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Original Article

Upregulation of Endothelin-1/Endothelin A Receptor Expression Correlates with Heparanase Expression in Ovarian Carcinoma

Nungki Anggorowati[1], MD, PhD;
Ahmad Ghozali[1], MD;
Irianiwati Widodo[1], MD, PhD;
Dwi Cahyani Ratna Sari[2], MD, PhD;
Muhammad Mansyur Romi[2], MD, MS;
Nur Arfian[2], MD, PhD;

[1]Department of Anatomical Pathology, Universitas Gadjah Mada/Sardjito Hospital, Yogyakarta, Indonesia;
[2]Department of Anatomy, Universitas Gadjah Mada/Sardjito Hospital, Yoyakarta, Indonesia

Correspondence:
Nungki Anggorowati, MD, PhD;
Department of Anatomical Pathology, Universitas Gadjah Mada, Farmako Street, Sekip Utara, Yogyakarta 55281, Indonesia
Tel: +62 274 540460
Fax: +62 274 540460
Email: nungki@ugm.ac.id
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What's Known

- Endothelin-1 and its receptors play such roles in tumor cell pathology as proliferation, migration, invasion, and vascular differentiation, especially in ovarian cancer.
- Heparanase, which plays a role in tumor progression and angiogenesis, correlates with poor survival in ovarian carcinoma.

What's New

- Serial immunostaining revealed similar localization of the endothelin A receptor (ETAR) and heparanase in ovarian cancer samples. Moderate correlation between heparanase and ETAR expression was also shown in this study. Findings may indicate interactions between endothelin and heparanase signaling.

Abstract

Background: Heparanase and endothelin-1/endothelin A receptor (ET-1/ET_AR) expressions increase in cancer. This condition enhances tumor progression and correlates with poor survival. Limited data are documented regarding the role of heparanase and ET-1/ET_AR in epithelial ovarian cancer (EOC). We sought to characterize the correlation between heparanase and ET-1/ET_AR in EOC.

Methods: Thirty patients with benign and malignant ovarian neoplasms were recruited in this study. Neoplasm subtypes were diagnosed by pathologists. RNA extraction was done in fresh frozen neoplasms while immunohistochemical (IHC) staining was done on ET_AR, heparanase, and proliferation (Ki-67 antigen) in paraffin sections. Reverse transcriptase PCR was done to quantify the expression of preproET-1 (ppET-1), ET_AR, and heparanase. ET_AR and heparanase histoscores were done based on IHC staining. The Independent Samples *t* Test, ANOVA, and correlations were used for statistical analysis.

Results: Heparanase and ET_AR histoscores, ppET-1 and ET_AR mRNA levels, and Ki-67 were significantly higher in the group with EOC than in the benign or borderline group, regardless of the histopathological types. The heparanase histoscore correlated with the ET_AR histoscore (r=0.484, P=0.007) and the ET_AR mRNA level (r=0.551, P=0.003). The level of ppET-1 mRNA correlated with both ET_AR mRNA level and ET_AR histoscore (r=0.603, P=0.001 and r=0.455, P=028, respectively). The ovarian neoplasms with high ppET-1 mRNA levels also tended to have high heparanase mRNA levels; however, the correlation was weak (r=0.354, P=0.07). Ki-67 correlated with the heparanase and ET_AR histoscores (r=0.381, P=0.038 and r=0.477, P=0.008, respectively).

Conclusion: Heparanase and ET_AR were upregulated in EOC, and the correlation between heparanase and ET_AR expressions was also elucidated in the current study.

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Keywords ● Ovarian neoplasms● Endothelin-1 ● Receptor, Endothelin A ● Heparanase ● Ki-67 antigen

Introduction

Ovarian carcinoma is the most frequent type of malignancy and causes mortality among women. [1] It accounts for 140,200

deaths worldwide.[1,2] The most common ovarian malignancy is epithelial ovarian carcinoma (EOC). There are 4 major histopathological subtypes of EOC: serous, mucinous, endometrioid, and clear cell.[2,3] All over the world, the most frequent subtype of EOC is the serous type, followed by mucinous, endometrioid, and clear cell subtypes.[1] The distribution of the subtypes varies according to regions or countries. They have distinctive molecular pathogeneses and different vulnerabilities to chemotherapeutic agents. However, the regulatory mechanisms underlying this heterogeneity remain vaguely known. At present, clinical trials do not discriminate these subtypes but treat them as a homogeneous cluster. Hence, the outcomes should be analyzed further to understand whether the therapy has different influences in each subtype.[1,4] Many substances may also influence the therapeutic approach based on the biological activities of those substances. They may have interactions with each other and influence tumor progression.

