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Unique expression of 35 KDa protein in serum and cystic fluid of women with malignant ovarian cysts substantiates its role in disease progression

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ABSTRACT

Objective: The present study was undertaken as a part of a continuing search for proteomic based approaches for the diagnosis of ovarian cancers. **Methods:** The study comprised four groups of women with: i): malignant ovarian cysts, ii) benign ovarian cysts, iii): breast cancer (positive controls) and, iv) healthy females (negative controls). Serum and cystic fluids were processed for gel electrophoresis (SDS-PAGE). Serum and cystic follicular fluid estradiol and testosterone concentrations were measured by ELISA. **Results:** Proteomics analysis revealed the presence of a uniquely expressed protein with MW of 35 kDa in the serum and cystic fluid of patients with malignant ovarian cysts. On the other hand a protein of 100 kDa was not expressed in malignant ovarian patients whereas it was differentially expressed in the cystic fluid and serum of other three groups. Although mean values of estradiol levels were discernibly higher in patients with benign ovarian cysts as compared to those with malignant ovarian cysts and of positive and negative controls, difference was not significant. Mean concentrations of estradiol were significantly higher in cystic fluid aspirated from benign cysts as compared to that from malignant cysts. **Conclusions:** Taken together these data indicate a uniquely expressed protein of 35 kDa in patients with malignant ovarian cysts that may serve as a specific protein biomarker for the differential diagnosis of the ovarian cancer. However, a much larger sample of subjects is required to validate and confirm these findings.

1. Introduction

Various forms of cystic disease of the ovaries are characterized by a lack of normal ovarian activity and a low conception rate, and are the main cause of female infertility. Mostly ovarian cysts occur in the childbearing years and are benign in nature. However, ovarian cysts can lead to malignancy that may lead to death of the patient[1, 2]. Reports on the geographical prevalence of the ovarian cancer show that ovarian malignancy is five-times more common in developed than in developing countries. Ovarian cancer has been regarded as the fourth most frequent cause of deaths from cancers in women in Europe and United States and mostly affects elderly and middle-aged women[2, 3]. In USA and Canada, 2 500 new cases are reported annually and of these 56%-60% die from this disease each year. According to reports, the risk of developing ovarian cancer in lifetime is 1.4% in the western world. The mortality from ovarian cancer disease is, therefore, much higher than due to breast cancer (19%) in Northern America[4, 5]. In local population of Pakistan overall malignancy rate has been reported to be relatively high (23%) as compared to the overall world incidences (15%) and the incidence of ovarian cancer has been reported to be 13.6%[6, 7].

Generally this has been reported that majority of the ovarian cysts are benign which accounts about 86.7% of the total ovarian masses and the rest 13.3% are malignant[8]. Due to the silent nature of the ovarian cancer the early differentiation between benign and malignant tumors is not evident. Consequently a number of possible biomarkers have been identified to differentiate between malignant and benign ovarian cysts. In this continuation aspiration cytology

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has been used to differentiate between neoplastic and benign ovarian cysts but this method alone is considered non reliable[9].

The advent of novel scientific approaches and more sophisticated equipments in cancer biomarker identification has increased the possibility of early detection, better monitoring of tumor progression and improved therapeutic management. Such markers are still wanting for ovarian cancer which is associated with advanced stage at presentation and poor survival[10, 11]. Some earlier approaches for cancer biomarker identification used tumor cells to immunize animals and screen for antibodies that could recognize the specific antigen. This approach has resulted in the identification of a high molecular weight (MW) glycoprotein CA-125 which is currently in use for ovarian cancer monitoring[12]. However, CA-125 overlooks 50% of early stage ovarian malignant cases and therefore suffers from certain draw backs[1, 13]. Moreover, the levels of CA-125 may also be elevated in normal individuals and in patients with benign cysts. Raised levels of CA-125 have also been reported in cases of benign and malignant tumors of tissues and organs such as prostate [14–18].

