RESEARCH ARTICLE

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Prognostic Outcome of Mesenchymal Transition Biomarkers in Correlation with EGFR Expression in Epithelial Ovarian Carcinoma Patients

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Abstract

Background: CD44 is an epithelial-mesenchymal transition (EMT) surface receptor that regulates the interactivity between the cells and the extracellular matrix, thereby promoting cell migration. The epidermal growth factor receptor (EGFR) family is a trans-membrane kinase-related protein. It regulates cell adhesion proteins, which may promote cell proliferation and invasiveness. Mesenchymal epithelial transition (MET) is another EMT receptor that stimulates cell proliferation, invasion, survival, and angiogenesis. This study aimed to evaluate the prognostic impact of CD44, EGFR expressions, and MET gene amplification in epithelial ovarian cancer (EOC). **Methods:** This is a retrospective cohort study, including 85 cases of EOC. CD44 and EGFR expressions were evaluated in both epithelial and stromal cells by immunohistochemistry. Tumor cells also underwent a cytogenetic analysis using fluorescent in situ hybridization (FISH) to detect MET gene amplification. **Results:** High CD44 expression in tumors was significantly associated with serous subtypes (P=0.001), peritoneal deposits (P=0.002), and advanced stage (P=0.002). EGFR high tumor expression demonstrated a significant association with lymph node metastasis (P=0.038) and the advanced stage of EOC (P=0.016). Increased copy number of the MET gene was significantly associated with partial therapy response (P=0.030). CD44 and EGFR tumor high expression was associated with poor overall survival (OS). In addition, MET gene gain in tumors was associated with a shorter OS (P=0.000). **Conclusion:** EMT biomarkers (CD44 and MET) and EGFR expression in EOC are independent prognostic factors for OS. MET gene increase copy number was detected in cases of serous neoplasm and associated with poor survival and minimal therapy response.

Keywords: Epithelial ovarian cancer- CD44- EGFR- MET- Survival

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Introduction

Ovarian cancer (OC) is a lethal cancer of the female genital system. According to the American Cancer Society, OC was assorted as the fifth cancer-related death among female genital cancers. Furthermore, about 19,880 cases of OC were newly diagnosed in the United States in 2022, and approximately 12,810 cases have died from OC (CTPbG, 2022). EOC represents the most frequent type of OC in adult women. Unfortunately, the EOC diagnosis was made at a late disease stage, which significantly renders the poor outcome of this malignancy (Gadducci et al., 2019).

EOCs were recently classified as types 1 and 2. Low-grade serous carcinomas, endometrioid, mucinous, and clear compromise type 1 EOC, while type 2 includes high-grade serous and transitional cell carcinoma (Kurman and Shih Ie, 2016). Type 2 EOCs are characterized by frequently advanced disease stages at diagnosis and harbored TP53 mutations, either a defect in the recombinant DNA repair pathway or CCNE1 gene amplification. Moreover, other unusual mutations may present as mismatched DNA repair genes, phosphatidylinositol 3-kinase (PI3K), K-Ras/B-Raf, and Wnt pathways (Shih et al., 2021).

EMT is an active process that regulates the biological characteristics of malignancies, especially drug resistance. CD44 is one of the EMT cell surface receptors that function as a co-receptor to receptor tyrosine kinases (RTK), such as mesenchymal-epithelial transition (MET) and the Epidermal Growth Factor (EGF) family. CD44 settles down specific RTK complexes, such as vascular endothelial growth factor receptors, which under pathological conditions promote angiogenesis with subsequently tumor progression and metastasis

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(Martincuks et al., 2020).

The epidermal growth factor receptor (EGFR) family is a transmembrane kinase-related protein. It has two domains, an extracellular ligand binding, and an intracellular tyrosine kinase domain. EGFR is activated through ligand binding to the extracellular side, leading to the receptor's homodimerization or heterodimerization. In turn, it induces the activation of intrinsic tyrosine kinase and finally stimulates several signaling pathways. These pathways consider key regulators for cancer behaviors, including cell proliferation, adhesion, invasiveness, angiogenesis, and apoptosis resistance. Several studies noted that EGFR increased expression is detected in EOC and is significantly related to poor prognosis and chemotherapy resistance (Zheng et al., 2017a).

MET is another EMT-RTK that can promote critical cellular activities, including cell proliferation, invasion, survival, and angiogenesis. MET overexpression is found in 7% to 27% of OCs and is associated with tumor progression and unfavorable outcomes (Kim et al., 2016).

Some studies showed that the expression of other members of the HER family (e.g., the EGFRvIII), the co-expression of other heterologous growth factor receptors (e.g., c-MET, IGF-1R), and the presence of cancer stem cells were suggested as potential mechanisms of resistance to therapy with the HER inhibitors and cytotoxic drugs (Puvanenthiran et al., 2018).

This study aims to evaluate CD44 and EGFR protein expressions using immunohistochemistry (IHC) and MET gene amplification via fluorescent in situ hybridization technique (FISH) as prognostic biomarkers in various types of EOC and their association with tumor response.

Materials and Methods

This study is a retrospective cohort conducted on patients with EOC. Eighty-five formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected. The study was approved by the Ethical Committee of the SECI -IRB, IORG0006563, Approval no: 472. Inclusion Criteria were: (1) Patients diagnosed with EOC by pathological assessment. (2) Patients who had complete record files. Exclusion Criteria were: (1) Patients had a history of benign or malignant tumors other than EOC. (2) Patient with mixed EOC and another ovarian neoplasm (e.g., germ cell tumor or sex cord-stromal neoplasm). (3) Metastatic carcinoma to the ovary.

Three expert pathologists did a review of H & E slides for histopathological parameters, including tumor laterality, tumor size, histological type, FIGO (The International Federation of Gynecology and Obstetrics) grade, FIGO stage, evidence of necrosis, lymphovascular tumor invasion (LVI), tumor regression grade in cases received neoadjuvant therapy, ovarian capsule integrity, peritoneal deposit, and lymph nodes metastasis (LNM). Clinical data were collected from the patient's archived records, such as patient age, laterality, tumor size, serum level of CA 125, history of neoadjuvant therapy, and survival data. Survival outcomes were defined as follows: overall survival (OS), time from the date of treatment initiation to the date of death from all causes; Progression-free survival (PFS) was defined as the time interval from the date of treatment initiation to the date of disease progression or, the date of death.

