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Immuno-reactivity of Engrailed (EN)-2 in Epithelial Ovarian Neoplasms

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Abstract

Background: Ovarian neoplasm refers to any tumor that originates primarily in the ovary. Epithelial ovarian neoplasms are the most common type of ovarian tumor and are classified based on their histogenetic and immunophenotypic features. However, treatment modalities based on this classification have shown limited improvements in patient outcomes. Engrailed (EN)-2 was first identified as a regulator of embryonic neural development and later found to be expressed in various non-neural epithelial tumors.

Aim: This study aimed to investigate the potential role of the EN-2 gene in epithelial ovarian tumors by comparing the expression of EN-2 protein in functional ovarian lesions and ovarian epithelial neoplasms. It also aimed to correlate EN-2 expression levels with factors such as patient age, tumor size, and carcinoma grade.

Methods: Archived formalin-fixed, paraffin-embedded ovarian tissue blocks from 47 women who underwent oophorectomies for various gynecological indications were examined. Two tissue sections were obtained from each block: one was stained with Hematoxylin and Eosin, and the other was immunohistochemically stained with an anti-human EN-2 antibody.

Results: EN-2 was overexpressed in malignant ovarian neoplasms compared to benign tumors and non-neoplastic lesions ($p<0.0001$). The expression of EN-2 was significantly associated with older patient age ($p=0.003$) and larger tumor size ($p<0.0001$).

Conclusion: EN-2 is aberrantly expressed in epithelial ovarian neoplasms, with increased expression observed in malignant tumors and larger tumor sizes, suggesting its potential as a biomarker for tumor progression in ovarian carcinoma.

Keywords: Engrailed-2, Epithelial ovarian neoplasms, Ovarian cancer

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Introduction

Ovarian neoplasm is a broad term that refers to all tumors arising from the ovary, regardless of the cell of origin. Ovarian neoplasms can be classified into four main categories, each encompassing a variety of neoplasms with different neoplastic potentials. These four categories are epithelial ovarian, germ cell, sex cord, and stromal neoplasms [1]. Epithelial ovarian neoplasms (EONs) are the most common accounting for approximately 80-85% of all ovarian neoplasms [2].

Epithelial ovarian neoplasms were once thought to originate from the overlying mesothelial cells; however, it has since been established that each subtype of EON arises from a distinct cell of origin [3]. Based on the cell of origin, EONs are classified into serous, mucinous, seromucinous, endometrioid, clear cell, and transitional (Brenner) tumors. Each of these subtypes includes benign ovarian tumors, borderline ovarian neoplasms, and epithelial ovarian carcinoma (EOC), which are distinguished by the presence or absence of

cytological atypia and stromal invasion [1].

Ovarian cancer accounts for approximately 3% of all gynecological malignancies, with a global annual incidence of around 220,000 new cases. Despite this, ovarian cancer is responsible for significant morbidity and mortality, primarily due to the lack of early detection and screening methods, as the symptoms in the early stages are often non-specific [4]. In Egypt, ovarian cancer ranks as the 12th most frequently diagnosed cancer among women. In 2020, it accounted for 4.1% of newly diagnosed malignant tumors in Egyptian women [5].

Risk factors for ovarian cancer can be classified into non-modifiable and modifiable categories. A family history of ovarian cancer is the most important non-modifiable risk factor [6]. Modifiable risk factors include hormone replacement therapy and cigarette smoking [7, 8].

Epithelial ovarian carcinoma accounts for more than 90% of all ovarian cancers. These tumors are histologically, immunophenotypically, and genetically heterogeneous. Despite this diversity, all EOCs share certain characteristics, including rapid tumor growth, aggressive behavior, late onset at diagnosis, and poor prognosis. For a long time, the classification of EOCs was based solely on tumor histogenesis and invasiveness. However, this classification has not led to the development of new, effective treatment modalities, and patient outcomes have shown little improvement. Consequently, there is a pressing need to establish new treatment strategies, primarily based on the tumor's genetic signature [9].

