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RESEARCH ARTICLE

HISTOLOGICAL GRADE, CA 125 LEVELS AND IHC EXPRESSION OF ER/ PR, HER-2/NEU, P53 AND KI 67 MARKERS IN EPITHELIAL OVARIAN NEOPLASMS: A CORRELATIVE STUDY.

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Abstract

Introduction:- Ovarian carcinoma accounts for a significant number of deaths from malignancies of the female genital tract and is the fifth leading cause of cancer fatalities in women. [1]. Incidence rates are higher in developed than developing countries. A female's risk at birth of having ovarian tumor sometime in her life is 6-7%. [2] In most of the population-based cancer registries in India, ovarian cancer is the third leading site of cancer among women, trailing behind cervix and breast cancer. The age-adjusted incidence rates of ovarian cancer vary between 5.4 and 8.0/100,000 population in different parts of the country. [2]

Methodology:- The present study was undertaken with aim to correlate serum CA-125 levels and IHC expression of ER/PR, HER2/neu, p53 and Ki67 markers with various histological types and grades of ovarian neoplasms and their role in prognosis.

Prospective histologically diagnosed epithelial ovarian neoplasm reported at Era's Lucknow Medical College & Hospital and other collaborating hospitals consenting to participate in this study.The blood samples were used for estimating serum CA-125 levels. Ovarian biopsy/excised tumors: The ovarian tumors were used for histopathological and immune-pathological studies. Any case which have any other associated malignancy even gynecological malignancies besides ovarian cancer in present or past or any case other than epithelial ovarian neoplasm of ovary were excluded from the study.

Results:- There was significant higher expression of ER and PR in serous ovarian neoplasm and among malignant cases with grade 3 tumors without any significant association with stage and grade. With increasing histopathological grade, an increase in mean serum CA-125 levels was observed but it was not significant statistically (p=0.088). Proportional differences in HER2/neu expression of cases with different clinical stages was not found to be statistically significant (p=0.443). Proportional difference in p53 expression and score in patients of different histological type was not found to be statistically significant (p=0.068). Proportional difference in Ki67 expression in patients of different histological type was found to be statistically significant (p=0.048). On follow up of serum CA-125 out of seven

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cases, in five cases serum CA-125 level returned to normal (<35 U/ml). However in 2 cases (both of stage IV) serum CA-125 levels were 96 U/ml and 74 U/ml respectively.

Conclusion:- Although ER PR and Her 2,p53 were higher in grade 3 tumors no significant association was seen with staging.ki67 showed a statistically significant association with staging. The follow yielded normalized CA-125 levels in 5 out of 7 cases post surgery.

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Introduction:-

Ovarian carcinoma accounts for a significant number of deaths from malignancies of the female genital tract and is the fifth leading cause of cancer fatalities in women. [1]. Incidence rates are higher in developed than developing countries. A female's risk at birth of having ovarian tumor sometime in her life is 6-7%. [2]Ovarian cancer is the third leading site of cancer among women, trailing behind cervix and breast cancer, in most of the population-based cancer registries in India. The age-adjusted incidence rates of ovarian cancer vary between 5.4 and 8.0/100,000 population in different parts of the country. [2]Each year over 22,000 women are diagnosed worldwide with epithelial ovarian cancer and 15,000 die of it [3].World Health Organization (WHO) classifies ovarian tumors according to their most probable cell of origin and histomorphological features.[3]More than 90% of ovarian tumors are "epithelial" in origin. Some evidence suggests that the fallopian tube epithelial lining is the precursor lesion of some ovarian tumors [4] However, the etiopathogenetic mechanisms are still under research. [5]

Ovarian neoplasm can arise from germinal epithelium, germ cell, sex cord, ovarian stoma. Surface epithelial tumors are most common followed by germ cell tumor. Surface epithelial stromal tumors account for 95% of all ovarian cancer. Tumors arising from surface epithelium include serous, mucinous, endometrioid, clear cell carcinoma, Brenner's and transitional cell carcinoma. Clinical presentation is quite variable. 70% of ovarian cancers present with disseminated disease and only 19% of tumors are organ confined at diagnosis.

Etiological factors involved in ovarian carcinogenesis remain poorly defined, and effective treatment protocols are limited. Some reports suggest that pregnancy and use of oral contraceptive is associated with diminished risk for development of ovarian neoplasms. The risk of developing ovarian cancer is increased fourfold in women with an affected first degree relative and the majority of familial ovarian cancers are due to mutation in BRCA1 gene BRCA2 gene. [1] Indian cancer registry data project ovary as an important site of cancer in women, comprising up to 8.7% of cancer in different parts of country. Nearly 2/3 of patients of ovarian cancer are diagnosed with advanced stage disease which is caused by an unspecific clinical appearance and the lack of effective early detection methods [6].Its mortality rate is greater than that of cervical and endometrial cancer together [7].Ultrasound is initial investigation followed by Serum tumor marker. Surgery and histopathological examination then determine the stage and benign or malignant nature of mass. Effective treatment protocols are limited specially for poorly defined ovarian tumors. There have been persistent efforts in the investigation of molecular markers in epithelial ovarian tumors by immunohistochemical (IHC) studies [3]. Steroid hormones such as estrogen and progesterone are thought to play an important role in the process of carcinogenesis of ovarian tumors. Ovarian neoplasms are characterized by changes in their receptor status and consequently, tumor can be either primary receptor negative or as a result of their progression, they may lose the receptors. [8]On account of this, few workers have studied estrogen receptors (ER) and progesterone receptors (PR) status in ovarian neoplasms and correlated with various variables. Ki-67 is a proliferation marker helpful in predicting disease outcome in many types of malignancies including ovarian neoplasms. [9]Previous studies have shown that the p53 gene is mutated in 30-80% of ovarian carcinomas. [10]The role of immunostaining is now employed not only for diagnosis but also for other parameters including prognosis, microscopic tumor staging, prediction of response to therapy, and for the selection of therapeutic agents.

CA-125 is the most frequently used biomarker for OC detection. Blood serum CA-125 is elevated 90% of women with advanced ovarian cancer. Monitoring CA-125 blood serum levels is also useful for determining the treatment response and prognosis. Monitoring CA 125 levels is important as high levels of CA-125 during therapy is associated with poor survival rates in patients. An increase in CA-125 levels within individuals in a remission is a strong predictor of the recurrence of ovarian cancer. In April 2011 the UK's National Institute for Health and Clinical Excellence (NICE) recommended that women with symptoms that could be caused by ovarian cancer

should be offered a CA-125 blood test. CA-125 is very useful method to discriminate between benign and malignant cases [7].A studyshowed that an increase of CA-125 was found in 81% of patients with serous, 60% with endometrioid and 30% with ovarian mucinous carcinoma[11]. Another study showed that CA-125 was positive in 89.1% woman with ovarian cancer and in 62% with neoplasm of low malignant potential. The higher value of CA-125 was detected in younger woman with low malignant tumor potential. Serous and metastatic tumor types were associated with higher values of CA 125[12] In this study we tried to correlate CA-125 levels and intensity of IHC expression of estrogen receptor, progesterone receptor, Her-2/neu, P53 and Ki 67 markers with various histological types and grade of ovarian neoplasm's and to assess the additional role of IHC expression of these markers in characterizing borderline and malignant cases and in their prognosis over CA-125 levels and histological grade in epithelial ovarian neoplasm and to predict targeted therapy.