In recent years, several serum markers have been identified and explored as possible adjuncts to CA-125 screening but some of these potential markers including CA19-9 and lysophaspatidic acid have not been shown to be clinically useful in large screening trials[19, 20]. Insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs) have been shown to play a role in regulation of the ovarian functions but little is known about their role in malignant disease of the ovary[21]. Among the IGF-binding proteins, IGFBP-2 has been shown to be elevated in ovarian cancer patients. In spite of the fact that this protein is elevated in ovarian cancer patients and its use as an adjunct tumor marker, the levels of this protein have also been shown to be raised in certain other types of cancer as well, such as the prostate cancer[22, 23]. Interestingly, another protein IGFBP-3 shows a decline in ovarian cancer patients and with a negative correlation with CA-125[21]. The expression of IGFBP-3 protein expression has been observed in 90% of ovarian cancers[24]. Although a significant role has been established for IGFBP-3 in breast cancer cell biology, no such role has yet been demonstrated in ovarian cancer. In contrast to breast cancer where high tissue IGFBP-3 levels may be associated with large tumours, low tissue IGFBP-3 levels have been found to be associated with ovarian tumors of large size and their active progression[25]. Recently two proteins namely calgranulins A and B have been reported in the serum and cystic fluid of the patients with malignant ovarian cysts [26].

The presence of high concentrations of certain proteins in biological sample (serum, plasma, CSF, pleural effusions) hinders the identification of specific protein biomarkers. Among the above mentioned biological samples, serum is considered to be a rich source of biological markers for disease and is believed to contain the largest set of proteins expressed. It is believed that about one million proteins or their isoforms, constitute only 1 % of the serum proteome whereas high abundance proteins like albumin

constitute 96%-97% of the proteome[27]. The abundant proteins like albumin and IgG, mask the low abundant proteins and obscure their identification and had posed a barrier in the identification of proteins that could serve the purpose of disease markers[17].

Keeping in view the importance of protein identification for the disease detection and the early diagnosis several attempts have been made to remove the abundant proteins (albumins) of serum or plasma by different methods based on the high affinity of albumin for certain textile dyes, such as Cibacron-blue and some other compounds[17, 26–28]. The removal of the abundant proteins allows the better comparison and correlation of the serum protein profiles in different diseased patients. Previously it has been demonstrated that serum proteins are differentially expressed in patients with benign and malignant ovarian cysts[26]. A recent study on bovine ovarian follicular fluid and cystic follicular fluid shows that the protein profiles of normal follicular fluid also differ from that of cystic fluid. A comparative proteomic analysis showed 8 increased protein spots in cystic follicular fluid[29].

Since most of the available biomarkers for the detection of ovarian cancer lack specificity and sensitivity for early detection of the disease, therefore, there is a need for a continuing search for more reliable and practical biomarkers for ovarian cancer disease. Furthermore, there is a possibility that certain hormones are over expressed in ovarian cysts whereas others may be under expressed. The level of estrogen hormone was shown to be low in the serum of patients with polycystic ovarian syndrome (PCOS) as compared to normal subjects[30]. However, in women with PCOS the levels of LH and testosterone were found to be higher than normal[31]. Rzapka-Gorska *et al*[32] provided evidence for the involvement of gonadotropins in ovarian carcinogenesis and found significantly higher concentrations of FSH and LH in malignant ovarian cystic follicular fluid as compared to serum of the corresponding patient. This study suggests that high concentration of FSH and LH in the cystic fluid could be used as a marker to differentiate between benign and malignant cysts. However, such an investigation would involve invasive techniques to collect cystic fluid especially in cases of malignant cysts where fluid aspiration is not recommended. The present study was, therefore, designed to attempt a comparison of protein profiles and steroid levels of serum and cystic fluid between samples from patients with malignant and those with benign ovarian cysts. The results of this study may help in the early screening of the ovarian cancer by just comparing the blood protein profiles without going for the intensive exploration.

