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Original Research Article

Observational study on serum markers and circulating tumor cells in ovarian cancer

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ABSTRACT

Aims: 1) To detect Cyclophilin, APC and SFRP5 genes associated with Epithelial ovarian cancer by PCR. 2) To study and compare the prognostic and diagnostic efficacy of serological markers like Ca 125 and HE4 and their correlation with epithelial/ non epithelial ovarian neoplasms.
Settings and Design: Comparative observational study, Prospective study
Methods and Materials: 64 cases fulfilling the inclusion criteria and giving their consent for inclusion in the study were enrolled as subjects of the present study over a period of one year. After DNA extraction (Invitrogen mini kit, USA) conventional PCR to amplify the extracted DNA and further subjected them to agarose gel electrophoresis for the identification(expression) of 3 genes i.e Cyclophillin, APC and SFRP5, was done; However, none expressed.
ELISA was used to assess CA125 and HE4 pre and post surgical intervention.
Results: The serum markers were raised more in malignant epithelial ovarian cancer cases and levels plummeted after surgical intervention, as compared to benign masses.
We could not establish correlation of the genes' expression with the serum markers and histopathology.
Conclusions: Combining HE4 and CA125 both might be more helpful than either of them using alone, in diagnosing as well as prognosticating ovarian diseases.
A panel of multiple genes on a larger sample size may be needed for CTC detection.
Key Messages: Early detection of ovarian tumors leads to early diagnosis and hence early institution of intervention and hence decreased morbidity and mortality.

1. Introduction

Ovarian malignancies constitute 3% of all malignancies in the females and is the fifth most common cause of death due to cancer in women.[1] 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 per 100,000 population in different parts of India.[2]

Primary ovarian tumors may arise from surface epithelial cells, germ cells, or sex cord stromal cells, so have been classified accordingly. Secondary tumours are implanted on to the ovary from somewhere else.

Dualistic model of epithelial ovarian cancer (EOC), which divides epithelial ovarian carcinomas into two broad categories, designated type I and type II. Prototypic type I tumour is low grade serous serous carcinoma, which has a high frequency of KRAS and BRAF mutations but no TP53 mutations and prototypic type II tumor is high grade serous carcinoma which is characterized by high level of genetic instability and harbours TP53 mutations in nearly all cases.

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The main problem with the epithelial ovarian cancers is that these patients (almost 75%) are diagnosed on advanced stages (FIGO III/IV). By the time these are diagnosed, metastases have already occurred by transperitoneal, hematogenous and lymphatic routes.[3,4]

1.1. Diagnostic tools

1.1.1. Molecular methods

A. Polymerase Chain Reaction: Molecular diversity exists within specific histological subtypes of EOC, between different tumors of an individual patient, as well as within individual tumors. EOC are classified as either type I or type II tumor on molecular basis with implications on disease pathogenesis and prognosis. Association of various genes alteration with the EOC I have been studied like BRAF, KRAS PIK3CA, CTNNB1, & CDKN2A and type II ovarian malignancies with BRCA1, BRCA2, MYC, MECOM, CCNE1, PRCK1, NOTCH3, KRAS, ERBB2, KIT & EGFR.[5–8]

For the early diagnosis of EOC, multiplex nested methylated specific polymerase chain reaction (PCR) is another method to detect the methylation level of serum and tissue samples in patients with ovarian carcinoma and the results compared with the detection with CA125.[9]

B. Circulating Tumor Cells (CTC) and Cell free DNA (cfDNA): Tumor-derived DNA has been identified in the body fluids of patients with a variety of cancers, including colorectal, head and neck, lung, bladder, kidney, and prostate.[10–14] Identifying tumor-specific molecular alterations in urine, saliva, sputum, and stool can be a noninvasive diagnostic test for cancer.[15–18]

Liquid biopsy, involves the collection and analysis of circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating cell-free microRNAs (cfmiRNAs), and exosomes.[16,17]

The use of cfDNA as a screening method has been evaluated in cancers of renal cell, lung, testicular, colorectal, and breast origin, suggesting that it could be used as a non-invasive biomarker for a wide variety of cancer types.[18–21]

These cfDNAs were found to range between 0.5 to 21 kb in length, with a plasma concentration ranging from 0.15 to 12 mg/mL, used as a diagnostic tool to detect different forms of cancer including hematological, colorectal and thoracic neoplasms.[22] This was followed by studies that focused on identifying micrometastases in form of CTCs.[23–25] Bettegowda et al. showed that these micrometastases signal can also be identified by measuring ctDNAs.[25]

1.1.2. Serum markers

A. CA125 (Carbohydrate or Carcinoma Antigen) is a high molecular weight glycoprotein. Serum CA125 levels are routinely ordered in women with adnexal masses, and levels less than 35 U/ml are considered to be normal. However, CA125 has low specificity as it is also increased in benign endometriosis, endometrial and cervical adenocarcinoma, breast and colon cancer etc.[26,27]

B. Serum HE4 (Human Epididymis protein) is used to monitor recurrence/progressive disease in patients with epithelial ovarian malignancy. Normally female genital tract and epididymal epithelium secrete HE4. Its over expression is seen in serous and endometrioid ovarian malignancies.[26]

1.2. Steps and methods

1.2.1. Study design

Comparative observational study.