OSEC participate in formation of ovarian cortex and it is seen that estrogen and possible progestin via nuclear receptors regulate their normal function[13]. These OSEC are tissue of origin in more than 90% of ovarian cancers. Epidemiological data suggest that endogenous and exogenous sex hormones play important role in pathogenesis of ovarian carcinoma[13].Estrogen taken as oral contraceptives during premenopausal years offer protection, but when used postmenopausal as hormone replacement therapies elevates the risk [13]. Excessive use of hormone replacement therapy and increase is a poor prognostic factor.

There are two isoforms of estrogen receptor, ERα and ERβ which are uniformly expressed in OSEC suggesting that estrogen via ER signaling, may play an important role in regulating normal functions of these cells. Studies show the classical estrogen receptor ERα, and the progesterone receptor (PR) were found in <50% of ovarian carcinoma specimens.[5] Some studies have stated that in normal ovaries, ERα mRNA was the predominant ER form .In benign and borderline tumors, ERβ mRNA was detected in 78% of tumors, whereas ERα mRNA was detected in 29%. In ovarian cancer cell lines ERα mRNA was markedly increased as compared with ER-beta. Thus overexpression of ERα relative to ERβ mRNA may be marker of ovarian carcinogenesis[14].

In relation to type of expression of receptors conflicting results are seen. In a study it was seen that there is loss of ERα but not ERβm RNA expression in ovarian cancer cells[15].Another study demonstrated that higher grade tumors had lower ERα expression[16].In contrast to the above study some authors showed ERα expression was higher in borderline and malignant tumors as compared to benign cases[17]. It has beenreported that benign tumors were negative for steroid receptors and that serous tumors had more expression as compared to mucinous tumors[17].Serous tumors, age >50 years, tumors with ascites and higher CA-125 levels had higher expression of ERα suggesting a mitogenic role for estrogen. Some authors found a decrease in PRA expression with grade 3 and advanced stage tumors [18]. A study showed that expression of PR was markedly down regulated in ovarian cancer cells [19]. This is in contrast to a previous study that showed PRA expression had higher expression in malignant, serous tumors, postmenopausal age group, advanced stage, and grade 3 tumors. However further studies are necessary to reveal the biological significance of ER/PR in ovarian tumorigenesis. This will further help to get a base line data for development of anti hormonal targeted therapy [20].

The HER-2/neu (*c-erbB2*) proto-oncogene encodes a transmembrane receptor protein which is structurally related to the epidermal growth factor receptor. This proto-oncogene is mainly expressed in epithelial tissue and activated due to its amplification [21].Over expression of extracellular domain of HER-2/neu is common as ovarian carcinoma progress. 25% of primary ovarian carcinomas express the HER-2/neu encoded receptor, and, unlike breast cancer, however it is controversial to what extent HER-2/neu amplification and protein overexpression correlates with prognosis. HER-2/neu expression is more frequent in ovarian carcinomas relapsing after chemotherapy [22].

p53 is a tumor suppressor gene and causes cell cycle arrest and apoptosis, located on chromosome 17p 13.1 and is the most common target for genetic alteration in human tumors. Inactivation of p53 a tumor suppressor has been seen to occur frequently in development of ovarian cancer. It has been shown that the p53 gene is mutated in 30-80% of ovarian carcinomas. P53 tumor protein accumulation is a marker of poor prognosis in a subset of patient with ovarian cancer. P53 expression in histologically low grade cancer was associated significantly with an increased risk for cancer relapse and death[23]. Studies have shown mutation in or inactivation of p53 in 46% of invasive ovarian tumors, but in only 8% of borderline (low malignant potential) tumors and virtually nonexistent in benign tumors or normal ovarian epithelium[24]

Ki-67 protein is a cellular marker for proliferation.Ki-67 is an excellent marker to determine the growth fraction of a given cell population. The fraction of Ki-67-positive tumor cells (the *Ki-67 labeling index*) is often correlated with the clinical course of cancer. Ki67 has been extensively studied in carcinomas of the prostate, brain and the breast. [24]Proliferative activity and steroid hormone receptor status along with clinical and morphological characteristics of serous ovarian carcinoma possess prognostic significance and may be used for evaluation of the disease course. We planned a study with the aim to **correlate CA 125 levels and IHC expression of ER/ PR, HER-2/neu, p53 and ki67 markers with various histological types and grades of ovarian neoplasms and their role in prognosis.**

Material and Methods:-

The present study was undertaken with aim to correlate serum CA-125 levels and IHC expression of ER/PR, HER2/neu, p53 and Ki67 markers with various histological types and grades of ovarian neoplasms and their role in prognosis.

Prospective histologically diagnosed epithelial ovarian neoplasm reported at Era's Lucknow Medical College & Hospital and other collaborating hospitals consenting to participate in this study.The blood samples were used for estimating serum CA-125 levels. Ovarian biopsy/excised tumors: The ovarian tumors were used for histopathological and immunopathological studies. Any case which have any other associated malignancy even gynecological malignancies besides ovarian cancer in present or past or any case other than epithelial ovarian neoplasm of ovary were excluded from the study.

Once ovarian sample was taken it was further subjected to routine H&E staining and immunohistochemical procedures. Specimen were grossed and processed including dehydration in increasing gradients of ethyl alcohol followed by cleaning in xylene and embedding in paraffin wax. Sections were cut and dewaxed and stained by H&E for routine histological diagnosis. Histopathological neoplastic lesions were classified into benign, borderline and malignant epithelial ovarian tumor (serous, mucinous, Brenner's, endometrioid and clear cell). Cases were subjected for immunohistochemistry for ER/PR, HER2/neu, p53 and Ki67 markers.

Immunohistochemical Evaluation:-

Immunohistochemistry was performed on formalin fixed paraffin embedded tissue blocks with adequate tumor. IHC was done by streptavidin biotin method as per protocol standardized in our laboratory. Primary antibody Monoclonal Mouse Anti-Human ER, PR, HER-2-neu, p53 ready to use from DAKO North America, Inc. Carpinteria, CA, USA and secondary antibody used was Dako REAL EnVision, HRP RABBIT / MOUSE (ENV)from DAKO North America, Inc. Carpinteria CA USA. Sections of 3um were cut and taken on poly L – Lysine coated slides. Slides were dried for 16 hours at 37oC followed by 1 hour at 60oC. Sections were deparaffinised by heating on the slide warming table at 60oc for 15-20 minutes and then passed through two changes of xylene for 5 minutes each. Sections were rehydrated by taking them through 3 changes of 99% alcohol for 5 minutes each, followed by 95% and 70% alcohol for 5 minutes each. Sections were then brought down to water for 10 minutes. Antigen retrieval was done by placing the slides in a coplin jar containing citrate buffer and processed in a microwave. Lid were placed on the coplin jars and heated to the maximum effect i.e. 800W till the time the fluid boils and then the microwave oven was set at mid effect i.e. 400W for 15 minutes. Containers were placed in a gentle water rinse for 5 minutes. Washed in Tris Buffer Saline (3 changes). Sections were treated with 3% H2O2 solution for 10 minutes to quench endogenous peroxidase activity. Washed in Tris Buffer Saline (3 changes) and incubated with primary antibody for 1 hour. Again washed with Tris Buffer Saline for (3 changes) and incubated with secondary antibody for 30 mins. Sections were washed in 3 changes of Tris Buffer Saline (TBS) for 5 minutes each and then covered with freshly prepared DAB chromogen solution for 1 minutes. The slides were then washed with water and counterstained with Harris hematoxylin for 1 minute, washed gently under running water. The sections were dehydrated, dipped in xylene bath and later were mounted using DPX, a non aqueous mounting medium. A positive and negative control was run with each batch of IHC staining.Positive control and negative control were run with each batch of staining to validate the result

Interpretation of IHC Staining:-

In each tissue section 10 representative fields were selected for ER, PR, HER2/neu, p53, Ki67. Average number of positive tumor cells were expressed as percentage/proportion and intensity of staining of each cell was noted. Positive reaction was indicated by a brown colored precipitate in the nucleus of the epithelial cells of ovary in cases

of ER/PR, p53, Ki67. The HER2/neu positive reaction was indicated by brown colored cell membrane (membranous positivity) of epithelial cells of ovary. The absence of staining reaction was interpreted as negative result. Results are considered valid if positive control specimen shows brown color at the antigenic site and the negative control specimen does not show brown color.