2. Materials and methods

2.1. Study population

A total of 50 subjects attending gynecology units were recruited in the study including positive and negative controls. Written informed

consent to participate in the study was obtained from each subject. A detailed questionnaire was designed to collect demographic data and complete family and medical history of the subjects. The presence of ovarian cyst was initially confirmed by ultrasound. The values of serum CA-125 levels were obtained from the clinical reports of the patients

2.2. Inclusion criteria

Strictly, the females who were of reproductive age and were not under any medication at present were included in the study. Females who had previously received or were currently on any medication were excluded from the study. Also post menopausal women were not included in the study.

2.3. Study design

The age matched females were grouped after the physical and clinical examination and on the basis of clinical diagnosis they were divided into four different groups:

1) Patients with known malignant ovarian cyst (n =10). Mean age: 36 years (range 20-50).

2) Patients with known benign ovarian cyst (n =10). Mean age: 25 years (range 18-40).

3) Patients with known history of breast cancer (positive control group) (n =10). Mean age: 42 years (range 37-50).

4) Normal healthy females with regular menstrual cycle (negative control group) (n =20). Mean age: 30 years (range 24-40).

2.4. Blood and cystic fluid

Blood samples were collected by venepuncture for serum collection. Blood was allowed to clot at room temperature for 30 minutes and subsequently samples were centrifuged at 5 000 rpm for 10 minutes. Serum was collected, aliquoted and stored at -80 ℃ until analyzed.

Cystic follicular fluid from benign ovarian cysts was collected during ultrasound guided aspiration whereas in patients with malignant ovarian cysts the fluid was aspirated after surgical removal of the cyst. Cystic fluids were also centrifuged to remove any solids and supernatants were frozen at -80 ℃. The surgically resected specimens of the ovaries were sent for histopathology. The histological and cytological examination of ovarian tissues from the patients was routinely carried out in the histopathology laboratory of the hospital. Grading of the cysts was carried out according to the FIGO classification. The benign and malignant ovarian cysts

included in this study were primarily of surface epithelial origin.

2.5. Gel electrophoresis

The serum and cystic fluid proteins were characterized by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [17, 27]. Briefly, cystic fluids of the benign and malignant ovarian tumors and serum of subjects of all the four groups were subjected to SDS-PAGE. Serum abundant proteins were removed by using routine protein purification kits (Aurum Serum Protein Mini Kit, Bio Rad, Hercules, CA, USA). In each well 5-8 μL of sample was loaded and samples were run at 100V for 90 minutes. A protein ladder (range 7 kDa - 240 kDa, REC003, Real Biotech Corporation, USA) was run in parallel to the studied samples. Gels were then placed in a destaining solution overnight. Multiple gels were run for each sample in order to document reproducible repeats of protein profiles.

2.6. Hormone analysis

Estradiol and testestosterone concentrations in serum samples and cystic fluid were measured by ELISA using commercial kits (BioCheck, Inc, Foster City, CA, USA). The levels of testosterone and estradiol in subjects of four groups were measured. In patients with benign and malignant ovarian cysts both cystic fluid and serum were tested while only serum was tested from positive (breast cancer patients) and negative controls (normal females). The samples were run in duplicate and the means of the concentrations were recorded.

2.7. Statistical analysis

Statistical analysis of the data was done using the computer software Statistical Package for Social Sciences (SPSS) Version 13. Mean levels of testosterone and estradiol among groups (malignant cystic ovarian, benign cystic ovarian, positive and negative controls) were compared by analysis of variance (ANOVA) followed by Post-Hoc Tukey's test. $P< 0.05$ was regarded as significant.

3. Results

3.1. Proteomic analysis

Grading of the cysts was carried out according to the FIGO classification (Tables 1 and 2).

Table 1
CA-125 levels, histological profile and tumor grading in patients with malignant ovarian cysts.