The identification of novel biological markers that show increased expression in EOCs compared to normal ovarian tissues and benign ovarian neoplasms could offer significant diagnostic and/or prognostic value in managing EOC patients.

Engrailed (EN)-2 is a member of the homeobox-containing engrailed gene family. Located on chromosome 7 in humans, EN-2 is expressed during early development, playing a role in the formation of cerebellar fissures and folia, as well as in neural connections and neuronal morphogenesis [10]. EN-2 protein has also been detected in non-neural tissues, such as the prostate and renal tubules. Several human neoplasms of epithelial origin, includingprostate, urothelial, and esophageal cancers, express EN-2 [11-14]. High levels of EN-2 expression in these tumors are associated with worse prognosis. Studying EN-2 expression in various human epithelial carcinomas and understanding its role in these tumors could offer promising diagnostic, prognostic, and/or therapeutic benefits for future anticancer treatment protocols.

The aim of this study was to investigate the role of the EN-2 gene in epithelial ovarian tumors by comparing the expression of EN-2 protein in normal ovarian stroma, functional ovarian lesions, benign epithelial ovarian tumors, and malignant EONs. Additionally, the study sought to correlate varying levels of EN-2 expression in ovarian lesions with clinical and pathological parameters, such as patient age, tumor size, and the grade of ovarian carcinoma.

Methods

This cross-sectional observational study was conducted using archived formalin-fixed, paraffin-embedded ovarian tissue blocks from 47 women who were admitted to the Obstetrics and Gynecology Department at Sohag University Hospital. These women underwent total hysterectomies with unilateral or bilateral salpingo-oophorectomies, or ovarian cystectomies for various benign or malignant gynecological indications. The specimens included all eligible cases from 1-January-2015 to 31-December-2021.

Clinical data and specimen collection

Tissue blocks and clinical data were retrieved from the archives of the Pathology Laboratory at Sohag University Hospital. The inclusion criteria encompassed all types of primary EONs (benign, borderline, and malignant), as well as functional, non-neoplastic ovarian cysts. Only cases with tissue blocks containing sufficient material for immunohistochemical analysis and with available clinical data were included. Cases of non-EONs, tissue blocks that were damaged or contained insufficient material, cases with incomplete clinical data, or those who had received pre-operative chemotherapy were excluded from the study.

From each formalin-fixed, paraffin-embedded tissue block, two 4 µm-thick tissue sections were obtained. One section was stained with Hematoxylin and Eosin (H&E) to confirm the

histogenesis of the ovarian lesions and categorize them as either neoplastic or non-neoplastic. Neoplastic epithelial ovarian lesions were further classified into benign, borderline, and malignant based on stromal invasion and cellular atypia. The second tissue section was stained immunohistochemically with an anti-human EN-2 antibody.

Immunohistochemical staining of Engrailed-2

The Avidin-Biotin Complex (ABC) technique was used for immunohistochemical staining. Tissue sections mounted on slides were first immersed in xylene to remove paraffin wax, then passed through a series of descending concentrations of ethyl alcohol to gradually rehydrate the tissues. Endogenous peroxidase activity was blocked by treating the sections with 3% hydrogen peroxide (H2O2) for 20 minutes. To unmask cell surface epitopes, the sections were boiled in sodium citrate buffer (pH 6) in a microwave for 10 minutes at mid-high power.

The sections were then incubated overnight with anti-engrailed (EN) homeobox-2 rabbit polyclonal primary antibody (diluted 1:100; Catalog Number: NBP3-10900, NOVUS). Afterward, they were incubated with goat serum as a secondary antibody, followed by treatment with streptavidin-biotin for 10 minutes. The reaction was visualized by immersing the sections in diaminobenzidine (DAB) for 15 minutes at room temperature (ScyTek, P.O. Box 3286, Logan, Utah 84323, USA). Harris' Hematoxylin was used for nuclear counterstaining.