Er/Pr Interpretation:-

Proportion Score (PS)*	PS Observation		IntensityScore (IS)**	IS Observation
0	None		0	None
1	>0 to 1/100		1	Weak
2	>1/100 to 1/10		2	Intermediate
3	>1/10 to 1/3		3	Strong
4	>1/3 to 2/3			
5	>2/3 to 1			

Total Score = PS+IS

Each **Proportion Score** encompasses a range represented by a whole number.

Total Score (TS)***	PS Observation
0, 2	Negative
≥ 3	Positive

* Proportion of tumor cells with positive nuclear staining

** Average intensity of all positive tumor cells

*** Sum of Proportion Score (PS) and Intensity Score (IS)

A) HER2/neu INTERPRETATION

Score	HER2/neu overexpression	Staining pattern
0	Negative	No staining is observed ,or membrane staining is observed in <10% of tumor cell
1+	Negative	Faint/barely perceptible membrane staining is detected in >10% tumor cell. Incomplete membrane staining
2+	Weakly positive (equivocal)	A weak to moderate complete membrane staining in >10% of tumor cell
3+	Strongly positive	A strong complete membrane staining is observed in >10% of tumor cell.

B) p53/Ki67 INTERPRETATIONError! Bookmark not defined..

Number of cells with nuclear positivity	Score
0	0
1-10%	1
10-50%	2
50-100%	3

The Ki67 staining reaction was considered positive only in the presence of immunostained nuclei in brown shades. A total of 25 microscopic fields were examined for Ki67 scoring. For each microscopic field, scoring was done using the following criteria (Giurgea et al., 2012) Quantitative assessment was done according to the number of stained cells:

- 0: score 0;
- 1-10%: score 1;
- 10-50%: score 2;
- 50-100%: score 3

For final score, the sum of 25 microscopic fields was taken in order to express the final score out of 100. The obtained score was expressed as % expression.

***METHOD FOR ELISA* for estimating serum CA-125 levels:-**

Components OF CALBIOTECH CA125 ELISA kit includes 96 microwells coated with Murine Monoclonal anti-CA 125, CA125 reference standards: 6 vials (ready to use), Enzyme conjugate Reagent, TMB Reagent, Stop

solution, Wash concentrate. Other material needed were Micropippet calibrated to deliver 50µ, 200µ, 500µ and disposable tips, Distilled water, Elisa washer - BIORAD PW40, Elisa reader- BIORAD imark microplate reader for absorbance at 450nm, MPM 6 software BIORAD for calculation of result. Storage of all reagents were stored at 2-8ºC. Working Wash buffer was prepared by diluting the concentrate 1:20 with distilled water (25 ml of wash buffer in 475 ml of distilled water).

Experimental Procedure:-

Bring all specimens and kit reagents to room temperature and gently mix. Secure the desire number of coated wells in the holder. Dispense 100 µl of CA125 standards, specimen, and controls into the appropriate wells. Dispense 100 µl enzyme conjugate reagent into each well. Mix gently for 30 sec. Incubate at 37ºC for 90 minutes. Remove liquid from all wells. Wash wells 3 times with 300µl of working wash buffer blot on absorbance paper or paper towel. Dispense 100 µl of TBM reagent into each well. Gently mix for 10 sec. Incubate at room temperature in dark for 20 min. Stop the reaction by adding 100 µl of stop solution to each well. Gently mix for 30 sec. it is important to make sure that all blue color changes to yellow color completely. Read the optical density at 450 nm with a microtitre plate reader within 15 minutes.

Follow up:-

- Serum CA1-25 levels before and after treatment, at 20th day and subsequently after three months was evaluated. Radiological imaging of thorax and abdomen before and after three months was done.
- The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical Analysis Software.
- There was significant higher expression of ER in serous ovarian neoplasm and among malignant cases with grade 3 tumors. However no association was found with neoplastic status and stage of tumor.
- There was significant higher expression of PR in serous ovarian neoplasm and neoplastic status and in grade III tumors. However no significant association was found in type of tumor and stage of tumor.
- There was significant higher expression of HER2/neu with neoplastic status only. However no significant association was found in type of tumor, grade and stage of tumor.
- Proportional differences in p53 expression and score of cases with different malignancy status was found to be statistically highly significant ($p<0.001$) & statistically significant ($p=0.008$) with grade of tumor.
- Proportional difference in Ki67 expression in patients of different histological type ($p=0.048$), status ($p<0.001$) & grade ($p<0.002$) was found to be statistically significant.

Result:-

Total	**40**		**14**	**12**						
N=66	**ER +**	**PR+**	**HER2/neu**	**P53 score**			**Ki 67**			
				0	2	3	0	1	2	3
Type	n=14	n=12	n=8	n=55	n=3	n=8	n=54	n=2	n=2	n=8
Serous(33)	13(39.4%)	11(33.3%)	7(21.21%)	24(72.7%)	2(6.1%)	7(21.2%)	23(69.7%)	2(6.1%)	1(3%)	7(21.2%)
Mucinous(25)	1(4%)	1(4%)	1(4%)	24(96%)	1(4%)	0(0%)	24(96%)	0(0%)	1(4%)	0(0%)
Transitional(4)	0(0%)	0(0%)	0(0%)	4(100%)	0(0%)	0(0%)	4(100%)	0(0%)	0(0%)	0(0%)
Clear cell(2)	0(0%)	0(0%)	0(0%)	1(50%)	0(0%)	1(50%)	1(50%)	0(0%)	0(0%)	1(50%)
Endometroid(2)	0(0%)	0(0%)	0(0%)	2(100%)	0(0%)	0(0%)	2(100%)	0(0%)	0(0%)	0(0%)
	P=0.001	**P=0.036**	**P=0.266**	**P=0.068**			**P=0.048**			
Status(n=66)	n=14	n=12	n=8	n=55	n=3	n=8	n=54	n=2	n=2	n=8
Benign(37)	4(10.8%)	2(5.4%)	1(2.7%)	37(100%)	0(0%)	0(0%)	37(100%)	0(0%)	0(0%)	0(0%)
Borderline	1(25%)	1(25	0(0%)	4(100	0(0%)	0(0%)	4(100	0(0%)	0(0%)	0(0%)