Heparanase is an endo-β-D-glucuronidase capable of cleaving heparan sulfate side chains at a limited number of locations.[5] A previous study showed an association between heparanase expression and worse prognosis in EOC.[6] Heparanase vitiates heparan sulfate and results in tumor progression, comprising adhesion, migration, invasion, metastasis, and angiogenesis.[7,8] The angiogenesis mechanism encompasses the release of heparan sulfate-bound angiogenic factors such as the vascular endothelial growth factor (VEGF) and the basic fibroblast growth factor.[7,9,10]

The endothelin (ET) pathway is used for targeting tumor vasculature as well as tumor cells.[11] ETs are a family of small peptides consisting of ET-1, ET-2, and ET-3.[12] These small peptides share structural homology and initiate signaling by binding to the G-protein-coupled receptors the endothelin A receptor (ET_AR) and the ET_BR.[13] Both ET-1 and ET_AR act in cancer cell proliferation and metastasis.[14,15] The ET axis has also been reported to be of significance in EOC.[14,16] The role of ETs and ET receptors in the biology and therapy for EOC has been previously delineated. The increased expression of ET-1 and the ET_AR in EOC cells and the ET_BR in intratumoral vessels has also been reported, as well as a relationship between the expression of ET-1 and the VEGF in the development of ascites and cell resistance to therapy.[17]

In EOC, ET-1 plays a role in the epithelial–mesenchymal transition. Tumor cells experiencing the epithelial–mesenchymal transition undergo epithelial morphology alterations and restructuring of their cytoskeleton; in addition, they attain a motile phenotype through the modification of the regulation of several molecules including tight and adherens junctions' proteins and mesenchymal markers.[14,18,19] Clarifying possible molecular interactions between heparanase and ET-1/ET_AR and their relation with histopathological subtypes and metastasis could help identify a new therapeutic approach. Nonetheless, there is a dearth of data in the existing literature on heparanase, ET-1/ET_AR axis, and histopathological subtypes of EOC. Hence, the present study aimed to elucidate the correlation between heparanase and ET-1/ET_AR among various histopathological subtypes of benign and malignant ovarian tumors.

Materials and Methods

Tissue Samples from the Patients

This was a cross-sectional study of the benign and malignant ovarian neoplasms. Thirty tissue samples of human ovarian neoplasms were obtained via surgical resection at Sardjito Hospital, Yogyakarta, Indonesia, between January and July 2014. The purpose of tissue sampling was to extract RNA and to process formalin-fixed paraffin-embedded tissues. The paraffin-embedded tissue blocks were placed in 10% buffer formalin, whilst the samples used for RNA extraction were retained in RNAlater (AM7021, Ambion) in a refrigerator at -30°C to -80°C. Next, 4-μm paraffin sections were deparaffinized and stained with hematoxylin–eosin for the histopathological examination of the cancer. Non-epithelial subtypes of ovarian cancer were excluded from this study.

The malignancy of the ovarian neoplasms was identified by pathologists at the Faculty of Medicine, Universitas Gadjah Mada. The study design was approved by the institutional Ethical Review Board of Universitas Gadjah Mada, and informed consent was obtained from the patients.

Immunohistochemistry

The paraffin-embedded tissues were used for immunohistochemistry with heparanase (AB85543, Abcam, dilution 1:200), ET_AR (SC33535, Santa Cruz, dilution 1:100), and Ki-67 (CRM 325 B, Biocare Medical, dilution 1:100) antibodies. Paraffin sections, 4 μm in thickness, were placed on poly-L-lysine-coated slides. After deparaffinization, endogenous peroxidase was reduced by incubation with 3% H_2O_2 in phosphate buffer saline for 5 minutes. The secondary antibodies used were EnVision+System-HRP anti rabbit (K4002, Dako) for heparanase and the

ET_AR and Histofine SAB-PO (MULTI) (414171F, Nichirei) for Ki-67. The chromogen used was 3,3'-diaminobenzidine. Hematoxylin was utilized for counterstaining.