The sections were dehydrated by passing through ascending grades of ethyl alcohol and cleared in xylene. Each staining run included both positive and negative control sections to ensure accurate staining and specificity of the positive signals. The positive control was human renal tissue, as recommended in the data sheet. The negative control was achieved by substituting the primary antibody with phosphate-buffered saline.

Evaluation of Engrailed-2 immunostaining

Engrailed-2 immunoreactivity was observed as brownish, granular, and nuclear staining (Figure 1). The immunostained ovarian tissue sections were evaluated using a well-established immunoreactivity scoring system (IRS). The intensity of EN-2 staining was scored as follows: 0 (negative staining), 1 (weak expression), 2 (moderate expression), and 3 (high expression). The percentage of EN-2-positive cells was categorized as: 0 (0%), 1 (<10%), 2 (10–50%), 3 (51–80%), and 4 (>80% positive cells). The final IRS score was determined by multiplying the intensity score by the percentage of positive cells. The resulting IRS was classified into four categories: negative (0), low (1–4), moderate (6, 8), and high staining (9, 12) [15].

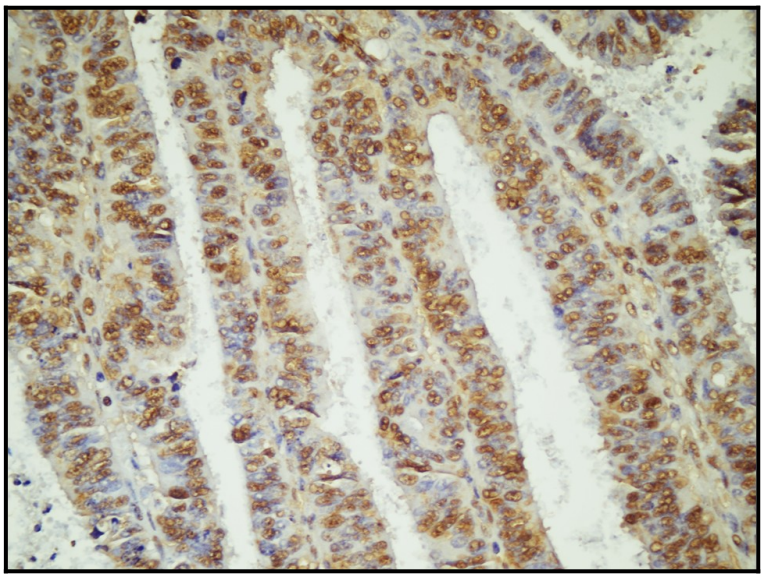

Figure 1: Immune-expression of engrailed-2 in high grade epithelial ovarian carcinoma showed nuclear staining, magnification: x200.

Statistical analysis

The data were analyzed using SPSS Statistical Software version 21.0 (SPSS Inc., Chicago, IL, USA). Descriptive analysis was conducted, and the normality of continuous variables was assessed using the Shapiro-Wilk test. Quantitative data were expressed as mean ± standard deviation (SD) for normally distributed data, while median and range or interquartile range (IQR) were used for non-parametric data. Qualitative data were reported as frequencies and percentages. Categorical data were analyzed using the Chi-square (X^2) test. A one-way ANOVA was used to compare the means of more than two groups. A p-value of < 0.05 was considered statistically significant.

Results

Patients' characteristics

The current study included 47 women, with ages ranging from 15 to 71 years (mean ± SD: 39.6 ± 15 years). The enrolled cases were categorized as follows: 28 cases (59.6%) of benign EONs, 14 cases (29.8%) of primary ovarian carcinoma, and 5 cases (10.6%) of functional (luteal) ovarian cysts. No cases of borderline EONs were included during the selected study period (Table 1).

The benign EONs comprised 21 cases of serous cystadenoma / cystadenofibroma and 7 cases of mucinous cystadenoma. Among the primary ovarian carcinoma cases, there were 3 cases of low-grade serous ovarian carcinoma and 11 cases of high-grade serous ovarian carcinoma (Figure 2). All studied cases of primary ovarian carcinoma were classified as ≥ FIGO stage 2.

