactivity of TGF-β1, Snail, and vimentin were consistently increased and that of E-cadherin reduced significantly in endometriotic epithelial cells. Furthermore, the eIF3e staining levels correlated positively with that of E-cadherin but negatively with those of TGF-β1, Snail, vimentin, and PCNA. The elevated TGF-β1 expression in adenomyotic lesions was in broad agreement with a previous study reporting increased TGF-β1 concentration in uterine washings.[32] In adenomyosis, platelet-derived TGF-β1 activates the TGF-β1/Smad3 signaling pathway, resulting in EMT, fibroblast-to-fibroblast, smooth muscle metaplasia, and fibrogenesis.[26] Previous reports also found that decreased eIF3e expression mediated EMT process in cancer cell invasion through activating the TGF-β1 signaling[33] and facilitated cellular proliferation through increasing angiogenesis in ectopic endometrium.[15] In fact, as 2 major regulators of EMT, the mRNAs of Snail and Zeb2 have been reported to be preferentially translated,[14] and the cap-independent translation of Zeb2 and vimentin is favored when eIF3e expression is reduced,[33] which may also be true for ectopic epithelial cells in adenomyosis. In addition, eIF3e downregulation activates stromal fibroblasts, leading to increased stromal-derived factor-1 (SDF-1) secretion,[34] while on the other hand SDF-1 secretion has been reported to be elevated in endometriosis.[35] In view of all these indications, it could be conclusive that eIF3e downregulation may mediate EMT *via* activating TGF-β1 signaling in adenomyosis.

In addition, some platelet adhesion molecules, such as certain integrins, are reported to be aberrantly expressed in adenomyosis.[36] It is suggested that adenomyotic lesions are just like endometriotic lesions and undergo platelet-induced EMT, and corroborated with the notion that women with adenomyosis are in a hypercoagulable state.[20] The present study found that the platelet count, but not other coagulation parameters, was the influential factor for adenomyosis. The platelet count negatively correlated with eIF3e staining levels. These observations raised the possibility that platelets may be responsible for the eIF3e downregulation.

In conclusion, we provided evidence that the eIF3e was downregulated in ectopic endometrium in adenomyosis, concomitant with increased expression of TGF-β1, Snail, and vimentin but reduced expression of E-cadherin in adenomyotic epithelial cells. These data suggested that reduced eIF3e expression may mediate EMT *via* activating the TGF-β1 signaling in adenomyosis. In addition, eIF3e downregulation may be the result of platelet activation in endometriotic lesions. Since few factors of translation control have been investigated systemically in adenomyosis, our study might provide novel insights into the development and treatment of adenomyosis.

Authors' Note

Xianjun Cai and Minhong Shen contributed equally to this work. This work was presented orally at the Fifth Asian Congress on Endometriosis, which was held in Osaka, Japan, on September 23, 2016.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material is available for this article online.

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