Evaluation of Immunostaining

The stains for heparanase and the ET_AR were assessed by calculating the positively marked carcinoma cells from 10 randomized representative fields (×400 magnification). The immunohistochemistry was scored based on the technique previously reported. The mean percentage of the positive cancer cells was calculated, and the staining strength was stratified from 0 to 3 (0, no staining; 1, slight staining; 2, medium staining; and 3, strong staining). The scores were obtained through the subsequent method: (mean percentage)×(intensity+1); range=0–400.[20,21]

Ki-67 was used to measure the proliferation index, and the score was calculated as stained nuclei over the total number of tumor nuclei in 10 randomized representative high-power fields (or ×400 magnification). The Ki-67 score was presented as a percentage ranging from 0% to 100%.

RNA Extraction and Semiquantitative Reverse Transcriptase PCR Assay

Twenty-seven fresh tissues from the 30 samples used for immunohistochemistry were available for RNA extraction, cDNA synthesis, and polymerase chain reaction (PCR). The purpose of RNA extraction was to quantify the expression of heparanase, ppET-1, and ET_AR. The level of ppET-1 expression was quantified to determine the ET-1 level. Total RNA was extracted from the ovarian neoplasm tissues using RNAiso PLUS (Takara Bio, Tokyo, Japan). The tissue was chosen from the area adjacent to the layer used for paraffin-embedded tissue processing. RNA (1 µg) was reverse-transcribed with ReverTra Ace reverse transcriptase (TRT-101, TOYOBO Co.) in a 20-µL mixture with a random primer. The cycling conditions were 30°C for 10 minutes, 42°C for 60 minutes, and 99°C for 5 minutes.

The reaction mixture (1 µL) was then used as a template in a conventional PCR assay. GoTaq Green Master Mix (M7122, Promega) was employed. The initial denaturation was performed at a temperature of 94°C for 2 minutes. The primers and PCR conditions used are presented in table 1.

The PCR products were subjected to 2% agarose (Agarose S; Nippon Gene, Tokyo, Japan) gel electrophoresis and gel red staining. The expression of the PCR products in gel electrophoresis was quantified using densitometry analysis by image J software. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used for normalizing the gene expressions.

Statistical Analysis

The variables were analyzed using ANOVA and the Independent Samples *t* test for the normally distributed data, while the Kruskal–Wallis test and the Mann–Whitney *U* test were applied for the data that were not normally distributed. The simple regression test and the Spearman test were drawn upon to test for correlation. Values of $P<0.05$ were considered statistically significant.

Results

Characteristics of the Patients

The age of the patients ranged from 15 to 71 years (median age=49.5 y) (table 1). The diagnoses of EOC were malignant in 18 (60%) samples and benign or borderline in 12 (40%). Based on the histopathological subtypes, the malignant tissue samples consisted of 8 (26.7%) serous, 3 (10%) mucinous, 4 (13.3%) endometrioid, and 3 (10%) clear cell carcinoma. The benign or borderline tissues were comprised of 5 (41.7%) serous and 7 (58.3%) mucinous subtypes (table 2). The mean age of the patients with benign or borderline tumors tended to be younger than that of the patients with malignant tumors (45.1±13.39 vs. 50.50±9.08; P=0.20 [mean±SD])

Immunohistochemical Characteristics of Heparanase, ET_AR, and Ki-67 in the Ovarian Neoplasms

The immunohistochemical staining of heparanase, ET_AR, and Ki-67 in the ovarian neoplasms is depicted in figure 1. The histoscores of heparanase and the ET_AR were higher in the ovarian carcinoma group than those in the benign or borderline group (363.2±35.99 vs. 127.6±170.46; P=0.004 and 306.8±103.11 vs. 56.2±114.32; P<0.001 [mean±SD], respectively) (figure 2A), regardless of the histopathological types (figure 2C). The mean percentage of Ki-67 was also significantly greater in the malignant cluster than in the benign or borderline cluster (31.9% vs. 5.4%; P<0.001) (figure 2B), irrespective of the histopathological types (figure 2D).

mRNA Level of Heparanase, ppET-1, and ET_AR in the Ovarian Neoplasms

The ovarian carcinoma tissues revealed a higher expression of ppET-1 and ET_AR mRNA