Regarding the size of the excised ovarian lesions, 20 cases (42.6%) were less than 8 cm. These included 5 functional (luteal) cysts, 14 benign ovarian cysts, and only 1 case of primary EOC. The remaining 27 cases (57.4%) had lesions exceeding 8 cm, comprising 14 benign ovarian cysts and 13 cases of primary EOC (Table 2).

Immunohistochemical detection of Engrailed-2

Engrailed-2 expression was detected in 21 out of 47 cases studied (44.7%). In these cases, EN-2 was scored using a semiquantitative immunoreactive score of 15. Low EN-2 expression was observed in 7 cases, while moderate and intense EN-2 expressions were found in 1 and 13 cases, respectively (Figure 3).

None of the tissue sections from the enrolled cases of luteal cysts, 18 cases of serous cystadenoma/cystadenofibroma, and 3 cases of mucinous cystadenoma showed EN-2 expression. Additionally, the ovarian stroma in all examined immunostained sections did not exhibit EN-2 expression (Figure 4).

Table 1: Clinical and pathological criteria of 47 ovarian lesion specimens

Variable		**Description**	
Patient's age (years)		Mean (SD)	39.6 (15)
		Range	15 - 71
Size of the lesion (cm)		Mean (SD)	8.9 (4)
		Range	2 - 17
	< 3	*n*(%)	2 (4.3)
	3 - < 8	*n*(%)	18 (38.3)
	8 - < 10	*n*(%)	6 (12.8)
	≥ 10	*n*(%)	21 (44.7)
Phenotype of the lesion	Luteal cyst	*n*(%)	5 (10.6)
	Serous cystadenoma/ cystadenofibroma	*n*(%)	21 (44.7)
	Mucinous cystadenoma	*n*(%)	7 (14.9)
	Primary epithelial ovarian carcinoma	*n*(%)	14 (29.8)
	Low grade serous ovarian carcinoma	*n*(%)	3 (6.4)
	High grade serous ovarian carcinoma	*n*(%)	11 (23.4)