Patients received first line neoadjuvant chemotherapy therapy (Taxol and Carboplatin), then pathological assessment of therapy response was done using evaluation of tumor cellularity as the following: score 1, represents no or minimal responese, score 2, encompasses partial response, while score 3, includes patients with complete pathologic response. Patient transformed to second line therapy, if progression occurred.

Immunohistochemistry

The selected FFPE blocks of EOC were sectioned at 4 μm thickness on positively charged coated slides and put in a microwave for 60 minutes for tissue adhesion.

For CD44 staining: Primary antibody CD44 (clone DF1485, code M7082 monoclonal mouse anti-human CD44, concentrated, Agilent company, Dako, Denmark) used freshly prepared in each run at 1:25 concentration incubated at room temperature for 60 minutes, then wash using PBS. Secondary antibody HRP was used for 20 minutes, then washed with PBS.

For EGFR staining: Antigen retrieval step was performed by enzymatic digestion technique using trypsin enzyme prepared. Tissue put 3-5 min in prepared trypsin enzyme. Then wash by PBS buffer was done. Primary antibody EGFR (monoclonal mouse anti-human antibody, Clone E30, Code M7239, concentrated, Agilent company, Dako, Denmark) was diluted in 1:25 and tissue incubated for 24 h- overnight- at 4c temperature, then washed by PBS. Secondary antibody (HRP) was put for 20 min, then washed with PBS.

Evaluation of CD44 and EGFR IHC expression

CD44 and EGFR expression was evaluated in the tumor's epithelial and stromal cells. Scoring of CD44 and EGFR expressions in tumor cells was done using a semiquantitative immunoreactivity score. The staining intensity score was multiplied by the staining percentage to obtain this score. The intensity score was assigned a value of 0, 1, 2, or 3 to represent weak, moderate, or intense staining. The staining percentage score was graded based on the proportion of positive tumor cells as follows: 0 to 5%, 6 to 25%, 26 to 50%, 51 to 75%, and more than 75% as scores 0, 1, 2, 3, and 4, respectively. The immunoreactivity score was calculated as follows: 0 to 2 deemed negative, 3 to 5 weakly positive, 6 to 8 moderately positive, and 9 to 12 intense positive We considered strong and moderate positive cases as high expression tumor for both CD44 and EGFR. While weak positive and negative cases considered as low expressing tumors (Vos et al., 2016; Cîrstea et al., 2017; Zheng et al., 2017b; Kar et al., 2021). Stromal expression was considered positive if any stromal staining was detected.

Fluorescence in situ hybridization study

MET gene amplification probe 7q31.2 was assessed using a dual-color FISH probe (Cat no. LPS004, CytoCell) consisting of a 278kb red probe spanning the MET gene. The centromeric probe was green and acts as a

control for chromosome 7, targeting MET and CEP7 on formalin-fixed paraffin-embedded tissue sections from tumor specimens. The procedure was done using a tissue pretreatment Kit [Cytocell Aquaris (Cat no. LPS 100)]. First, we started with deparaffinization of the FFPE slides by immersion in xylene for 15 minutes. Then the deparaffinized slides were immersed in absolute ethanol for 10 minutes at room temperature for dehydration. After that, the slides were put in a jar containing pretreatment reagent in the water bath at 98-100°C for 20 minutes. Then the slides were washed with distilled water twice for 3 minutes each at room temperature. By the end of the pretreatment of the tissue, we covered it with an enzyme reagent for 10 - 20 minutes at 37°C hotplates, and then the tissue was washed and dehydrated using ascending alcohol solutions 70%, 85%, 100% each for 2 minutes. Then we started the hybridization procedure by applying five µL of the probe to the slides and overlayed with a glass coverslip which was sealed with rubber glue. The slides were put in a hybridizer which was set at 80°C for 5 minutes for denaturation and then at 37°C for 12-18 hours for hybridization. Then the slides were washed using solutions and were left in dark, dry air. After that, five µL of DAPI counterstain were applied. We analyzed the prepared slides under an oil immersion objective (x63) with a fluorescence microscope (MetaSystems GmbH, Altlussheim, Germany) equipped with appropriate filters and a charge-coupled device camera using FISH imaging with the capturing software Metafer 5 (a Metafer slide scanning system [Metasystems]).

Assessment of MET gene alterations using FISH technique

Tumor was considered MET gene amplified; if>10% tumor cells showed MET-copy number (CN) ≥ 5. If MET-CN was ranged from ≥ 4 to < 5 in ≥10% of cells represented MET–copy number gain, while the presence of MET-CN < 4, the tumor considered negative (Fang et al., 2018).

Statistical analysis

All statistical calculations were done using SPSS (statistical package for the social science; SPSS Inc., Chicago, IL, USA) version 22. Quantitative data were statistically described in terms of mean ± SD and median (range) when not normally distributed. Qualitative data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages) when appropriate. A comparison of quantitative variables was done using student t test for normally distributed data and Mann Whitney U test for non-normally distributed data. For comparing categorical data, Chi square (χ^2) test was performed. Fisher Exact test was used instead when the expected frequency is less than 5. Correlation between various variables was done using Spearman rho correlation test. Kaplan-Meier's method with log rank test was used for overall and progression free survival analysis. Hazard ratio (HR) with95% Confidence Interval (CI) and COX regression analysis was calculated to determine significant factors associated with mortality. P-value is always a two-tailed set significant at 0.05 level. III.