Table 1: Primers and conditions used

No	Gene	Primer	PCR Conditions
1	Heparanase	5'-GTAGTGATGCCATGTAACTGAATC-3'(forward); 5'-TTCGATCCCAAGAAGGAATCAAC-3' (reverse)	30 cycles: 94°C for 10 s, 60°C for 20 s, 72°C for 1 min, extension 72°C for 10 min
2	ppET-1	5'-CAGAGGAACACCTAAGACAAACC-3' (forward); 5'-GTGGGTCACATAACGCTCTC-3' (reverse)	30 cycles: 94°C for 10 s, 60°C for 20 s, 72°C for 1 min, extension 72°C for 10 min
3	ET_AR	5'-GCACCGTCAAGGCTGAGAAC-3' (forward); 5'-TGGTGAAGACGCCAGTGGA-3' (reverse)	30 cycles: 94°C for 10 s, 60°C for 20 s, 72°C for 1 min, extension 72°C for 10 min
4	GAPDH	5'-GCACCGTCAAGGCTGAGAAC-3' (forward); 5'-TGGTGAAGACGCCAGTGGA-3' (reverse)	30 cycles: 94°C for 10 s, 60°C for 20 s, 72°C for 1 min, extension 72°C for 10 min

ppET-1: PreproET-1; ET_AR: Endothelin A receptor; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Table 2: Baseline characteristics of the patients (N=30)

Characteristics	No. of patients (%)
Age (y)	15–71 (median 49.5)
<45	5 (16.7)
≥45	25 (83.3)
Malignancy	
Benign/borderline	12 (40.0)
Serous cystadenoma	5 (16.7)
Mucinous cystadenoma	7 (23.3)
Malignant	18 (60.0)
Serous carcinoma	8 (26.7)
Mucinous carcinoma	3 (10.0)
Endometrioid carcinoma	4 (13.3)
Clear cell carcinoma	3 (10.0)

than the tissues with benign or borderline neoplasms (0.93±0.19 vs. 0.68±0.25; P=0.018 and 1.20±0.45 vs. 0.68±0.38; P=0.001 [mean±SD], respectively) (figure 3). The mRNA level of heparanase was revealed to be more elevated in the ovarian carcinoma tissues than in the tissues with benign or borderline tumors, even though the statistical test was not significant (0.82±0.65 vs. 0.74±0.69; P=0.46) (figure 3). There were no significant dissimilarities in terms of heparanase, ppET-1, and ET_AR mRNA levels between the histopathological subtypes of ovarian carcinoma (data not shown).

Correlation Between Heparanase, ppET-1, and ET_AR

The heparanase immunohistochemical histoscore was correlated with the ET_AR histoscore (r=0.484, P=0.007 [n=30]) and the ET_AR mRNA level (r=0.551, P=0.003 [n=27]) (figures 4A and 4B). A correlation was also detected between the ppET-1 mRNA level and both ET_AR mRNA level and ET_AR histoscore (r=0.603, P=0.001[n=27] and r=0.455, P=028 [n=27], respectively) (figures 4C and 4D). The ovarian neoplasms with high ppET-1 mRNA levels tended to have high heparanase mRNA levels, but a weak correlation was detected between ppET-1 and the heparanase mRNA level (r=0.354, P=0.07 [n=27]) (figure 4E). A correlation was also found between the Ki-67 percentage and the heparanase and ET_AR histoscores (r=0.381, P=0.038 [n=30] and r=0.477, P=0.008 [n=30]) (data not shown).

Discussion

The current study found a higher expression of heparanase in malignant ovarian tumors than in benign ovarian tumors. This enzyme is also related to the rapid progression and poor postoperative survival of cancer patients.[6,22,23] Heparanase relatively remains unexpressed in common normal tissues.[23] Thus, heparanase has become an appropriate therapeutic target for advanced cancers as well as for the prevention of metastasis.[10,22,23] The present study confirmed those findings. The higher histoscore of heparanase correlated also with a higher proliferation number in the malignant ovarian neoplasms. This outcome is consistent with the previous reports.[6,24] Heparanase upregulation has been correlated with the dissemination or metastatic capability of various cancer cells such as ovarian cancer cells.[5,25] Our immunohistochemical staining result also revealed high positive of heparanase signals in the cancer cells. Regarding this result, heparanase is believed to play a role in ovarian neoplasm progression. Extracellular matrix positive area of heparanase might elucidate the function of heparanase as an angiogenesis inducer. Heparanase has a role in angiogenesis through several mechanisms such as degradation of the subendothelial basement membrane and enabling of endothelial cell invasion, propagation, and adhesion.[26] In addition, the molecular processes also involve the discharge of the VEGF-A, a heparan sulfate-bound growth factor, and the basic fibroblast growth factor.[5]

The present study showed that the ET_AR and ppET-1 were expressed more in the malignant ovarian tumors, as detected by reverse transcriptase PCR and immunohistochemical staining. Similar to heparanase, ETs and their