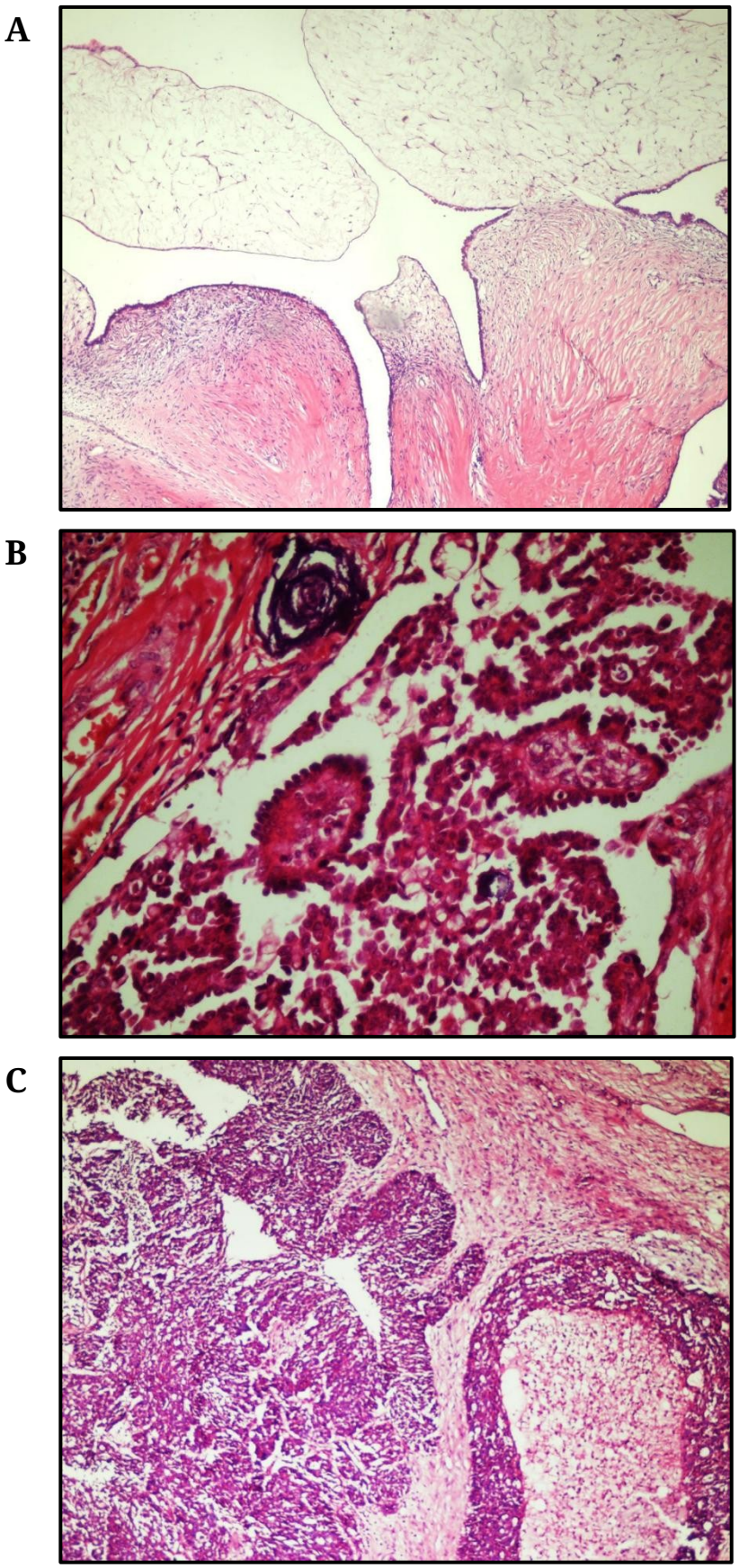

Figure 2: **Hematoxylin and Eosin-stained sections** for; **A:** Ovarian serous cystadenoma/ cystadenofibroma X100, **B:** Low grade serous ovarian carcinoma showing low grade atypical cytological features and psammoma bodies X200, **C:** High grade serous ovarian carcinoma showing high grade cytological atypia X100.

Table 2: Patient's age and lesion size according to the lesion phenotype

Variable			Luteal cysts (*n*=5)	Benign ovarian cysts (*n*=28)	Epithelial ovarian carcinoma (*n*=14)
Patient's age (years)		Mean (SD)	28.8 (9)	51.9 (18.5)	51.4 (10.8)
		Range	19 - 42	15 - 70	33 - 71
Size of the lesion (cm)	< 8	*n*(%)	5 (100)	14 (50)	1 (7.1)
	≥ 8	*n*(%)	0	14 (50)	13 (92.9)

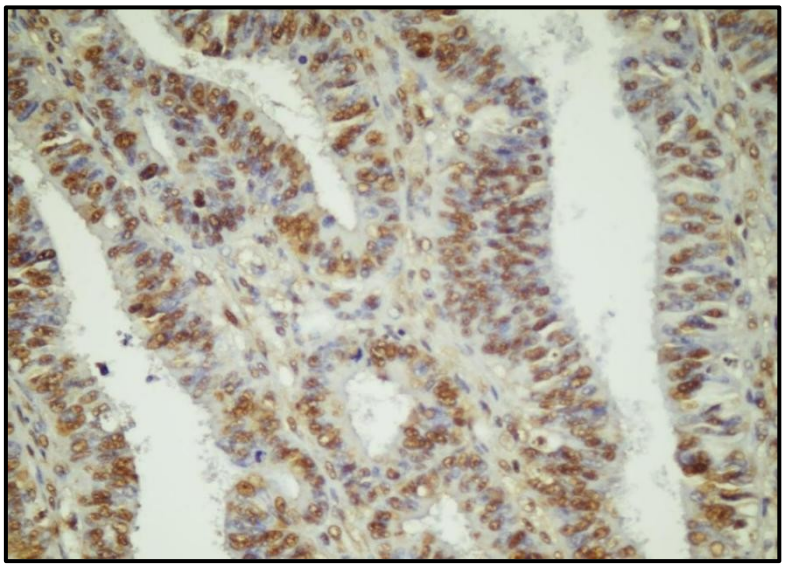

Figure3: Immune-expression of Engrailed-2 in high grade serous ovarian carcinoma, X400

