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RESEARCH ARTICLE

IMMUNOHISTOCHEMICAL EXPRESSION OF GLUT-1 IN EPITHELIAL OVARIAN TUMORS: CORRELATION WITH THE CLINICOPATHOLOGICAL FACTORS AND TUMOR PROLIFERATIVE MARKER PCNA.

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Abstract

Background: Ovarian carcinomas are frequently diagnosed at advanced-stage due to lack of distinct symptoms and reliable procedure for early detection. The applying of immunohistochemistry has become an important tool improving the prognosis of patients with ovarian carcinomas. **Aim:** To assess the expression of Glut-1 in epithelial ovarian tumors and study its correlation with PCNA to detect their usefulness in the diagnosis and prognosis of such tumors. **Methods:** Glut-1 immunoexpression was analyzed and correlated with PCNA in 45 epithelial ovarian tumors (7 benign, 10 borderline and 28 malignant tumors) **Results:** Glut-1 was expressed in 80% and 92.85% of the studied borderline and invasive carcinomas respectively, but not expressed in any benign tumors. These differences in Glut-1 expression among the benign, borderline and malignant cases, were statistically significant (p= 0.000). Analysis of Glut-1 immunoexpression with the clinicopathological criteria of ovarian carcinomas revealed that Glut-1 expression is moresignificantly expressed in high grade carcinoma and in tumors with an advanced FIGO stage (p=0.043 and p=0.005 respectively). Glut-1 was more significantly expressed in lymph node metastases positive group and in those with intraperitoneal implants (p=0.011 and 0.016 respectively).There was a strong positive significant correlation between Glut-1 and PCNA among the studied 45 ovarian tumors (Spearman correlation (r) = 0.603, p value= 0.000).**Conclusion:** Glut-1 can increase the diagnostic accuracy of ovarian tumors by help in differentiating between benign, borderline and malignant tumors. Glut-1 correlated with poor prognostic factors and can be used with PCNA as prognostic markers for epithelial ovarian tumors.

Introduction:-

In Egypt, tumors of the reproductive organs account for 4% of the overall malignancies in females and ovarian malignanciesconstituted about 1.4% of them [1]. Ovarian carcinomas are frequently diagnosed at advanced-stage due to lack of distinct symptoms and lack of reliable procedure for early detection [2]. Epithelial ovarian tumors

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constitute the majority of ovarian tumors [3] WHO classified ovarian surface epithelial tumors histologically into: serous, mucinous, transitional cell, endometrioid, clear cell, mixed, squamous, and undifferentiated subtypes [4].

Many clinicopathological variables are considered to be of great importance in determining the prognosis of patients with ovarian carcinoma, including tumor staging, histological typing, presence of ascites positive for malignancy, residual tumor mass, and the chemoresponsiveness of the tumor cells [5]**.** Beside these well-known clinical prognostic factors, the applying and usage of molecular techniques is more accurately related to the intrinsic behavior of the tumors and the pathway of carcinogenesis and may be supportive in improving the prognosis of patients with ovarian carcinomas [6].

There have been significant advances in understanding ovarian carcinoma based on immunohistochemistry and molecular analysis [7]. Immunohistochemistry has become an important tool not only in the diagnosis of ovarian tumors, but also represent markers of prognostic significance[8].Most of the identified tumor markers in ovarian epithelial tumors have not shown satisfactory sensitivity and specificity. Therefore, new candidate markers are needed that can be used to improve the diagnostic accuracy of the screening strategies [9].

The cells of the tumors show a considerable increase in the metabolism of glucose in contrast to the normal tissue. This great increase in the demand of glucose by tumor cells signifying a need for a corresponding enhancement glucose transport through the cellular membrane [10]. The active passage of glucose through the membrane of the cell is controlled via a group of proteins named glucose transporters, they are 14 types (from Glut 1 to Glu-14). They show variable differences in the affinity for glucose, tissue allocation and physiological control [11].

The glucose transporter-1 (Glut-1), is normally expressed and detected by immunohistochemical staining in the membranes of red blood cells (RBCs), endothelium of the capillary of the brain and the perineurium of the peripheral nerves [12]. The Glut-1 is up regulated in situations with decreased oxygen concentrations and hypoxia [13]. Moreover,Glut-1 is accompanied by a rise in the expression of many proteins that have the ability to help the survival of cellular tumor in the adverse microenvironment and enhance the metabolism of glucose [14]. Tumors that overexpress Glut-1 tend to show better and complete response to chemotherapy. So, Glut-1 may be regarded as an independent factor of prognosis that can predict the response of the patient to treatment with chemotherapy, it addition to its early diagnostic role [15]. There has been a great attention to find a link between the expression of Glut-1 in epithelial neoplasms, and the association of Glut-1 expression with the carcinogenesis and patient prognosis in ovarian tumors [16]. However, many researches have detected an association between the expression of Glut-1 and neoplastic progression, development, and the bad outcome of many neoplasms [17, 18].