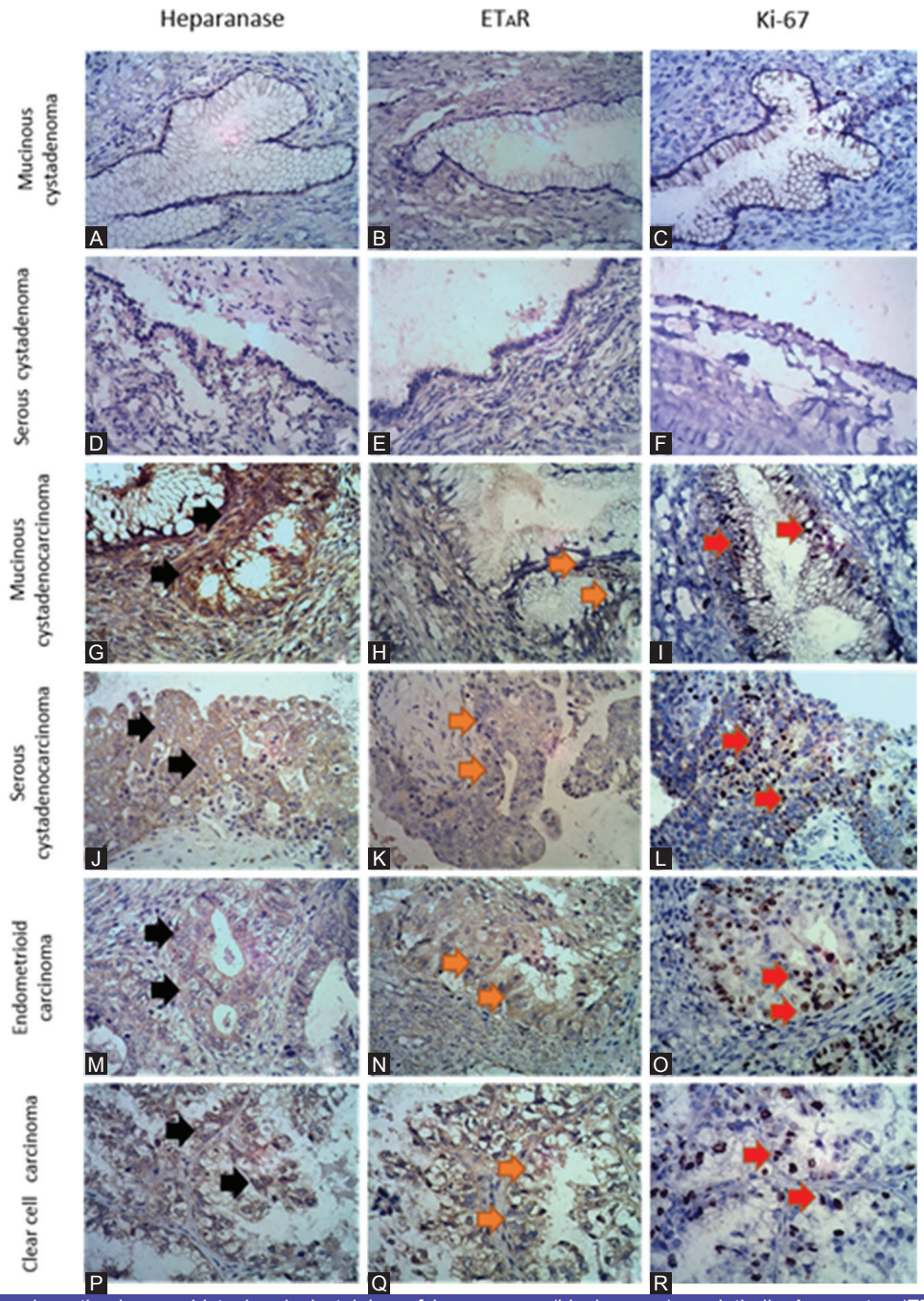

Figure 1: Based on the immunohistochemical staining of heparanase (black arrow), endothelin A receptor (ET_AR) (orange arrow), and Ki-67 (red arrow), overexpression of heparanase and ET_AR was detected in the malignant ovarian epithelial tumors (i.e., mucinous [G-I] and serous cystadenocarcinoma [J-L], endometrioid [M-O], and clear cell carcinoma [P-R]). Nevertheless, lesser expression was shown in the benign ovarian epithelial tumors (i.e., mucinous [A-C] and serous [D-F] cystadenoma (magnification ×400).

receptors also play a role in tumor growth. ET-1 upregulation contributes to the growth and progression of various tumors such as male and female genital epithelial carcinoma as well as bladder, pulmonary, and colorectal cancer. The roles of ET-1 in malignancy include mitogenesis regulation, angiogenesis, invasion and metastatic spreading, cell persistence, tumor-infiltrating immune cells, and epithelial–mesenchymal transition.[2,27,28] An increased expression of ET-1 has also been detected in the ascitic fluid of patients with ovarian carcinoma,[28] which may denote the progression and spreading of the carcinoma.