1.2.2. Sample size

64 (95% CI; 5% error; prevalence of 5%).

1.2.3. Duration of study

One year.

The study was carried out in the department of Pathology, in collaboration with Microbiology and Obstetrics and Gynaecology, was approved by the Institutional Ethical Committee and review board. Patients of clinically diagnosed and radiologically suggested ovarian mass were included in study. Final histopathological diagnosis was recorded in all cases.

Patients with a history of cancer, either gynecological or from other systems or inflammatory diseases as a side diagnosis were excluded.

The blood samples were collected twice, once before any invasive procedures or before start of any medical treatments, and second between 4^{th}-7^{th} day post surgery. Clinical information including demographic information, serum CA 125 and HE4 levels, tumor stage, histology, and ultrasonographic finding was obtained from the patients' medical records and pathology reports. Serum markers were tested and analysed in pre-operative and post-operative samples, in all cases.

The tumor volume in epithelial ovarian cancers was obtained before any tumor reduction by Radiology. CA 125 was measured before any treatment or surgery. The cut off value of 35 U/mL was used to distinguish between normal and pathologic cases.

1.3. Sample collection, storage and steps

A total of 5 mL of venous blood sample were collected. These samples were transferred to an 8 mL gel serum separator tube (BD—Becton, Dickinson and Company) and kept at room temperature for coagulation. Subsequently, the samples were centrifuged for 10 min at 1000 g. The samples were processed within 2 hours after collection to prevent loss of cell-free DNA for PCR.[23,24]

The serum of all patients were collected and DNA was extracted by Invitrogen DNA Mini Kits (USA, Invitrogen company), with all operations being conducted strictly

according to the kit instructions. PCR was performed for 3 genes that is Cyclophilin, SFRP5 and APC.

Steps performed were DNA Extraction, Preparation of lysate: (Manufacturer's instructions were followed), Binding of DNA, Washing of DNA, Elution of DNA and finally Agarose gel electrophoresis was performed to analyse results.

1.3.1. Conventional PCR cycles and thermal conditions

Conditions used for PCR were "initial denaturation at 95°C for 10 min followed by 40 cycles of 1min denaturation at 95°C, 1min annealing at 55°C, and 1min elongation at 72°C. The last cycle was followed by a final extension of 10min at 72°C.

PCR was performed with prepublished primers for 3 genes, cyclophilin with forward sequence 5'-TTCTTCATCACCTATGGCAAAC-3' & reverse primer 5'- GCAACTTCTCCAACTCATCTAG-3', SFRP5 WITH FORWARD primer 5'-CAGATGTGCTCCAGTGACTTTG-3' & reverse primer 5'- AGAAGAAAGGGTAGTAGAGGGAG-3' and APC with forward primer 5'-GAGACAGAATGGAGGTGCTGC-3' &reverse primer 5'-GTAAGATGATTGGAATTATCTTCT-3'. PCR products were identified by 2% agarose gel electrophoresis (AE), and DNA was regarded to be qualified when there were significantly visible products. (seephotoplates).

1.3.1.1. Agarose gel electrophoresis. To visualize, 5μl of the PCR amplicon was loaded with gel loading dye (Thermo Fisher Scientific, India) in 1.5 % agarose gel containing 0.5μl/ml of ethidium bromide (0.5mg/ml, Medox biotech India Pvt Ltd) along with molecular weight marker (100bp DNA ladder; Bangalore Genei, India) followed by electrophoresis at 80 V for 2 h and multiple amplified DNA was analysed by 264nm wavelength UV transilluminator and gel was documented.(images attached at photoplates).

A sample would be labelled as CTC positive if at least one of 3 gene marker panel is found to be over expressed.

1.3.2. Detection of serum CA125

Serum CA125 concentration was detected by ELISA CA125 Test principle: The CA125 quantitative test is based on a solid phase enzyme linked immunosorbent assay, measured spectrophotometrically at 450nm. The concentration of CA125 is directly proportional to intensity of the sample.