Detection of proliferating cells by immunohistochemistry is a method to determine the proliferative potential of a tumor[19].Proliferating Cell Nuclear Antigen (PCNA) is a protein cofactor of DNA polymerase that is expressed in the cell cycle during the replication of the DNA and often regarded as an index of cell proliferation**.**Ki67 is thought to be a more expressive marker of proliferation than PCNA, and as result of that PCNA was less routinely utilized [20]. Interestingly, many researches that have studied the expression of both proliferative markers (Ki67 and PCNA) in the same patient group reported a concordant score with the use of both markers [6, 21, and 22]. This signifies that the usefulness of cellular proliferative markers and the prognosis of cases is more dependent on the selected group of cases rather than the proliferative marker used [20].

The aim of the present work was to assess the immunohistochemical expression of Glut-1in epithelial ovarian tumors and studyits correlation with tumor proliferative marker PCNA and other clinicopathological factors of ovarian carcinomas to detect the usefulness of Glut-1 expression in the diagnosis and prognosis of ovarian epithelial tumors.

Material And Methods:-

Patients and clinical data:-

The present study was performed at the Departments of Pathology, Obstetrics and Gynecology, and Surgery, Zagazig University Hospital, Egypt in the period from May 2015 to November 2016. The study included 7 patients with benign tumors, 10 patients with borderline tumors and 28 patients with malignant tumors. Complete surgical staging that includes total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, peritoneal washing with cytology and appendectomy for mucinous neoplasms was performed. Conservative surgery that includes either (ophorectomy, unilateral salpingo-oophorectomy or cystectomy) was performed for young aged

patients with apparently benign neoplasm by radiological and clinical data and patients with stage I (with no peritoneal implants) borderline neoplasm who wish to preserve their fertility. For patients with advanced stage disease, cytoreductive surgery was performed.

We collected the clinical and pathological information from the medical records of the patients. All the patients did not receive chemotherapy or radiation prior to the surgical interference. Tumors were staged according to the FIGO (The International Federation of Gynecology and Obstetrics staging system) [23]. Histological typing and grading have followed the World Health Organization classification (WHO) criteria [4]. The ethical committee of Zagazig University approved this study and all patients wrote a consent of agreement prior to their inclusion in this study. All tissue samples were formalin-fixed and paraffin-embedded, the blocks were cut at 4-5 microns and stained with ordinary H&E stain to diagnosis and grade the tumors.

Immunohistochemical staining:-

The sections (4–5 μm) cut from the corresponding tissue sample blocks were deparaffinized with xylene, placed in graded alcohols for rehydration, and deposited in 0.5% hydrogen peroxide in methanol for 10 min to stop endogenous peroxidase activity. Antigen retrieval was achieved by keeping in 0.01 M citrate buffer (pH 6.0) for 5 minutes in a pressure cooker. The primary antibodies were added to the sections at room temperature for 60 min. Thenorm strept avidin-biotin-peroxidase complex technique was applied for Glut-1 (Rabbit Polyclonal Antibody, Dilution 1:200, catalog no. PA1-1063Thermo Fisher Scientific/Lab Vision Corporation, Rockford, USA.) and PCNA (Mouse monoclonal antibody, clone PC10, Dilution 1:50, catalog no. MA5-11358, Thermo Fisher Scientific/Lab Vision Corporation, Rockford, USA.) byutilizing diaminobenzidine (DAB) as the chromogen.RBCs were used as internal positive control for Glut-1 and breast carcinoma were used as positive control for PCNA. Negative controls for both markers were performed by excluding the primary antibody.

Evaluation of immunohistochemical staining:-

Glut-1 expression was considered positive only if membrane staining is present. The expression was semi-quantitatively evaluated by analyzing the intensity and the percentage of stained cells. The intensity was scored as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). A combined score depending on the intensity of staining and the proportion of stained cells was applied as the final score as follow: Low expression was defined as an intensity of 1, 2, or 3 and < 10% stained cells or an intensity of 1 and <50% stained cells; and high expression was defined as an intensity of 2 or 3 and >10% stained cells or an intensity of 1, 2, or 3 and > 50% stained cells [24]. Finally for statistical evaluations we have 3 groups, negative, low expression and high expression groups.