In the current study, correlations were also detected between the ppET-1 mRNA level and both ET_AR mRNA level and ET_AR histoscore ($r=0.603$, $P=0.001$ [$n=27$] and $r=0.455$, $P=0.28$ [$n=27$], respectively) (figures 4C and 4D), which might indicate an activation in the ET-1/ET_AR

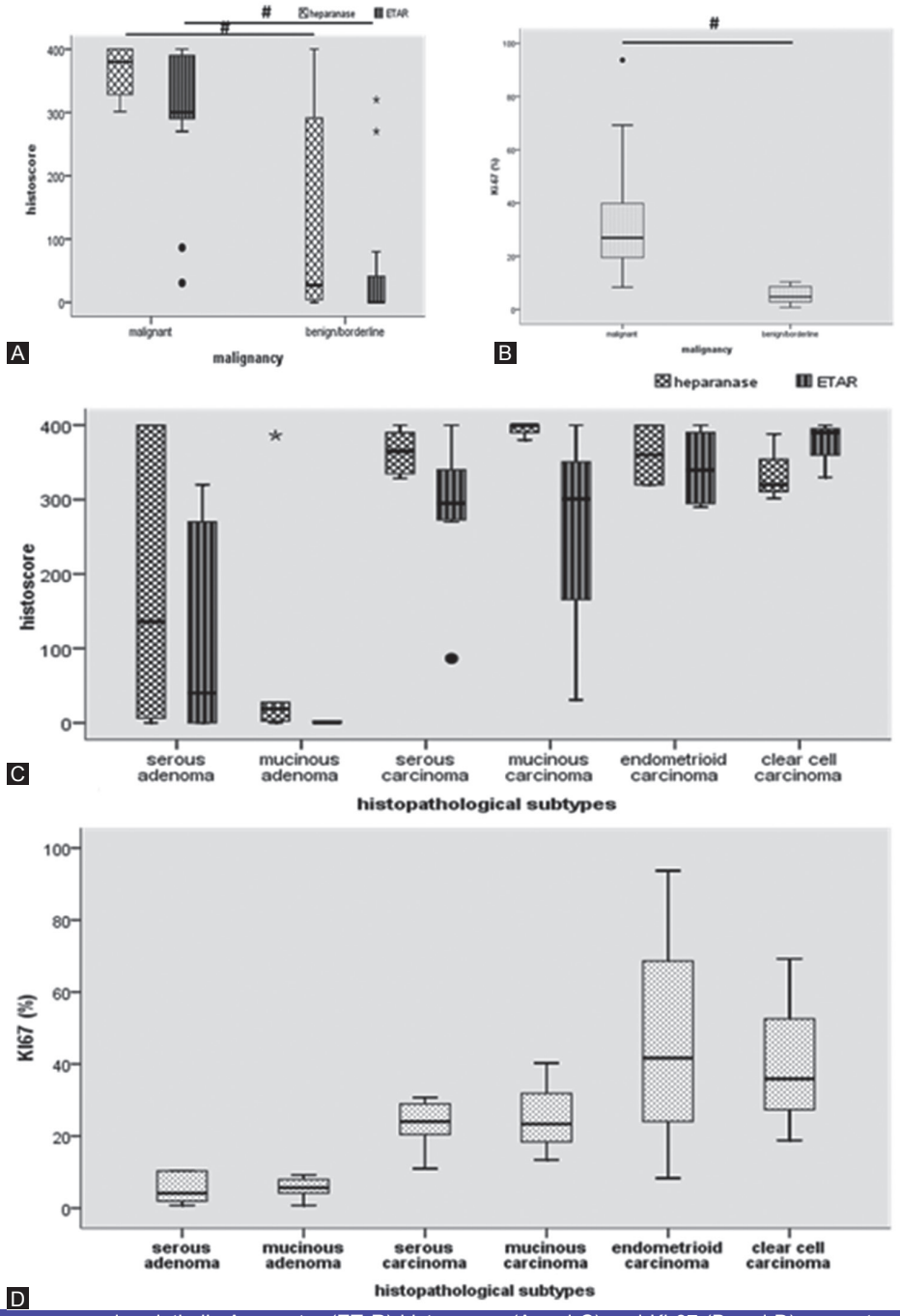

Figure 2: Heparanase and endothelin A receptor (ET_AR) histoscores (A and C) and Ki-67 (B and D) percentage in the ovarian neoplasms, which were classified according to malignancy and histopathological subtypes. #P<0.001.