Patient no.	Patient ID	Age (yrs)	CA-125 level U/mL	Histological diagnosis	Grading
1	G1	50	7252	Serous cystadenocarcinoma	III
2	G4	22	188	Serous cystadenocarcinoma	II
3	G6	30	NAa	Borderline Serous cystcarcinoma	NA
4	G12	50	92	Mucinous cystadenocarcinoma	I
5	G13	45	588	Mucinous cystadenocarcinoma	III
6	G17	40	138	Mucinous cystadenocarcinoma	II
7	G19	32	47.94	Serous cystadenocarcinoma	I
8	G23	45	108	Mucinous cystadenocarcinoma	NA
9	G27	20	414	Serous cystadenocarcinoma	II
10	G29	25	85	Serous cystadenocarcinoma	I

NA =Not available; Ovarian cysts were graded according to guidelines by the FIGO.

Table 2
CA-125 levels, histological profile and tumor grading in patients with benign ovarian cysts.

Patient no.	Patient ID	Age (yrs)	CA-125 level	Histological diagnosis
1	G2	32	NA	Serous cystadenoma
2	G3	28	23	Serous cystadenoma
3	G8	22	27.5	Serosa cyst
4	G14	22	NA	Serous cystadenoma
5	G16	27	NA	Serous cystadenoma
6	G20	21	6.4	Follicular cyst
7	G22	22	NA	Hemorrhagic cyst
8	G28	40	18	Serous cystadenoma
9	G31	18	10	Mucinous cystadenoma
10	G32	20	22	Mucinous cystadenoma

NA=Not available; Ovarian cysts were graded according to guidelines by the FIGO.

Figure 1. Protein profile of the serum and cystic follicular fluid of patients with malignant ovarian cysts.

Marker: Protein ladder (range 7 kDa - 240 kDa); MS: Protein profiles of the serum of patients with malignant ovarian cysts; MF: Protein profile of the cystic follicular fluid of patients with malignant ovarian cysts; Note: The adjacent MS and MF lanes are of the same patient exhibiting serum and cystic fluid protein profiles. This figure shows the protein profile of serum and cystic follicular fluid of 4 selected patients of malignant ovarian cysts.

The protein profiles showed a similar expression in serum and cystic fluid of the corresponding patients in the two cystic groups. Four protein bands (70, 50, 35 and 25 kDa) were detected in patients with malignant ovarian cysts (Figure 1). A 35 kDa protein was uniquely expressed in this group whereas it was absent in the other three groups. In addition, a 25 kDa protein was also uniquely expressed in two (2/10) patients of the group (Figure 2).

A 50 kDa protein was over-expressed in serum and cystic fluid of patients with ovarian malignancy in comparison with other groups (Figure 2). However, it was also over expressed in one patient with benign ovarian cysts (Figure 3). In patients with benign ovarian cysts three protein bands were identified (100, 70 and 50 kDa) (Figure 3). The 100 kDa protein though present in serum and cystic fluid of patients with benign ovarian tumors and the serum of the two control groups, was unusually lacking in samples from patients with ovarian malignancy. In breast cancer patients (positive control) six protein spots were visible (240, 240-140, 100, 70 and 50-35 kDa) (Figure 4). Two proteins present between MW range of 240-140 kDa and 50-35 kDa, were differentially expressed in positive control group. The 240 kDa protein identified in the positive control group was also present in the serum of normal subjects (negative control). Serum protein profiles of the negative controls revealed the presence of six protein bands (240, 140, 100, 70, 50, 25-20 kDa) (Figure 5). A140 kDa protein and a protein present between MW range of 25-20 kDa that was differentially expressed in this specific group.
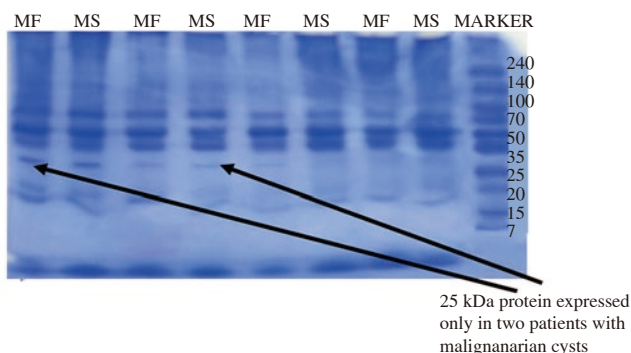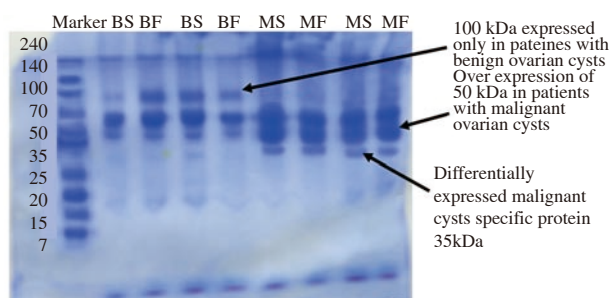