PCNA was considered positive if there was any brown nuclear staining present. To assess proliferation, the PCNA labelling index (PCNA LI), was calculated following the previously described method by Ino et al. [6]. The PCNA LI was defined as the number of tumor cells with nuclear PCNA immunostaining divided by the total number of tumor cells, and expressed as a percentage. A total of 1000 nuclei in the selected area were counted under a light microscope at high magnification (X 400 fields) and the mean percentages were recorded as the PCNA LI. For statistical evaluation, tumors with a PCNA LI ≥ 50, were considered as high proliferative index group, while cases with a PCNA LI of < 50 were defined as low proliferative index group.

Statistics:-

The results from the continuous variables analysis were expressed as a means ± standard deviation (SD). Categorical data analysis was performed using the x^2 or Fisher's exact test, spearman correlation was done to detect and measure the correlation between Glut-1 and PCNA. The statistical analyses were done using SPSS software (version 19.0; SPSS, Chicago, IL) and $P \leq 0.05$ was regarded as indicator of a statistically significant difference.

Results:-

Clinicopathological results:-

The mean age of the studied 45 ovarian tumor patients at initial surgery was 53.16± 12.01 years (range 23- 72 years). Among these 45 patients, 7 (15.55%) cases were benign cystadenomas, 10 (22.22%) cases were borderline, and 28 (62.22%) cases were malignant (invasive) carcinoma. Serous type was the predominant histological type (62.22%, 28/45) among all the studied benign, borderline and malignant tumors (Fig. 1). Grading of the studied 28 ovarian carcinomas according to WHO grading system revealed that GI was the most frequent grade of the studied ovarian carcinoma (53.57%, 15/28), while staging of ovarian carcinomas according to FIGO staging system revealed that the majority of carcinomas (60.7%, 17/28) were diagnosed at advanced stage (Stage III-IV). Lymph node metastases, intraperitoneal implants and ascites were detected in 35.71% (10/28), 57.14% (16/28) and 75%

(21/28) respectively of the studied ovarian carcinomas. All ovarian carcinoma patients' clinicpathological variables are outlined in Table 1.

Immunohistochemical expression of Glut-1:-

Glut-1 memberanous immnuoreactivity was detected in 80% (8/10) and 92.85% (26/28) of the studied borderline and invasive carcinomas respectively and showed progressively more increase in staining intensity in invasive tumors as compared to borderline tumor. Glut-1 immunoreactivity was absent in all the studied benign ovarian tumors (Fig. 2, a, c. Fig. 3, a, c). These differences in Glut-1 expression among the studied benign, borderline and malignant cases, were statistically highly significant (p= 0.000) (Table 2). Analysis of Glut-1 immunoexpression with the clinicopathological criteria of the studied 28 ovarian carcinomas revealed that Glut-1 expression is more intensely expressed in high grades carcinoma (G3) and in tumors with advanced FIGO stage (Stage III-IV) with a significant relationship (p=0.043 and p=0.005 respectively). Glut-1 also tend to be expressed with more intensity in lymph node metastases positive group and those with intraperitoneal implants with statistically significant relationship from the negative groups (p=0.011 and 0.016 respectively). However, no correlation was found between Glut-1 immunoexpression and age of the patient, histological types of the tumor or the presence or absence of ascites. (p>0.05) (Table 3).

PCNA immunoreactivity and the result of correlation analysis between the expression of Glut-1 and the expression of PCNA (PCNA LI) among the studied 45 ovarian epithelial tumors:

PCNA positive immunoreactivity was detected as brown nuclear staining (Fig. 2, b, d. Fig. 3, b, d). Calculation of PCNA labelling index revealed that 37.77% (17/45) of cases had PCNA LI ≥ 50. Based on the correlation analysis between the results of the expression of Glut-1 and the tumor proliferative marker PCNA LI, a significant strong positive correlation was found between the expressions of the two markers among the studied 45 epithelial ovarian tumors (Spearman correlation (r) = 0.603, p value= 0.000) (Table 4).