1.3.3. Detection of Human Epididymal Protein 4

Serum HE4 was detected by sandwich ELISA

1.3.3.1. Testprinciple. The optical density (OD) measured with spectrophotometry at a wavelength of 450 nm ± 2 nm is proportional to the concentration of Human HE4 calculated in samples by comparing the OD of the samples with the standard curve.

2. Results

64 cases fulfilling the inclusion criteria and giving their consent for inclusion in the study were enrolled as subjects of the present study. However, 5 samples were discarded due to either inadequacy or hemolysis. All the calculations are based on 59 samples studied for all parameters.

On the Serum samples which we procured, after DNA extraction (Invitrogen mini kit, USA) we performed conventional PCR to amplify the extracted DNA and further subjected them to electrophoresis for the identification(expression) of 3 genes i.e Cyclophillin, APC and SFRP5, however, none of the genes expressed in either benign or malignant cases, in either pre or post operative samples.

Age of patients enrolled in the study ranged between 12 to 85 years, mean age of patients was 38.24±16.06 years. Majority of the cases enrolled in the study were aged above 30 years (66.1%).

50(84.7%) were married and only 9(15.3%) cases were unmarried.

Majority of the cases enrolled in the study were multipara to grandmultipara (P2 and above; n=39, 66.1%), only 6.8% were Unipara and rest were Nullipara (27.1%).

Size of lesions of the patients enrolled in the study ranged from 1.78 to 31.0 cm, mean size of lesions was found to be 8.64±5.91 cm. Only 35.6% cases had lesion size ≤5 cm, 32.2% each of the patients had lesion size 5-10 cm and >10 cm.

Majority of the patients had unilateral involvement (71.2%) rest 28.8% had bilateral involvement.

Cystic echotexture on imaging was observed in majority of the cases (54.2%), solid echotexture was observed for 13.6% in rest 32.2% cases mixed echotexture was observed.

Pre-operatively, patients with Malignant lesions as compared to Benign lesions had significantly higher CA-125 levels (902.39±1792.10 vs. 62.49±109.91 U/ml) as well as higher HE-4 levels (744.31±405.21 vs. 72.68±69.69 pmol/l).Table 2A.

Post-operatively too, patients with Malignant lesions as compared to Benign lesions had significantly higher CA-125 levels (54.09±39.93 vs. 27.60±25.86 U/ml) as well as higher HE-4 levels (150.11±88.20 vs. 35.82±28.02 pmol/l).Table 2B.

Decline in pre-operative CA-125 and HE-4 was observed in patients with benign as well as malignant lesions.Table 2C.

Changes were found to be significant statistically for both the markers, in benign lesions while significant statistically only for HE-4 marker, in malignant lesionsFigure 1

On receiver-operator characteristic analysis, the area under curve was found to be 0.782 for CA-125 and 0.926 for HE-4. The difference between two was not significant

Table 1: Histopathological Diagnosis

S.No	Diagnosis		No.	%
	Benign		**43**	**72.9**
	Cystic lesion	5/43 (11.6%)	5	8.5
	Serous cyst	15/43 (34.9%)	15	25.4
1.	Mature cystic	7/43 (16.3%)	7	11.9
	Follicular cystic	5/43 (11.6%)	5	8.5
	Hemorrhagic cyst	4/43 (9.34%)	4	6.8
	Others (Benign)	7/43 (16.3%)	7	11.9
	Malignant		**16**	**27.1**
2.	Serous cystadenoCa	10/16 (62.5%)	10	16.9
	Others (Malignant)	6/16 (37.5%)	6	10.2

Table 2: A. Evaluation of mean preoperative HE-4 and CA-125 levels for their diagnostic efficacy

Diagnostic domain	N	CA-125 (U/ml)		HE-4 (pmol/l)	
		Mean	SD	Mean	SD
Benign vs Malignant					
Benign	43	62.49	109.91	72.68	69.69
Malignant	16	902.39	1792.10	744.31	405.21
Statistical significance		't'=3.104; p= 0.003 (Sig)		't'=10.603; p<0.001	

Table 2B: Evaluation of mean Post-operative HE-4 and CA-125 levels for their diagnostic efficacy

Diagnostic domain	n	CA-125 (U/ml)		HE-4 (pmol/l)	
		Mean	SD	Mean	SD
Benign vs Malignant					
Benign	43	27.60	25.86	35.82	28.02
Malignant	16	54.09	39.93	150.11	88.20
Statistical significance		't'=2.994; p=0.004 (Sig)		't'=7.616; p<0.001 (Sig)	

Table 2C: Change in Pre-Operative HE4 and CA-125 levels (Paired 't' test)