Figure 2. Comparison of the protein profiles of the serum and cystic follicular fluid of patients with benign and malignant ovarian cysts.

Marker: Protein ladder (range 7 kDa - 240 kDa); BS: Protein profiles of the serum of patients with malignant ovarian cysts;   BF: Protein profile of the cystic follicular fluid of patients with malignant ovarian cysts; MS: Protein profiles of the serum of patients with malignant ovarian cysts MF:Protein profile of the cystic follicular fluid of patients with malignant ovarian cysts. Note: The profiles of two patients of each group are shown here.
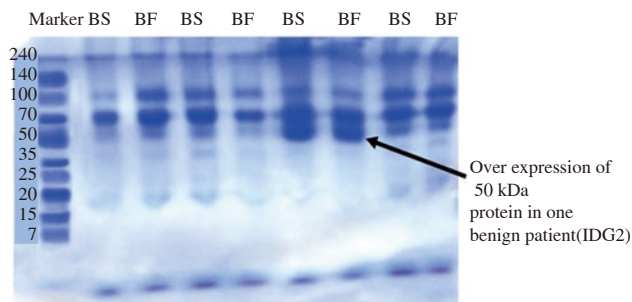
Figure 3. Protein profile of the serum and cystic follicular fluid of patients with benign ovarian cysts.

Marker: Protein ladder (range 7 KDa - 240 KDa); BS: Protein profiles of the serum of patients with benign ovarian cysts;   BF: Protein profile of the cystic follicular fluid of patients with benign ovarian cysts; Note: The adjacent BS and BF lanes are of the same patient exhibiting serum and cystic follicular fluid protein profiles. This figure shows the protein profile of serum and cystic follicular fluid of 4 selected patients of benign ovarian cysts.
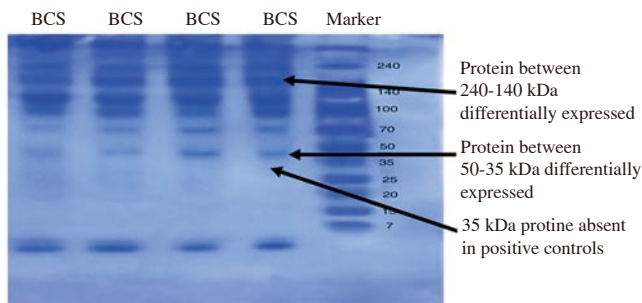