Table 1:-Clinicopathological characteristics of the studied 28 ovarian carcinoma

Variable	N (%)
Age at surgery (y)	
< 55	9 (32.14%)
≥ 55	19 (67.85%)
Histological type	
Serous	17 (60.71%)
Mucinous	7 (25%)
Other types (including mixed type)	4 (14.28%)
Histological grade	
G1	15 (53.57%)
G2	7 (25%)
G3	6 (21.4%)
Lymph node metastases	
Negative	18 (64.28%)
Positive	10 (35.71%)
FIGO stage	
I-II	11 (39.2%)
III-IV	17 (60.7%)
Intraperitoneal implants	
No	12 (42.8%)
Yes	16 (57.14%)
Ascites	
No	7 (25%)
Yes	21 (75%)
Total cases	28 (100%)

Table 2:-Glut-1 immunohistochemical expression among the tumor type of the studied 45 ovarian tumors**.**

	Expression	**Benign** n = (7)	**Borderline** n = (10)	**Invasive** n = (28)
Glut-1	Negative	7 (100%)	2 (20%)	2 (7.14%)
	Low	0 (0%)	5 (50%)	11 (39.28%)
	High	0 (0%)	3 (30%)	15 (53.57%)
P value	0.000			

Table 3:-Correlation of clinicopathological parameters of the studied 28 ovarian carcinoma with Glut-1 expression.

Variable	Total (n= 28)	**Glut-1 expression** Absent (2)	Low (11)	High (15)
Age at surgery (y)				
< 55	9 (32.14%)	2 (22.2%)	4 (44.4%)	3 (33.3%)
≥ 55	19 (67.85%)	0 (0.0%)	7 (36.8%)	12 (63.2%)
P value	0.092			
Histological type				
Serous	17 (60.71%)	0 (0.0%)	6 (35.3%)	11 (64.7%)
Mucinous	7 (25%)	1 (14.3%)	3 (42.3%)	3 (42.3%)
Other types (including mixed type)	4 (14.28%)	1 (25%)	2 (50%)	1 (25%)
P value	0.231			
Histological grade				
G1	15 (53.57%)	2 (13.3%)	9 (60%)	4 (26.6%)
G2	7 (25%)	0 (0.0%)	1 (14.3%)	6 (85.7%)
G3	6 (21.4%)	0 (0.0%)	1(16.7%)	5 (83.3%)
P value	0.043			
Lymph node metastases				
Negative	18 (64.28%)	2 (11.1%)	10 (55.6%)	6 (33.3%)
Positive	10(35.71%)	0 (0.0%)	1 (10%)	9 (90%)
P value	0.011			
FIGO stage				
I-II	11(39.2%)	1 (9.1%)	8 (72.7%)	2 (18.2%)
III-IV	17 (60.7%)	1 (5.9%)	3 (17.6%)	13 (67.5%)
P value	0.005			
Intraperitoneal implants				
No	12 (42.8%)	2 (16.7%)	7 (58.3%)	3 (25%)
Yes	16 (57.14%)	0 (0.0%)	4 (25%)	12 (75%)
P value	0.016			
Ascites				
No	7 (25%)	0 (0.0%)	4 (57.1%)	3 (42.9%)
Yes	21 (75%)	2 (9.5%)	7 (33.3%)	12 (57.1%)
P value	0.416			

Table 4:-Correlation analysis between the expression of Glut-1 and PCNA (PCNA LI) among the studied 45 cases of ovarian tumors.

		PCNA LI		Total
		< 50	≥ 50	
Glut-1	Absent Count (% of total)	11 (24.44%)	0 (0%)	11 (24.44%)
	Low expression Count (% of total)	12 (26.66%)	4 (8.88%)	16 (35.55%)
	High expression Count (% of total)	5 (11.11%)	13 (28.88%)	18 (40%)
Total Count (% of total)		28 (62.22%)	17 (37.77%)	45 (100%)