Table 1. Clinicopathological Characteristics o of the Studied Participants (n=85)

Variable Name	N	(%)
Age (years)		
Mean ± SD	53.86 ± 9.73	
Median (range)	55 (31 – 75)	
≤ 50	28	32.90%
> 50	57	67.10%
Laterality		
Unilateral	28	32.90%
Bilateral	57	67.10%
Tumor size (cm)		
Mean ± SD	7.14 ± 5.02	
Median (range)	5 (2 – 20)	
Subtypes		
Serous	62	72.90%
Non serous	23	27.10%
Capsule		
Intact	54	63.50%
Infiltrated	31	36.50%
Necrosis		
No	30	35.30%
Yes	44	64.70%
LVI		
No	24	28.20%
Yes	61	71.80%
LNM		
Free	36	42.40%
Positive	35	41.20%
N/A	14	16.50%
Peritoneal		
Free	14	16.50%
Positive	62	72.90%
N/A	9	10.60%
FIGO stage		
Stage I	21	24.70%
Stage II	5	5.90%
Stage III	55	64.70%
Stage IV	4	4.70%
Grade		
Grade 1	5	5.90%
Grade 2	35	41.20%
Grade 3	45	52.90%
Therapy		
No	40	47.10%
Positive	45	52.90%
Response to therapy		
Score 3	23	51.10%
Score 2	16	35.60%
Score 1	6	13.30%

Quantitative data are presented as mean ± SD and median (range), qualitative data are presented as n (%).

Table 2. Relation between CD44 Expression and Clinicopathological Data

Variable name	Low expression (n=44)		High expression (n=41)		P value	Stromal negative (n= 31)	Stromal positive (n=54)	P value
Age (years)								0.183
Mean ± SD	54.14 ± 10.21		53.56 ± 9.29		0.787	52.00 ± 11.33	54.93 ± 8.60	
Median (range)	54.5 (31 – 75)		55 (32 – 73)			52 (31 – 75)	55.5 (32 – 70)	
≤ 50	15	34.10%	13	(31.70)	0.815	13 (41.9)	15 (27.8)	0.181
> 50	29	65.90%	28	(68.30)		18 (58.1)	39 (72.2)	
Laterality					0.487			0.391
Unilateral	16	36.40%	12	29.3)		12 (38.7)	16 (29.6)	
Bilateral	28	63.60%	29	70.7)		19 (61.3)	38 (70.4)	
Tumor size (cm)					0.326			0.807
Mean ± SD	7.89 ± 5.42		6.34 ± 4.47			7.76 ± 5.67	6.79 ± 4.61	
Median (range)	7 (2 – 20)		5 (2 – 17)			5 (2 – 20)	5 (2–20)	
Subtypes					0.001*			0.019*
Serous	25	56.80%	37	(90.20)		18 (58.1)	44 (81.5)	
Non serous	19	43.20%	4	(9.80)		13 (41.9)	10 (18.5)	
Capsule					0.000*			0.541
Intact	36	81.80%	18	(43.90)		21 (67.7)	33 (61.1)	
Infiltrated	8	18.20%	23	(56.10)		10 (32.3)	21 (38.9)	
Necrosis					0.042*			0.618
No	20	45.50%	10	(24.40)		12 (38.7)	18 (33.3)	
Yes	24	54.50%	31	(75.60)		19 (61.3)	36 (66.7)	
LVI					0.085			0.261
No	16	36.40%	8	(19.50)		11 (35.5)	13 (24.1)	
Yes	28	63.60%	33	(80.50)		20 (64.5)	41 (75.9)	
LNM					0.055			0.417
Free	21	47.70%	15	(36.60)		16 (51.6)	20 (37.0)	
Positive	13	29.50%	22	(53.70)		11 (35.5)	24 (44.4)	
N/A	10	22.70%	4	(9.80)		4 (12.9)	10 (18.5)	
Peritoneal					0.002*			0.041*
Free	12	27.30%	2	(4.90)		9 (29.0)	5 (9.3)	
Positive	25	56.80%	37	(90.20)		18 (58.1)	44 (81.5)	
N/A	7	15.90%	2	(4.90)		4 (12.9)	5 (9.3)	
FIGO stage					0.002*			0.155
Stage I	17	38.60%	4	(9.80)		11 (35.5)	10 (18.5)	
Stage II	4	9.10%	1	(2.40)		3 (9.7)	2 (3.7)	
Stage III	22	50%	33	(80.50)		16 (51.6)	39 (72.2)	
Stage IV	1	2.30%	3	(7.30)		1 (3.2)	3 (5.6)	
Grade					0.012*			0.017*
Grade 1	5	11.40%	0	0.00		4 (12.9)	1 (1.9)	
Grade 2	21	47.70%	14	(34.10)		16 (51.6)	19 (35.2)	
Grade 3	18	40.90%	27	(65.90)		11 (35.5)	34 (63.0)	
Therapy					0.006*			0.276
No	27	61.40%	13	(31.70)		17 (54.8)	23 (42.6)	
Positive	17	38.60%	28	(68.30)		14 (45.2)	31 (57.4)	
Response					0.827			0.326
Score 3	8	47.10%	15	(53.60)		8 (57.1)	15 (48.4)	
Score 2	7	41.20%	9	(32.10)		3 (21.4)	13 (41.9)	
Score 1	2	11.80%	4	(14.30)		3 (21.4)	3 (9.7)	

Quantitative data are presented as mean ± SD and median (range), qualitative data are presented as n (%). Significance defined by $p < 0.05$

Results

Our study is a retrospective study, including 85 cases of ovarian carcinoma. The patient's age ranged from 31 to 75 years, with a mean age of 53.86 ± 9.73 years. About