(4)		%)		%)			%)			
Malignant(25)	9(36%)	9(36%)	7(28%)	14(56%)	3(12%)	8(32%)	13(52%)	2(8%)	2(8%)	8(32%)
	P=0.058	**P=0.009**	**P=0.008**	**P<0.001**			**P<0.001**			
Grade (n=25)	n=9	n=9	n=7	n=14	n=3	n=8	n=13	n=2	n=2	n=8
I(7)	1(14.3%)	1(14.3%)	1(14.3%)	4(57.1%)	2(28.6%)	1(14.3%)	5(71.4%)	1(14.3%)	1(14.3%)	0(0%)
II(7)	1(14.3%)	1(14.3%)	3(42.9%)	7(100%)	0(0%)	0(0%)	6(85.7%)	1(14.3%)	0(0%)	0(0%)
III(11)	7(63.6%)	7(63.6%)	3(27.3%)	3(27.3%)	1(9.1%)	7(63.6%)	2(18.2%)	0(0%)	1(9.1%)	8(72.7%)
	P=0.039	**P=0.039**	**P=0.491**	**P=0.008**			**P=0.002**			
Stage (n=25)	n=9	n=9	n=7	n=14	n=3	n=8	n=13	n=2	n=2	n=8
I(5)	1(20%)	1(20%)	0(0%)	4(80%)	0(0%)	1(20%)	3(60%)	0(0%)	1(20%)	1(20%)
II(6)	1(16.7%)	0(0%)	2(33.3%)	4(66.6%)	1(16.6%)	1(16.6%)	5(83.3%)	1(16.7%)	0(0%)	0(0%)
III(12)	5(41.7%)	7(58.3%)	4(33.3%)	6(50%)	2(16.6%)	4(33.33%)	5(41.7%)	1(8.3%)	1(8.3%)	5(41.7%)
IV(2)	2(100%)	1(50%)	1(50%)	0(0%)	0(0%)	2(100%)	0(0%)	0(0%)	0(0%)	2(100%)
	P=0.154	**P=0.082**	**P=0.443**	**P=0.191**			**P=0.073**			

N=Total no. of epithelial ovarian cases
n=Total no. of positive cases

ER expression:-
ER positive expression was found to be significantly higher in Serous type of epithelial ovarian neoplasm (p=0.001). Though proportion of ER positive expression was higher in Malignant (36.0%) and Borderline cases (25.0%) s compared to Benign (10.8%) cases. Difference in prevalence of ER positive expression with malignancy status was not found to be statistically significant (p=0.058).

ER expression was positive in higher proportion of histopathological Grade III cases (63.6%) as compared to Grade II (14.3%) and Grade I (14.3%) cases. Proportional difference in ER positive expression in patients of different histopathological grades was found to be statistically significant (p=0.039).

ER expression was positive in all the cases of Stage IV (n=2; 100.0%). Apart from Stage IV, ER expression was positive in higher proportion of Stage III (41.7%) followed by Stage II (16.7%) and Stage I (13.0%). Proportional difference in ER positive expression in patients of different clinical stage was not found to be statistically significant (p=0.154).

There was significant higher expression in serous ovarian neoplasm and among malignant cases with grade 3 tumors. However no association was found with neoplastic status and stage of tumor.
Pr Expression:-
Proportion of PR negative expression were higher in Serous (33.3%) and Mucinous (4.0%) cases, and this difference was not found to be statistically significant (p=0.036).

Prevalence of PR positive expression was higher in Malignant cases (36.0%) as compared to Borderline (25.0%) and benign cases (5.4%) and difference in prevalence of PR expression in different status of malignancy was found to be significant (p=0.009).

Prevalence of PR positive expression was higher in histopathological Grade III cases (63.6%) as compared to Grade II (14.3%) and Grade I (14.3%) cases. Proportional difference in prevalence of PR positive expression in patients of different histopathological grades was found to be statistically significant (p=0.039).

No statistically significant association of PR positive expression with clinical staging was found (p=0.082).

There was significant higher expression of malignant cases in neoplastic status and in grade III tumors. However no significant association was found in type of tumor and stage of tumor.

Serum CA-125 levels:-

Serum CA-125 levels ranged from 6.8 to 1440 U/ml with a mean value of 230.7±342.7 U/ml. A total of 32 (48.5%) patients had serum CA-125 levels above 35 U/ml. Mean serum CA-125 levels were found to be maximum in malignant cases (487.46±345.73 U/ml) followed by benign lesions (75.55±241.84 U/ml). Mean CA-125 levels were found to be minimum in cases diagnosed as borderline (61.00±34.97 U/ml).

With increasing histopathological grade, an increase in mean serum CA-125 levels was observed but it was not significant statistically (p=0.088). ER positive expression was found to be significantly higher in Serous type of epithelial ovarian neoplasm (p=0.001).

ER Expression:-

Though proportion of ER positive expression was higher in Malignant (36.0%) and Borderline cases (25.0%) as compared to Benign (10.8%) cases. Difference in prevalence of ER positive expression with malignancy status was not found to be statistically significant (p=0.058).

ER expression was positive in higher proportion of histopathological Grade III cases (63.6%) as compared to Grade II (14.3%) and Grade I (14.3%) cases. Proportional difference in ER positive expression in patients of different histopathological grades was found to be statistically significant (p=0.039). ER expression was positive in all the cases of Stage IV (n=2; 100.0%). Apart from Stage IV, ER expression was positive in higher proportion of Stage III (41.7%) followed by Stage II (16.7%) and Stage I (13.0%). Proportional difference in ER positive expression in patients of different clinical stage was not found to be statistically significant (p=0.154).

PR Expression:-

Proportion of PR negative expression were higher in Serous (33.3%) and Mucinous (4.0%) cases, and this difference was not found to be statistically significant (p=0.036). Prevalence of PR positive expression was higher in Malignant cases (36.0%) as compared to Borderline (25.0%) and benign cases (5.4%) and difference in prevalence of PR expression in different status of malignancy was found to be significant (p=0.009).

Prevalence of PR positive expression was higher in histopathological Grade III cases (63.6%) as compared to Grade II (14.3%) and Grade I (14.3%) cases. Proportional difference in prevalence of PR positive expression in patients of different histopathological grades was found to be statistically significant (p=0.039). No statistically significant association of PR positive expression with clinical staging was found (p=0.082).

Her2neu Expression:-

Positive HER2/neu expression was found in only 8 cases. Proportional difference in HER2/neu expression in patients of different histological type was not found to be statistically significant (p=0.266).

Proportional differences in HER2/neu expression of cases with different malignancy status was found to be statistically significant (p=0.008). Proportional differences in HER2/neu expression of cases with different histopathological grades was not found to be statistically significant (p=0.491).

Proportional differences in HER2/neu expression of cases with different clinical stages was not found to be statistically significant (p=0.443). Proportional difference in p53 expression and score in patients of different histological type was not found to be statistically significant (p=0.068).

p53 Expression:-

Proportional differences in p53 expression and score of cases with different malignancy status was found to be statistically highly significant (p<0.001). Proportional differences in p53 expression score of cases with different

histopathological grades was found to be statistically significant (p=0.008).Proportional differences in P53 expression of cases with different clinical stages was not found to be statistically significant (p=0.191).

Ki67 Expression:-
Proportional difference in Ki67 expression in patients of different histological type was found to be statistically significant (p=0.048). Proportional differences in Ki-67 expression of cases with different malignancy status was found to be statistically significant (p<0.001).

Proportional differences in Ki-67 expression and score of cases with different histopathological grades was found to be statistically significant (p=0.002). Proportional differences in Ki-67 expression of cases with different clinical stages was not found to be statistically significant (p=0.073).