Figure 4. Serum protein profiles of breast cancer patients (positive controls). Marker: Protein ladder (range 7 kDa - 240 kDa); BCS: Protein profile of serum of breast cancer patients. Note: Protein profiles of four breast cancer patients are exhibited in this gel photograph.
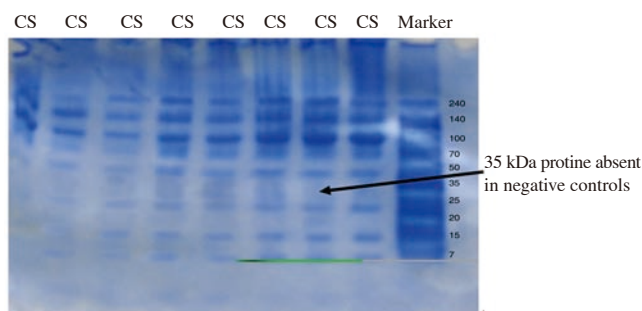

Figure 5. Serum protein profile of the negative controls (healthy females). Marker: Protein ladder (range 7 kDa - 240 kDa); CS: Protein profiles of the serum of healthy females with normal menstrual cycle (negative controls); Note: Eight negative control samples are presented in above gel photograph.

3.2. Hormone analysis

No statistically significant differences in estradiol concentrations were observed amongst the four groups although mean values of estradiol levels were markedly higher in patients with benign ovarian cysts as compared to that with malignant ovarian cysts and negative controls (Table 3). Similarly no significant difference in serum

testosterone levels was found between patients with and without ovarian cysts (Table 3).

Mean concentrations of estradiol were significantly higher in cystic fluid aspirated from benign cysts as compared to that from malignant cysts. However, testosterone concentrations were not significantly different between the two groups (Table 4).

Table 3
Serum estradiol and testosterone levels in subjects of four groups (Mean ±SEM).

Groups	n	Serum estradiol (pg/mL)	Serum testosterone (ng/mL)
With malignant ovarian cysts	10	64.71±11.36	1.30±0.26
With benign ovarian cysts	10	139.38±51.74	1.61±0.43
With breast cancer (positive control)	10	59.56±5.33	0.83±0.22
Normal subjects (negative controls)	20	73.74±5.65	2.00±0.29

Table 4
Estradiol and testosterone levels in cystic follicular fluid of patients with malignant and benign ovarian cysts (Mean ± SEM).

Groups	n	Cystic fluid estradiol (pg/mL)	Cystic fluid testosterone (ng/mL)
With malignant ovarian cysts	10	68.06±12.34*	1.18±0.28
With benign ovarian cysts	10	508.46±154.03	4.00±2.00

*$P<0.05$ comparing with the patients with benign ovarian cysts.

4. Discussion

Recent advances in proteomics have provided new insights in disease diagnosis as well as helped in development of convenient and effective diagnostic tools. The CA-125 antigen is often used as a preliminary test to diagnose various forms of cancer including ovarian malignancy and to monitor disease progression and recurrence. However circulating CA-125 has limited sensitivity for the detection of the early stage of the ovarian cancer. In the last few years considerable efforts have been made to identify other potential biomarkers that might substitute or complement CA-125 in disease management and in the design of new screening strategies [11, 14, 15, 33–36].

The present study was designed primarily to identify differences if any in the protein profiles of serum and cystic follicular fluid among patients with malignant ovarian cysts and benign ovarian cysts. In addition we have measured serum estradiol and testosterone concentrations in serum and cystic fluid to assess ovarian steroidogenic functions

The proteomic analysis exhibited a similar expression of protein bands in serum and cystic follicular fluid of the corresponding patients which confirms the previous findings of Ott *et al* [26]. Our results revealed an absence of 100 kDa protein in serum and cystic fluids of patients with malignant ovarian tumors. However, a protein with MW of 50 kDa was found over expressed in patients with