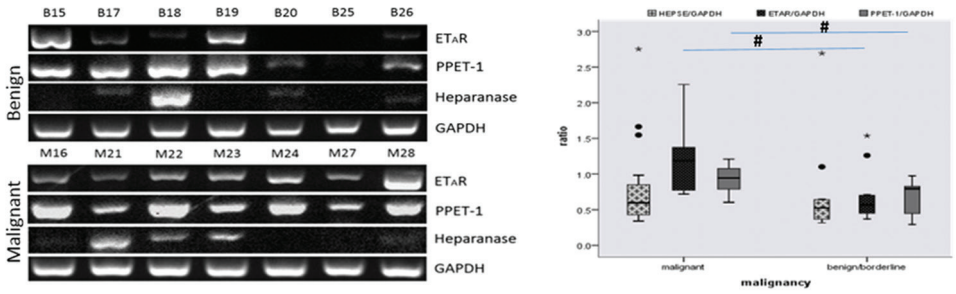

Figure 3: Endothelin A receptor (ET_AR), preproET-1 (ppET-1), and heparanase mRNA levels in the ovarian tumors classified as benign/borderline and malignant. #P<0.05.

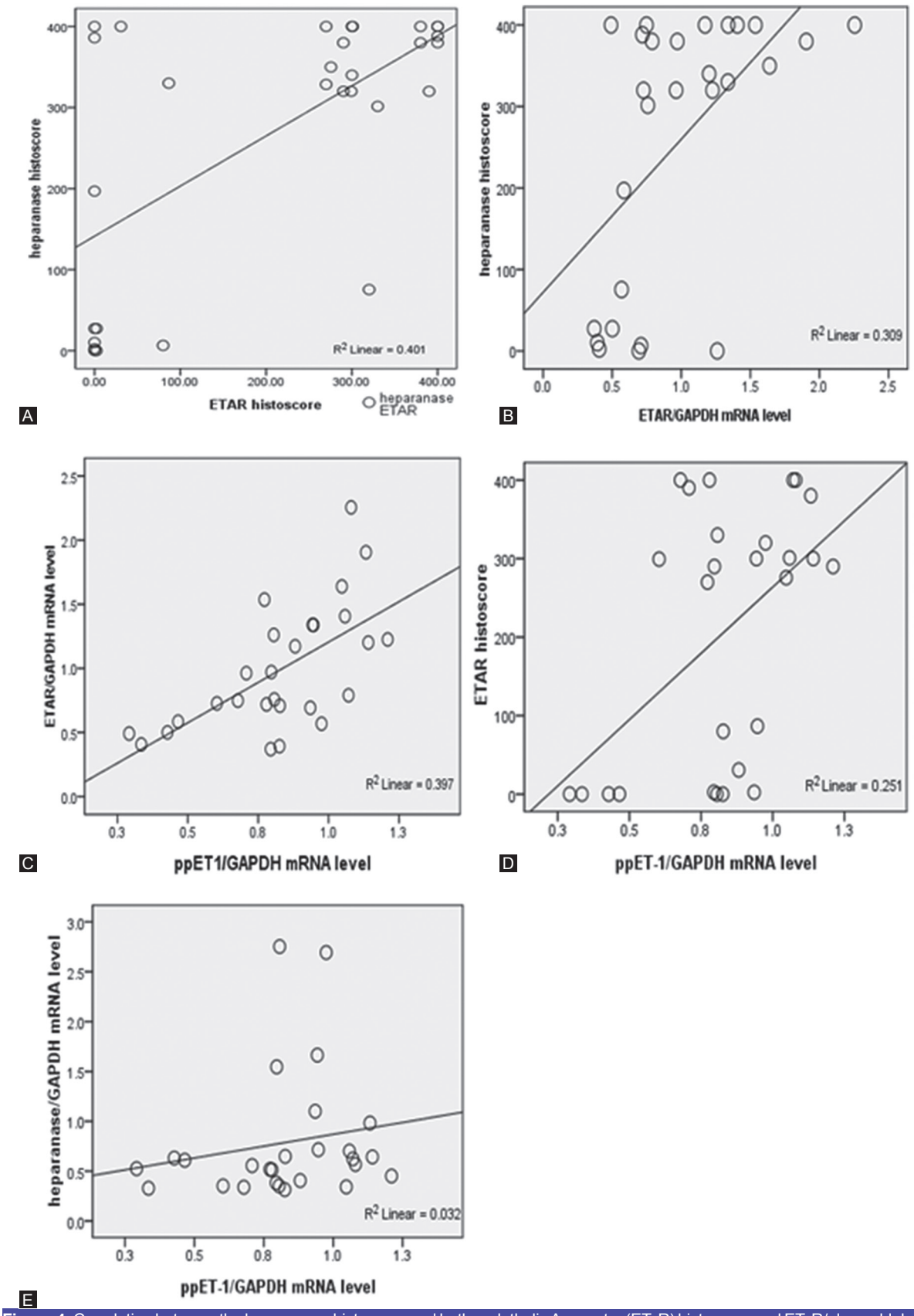

Figure 4: Correlation between the heparanase histoscore and both endothelin A receptor (ET_AR) histoscore and ET_AR/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level (A and B). In addition, correlation between preproET-1(ppET-1)/GAPDH mRNA and ET_AR/GAPDH mRNA levels (C), ppET-1/GAPDH mRNA level and ET_AR histoscore (D), and ppET-1/GAPDH mRNA and heparanase/GAPDH mRNA levels (E).

axis. Moreover, the increased ET-1/ET_AR was associated with higher proliferation in this study. Furthermore, ET_AR has been also known as one of the genes highly expressed in post-chemotherapy samples compared to untreated primary tumors. Combined treatment of ET_AR antagonist and cytotoxic drugs such as paclitaxel or with molecular inhibitors such as gefitinib displays evident inhibition on tumor growth.[29] Elevated ET-1 levels are correlated with increased VEGF and transactivation of the epidermal growth factor receptor (EGFR).

The current study illustrated that the heparanase immunohistochemical histoscore was correlated with the ET_AR histoscore and the ET_AR mRNA level (figures 4A and 4B). Both heparanase and the ET_AR play roles in metastasis and tumor growth with particular mechanisms which have previously been explained. We also revealed a positive correlation between ppET-1 expression and heparanase expression (figure 4). The possible crosstalk between these 2 molecules is a scintillating issue to be investigated. A preceding study elucidated the crosstalk between the EGFR and the ET_AR. In this study, heparanase and ET_AR overexpression was also correlated with the proliferation index, expressed as the Ki-67 percentage (r=0.381, P=0.038 [n=30] and r=0.477, P=0.008 [n=30], respectively). Several serial immunostainings have revealed positive signals for heparanase