Follow-up:-
Malignant cases, post-op were subjected to chemotherapy and kept for follow up. CA-125 levels at baseline ranged between 6.8 U/ml and 1440.0 U/ml and mean levels were found to be 230.70±342.65 U/ml.
On follow up of serum CA-125 values at 20th post-operative day. Among the malignant cases (n=25) 18 cases had serum CA-125 levels <35 U/ml while 7 cases had serum CA-125 levels >35 U/ml. Follow up at 3rd month post-operative, out of 7 cases, all cases were subjected to serum CA-125 level and radiological assessment via X-Ray chest and ultrasonography abdomen. There was no significant radiological finding in any case at 3rd month postoperative. Out of seven cases, in five cases serum CA-125 level returned to normal (<35 U/ml). However in 2 cases (both of stage IV) serum CA-125 levels were 96 U/ml and 74 U/ml respectively.

Discussion:-

Epithelial ovarian neoplasms have a high probability of converting into malignant tumors and hence an early diagnosis and intervention is key to successful management and treatment. Hence, the present study was carried out with an aim to find out a correlation between histological grade, serum CA-125 levels and IHC expression of ER/PR, HER2/neu, p53 and Ki67 markers in epithelial ovarian neoplasms in order to identify them properly and provide a basis for treatment planning. For this purpose, a total of 66 epithelial ovarian neoplasm specimens obtained from patients admitted to Department of Obstetrics & Gynaecology and Oncosurgery were subjected to evaluation.

Among the specimens obtained serous neoplasms were most common (50%) followed by mucinous (37.9%) neoplasms. Transitional, clear cell and endometrioid neoplasms comprised 6%, 3% and 3% of total specimens respectively. Serous type is reported to be the most common ovarian neoplasm [25,26,27] In the present study too it was the most common type encountered. Although most of the studies analyzing the global burden of ovarian cancer place mucinous type as the uncommon type [28] however, a systematic review analyzing the global distribution pattern of histological subtypes of epithelial ovarian cancer has shown South Africa, Greece, and India as the three highest countries; for mucinous subtype [29].

Table 1 below shows the distribution of different histopathological types of neoplasms reported in some contemporary studies:

Table 1:- Distribution of different histopathological types of ovarian neoplasms reported in some contemporary studies from Asian countries[@]

SN	Author (Year)	N	Serous	Mucinous	Others
1.	Jha and Karki (2008)	161	77 (47.8%)	50 (31.1%)	34 (21.1%)
2.	Mondal *et al.* (2011)	702	447 (63.68%)	158 (22.51%)	97 (13.8%)
3.	Abdullah *et al.* (2012)	84	28 (33.3%)	13 (15.4%)	43 (51.2%)
4.	Jindal (2014)	53	23 (43.4%)	3 (5.7%)	27 (50.9%)
5.	Vaidya *et al.* (2014)	363	158 (43.5%)	92 (25.3%)	113 (31.1%)
6.	Makwana *et al.* (2014)	135	60 (44.4%)	18 (13.3%)	57 (42.2%)

A perusal of Table 1 above shows that all the contemporary case series from Asian countries depict serous and mucinous types to be most common. The findings in present study also followed a similar pattern. In present study, majority of neoplasms were diagnosed as benign (56.1%). A total of 4 (6.1%) were diagnosed as borderline and remaining 25 (37.9%) were diagnosed as malignant. Thus the malignancy rate was 37.9%.

Epithelial ovarian lesions are the biggest contributor to the burden of total primary malignant ovarian tumors [1]. Malignancy rate is slightly higher in Asian countries where in the absence of a regular screening programme, progression to malignancy is higher. A number of studies have supported this view. Table D2 shows distribution pattern of ovarian epithelial tumors in different case series specifically from South Asia:

Table 2:- Distribution of different clinical types of ovarian neoplasms reported in some contemporary studies from Asian countries

SN	Author (Year)	N	Malignant	Borderline	Benign
1.	Jha and Karki (2008)	161	26 (16.1%)	0	135 (83.9%)
2.	Mondal *et al.* (2011)**Error! Bookmark not defined.**	702	225 (32.1%)	70 (10.0%)	407 (58.0%)
3.	Khatri (2011)	56	18 (32.1%)	2 (3.6%)	36 (64.3%)
4.	Abdullah *et al.* (2012)**Error! Bookmark not defined.**	84	84 (22%)	20 (5.2%)	278 (72.8%)
5.	Jindal (2014)	53	10 (18.9%)	0	43 (81.13%)
6.	Iqbal *et al.* (2013)	63	24 (38.1%)	0	39 (61.9%)
7.	Vaidya *et al.* (2014)	158	28 (17.7%)	13 (8.2%)	117 (74.1%)
8.	Makwana *et al.* (2014)	140	27 (19.3%)	5 (3.57%)	108 (77.1%)
9.	Present study (2014)	66	25 (37.9%)	4 (6.1%)	37 (56.1%)
10	Naik et al(2016)	82	41(50%)	4(5)%	437(5.1%)

Out of nine case series in Table 2, a total of 4 series have shown the malignancy rate between 30 and 40 percent. The malignancy rate in different series ranged from 16.1 to 38.1%. In all the series borderline tumors were least commonly found with its prevalence ranging from 0% to 10%. Rate of benign pathologies was minimum in present study (56.1%) and maximum in a review series (83.9%)[29]. In general, all the series have shown maximum ovarian pathologies to be benign and minimum to be borderline.

In present study, majority of specimens obtained were from women above age of 40 years (n=38/66; 57.6%). Among these, 23 were malignant (60.53%), 3 were borderline (7.89%) and remaining 12 (31.58%) were benign. In the remaining 28 cases, with age <40 years, only 2 (7.1%) were malignant, 1 (3.6%) was borderline and remaining 25 (89.3%) were benign. These findings are similar to some studies which reported that 36/56 (64.3%) of their cases to be aged >40 years and 16/36 (44.4%) of these to be malignant as compared to only 2/20 (10%) of those aged <40 years[30]. In another study, it was found that the malignancy rate in ovarian neoplasms to be increasing from 15.8% in cases <50 years of age to 49.3% in cases above 50 years of age[31]. A study reported 76/158 (48.1%) of their cases to be <40 years of age and found 8/76 (10.5%) of them to be malignant as compared to 24/82 (29.3%) of those aged >40 years.[32] All these studies indicate that age is a significant predictor of malignancy and with increasing age malignancy rate increases substantially. In present study, out of 25 malignant cases, a total of 11 (44%) were histopathologically diagnosed as Grade III and 7 (28%) each were diagnosed as Grades I and II. These findings are similar to another [30] who also found 60% of malignant ovarian neoplasms to be grade III and 20% each to be grade II and I respectively.

Serum CA 125 Levels in Epithelial Ovarian Neoplasms:-

In present study Serum CA-125 levels of malignant neoplasms were found to be 487.46±343.73 U/ml (range 29.4-1120 U/ml) as compared to 61.00±34.97 (range 26.0-94.0 U/ml) and 75.55±241.84 U/ml respectively in borderline and benign neoplasms. Thus showing that serum CA-125 levels in malignant neoplasms were significantly higher as compared to those in benign and borderline tumors. With respect to association between serum CA-125 levels and histopathological grades of malignant tumors too, an incremental trend in mean serum CA-125 levels was observed with increasing grade of malignancy, however, this association was not significant statistically, owing to fewer number of cases in each grade. These observations are similar to another who also observed CA-125 levels to be significantly higher in malignant as compared to benign and higher grades as compared to lower grades of malignancy [33]. In another study it was seen that serum CA-125 levels of malignant epithelial ovarian tumors to be significantly higher. We encountered some extreme values for CA-125 in benign cases [34]. These might be a precursor of a malignancy in next few months. In a more recent study, they saw that a significant association between histological grade and CA-125 levels of 87 cases of epithelial ovarian carcinoma[35].