* Spearman correlation (r) = 0.603 p value= 0.000

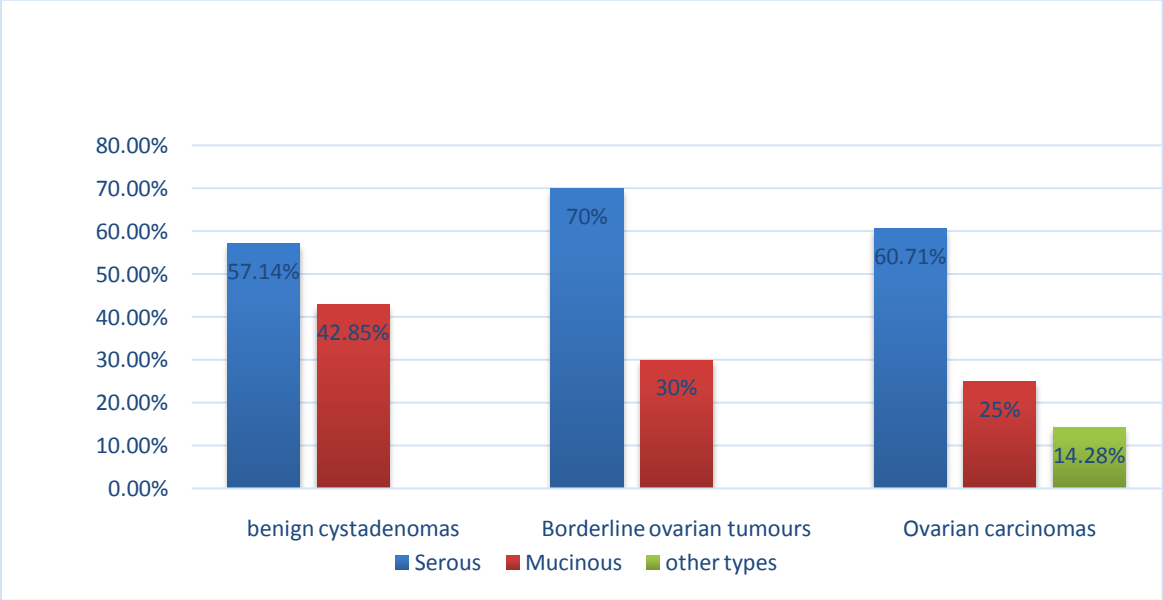
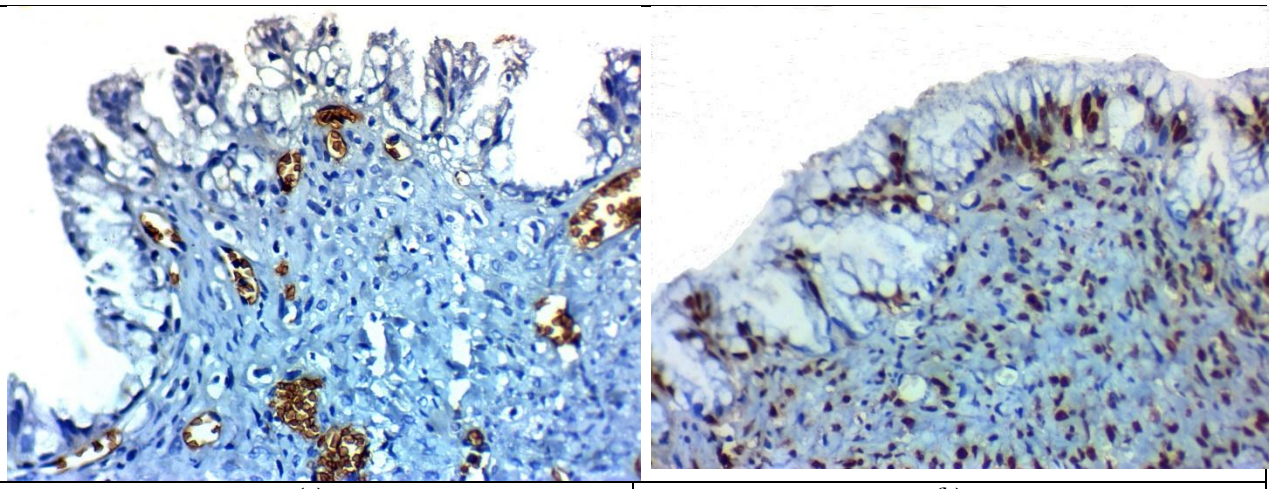
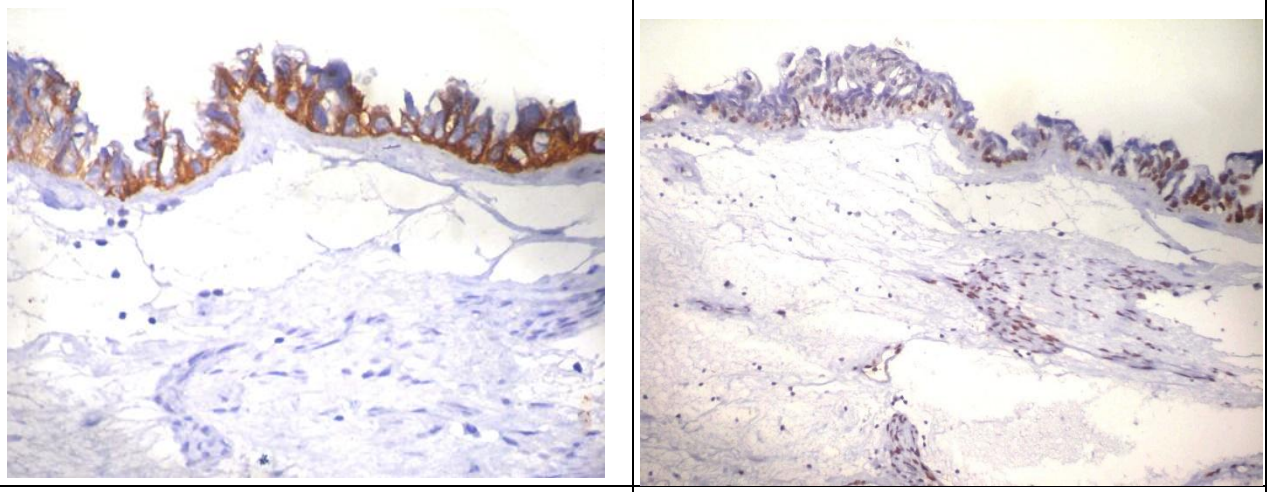