Different levels of EN-2 expression were compared with the tumor characteristics of the studied cases (Table 3). Regarding the age of the studied ovarian neoplasm cases, EN-2 was found to be over-expressed in older age groups ($p = 0.003$). A statistically significant relationship was observed between the size of the excised ovarian tumors and the intensity of EN-2 expression. All ovarian tumors smaller than 8 cm either did not express EN-2 or showed low levels of expression. However, half of the ovarian neoplasm cases with a largest diameter ≥ 8 cm exhibited moderate to intense EN-2 expression in their tissue sections ($p < 0.0001$).

A statistically significant relationship was found between the intensity of EN-2 expression and tumor histogenesis. All the examined tumor tissue sections that did not express EN-2 or showed low levels of expression were from benign neoplastic ovarian lesions (serous cystadenoma / cystadenofibroma and mucinous cystadenoma). In contrast, all the tissue sections from primary EOC exhibited moderate to high EN-2 expression ($p < 0.0001$).

When comparing EN-2 expression levels in primary EOC to tumor grades, no significant relationship was detected ($p = 0.59$).

Discussion

Ovarian cancer is a broad term used to describe any malignant neoplasm arising primarily from ovarian tissue, regardless of the cell of origin within the affected ovary [1]. One of the main challenges in managing ovarian cancer is the late stage at diagnosis, compounded by the lack of specific and reliable screening tools, along with progressive genomic instability. This instability contributes to the variable patient outcomes, even among those within the same category [4].