ER in Epithelial Ovarian Neoplasms:-
In present study, ER positivity was observed in 14/66 (21.2%) cases. ER positivity was found to be higher in serous types (39.4%) as compared to mucinous type (4.0%). None of the cases with transitional, clear cell and endometroid types were ER positive. Association between histopathological type was found to be significant statistically. In a study of 155 patients with ovarian cancer found the ERα positivity rate of 31.4%.[36] Another study of 37 epithelial ovarian tumors found the positivity rate to be 21.6% which is similar to that reported in present study[37]. The findings in general indicate that positivity rate among epithelial ovarian tumors is around 20-30% and it is predominantly in serous types as compared to other types.

If we relate the estrogen positivity rate with malignancy rates then we find that ER positivity rate was higher in malignant and borderline cases (10/29; 34.5%) as compared to benign (4/37; 10.8%), thus showing a significant difference (p=0.020). However, on evaluating the sensitivity and specificity of ER for diagnosis of malignancy it was found to be only 34.5% sensitive but 89.2% specific, thus showing it to be a specific rather than sensitive tool for diagnosis of malignancy (including borderline cases). Another study also found the positivity rate of ER to be 38% in malignant cases.[38] All these findings imply that ER positivity is specific in nature and findings of our study also supported the same point of view.

In present study a significant increase in ER expression rate was observed with increasing grade of malignancy. Although a similar trend was also observed for clinical stage but the association was not found to be significant statistically.

PR in Epithelial Ovarian Neoplasm:-
With respect to PR positivity, the positivity rate was 12/66 (18.2%) cases. This expression was also observed to be more common in serous type (11/33; 33.3%) as compared to mucinous and other types (1/25; 4.0%), thus indicating that like ER, they are also more common in serous types as compared to other histopathological types, were present in only 2/37 (5.4%) of benign tumors as compared to 10/29 (34.5%) of borderline/malignant tumors.

In the present study, PR positivity rates were found to be higher in histopathological grade 3 (63.6%) as compared to grades 1 and 2 (14.3% each) and this association was found to be significant statistically (p=0.039). However, higher clinical stages were not found to be significantly associated with higher PR positivity (p=0.082). These observations are in agreement with the observations of another study that also showed a higher PR expression in malignant, serous tumors and grade 3 tumors. In present study though PR expression was higher in advanced clinical stages as compared to early stages yet the difference could not be proven significantly owing to fewer number of cases in clinical stage IV (n=2).[39]

HER2/neu in Epithelial Ovarian Neoplasms:-
Similar to ER/PR expression, HER2/neu expression also followed the same trend with a positivity rate of 8/66 (12.1%) and higher expression in serous (21.21%) as compared to mucinous (4%) tumors, was more common in malignant (28%) as compared to benign (2.7%) and borderline (0%) cases (p=0.008). In the present study, no significant association between HER2/neu and histopathological grading and clinical staging of tumor was observed (p=0.491; p=0.433), probably owing to fewer number of cases.

HER2/neu expression has a limited discriminant role as it has also been reported to express in majority of normal ovaries and may be rarer in ovarian carcinoma cases[40,41,42]. 25% of primary ovarian carcinomas express the HER2/neu encoded receptor, and, unlike breast cancer, it is controversial to what extent HER2/neu amplification and protein overexpression correlates with prognosis. However it has been reported that HER2/neu expression is more frequent in ovarian carcinomas relapsing after chemotherapy**Error! Bookmark not defined.**. The findings in present study supported these observations. This kind of expression indicated that HER2/neu was neither sensitive nor specific for diagnosis of malignancy in ovarian epithelial neoplasia.

p53 in Epithelial Ovarian Neoplasms:-
In the present study, p53 expression was observed in only 11/66 (16.7%) cases. It was expressed only in malignant cases. However, among malignant cases too, the expression was only 25% (11/25 cases), thus showing it to be a specific marker rather than being a sensitive marker. The expression rate was higher in grade III (8/11; 72.7) as

compared to grades I and II (3/14; 21.4%) and did not correlate with the clinical staging. A study reported only 44% of ovarian epithelial carcinoma cases to be positive. Similar to our study, they also did not reveal a significant association between p53 expression and clinical stage. The findings of present study reaffirmed the findings of previous studies that the p53 gene is mutated in 30-80% of ovarian carcinomas [43].

In present study, we observed p53 expression rate to be 27.3% in serous type and 4% in mucinous type. We utilized a scoring criteria for the purpose of differentiation, however, as most of the p53 positive cases had higher score (8/11; 72.73%), it was only of limited help owing to fewer number of cases.

Ki67 in Epithelial Ovarian Neoplasms:-

In present study, Ki67 expression was observed in 12/66 (18.2%) cases. The expression was higher in serous 10/33 (30.3%) as compared to mucinous 1/25 (4%) and other types 1/8 (12.5%) cases. All the Ki67 positive findings were obtained in malignant tumors only (p<0.001), predominantly in grade III (9/12; 75%) (p=0.002) and in clinical stages 3 and 4 (9/12; 75%). It was observed that 9/11 (81.8%) of Grade III and 9/14 (64.3%) of Clinical stages 3 and 4 were positive for Ki67 as compared to 3/14 (21.4%) of Grades I and II and 3/11 (27.3%) of Clinical stage 1 and 2. These findings indicated that Ki67 was highly specific for malignancy and serous types, sensitive as well as specific for high histopathological grade and clinical stage. With respect to higher overexpression and in malignant and serous subtypes, our findings are in agreement with the observations of other authors. [44,45]

In a study done by Naik et al. 2016 [46] they saw that the expression of ER was more in malignant tumors (13/16, 81.25%) than borderline (9/12, 75%) and benign (20/82, 24.39%). The expression of PR was more in benign (51/82, 62.19%) than borderline (8/12, 66.67%) and malignant tumors (9/16, 56.25%) as compared to ER. The PR expression was more in benign tumors than borderline and malignant tumors. The expression of p53 was less in benign (5/82, 6.1%) than borderline (9/12, 75%) and malignant tumors (13/16, 81.25%). While the expression of Ki-67 was more in malignant (4/82, 4.88%) than borderline and benign tumors . There was statistically significant difference in the expression of ER, PR, p53, and Ki-67 in the patients with age <40 years and above 40 years (P = 0.912). In the case of serous tumors, ER was expressed in all high- and low-grade tumors. The expression of PR was more in low-grade tumors than high-grade ones. P53 expression was seen in all high-grade tumors and 33.34% of low-grade tumor. The Ki-67 Li was more in high-grade tumors than low-grade tumors. Expression of ER, p53, and Ki-67 was higher in tumor showing metastasis. The mean Ki-67 Li was also higher in metastasizing tumors. However, PR expression was less in metastasizing tumors than non metastasizing tumors.