Fig. 1:-A histogram showing histopathological diagnosis and percentage of the studied 45 ovarian tumors. Note: serous type is the predominate type

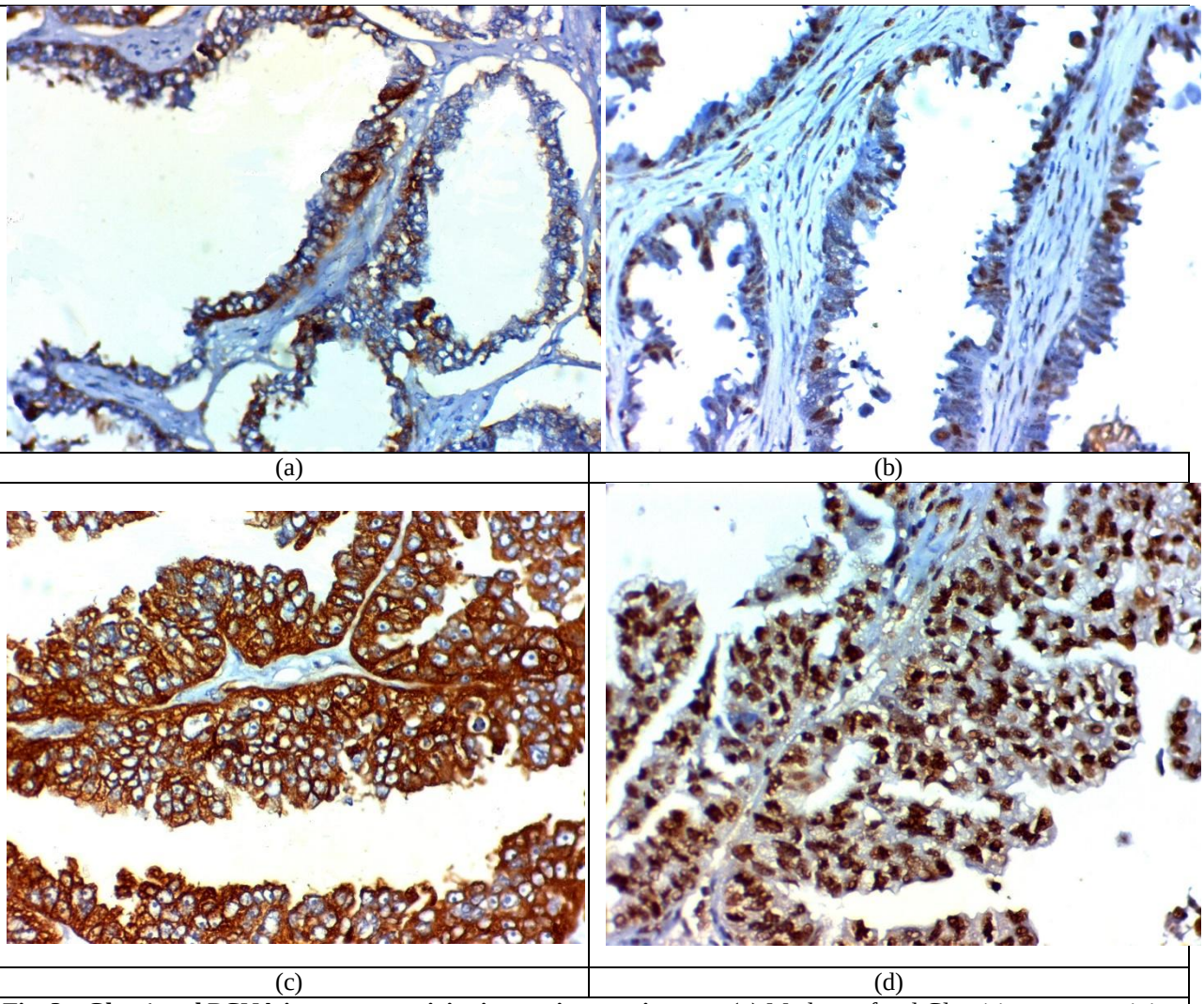


(a) (b)



(c) (d)

Fig. 2:- Glut-1 and PCNA immunoreactivity in benign and borderline ovarian tumors. (a) Benign mucinous cystadenoma showing negative immunoexpression for Glut-1. Note: RBCs in the vascular spaces show Glut-1 immnuoreactivity, which were used as an internal positive control (x400). **(b)** The previous cystadenoma showing focal nuclearPCNA immnuostaining (PCNA LI< 50) (x 400)**. (c)** Serous border line ovarian tumor showing moderate Glut-1 staining(x 200). **(d)** The previous borderline tumor showing weak to moderate focal PCNA staining (PCNA LI< 50) (x 200)



(a) (b)

(c) (d)

Fig. 3:- Glut-1 and PCNA immunoreactivity in ovarian carcinomas. (a) Moderate focal Glut-1 immnuoreactivity in G I serous ovarian carcinoma**.(b)** The previous low grade adenocarcinoma showing weak to moderate PCNA immunostaining (PCNA LI> 50)**.(c)** Grade III mucinous adenocarcinoma showing diffuse strong Glut-1 immnuoreactivity. **(d)** The previous high grade adenocarcinoma showing strong nuclear PCNA immnuoreactivity (PCNA LI> 50). (X 400).

Discussion:-

Glut-1 is a prototype of the Glut family and is widely distributed in normal tissues including RBCs [25]. Studies on ovarian cancer have suggested that Glut-1 is overexpressed and correlated with tumor aggressiveness and poor prognosis [15, 26]. PCNA is a validated specific cellular proliferative marker. In the cell cycle, it is expressed in the cellular nuclei during the phase of DNA synthesis. It is suitable for estimation of different tumors and benign lesions with proliferative potential [27]

In the present study, we examined the immuoexpression of Glut-1 in 45 ovarian epithelial tumors, including 7 benign cystadenomas, 10 borderline tumors and 28 ovarian carcinomas. We correlated the Glut-1 expression with the expression of tumor proliferative marker PCNA and with otherclinicopathological factors of the ovarian carcinoma to detect the usefulness of Glut-1 expression in diagnosis and prognosis of ovarian epithelial tumors.