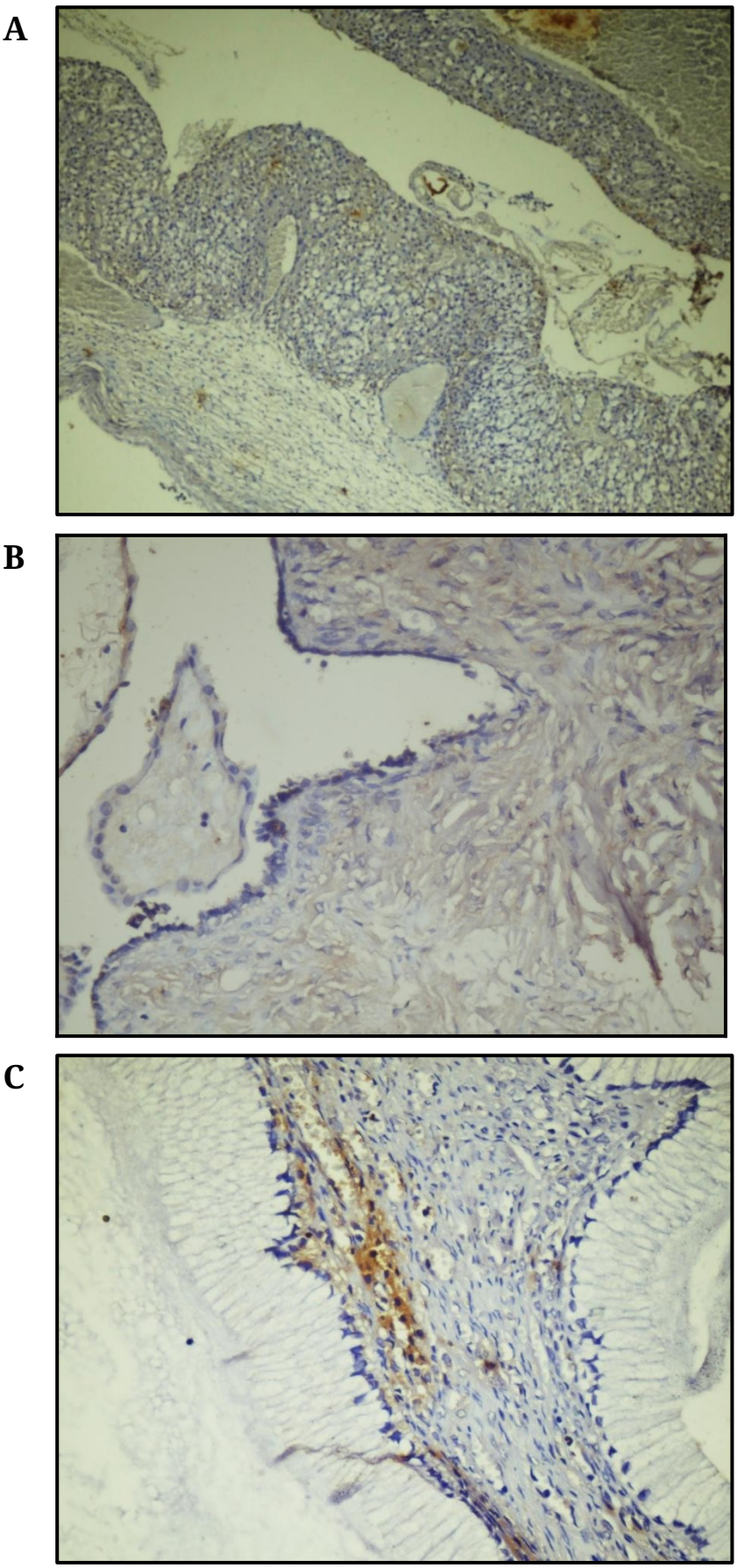

Figure 4: **Negative expression of Engrailed-2** in **A:** luteal cyst X100, **B:** serous cystadenoma/ cystadenofibroma X200, and **C:** mucinous cystadenoma X200.

Table 3: Clinicopathological characteristics in relation to Engrailed-2 expression

Variable	*n*	Engrailed-2 expression				P value
		Negative	Low positive	Moderate positive	Strong positive	
		Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
Age (years)	47	34.6 (13.8)	34.6 (14.8)	46 (0)	51.8 (11)	0.003
		n(%)	*n*(%)	*n*(%)	*n*(%)	
Size of the lesion (cm)						
< 8	19	16 (84.2)	3 (15.8)	0 (0)	0 (0)	0.012
≥ 8	28	10 (35.7)	4 (14.3)	1 (3.6)	13 (46.4)	
Histeogenesis of the lesion						
Luteal cyst	5	5 (100)	0 (0)	0 (0)	0 (0)	< 0.0001
Serous cystadenoma/ cystadenofibroma	21	18 (85.7)	3 (14.3)	0 (0)	0 (0)	
Mucinous cystadenoma	7	3 (42.9)	4 (57.1)	0 (0)	0 (0)	
Low grade serous ovarian carcinoma	3	0 (0)	0 (0)	0 (0)	3 (100)	
High grade serous ovarian neoplasm	11	0 (0)	0 (0)	1 (9.1)	10 (90.9)	
Tumor grade (*n*=14)						
Low	3	0 (0)	0 (0)	0 (0)	3 (100)	0.59
High	11	0 (0)	0 (0)	1 (9.1)	10 (90.9)	

Studying the biological characteristics of a given tumor to develop targeted therapies that specifically attack the oncogenes driving tumor growth may lead to better results, improved patient outcomes, reduced metastasis risk, and delayed recurrence.

Engrailed-2 is a homeodomain-containing transcription factor. During intrauterine development, EN-2 plays a role in neural development and neural connections [10]. EN-2 has also been found to be aberrantly expressed in prostatic adenocarcinoma and urinary bladder carcinoma [11, 13]. Although the role of EN-2 in ovarian cancer has not been extensively studied, preliminary research has shown promising results regarding its involvement in the initiation and progression of ovarian cancer, making it an intriguing subject for further investigation.