Follow Up:-

On follow up of serum CA-125 values at 20th post-operative day. Among the malignant cases (n=25) 18 cases had serum CA-125 levels <35 U/ml while 7 cases had CA-125 levels >35 U/ml. Follow up at 3rd month post-operative, out of 7 cases, all cases were subjected to serum CA-125 level and radiological assessment via X-Ray chest and ultrasonography abdomen. Out of seven cases, in five cases CA-125 level returned to normal (<35 U/ml). However in 2 cases (both of stage IV) CA-125 levels were 96 U/ml and 74 U/ml respectively. There was no significant radiological finding in any case. A study observed significant decrease irrespective of tumor stage and grade only two weeks after the surgery in 50 patients with epithelial ovarian cancer[47]. In another study, it was observed a significant fall in postoperative CA-125 levels in 56% of ovarian cancer patients within first week after the surgery. Some of the authors have also evaluated the significance of preoperative CA-125 and postoperative decline in CA-125 levels as a prognostic marker [48].In present study, we observed that none of the markers used by us except serum CA-125 were capable of differentiating completely between malignant and benign/borderline cases. Serum CA-125 proved to be an efficient marker for malignancy. A number of studies have indicated use of all of these markers for prognostic rather than diagnostic purposes, however, for the purpose of diagnosis, none of these markers, except serum CA-125, has a practical accuracy when used alone. The combined use of these markers might be of use for which larger studies are required to substantiate these preliminary finding.

IMAGES IN TIFF FORMAT

Figure1a:- Gross of clear cell ovarian carcinoma

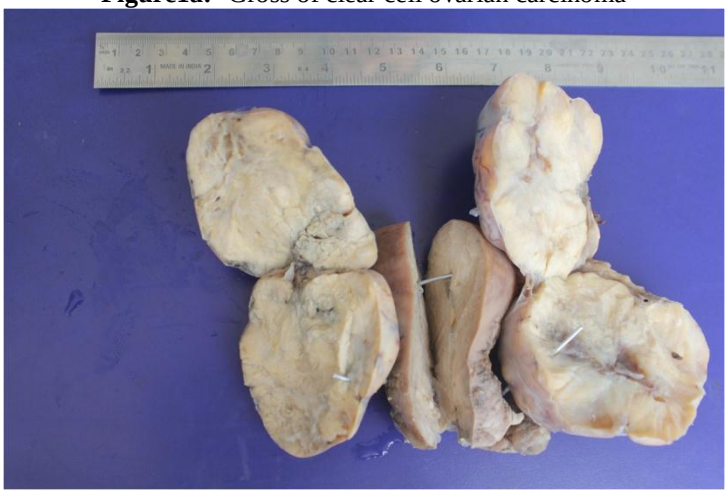


Figure 1b:- Gross of mucinous adenocarcinoma ovary.

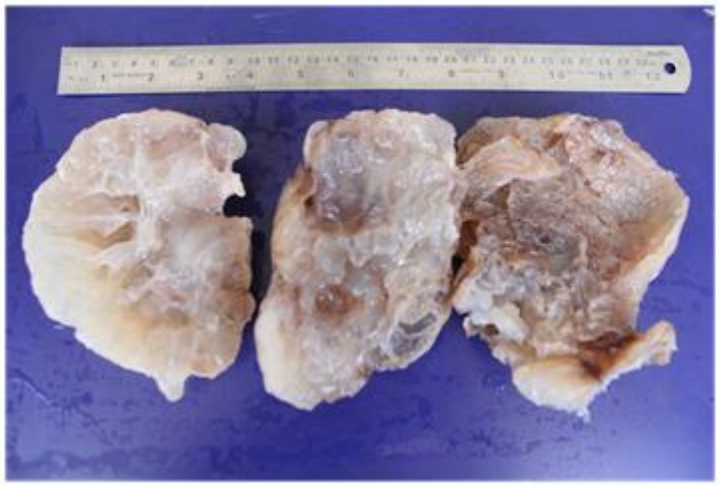


Figure 1d:Gross of Transitional cell carcinoma ovary

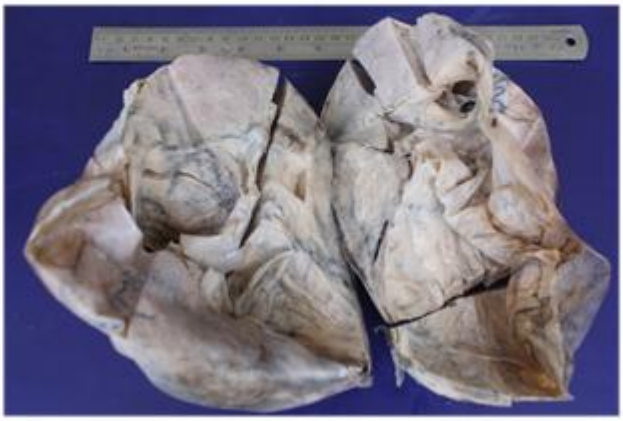


Figure1c:Gross of serous adenocarcinoma ovary

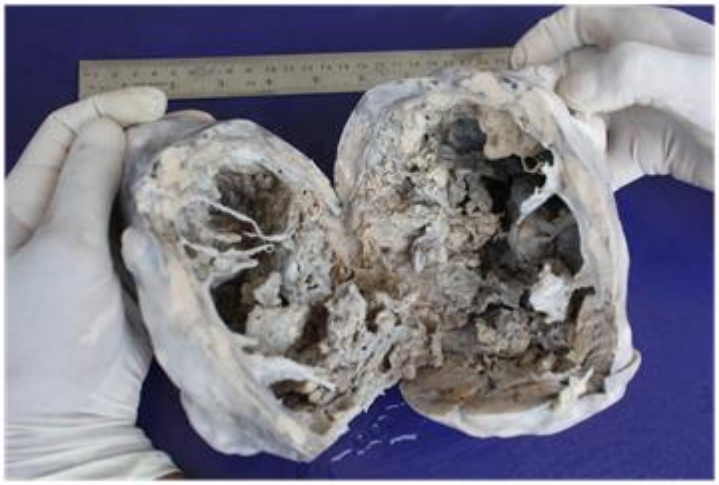


Figure 2b: Microscopy of clear cell ovarian carcinoma(H&E;400X)

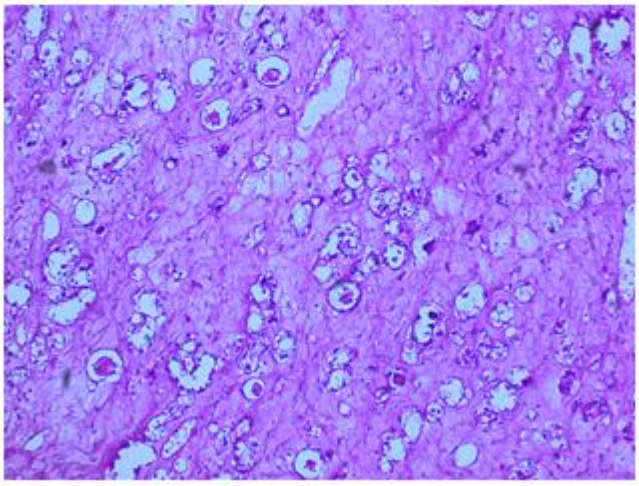


Figure 2a:Microscopy clear cell ovarian carcinoma(H&E;100X)