Our analysis revealed that serous type was the predominant histological type (62.2%, 28/45) among all the studied benign, borderline and malignant ovarian neoplasms. This is closely similar to the study of Choetal. [28],they reported 59.32% (35/59) serous type among their studied ovarian neoplasms.

Grading of the studied 28 ovarian carcinomas, revealed that 53.57% (15/28) of carcinoma were GI. This is in agreement with previous related studies [29, 30], whilestaging revealed that 60.7% (17/28) of ovarian carcinoma, were at stage III - IV.These results revealed that most of ovarian carcinomas are diagnosed at an advanced stage, this is in concordance with many previous related studies [31, 32].

In the present study, analysis of Glu-1 expression among the studied 45 ovarian tumors, revealed that Glut-1 staining was absent in all benign ovarian tumors, and showed progressively more staining in invasive tumors as compared to borderline tumors. These differences in Glut-1 expression among the studied benign, borderline and malignant tumors, were statistically highly significant (p= 0.000). These findings are in concordance with many previous related studies [15, 16, 26, 28, and 33].

Also, Ma et al [34] reported nearby results on endometrium, where there was a progressive increase in the expression of Glut-1 among normal, hyperplastic and endometrial carcinomas (3.3%, 25.0% and 70.0% respectively). However, in contrast to our finding, Iida et al. [35], reportedGlut-1 expression in 68% of benign tumors, 95% of borderline and in all cases of ovarian carcinomas (100%). These differences in the finding may be due to differences in the cohort number (Iida: 102 ovarian tumors, ours: 45), different staining technique, the use of different primary antibody types and the use of different methods of evaluation of marker immunoreactivity.