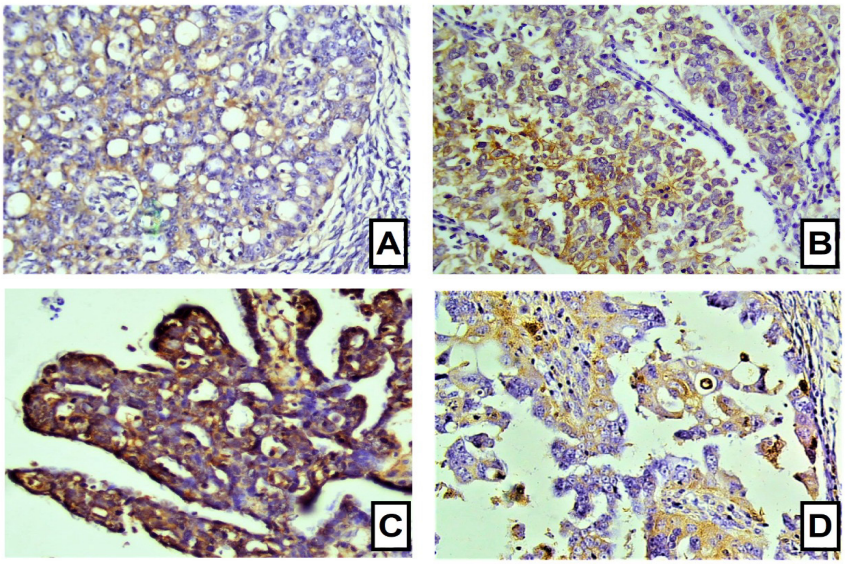

Figure 1. CD44 Expression in EOC Tumor Cells and Stroma. (A),Weak CD44 expression in 30% of tumor cells and positive in stroma (x20); (B), Moderate CD44 expression in 60% of tumor with negative stroma (x20); (C), Strong CD44 expression in tumor (x20); (D), Negative CD44 tumor expression with positive stroma (x20).

53% of cases received neoadjuvant therapy. Concerning histopathological types, serous carcinoma represents about 72% of all cases. Clinicopathological data were summarized in Table 1.

Association between CD44 protein expression and clinicopathological parameters

In our study, 47 out of 85 cases were positive for CD44 (55.3%), and 44.7% of cases were negative for CD44 staining. Of positive CD44 cases, 41 cases (48.2%) were high expressing CD44, and the rest were low positive CD44 expressing tumors (Figure 1).

CD44 tumor high expression was significantly associated with serous histopathological type (P=0.001), infiltration of the ovarian capsule (P=0.000), positive peritoneal deposits (P=0.002), advanced pathological stage (P=0.002), and FIGO grade 3 (P=0.012). The association between CD44 expression and clinicopathological parameters is illustrated in Table 2

Regarding CD44 expression in tumor-supporting stroma, CD44 was expressed in 63.3% of cases in tumor stroma (Figure 1). A significant relation was found between CD44 stromal expression and serous carcinoma subtype (P=0.019), peritoneal tumor deposits (P=0.041), and grade 3 EOC (P= 0.017).

Association between EGFR protein expression and clinicopathological parameters

This study exhibited 56 out of 85 cases (66%) EGFR-

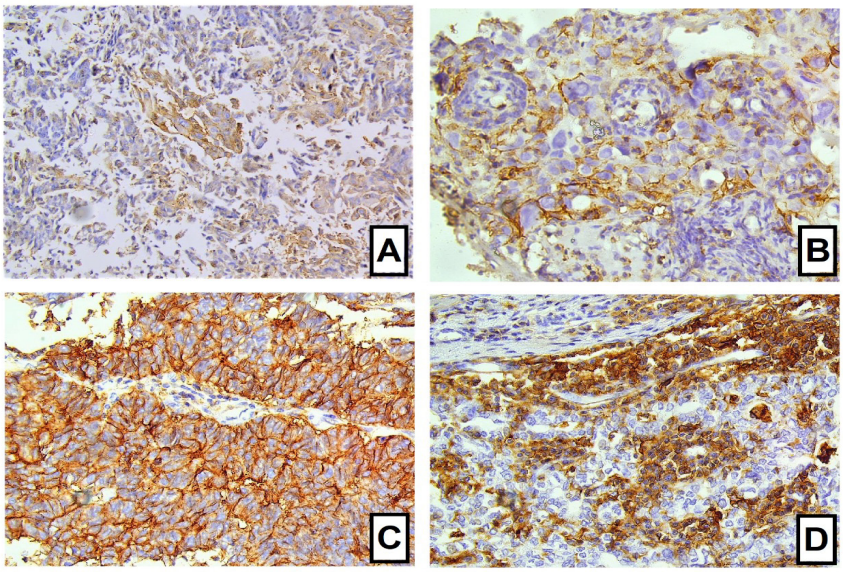

Figure 2. EGFR Expression in EOC Tumor Cells and Stroma. (A), Weak EGFR expression in 70% of tumor cells and negative in stroma (x20); (B), Moderate EGFR expression in 80% of tumor with positive stroma (x20); (C), Strong EGFR expression in tumor (x20); (D), Negative EGFR tumor expression with positive stroma (x20).