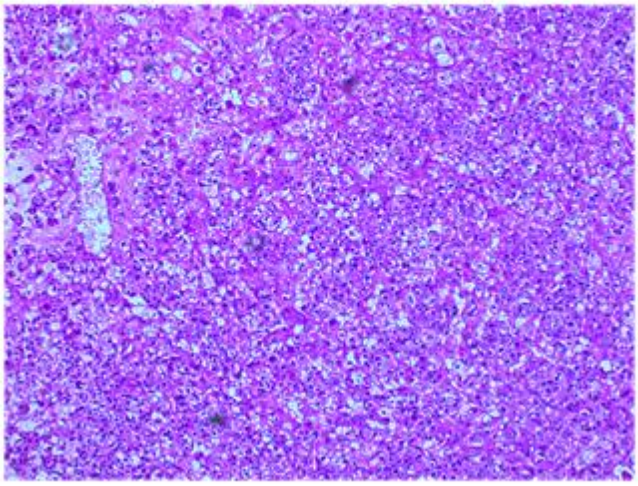


Figure2c:Microscopy of mucinous carcinoma ovary (H&E;100x)

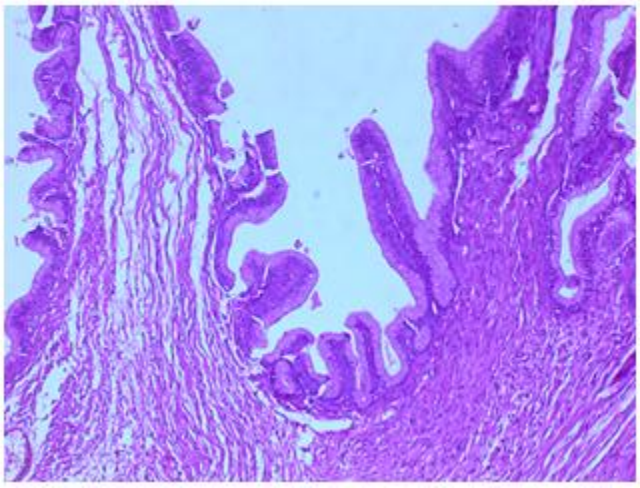


Figure2d: Microscopy of serous carcinoma ovary showing psammoma bodies (H&E;400X)

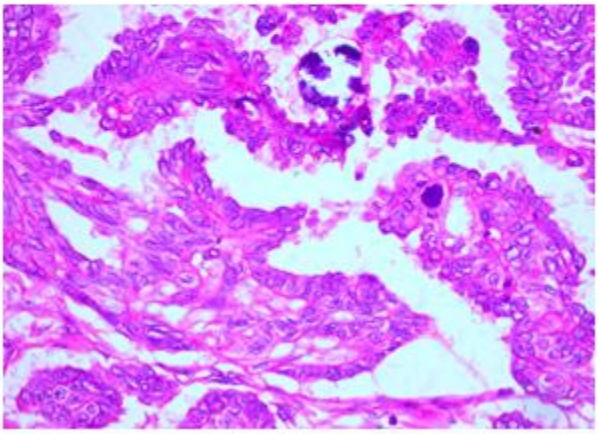
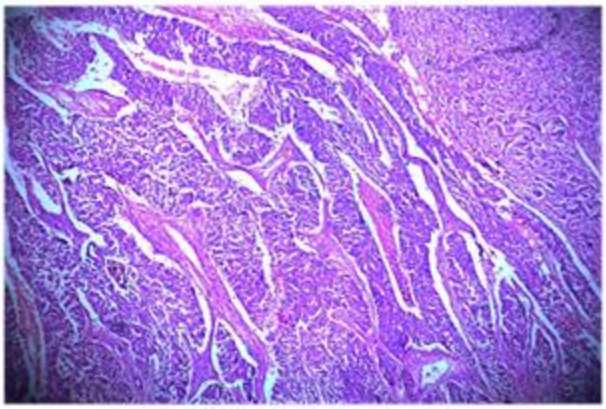
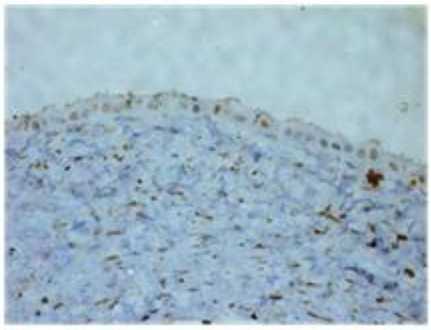


Figure 2e:Microscopy of Transitional Cell Carcinoma ovary(H&E;100X)

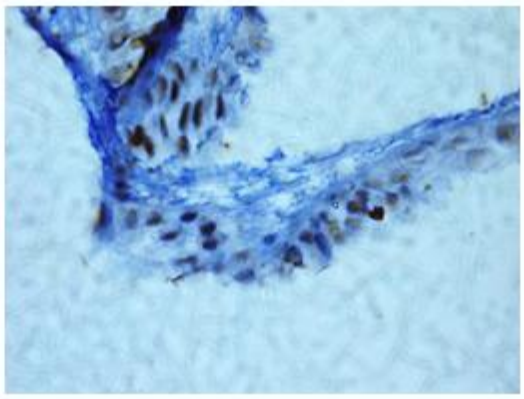


IHC

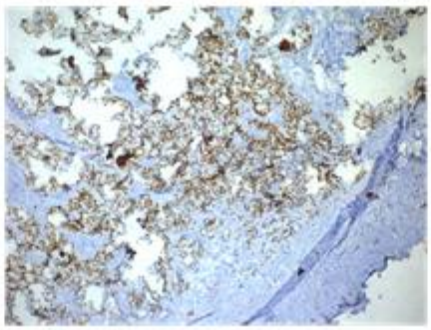
- Figure 3a:Benign Serous Ovarian Tumor showing positive nuclear stain(IHC for ER; 400x)



- Figure 3b: Borderline Serous Ovarian Tumor showing positive nuclear stain(IHC for p53; 400x)



- Figure 3c: Serous Carcinoma Ovary showing nuclear positivity (ER;100x)



- Figure 3c: Serous Carcinoma Ovary showing nuclear positivity (ER;100x)

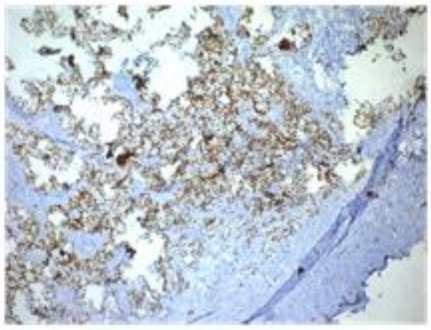
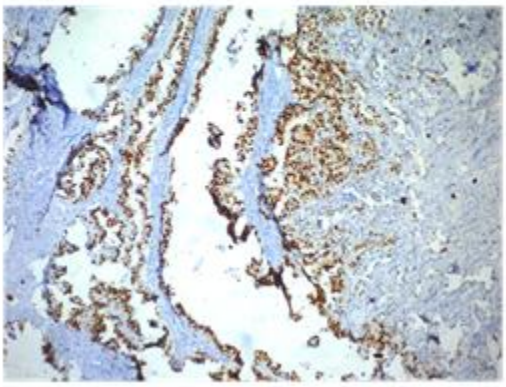


Figure 3d:Serous carcinoma ovary showing nuclear positivity (PR;100x)



Conclusion:-

The present study emphasized the role of serum CA-125 in diagnosis of different ovarian epithelial tumors and also evaluated the role of IHC markers(ER/PR, HER2/neu, p53 and Ki67) and came to the conclusion that IHC markers have limited utility in the diagnosis and prognosis of epithelial ovarian neoplasm. However combined use of these markers might be of use for which larger studies are required to substantiate these preliminary findings.

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