	CA-125 (U/ml)					HE-4 (pmol/l)				
	Mean Ch.	SD	% Ch.	't'	'p'	Mean Ch.	SD	% Ch.	't'	'p'
Benign	-34.89	91.56	-55.83	-2.499	0.016	-36.85	53.43	-50.70	-4.523	<0.001
Malignant	-848.3	1766.3	-94.01	-1.921	0.074	-594.2	347.1	-79.83	-6.848	<0.001

Table 2D Comparative parameters of HE4 and CA125 at the expected cut off values **(Receiver Operator Characteristic Curve)**

Parameter	Area under curve (95% CI)	Projected cut-off value	Projected Sensitivity	Projected Specificity
CA-125	0.782 (0.63-0.94)	≥92.35	75.0%	81.4%
HE-4	0.926 (0.84-1.01)	≥102.62	87.5%	70.1%

statistically (p>0.05).Table 2D.

On evaluating the regressed coordinates for different sensitivity/specificity trade-off, the cut-off value >92.35 U/ml for CA-125 and or HE-4 and cut-off value >102.62 p/mol. Sensitivity and specificity for CA-125 were found to be 75.0% & 81.4% while that for HE-4 were 87.5% & 70.1% in differentiating malignant masses and benign lesions.

3. Discussion

3.1. Molecular methods

Since none of our samples came out to be positive for either of the three genes (cyclophyllin, SFRP5 and APC), we probed further into literature to compare our results. Most of the related studies conclude that concentration of CTCs presented in ovarian cancer are extremely low, $1/10^9$ blood cells or $1/10^6$ nucleated blood cells, and hence difficult to detect. Moreover stringent procurement, storage and test conditions are demanded.[3]

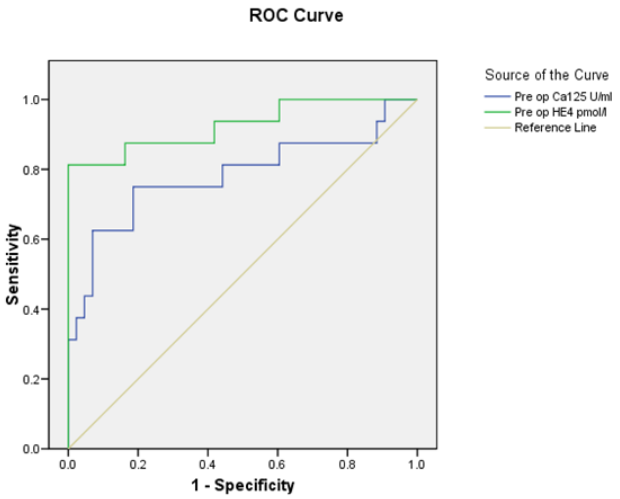

Fig. 1:

Board et al in 2010, to detect SNV (PIK3CA) alteration by allele specific PCR in operable stage of breast cancer reported 0% (0/14) sensitivity in patients with detected ctDNA with marker positive breast cancers.[28]

However Shao et al. in 2015 suggested that there is a definite correlation between levels of serum cfDNA and development of ovarian cancer, but their detection is challenging.[29]

Ignatiadis et al. in 2015 said that ctDNA may represent an exceedingly rare subpopulation within total cell-free DNA, at levels corresponding to one genome equivalent in 5 mL of plasma (0.01% allele fraction), and may be undetectable in plasma volumes typically sampled.[30]

3.2. Serum markers

CA-125 levels ranged from 6.10 to 7087 units. A mean of 62.49 units/ml and SD of 109.91 in benign cases, and a mean of 902.39 units/ml and SD of 1792.10 in malignant cases was calculated, as is shown in Table 2A. The ability of CA125 in differentiating benign from malignant cases was found to be statistically significant (p=0.003). Along with USG findings, it proves to be a sensitive method to differentiate benign from malignant masses. Some studies however show that CA125 can sometimes over-diagnose benign cases like endometriosis and hence lead to unnecessary operation.

Human Epididymis 4 levels: HE4 levels ranged from 10.04 pmole/litre to 1391.08 pmole/litre.

In benign cases, a mean of 72.68 and a standard deviation of 69.69 was obtained while in malignant cases, mean was 744.31 and SD was 405.21. HE-4 lvels were found to be significantly higher in malignant cases as compared to benign (p<0.001).

4. Conclusion

Detection of circulating tumor DNA in ovarian cancer sera is difficult, and requires meticulous draft of multiple genes in panel to get a significant yield Serum markers must be used in conjunction with clinico-radiological findings.

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6. Conflict of Interest

The authors declare that there are no conflicts of interest in this paper.

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None.

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