Table 3. Association between EGFR Expression and Clinicopathological Parameters

Variable name	Low expression (n=35)		High expression (n=50)		P value	Stromal negative (n=30)	Stromal positive (n=55)	P value
Age (years)								
Mean ± SD	53.60 ± 10.01		54.04 ± 9.62		0.839	54.37± 11.29	53.58±8.86	0.724
Median (range)	54 (31 – 75)		55 (32 – 73)			54.5 (32-75)	55 (31-73)	
≤ 50	12	(34.30)	16	32%	0.825	10 (33.3)	18 (32.7)	0.955
> 50	23	(65.70)	34	68%		20 (66.7)	37 (67.3)	
Laterality					0.49			0.363
Unilateral	13	(37.10)	15	30%		8 (26.7)	20 (36.4)	
Bilateral	22	(62.90)	35	70%		22 (73.3)	35 (63.6)	
Tumor size (cm)					0.044*			0.705
Mean ± SD	8.70 ± 5.80		6.05 ± 4.10			7.42 ± 5.59	6.99 ± 4.72	
Median (range)	10 (2 – 20)		5 (2 – 17)			5 (2 – 20)	5 (2 – 20)	
Subtypes					0.025*			0.047*
Serous	21	(60.00)	41	82%		18 (60.0)	44 (80.0)	
Non serous	14	(40.00)	9	18%		12 (40.0)	11 (20.0)	
Capsule					0.029*			0.978
Intact	27	(77.10)	27	54%		19 (63.3)	35 (63.6)	
Infiltrated	8	(22.90)	23	46%		11 (36.7)	20 (36.4)	
Necrosis					0.448			0.219
No	14	(40.00)	16	32%		8 (26.7)	22 (40.0)	
Yes	21	(60.00)	34	68%		22 (73.3)	33 (60.0)	
LVI					0.127			0.79
No	13	(37.10)	11	22%		9 (30.0)	15 (27.3)	
Yes	22	(62.90)	39	78%		21 (70.0)	40 (72.7)	
LN					0.038*			0.133
Free	20	(57.10)	16	32%		16 (53.3)	20 (36.4)	
Positive	9	(25.70)	26	52%		12 (40.0)	23 (41.8)	
N/A	6	(17.10)	8	16%		2 (6.7)	12 (21.8)	
Peritoneal					0.379			0.871
Free	8	(22.90)	6	12%		6 (20.0)	8 (14.5)	
Positive	23	(65.70)	39	78%		21 (70.0)	41 (74.5)	
N/A	4	(11.40)	5	10%		21 (70.0)	6 (10.9)	
FIGO stage					0.016*			1
Stage I	13	(37.10)	8	(16)		7 (23.3)	14 (25.5)	
Stage II	0	0.00	5	(10)		2 (6.7)	3 (5.5)	
Stage III	22	(62.90)	33	(66)		20 (66.7)	35 (63.6)	
Stage IV	0	0.00	4	(8)		1 (3.3)	3 (5.5)	
Grade					0.937			0.1
Grade 1	2	(5.70)	3	(6)		1 (3.3)	4 (7.3)	
Grade 2	15	(42.90)	20	(40)		17 (56.7)	18 (32.7)	
Grade 3	18	(51.40)	27	(54)		12 (40.0)	33 (60.0)	
Therapy					0.264			0.611
No	19	(54.30)	21	(42)		13 (43.3)	27 (49.1)	
Positive	16	(45.70)	29	(58)		17 (56.7)	28 (50.9)	
Response					0.909			0.835
Score 3	9	(56.30)	14	(48)		8 (47.1)	15 (53.6)	
Score 2	5	(31.30)	11	(38)		6 (35.3)	10 (35.7)	
Score 1	2	(12.50)	4	(14)		3 (17.6)	3 (10.7)	

Quantitative data are presented as mean ± SD and median (range), qualitative data are presented as n (%). Significance defined by $p < 0.05$

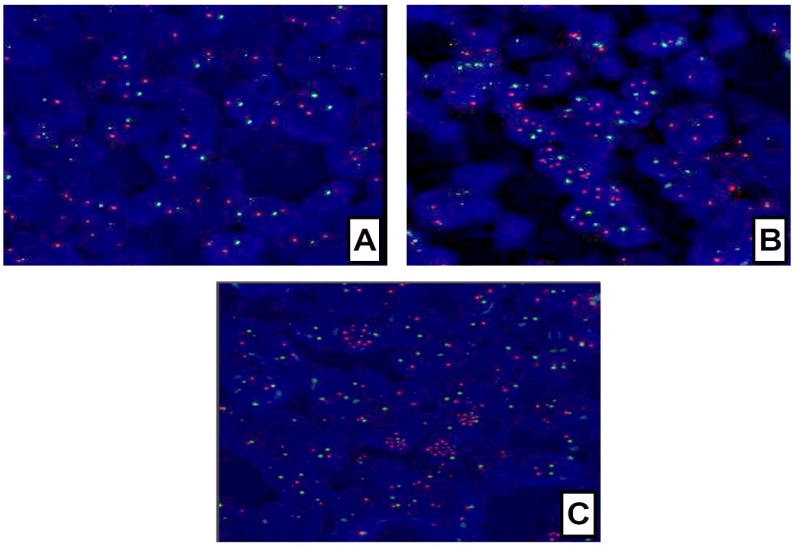

Figure 3. MET Gene Analysis by FISH Technique in EOC. (A), MET Negative tumor with MET-CN < 4 (X63); (B), MET–copy number gain with MET-CN ranged from ≥ 4 to < 5 in ≥ 10% of cells (X63); (C), MET amplified tumor with MET-CN ≥ 5 in >10% tumor cells (X63).

positive cases and 34.1 % negative EGFR cases. Of fifty-six EGFR-positive patients, fifty (59%) had high-expressing tumors (Figure 2). A significant association was noted between EGFR low tumor expression and large tumor size (P=0.044) and intact ovarian capsule (P=0.025). EGFR high tumor expression was significantly associated with LNM (P=0.038) and advanced tumor FIGO stage (P=0.016). There was no significant correlation between EGFR tumor expression and other clinicopathological variables. It is shown in Table 3. Regarding stromal EGFR expression, 64.7% of cases were positive and exhibited significant relation to pathological serous subtypes (P= 0.047).

Association between MET gene cytogenetic study and clinicopathological parameters

Seven out of 85 cases were acquired alterations in MET gene using cytogenetics FISH technique. Four cases were implied MET gene amplification while 3 cases exhibited MET gene copy number gain (Figure 3). MET gene increase copy number (gain and amplification) was significantly related to tumor size, ovarian capsule infiltration and partial therapy response (P value = 0.007, P=0.042 and P=0.030) respectively. It is shown in Table 4.

Correlation between MET gene alterations, CD44, and EGFR IHC protein expression

The expression of CD44 protein was found to be positively correlated with that of EGFR protein (P = 0.012, r = 0.271). MET gene amplification was positively correlated with EGFR protein expression (P = 0.013, r = 0.269). There was no link found between CD44 protein expression and MET gene amplification (Figure 4).