In the current study, we evaluated the immunohistochemical expression of EN-2 in various ovarian neoplastic and non-neoplastic lesions and correlated different levels of EN-2 expression with various clinical and pathological parameters.

Regarding the relationship between EN-2 expression and the sizes of excised ovarian lesions, we observed a direct correlation between lesion size and EN-2 expression. This finding is consistent with that of Li et al., who evaluated the role of EN-2 in colorectal carcinoma. They found that EN-2 expression increased with larger tumor sizes, suggesting that EN-2 may promote the proliferation of colorectal carcinoma cells, thereby increasing tumor size [16]. Furthermore, Pandha et al. correlated urinary levels of EN-2 in patients with prostatic adenocarcinoma before and after radical prostatectomies. They found that urinary EN-2 levels were associated with tumor volumes and recommended EN-2 as a novel biomarker for the detection and follow-up of prostatic carcinoma patients [17].

Intense expression of EN-2 was detected in malignant ovarian tumors, while absent or low levels of EN-2 expression were found in functional ovarian cysts, ovarian stroma, and benign ovarian neoplasms. These findings align with those of McGrath et al., who reported that EN-2 expression was significantly lower in benign and borderline ovarian tumors compared to malignant ovarian neoplasms. They also found that EN-2 expression was highest in high-grade serous ovarian carcinoma (HGSOC) [18]. This could be explained by EN-2 acting as a transcription factor that stimulates tumor cell proliferation, resulting in the accumulation of aberrant genes that lead to higher-grade ovarian carcinoma.

The current study also found that EN-2 expression increased with age, a parameter that has not been extensively explored in previous research on EN-2 in human malignancies.

The higher expression of EN-2 in older age groups could be explained by the increased incidence of EOC in these groups, as it is characterized by cumulative and progressive genetic mutations.

Although no significant association was detected between EN-2 expression and tumor grades in the EOC cases examined, this result is of limited value due to the small number of low-grade cases (n = 3) compared to high-grade cases (n = 11), in which 90.9% of the examined sections exhibited strong EN-2 expression. Therefore, future studies should include a larger number of EOC cases.

Moreover, all the retrospective EOC cases in this study lacked sufficient data on their corresponding omental and nodal statuses. As a result, the cases were grouped into two categories: FIGO stage I and FIGO stage ≥ 2, based on the presence or absence of pelvic extension. Since all cases were FIGO stage ≥ 2, this limited the ability to correlate EN-2 expression with FIGO stage. In a study by Laven et al. on 50 retrospective cases of early-stage EOC (FIGO stages I and IIa), several challenges to accurate surgical staging of ovarian cancer were identified, including non-representative omental biopsies and insufficient regional lymph node excision [19].

Conclusion

In conclusion, the current study suggests that EN-2 expression tends to be higher in a subset of EON patients with more advanced disease. As an important transcription factor, targeting EN-2 through future immunotherapeutic approaches could potentially improve the management of epithelial ovarian cancers. Further research, particularly with larger sample sizes and more comprehensive staging, is needed to better understand EN-2's role in ovarian cancer progression and its potential as a therapeutic target.

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Authors' contribution
Conception & Design: Hassab El-Naby NED, Abdelkareem AO, Abdelall AH, Mohammed MH, ; Acquisition, analysis, or interpretation of data: Awaga HA, Abdelall AH, Mohammed MH, Hassab El-Naby NED; Drafting / revising the manuscript: Mohammed MH, Abdelkareem AO, Awaga HA; Approval of the final version of the manuscript: All authors; Agreement to be accountable for all aspects of the work: All authors.

Conflict of interest
The authors declare that they have no conflict of interest to disclose.

Data availability
Data supporting this study is available from the Pathology Department, Faculty of Medicine, Sohag University. Access to the data is subject to approval of the local Research Ethics Committee.

Ethical considerations
The study was approved by the Research Ethical Committee at Sohag Faculty of Medicine, Sohag University (Registration ID: Soh-Med-22-09-13).

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Study registration
The study is registered with ClinicalTrials.gov PRS (Clinical Trials.gov ID: NCT05553067).

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