and the ET_AR in the same tumor areas (figures 1P and 1Q). These areas have also shown higher positive results for Ki-67 staining. This could indicate a possible interaction between ET-1/ET_AR and heparanase in EOC. This interaction might play a role in the proliferation of EOC. Recently, another study explained the presence of an interaction between ET-1 and heparanase in proteinuria condition in chronic kidney diseases.[30] ET-1 might promote proteinuria through heparanase modulation and glycocalyx effacement.[31] Furthermore, in neoplasm condition, this condition may also augment the metastatic potential of EOC cells and trigger different pathways. An interaction between ET_AR and β-catenin also has been known in EOC. β-Catenin is an ET_AR/EGFR downstream pathway in the invasive manners of EOC cells and co-targeting the ET_AR and the EGFR may serve as a therapeutic potential.[32] Based on our study, it is still far away to conclude the crosstalk between ET-1/ET_AR and heparanase before further research is undertaken. Double immunofluorescence or in vitro assay with the ET_AR or the heparanase inhibitor in ovarian cancer cells can provide an important result to confirm possible crosstalk. Further in vitro studies to asses our hypothesis are needed using tumor cell lines with both ET-1 and heparanase inhibition. Combining this study with the history of the patients such as survival rates or prognoses may yield important data as well.

Conclusion

In conclusion, the correlation between heparanase and ET-1/ET_AR in the present study marks the possible enhancing antimetastatic activity of the ET_AR and heparanase inhibitors. It is suggested that those systems might perform crosstalk in the mechanism underlying EOC. The inhibition agents of each system might consider the contribution of the other system in the experiment or therapeutic approach in a clinical setting.

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Conflict of Interest: None declared.

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