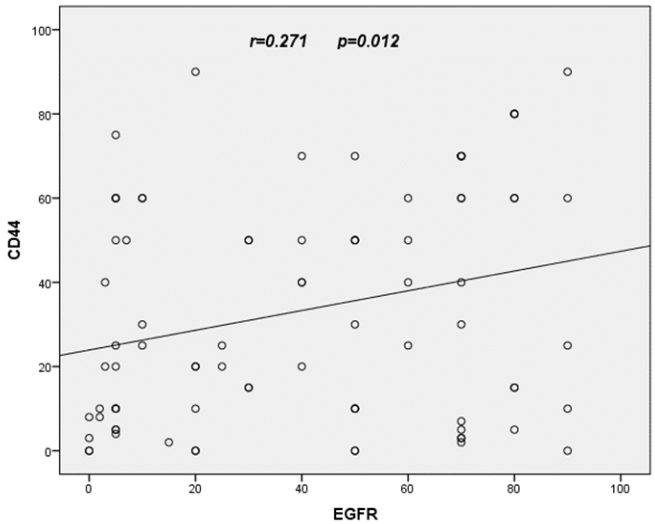

Figure 4. Scatter Plot Explains the Correlation between the CD44 and EGFR Expression CD44 Protein Expression was Positively Correlated with EGFR Protein Expression.

Table 4. Relationship between the MET Gene Amplification and Clinicopathological Parameters

Variable Name	Negative (n=78)		Gian (n=3)		Amplified (n=4)		P value
Age (years)							
Mean ± SD	53.59 ± 9.51		60.67 ± 11.59		54.00 ± 13.86		0.844
Median (range)	54.5	(31 – 75)	59	(50 – 73)	54	(42 – 66)	
≤ 50	25	(32.10)	1	(33.30)	2	(50.00)	0.817
> 50	53	(67.90)	2	(66.70)	2	(50.00)	
Laterality							0.383
Unilateral	27	(34.60)	1	(33.30)	0	0.00	
Bilateral	51	(65.40)	2	(66.70)	4	(100.00)	
Tumor size (cm)							0.007*
Mean ± SD	7.46 ± 5.04		5.17 ± 5.06		2.50 ± 0.58		
Median (range)	5	(2 – 20)	2.5	(2 – 11)	2.5	(2 – 3)	
Subtypes							0.421
Serous	55	(70.50)	3	(100.00)	4	(100.00)	
Non serous	23	(29.50)	0	0.00	0	0.00	
Capsule							0.042*
Intact	52	(66.70)	0	0.00	2	(50.00)	
Infiltrated	26	(33.30)	3	(100.00)	2	(50.00)	
Necrosis							0.484
No	28	(35.90)	0	0.00	2	(50.00)	
Yes	50	(64.10)	3	(100.00)	2	(50.00)	
LVI							0.43
No	22	(28.20)	0	0.00	2	(50.00)	
Yes	56	(71.80)	3	(100.00)	2	(50.00)	
LN							0.597
Free	32	(41.00)	1	(33.30)	3	(75.00)	
Positive	33	(42.30)	1	(33.30)	1	(25.00)	
N/A	13	(16.70)	1	(33.30)	0	0.00	
Peritoneal							0.801
Free	13	(16.70)	1	(33.30)	0	0.00	
Positive	56	(71.80)	2	(66.70)	4	(100.00)	
N/A	9	(11.50)	0	0.00	0	0.00	
FIGO stage							0.373
Stage I	21	(26.90)	0	0.00	0	0.00	
Stage II	4	(5.10)	1	(33.30)	0	0.00	
Stage III	49	(62.80)	2	(66.70)	4	(100.00)	
Stage IV	4	(5.10)	0	0.00	0	0.00	
Grade							0.617
Grade 1	5	(6.40)	0	0.00	0	0.00	
Grade 2	33	(42.30)	0	0.00	2	(50.00)	
Grade 3	40	(51.30)	3	(100.00)	2	(50.00)	
Therapy							0.020*
No	37	(47.40)	3	(100.00)	0	0.00	
Positive	41	(52.60)	0	0.00	4	(100.00)	
Response							0.030*
Score 3	23	(56.10)	0	0.00	0	0.00	
Score 2	12	(29.30)	0	0.00	4	(100.00)	
Score 1	6	(14.60)	0	0.00	0	0.00	

Quantitative data are presented as mean ± SD and median (range), qualitative data are presented as n (%). Significance defined by $p < 0.05$

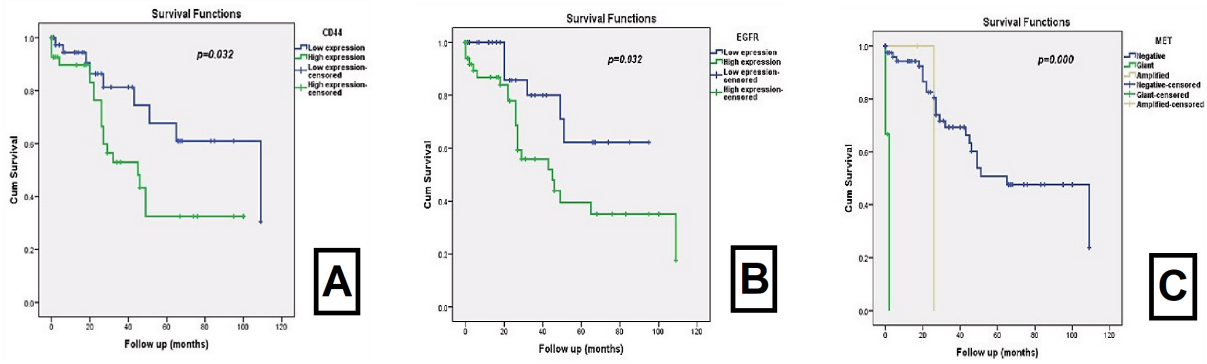

Figure 5. Relationship between Survival and CD44, EGFR, MET. (A), High C44 expression was significantly associated with poor OS; (B), High EGFR expression was significantly associated with poor OS; (C), MET gene increase copy number (copy number gain and amplification) was associated with marked short OS.