Based on our finding that Glut-1 immunoreactivity showed a gradual increase in the staining intensity from borderline to frankly malignant ovarian tumors and absence in benign tumors, we could infer that Glut-1 plays an important role in pathogenesis of ovarian carcinomas by supporting their increased need for glucose metabolism. So, Glut-1 increases the diagnostic accuracy of ovarian tumors by help in differentiating between benign, borderline and malignant tumors. This differentiation is of great significance in planning therapeutic strategy.

In the present study, analysis of Glut-1 immunoexpression with clinicopathological criteria of the studied 28 ovarian carcinomas revealed that Glut-1 expression is more intensely expressed in high grade carcinomas with a significant relationship (p=0.043). This observation was inconcordance with many previous related studies [15, 28, 33, and 36], they reported that poorly differentiated tumors tend to significantly overexpress Glut-1 compared to well and moderately differentiated tumors. Moreover, Centuaria et al [15] concluded that tumors with overexpression of Glut-1, had more possibility to get benefit from chemotherapy, so Glut-1 may play a role not only in diagnosis but also it is an independent prognostic factor which determine the response to therapy.

However, in contrast to our finding,Kim et al. [37] did not find anystatistical relationship between the grade of ovarian carcinoma and the expression of Glut-1. This difference may be due to the different immunohistochemical clones, different technique used, different cohort number and difference in the selection criteria. This indicates further study on a larger cohort.

Our finding that high grade tumors tend to overexpress Glut-1 than low grade tumors may be due to increase demand for glucose uptake in poorly differentiated tumors. So we could infer that Glut-1 plays a role in tumor differentiation, as well as supplying energy to rapidly proliferating tumor cells. Furthermore, analysis of Glut-1 expression among the studied ovarian carcinomas, detected that Glut-1 tend to be expressed more intensely in tumors with advanced FIGO stage (Stage III-IV) with a statistically significant relationship (p=0.005). This is consistent with many previous related studies [24, 28, 33, 36, and 37].

Glut-1 also tends to be expressed with more intensity in lymph node metastases and intraperitoneal implants positive groups with statistically significant relationship from the negative groups (p=0.011 and 0.016 respectively). This is consistent with the finding of Cai et al [16], and also consistent with Zhao et al [36] who found that Glut-1 staining was positively correlated with the cancer invasion and lymph node metastasis. The correlation of Glut-1 expression with the grade and stage of studied ovarian carcinomas and with lymph node metastasis and intraperitoneal implants can spotlight on its advantage as prognostic marker in targeted therapy

In the present study, a significant strong positive correlation was detected between the expressions of Glut-1 and the expressions of tumor proliferative marker PCNA among the studied 45 ovarian epithelial tumors (Spearman correlation (r) = 0.603, p value= 0.000). This finding is nearly similar to the finding obtained by Mamede et al. [38] who found a significant positive correlation between the expressions of Glut-1 and the expressions of PCNA

(Spearman correlation (r) = 0.58, P < 0.01) among the studied pulmonary malignant lesions (41 primary lung cancers and 5 pulmonary metastatic lesions). This finding is also consistent with the finding of Zhao et al. [36] who reported that the score of PCNA was significantly higher in malignant ovarian tumors with strong Glut-1 staining, but this relationship was not significant with moderate and low Glut-1 staining. This difference may be due to the usage of different immunohistochemical clones and technique and different method of interpretation of markers immnuoreactivity.

Our finding that there was a strong positive correlation between Glut-1 and tumor proliferative marker PCNA among our studied cases, indicates that tumors with high proliferative activity, need a comparable increase in glucose uptake to have sufficient energy for rabid cellular division.

This supports that both markers play important role in the progression of epithelial ovarian carcinoma and further support their value as a predictive marker of poor prognosis in targeted therapy.

In conclusion, Glut-1 increasing the diagnostic accuracy of ovarian tumors by help in differentiating between benign, borderline and malignant tumors. This differentiation is of great significance in planning therapeutic strategy. The correlation of Glut-1 expression with poor prognostic factors such as high grade, advanced stage, lymph node metastasis and intraperitoneal implants can spotlight on its advantage as prognostic marker in targeted therapy together with PCNA which showed high strong correlations with Glut-1 among the studied epithelial ovarian tumors.

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