Survival analysis

The median follow-up duration of the 85 ovarian cancer patients was 23 months (range, 0 to 109 months). During follow-up, 28/85 patients (32.9%) died because of tumor progression. According to Kaplan-Meier analysis, the median OS was 51 months (95% confidence interval [CI], 26.700 to 75.300), while the 109-month (9 years) OS rate was 22.2%. A total of 39/85 patients (45.9%) developed disease progression. The median time to progression was 18 months (range, 0 to 95 months). According to Kaplan-Meier analysis, the median PFS was 36 months (95% CI, 20.836 to 51.164). The PFS rate at 95 months was 13.3%.

CD44 high expressing tumors were associated with poor OS (P =0.032), in which three-year free OS was 81.3% in patients with low CD44 expression vs. 52.9% of CD44 high expressing cases. Furthermore, EGFR high expression significantly had shorter OS (P= 0.032); in 3- years, free OS was 80% in low expressing tumors; nevertheless, 55.9 % in high expressing tumors to EGFR (Figure 5).

Regarding MET gene alterations, cases with MET gene copy number gain showed a shorter OS (P=0.000), two cases with amplified MET gene showed progression, and follow-up ended at 18 months (Figure 5). High CA 125 serum level was significantly associated with shorter PFS (P= 0.003). It was observed that about 60% of patients with serum levels of CA 125 < 286 U/ml at the first presentation had better three years PFS, while 37 % of patients with serum levels> 286 U/ml showed worse three-year PFS (Figure 5).

Clinicopathological characteristics that were significant in univariate analysis were subjected to multivariate analysis to account for confounders. Our results revealed that CD44, EGFR proteins expression and MET gene amplification were independent predictors for OS, (P<0.040, HR=2.368, 95% CI: 1.039 – 5.477), (P=<0.041, HR=2.576, 95% CI: 1.039 – 6.390) and (P<0.000, HR=38.833, 95% CI: 5.178 – 291.228), respectively, as depicted in (Table 5).

Discussion

OC is one of the women's leading causes of cancer deaths (CTPbG, 2022). OC deaths in Egypt reached 0.33% of total deaths (Gadducci et al., 2019). This research aimed to study cancer CD44 and EGFR protein expression in EOC, their correlation with MET gene amplification, and their impact on patient outcomes.

We observed that the mean age of patients included was 53 years, comparable with the study of Chao et al., (2019) which demonstrated the mean age of EOC was 53 years. However, older patient age was observed in the study by Achlaug et al., (2021), which showed that 62

Table 5. COX Regression Analysis for Predicting Likelihood of Death According to Clinic-Pathological Characteristics of the Study Participants (n=85)

Variable Name	N	B	S.E.	P value	HR	95% C.I. for HR
CD44						
Low expression	44				ref	
High expression	41	0.87	0.424	0.040*	2.368	1.039 – 5.477
EGFR						
Low expression	35				ref	
High expression	50	0.946	0.646	0.041*	2.576	1.039 – 6.390
MET						
Negative	78				ref	
Positive	7	1.838	0.589	0.002*	6.283	1.981 – 19.928

B, regression coefficient; SE, standard error; HR, hazard ratio; CI, confidence interval; p value is significant £0.05

years was the mean age of ovarian epithelial neoplasm. This difference may be due to decreased co-morbidity factors in patients such as diabetes and hyperlipidemia.

The current study showed that serous tumor type represents about 73% of total cases. This finding matched the study by Momenimovahed et al., (2019) and Badmann et al., (2020), with 70% of cases typed as serous neoplasm (Momenimovahed et al., 2019; Badmann et al., 2020). On the other hand, Yamada et al., (2021) study demonstrated less incidence of serous neoplasm was 52% of total cases and 40% of unilateral ovarian cancer. Sixty-five percent of cases in our study were presented at FIGO stage III. This finding is relatively similar to the study of Doa et al., (2016) and Ye et al., (2022) that 57% of cases come at stage III in the first visit. However, Takeshima et al., (2005) and Hanatani et al., (2020) showed that 75% of cases were at stage I. This discrepancy may be explained by lacking screening programs in our country for OC.

Regarding tumor grade, 53% of cases presented with grade 3 ovarian carcinoma at diagnosis. Similarly, Matsuno et al., (2013) study showed that 54 % of cases had high-grade carcinoma. This finding disagreed with Gockley et al., (2017) study in which 96% of total cases were high-grade ovarian carcinomas. Cheng et al., (2020) study revealed that only 38% of cases had grade 3 EOC. Paes et al., (2011) and Edrem et al., (2018) also showed that 41% of cases were grade 2.

LNM was present in 41.2 % of our cases at the presentation. This finding matched with Cheng and Lang, (2020) study that proved 55% of cases were positive LNM. This result disagreed with Takeshima et al., (2005) study, which showed that 22% of cases had positive LNM. This variation may be explained by some of the cases in the current study that did not have regional lymph node dissection. Positive peritoneal metastasis was accounting 73% of cases, comparable with 80% of positive peritoneal deposits in the study by Kojima et al., (2019). However, Pavatherad et al., (2021) work showed that only 46% of cases had positive peritoneal metastasis in EOC.

Our study noted that CD44 expression was significantly associated with serous type of carcinoma. This finding was compatible with Bartokova et al., (2018) study reported significant concordance between CD44 expression and serous histopathological type. Kar et al., (2021) did't exhibit considerable association. This discrepancy may be explained by the low number of cases in his study, most of which were borderline neoplasm.

We found a significant association between CD44 expression with tumor stage, grade, and peritoneal tumor deposits. Also, the study of Kar et al., (2021) resulted in significant concordance between CD44 and tumor grade. Tajhy et al., (2015) exhibited a significant association between CD44 and peritoneal dissemination. In contrast, Bartokova et al (2018) showed no meaningful relationship between CD44 expression tumor stage and peritoneal deposits.

The present study demonstrated positive EGFR expression in 66% of cases. A finding is nearly harmonized with Mehner et al., (2017) study that tumor expressing EGFR constitutes 52% of ovarian carcinoma cases. A significant association between EGFR expression and tumor stage was noted as most high-expressing tumors were stage III. This result matched Wang et al., (2016) study that showed a significant relationship between high expressing tumor and tumor staging. However, Mehner et at., (2017) study didn't find a significant association between EGFR expression and tumor stage. The contradiction may be referred to different methods used for EGFR expression evaluation in both studies. The present research impressed a significant relationship between EGFR and histopathological type, tumor size, capsule integrity, and LNs metastasis, in comparison with Wang et al., (2012); Wang et al.,(2016) and Zheng et al., (2017) that showed a significant association between EGFR and LN metastasis.

MET gene amplification constitutes 4.7 % of cases in this study; all of them were of the serous type, and 3.5% showed copy number gain of MET gene. At the same time, most cases were negative and accounted for about 92%. Yamamoto et al., (2011) study of MET gene alterations in clear cell carcinoma showed only 2% of their patients had amplified MET gene, and 18% showed copy number gain of MET gene. A significant association was noted between MET gene alterations and tumor size, ovarian capsule infiltration, and poor therapeutic response to treatment. A result agrees with Tang et al., (2014) who showed that cases with MET gene amplification were of high-grade serous carcinoma and had no response to therapy.

Regarding outcome analysis, high CD44 was associated with poor OS. A finding is compatible with Zhu et al., (2019), which also exhibited a significant impact of CD44 expression and OS. However, Puvanenthiran et al., (2018) showed that high CD44 expression was not significantly correlated with OS.

A significant association was noted between EGFR and OS but was non-significant with PFS. Fujiwara et al., (2012) study of EGFR expression in EOC revealed that EGFR-positive tumors had worse PFS. On the other hand, The American research by Mehner et al., (2017) did not find a significant association between OS and EGFR expression of EOC.

A study showed that MET gene alterations in EOC were significantly associated with poor outcome (Zhou et al., 2020). Also, the study by Kim et al., (2018) indicated that patients with high MET protein expression EOC showed significantly worse OS than those with MET-low tumor expression. A meta-analysis of MET gene alterations was done by Li et al., (2021) that showed MET gene alterations were found to be associated with a better prognosis in uterine corpus adenocarcinoma but associated with poor prognosis in lung adenocarcinoma. These findings draw attention to MET gene alterations that varied among different tumor types.

A significant poor impact of a high serum level of CA 125 on three years of PFS was found in the current study, congruent with Van Altena et al., (2010) study. Bachmann et al., (2022) study resulted in serum CA 125 levels below 1,404.5 U/ml compared to CA 125 levels>1,404.5 U/ml, leading to longer 3 years of PFS and OS without a significant difference; this is explained by a high median value of CA 125 in this study. Another Taiwanese study by Kim et al., (2018a) demonstrated elevated serum

preoperative CA 125 level in ovarian germ cell neoplasm (>249.5 U/mL) was associated with poorer DFS, but this was not statistically significant. However, elevated preoperative CA 125 (>249.5 U/mL) was significantly associated with short OS. The study by Piatek et al., (2021) lightened the prognostic role of CA 125 level in recurrence rate but didn't show a significant relationship with OS.

Using multivariate analysis for OS, we found that CD44, EGFR protein expression, and MET gene amplification were independent predictors for OS. In contrast, Pauvanantherian et al., (2018) study showed that EGFR and MET co-expression in OC wasn't a significant independent factor with DFS. The contradiction may be attributed to the different categorization of CD44, EGFR expressing tumor into cytoplasmic and membranous positivity, along with MET evaluation was done by immunohistochemical technique. Study of CD44 and EGFR as prognostic role in breast cancer was done by Zheng et al., (2014) and implied that EGFR and CD44 positive tumors had worsen OS and DFS than negative cases.

The current work indicated a positive correlation between EMT biomarkers (CD44 and MET) and EGFR protein expression. Recent research conducted by Yin et al., (2020) on the H460 cell line of small cell carcinoma of the lung demonstrated that CD44 knockdown leads to direct suppression of EGFR signaling via reduction of EGF mediated activation of EGFR signaling. These effects were augmented when associated with cisplatin treatment. Consequently, the binding of EGFR ligand followed by EGF boosted the expression of CD44 and the activation of ERK, STAT3, and AKT in SKBR3 cancer cells. Another study discovered that CD44 increased tumorigenesis and progression in head and neck squamous cell carcinoma by interacting with EGFR (Perez et al., 2013). Also, Zheng et al., (2015) study showed that EGFR and CD44 expressions in breast carcinoma were significantly positively correlated. Another study presented that CD44 expression was significantly associated with EGFR at the mRNA level in breast cancer (Xu et al., 2016). Suda et al., (2017) study showed that CD44 expression was significantly correlated with EGFR mutated lung carcinoma.

Regardless of EGFR presence, an activation of EGFR-independent phosphorylation of ErbB3 and subsequent PI3K/AKT pathway activation is linked to MET gene amplification, allowing a signaling pathway bypass (Wang et al., 2019). CD44, EGFR, and MET expression significantly correlated in head and neck SCC as reported in Baschngel et al., 2016 study (Baschnagel et al., 2017).

In conclusion, this study highlights the role of EMT biomarkers (CD44 expression and MET gene alterations) and EGFR expression in EOC as an independent prognostic factor for OS. A significant positive correlation between EMT biomarkers and EGFR protein expression, hence sharing common molecular pathways. MET gene increase copy number (gain and amplification) was detected only in cases of serous neoplasm and was significantly related to poor therapy response. Comparative studies with large patient cohorts are highly recommended to introduce the MET targeting therapy in the treatment plan for EOC to improve the response to therapy.

Author Contribution Statement

Conceptualization: IMK MTH, EHY; Data curation: IMK MTH, HA; Formal analysis: IMK MTH, HA; Methodology: IMK MTH, DFT, EM; Resources: IMK MTH, DFT, EM; Writing – original draft: IMK, MTH; Writing – review & editing: all authors; Approval of final manuscript: all authors.

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Conflict of interest

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

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