malignant ovarian cysts compared to its expression in patients with benign ovarian cysts. Cancer specific reactivity to 50 kDa protein has also been reported in patients with cancer of the cervix[37]. In our study one of the patient with benign ovarian cyst also showed an over expression of 50 kDa proteins similar to that of patients with malignant ovarian cysts. Interestingly we found a protein of 35 kDa uniquely expressed in the serum and cystic fluid of the patients with malignant ovarian cysts. In another study the same MW (35 kDA) protein has also been demonstrated to be enhanced in the sera of patients with epithelial ovarian cancer and germ cell ovarian carcinoma. But this study also demonstrated the elevation of this protein in the serum of the patients with breast cancer[38] which we did not. Moreover, a protein with almost similar MW of 36 kDa has previously been reported in the sera of the patients with ovarian cancer and its overexpression in human ovarian cell cultures has also been established[39, 40]. Also, we demonstrate the expression of a low MW protein (25 kDa) in 2 of the 10 patients with malignant ovarian cysts. A protein of exactly the same MW (25 kDa) has been reported in the malignant tissues of the female genital tract. However, the expression of this protein was also observed in the benign tissues of the female genital tract [41]. Furthermore, presence of a protein with a very similar MW (27 kDa) has previously been reported in ovarian cancer patients[42]. Moreover, glycodelin A, a protein of 28 kDa MW has been reported significantly elevated in the sera, cystic fluid and amniotic fluid in patients with the malignant ovarian cysts compared to the patients with benign ovarian cysts[43]. In another study two low MW (10-20 kDa) proteins calgranulins A and B have been identified in the serum and fluid of the patients with malignant ovarian cysts[26]. In a more recent study the higher expression levels of a low MW protein (11.3 kDa, A100A7) have been demonstrated in ovarian cancer patients [44]. The previously reported findings and our results indicate a higher expression of specific low MW proteins in the serum and cystic fluid of patients with malignant ovarian cysts. However, identification of these proteins and their association with ovarian malignancy needs further systematic investigations.

In our study the serum estradiol levels were not significantly different among the four groups of subjects although mean estradiol concentrations were relatively higher in patients with benign ovarian cysts compared to mean levels in the other three groups. On the other hand a study by Agarwal *et al* [30] showed a decrease in the serum estrogen levels in patients of PCOS. Furthermore, low levels of estrogen in patients with ovarian serous cystadenoma compared to that of the estrogen levels in follicular fluid have been reported[45]. Moreover, the lower levels of estrogen have also been reported in the ovarian cancer tissue, ovarian cystic fluid and serum in patients

with FIGO stages 3 and 4 than in stages 1 and 2 [46] which support our findings of low estradiol levels, although non-significant, in malignant patients compared to the benign patients. Also, in our study significantly higher estradiol concentrations were found in follicular fluid aspirated from benign cystic follicles as compared to that of malignant cysts (508.46±154.03 *vs.* 68.06±12.34). The possible reason for the higher levels of estradiol in case of benign ovarian cysts could be that the steroid producing cells are functioning normally or even slightly at a higher level whereas in malignant cyst their normal function of androgen aromatization is affected resulting in decreased ovarian estrogen synthesis.

Our data failed to demonstrate a significant difference in the serum testosterone concentrations among the four groups. Our findings of testosterone are in accordance to the study of Heiononen PK [47] which also demonstrates no difference in the androgen levels in patients with malignant, borderline, benign ovarian tumors and the control subjects. However, an in vitro study showed increase production of testosterone by the benign tumor cells compared to malignant ovarian cells[48]. Our results of serum testosterone levels in patients with benign ovarian cysts also differ from those reported in patients with PCOS where an increase in the testosterone levels as compared to the controls, has been demonstrated[31]. The mean cystic fluid testosterone concentrations of patients with benign and malignant ovarian cysts were also not significantly different from each other although testosterone concentrations were lower in malignant cystic fluid compared to that of benign ovarian cystic fluid. These observations suggest that at this stage of malignancy steroidogenic function of the thecal cells of the ovary is not greatly impaired. However, these results need further verification in future studies.

In summary our results demonstrate unique expression of a protein with MW of 35 kDa in the serum and cystic fluid of patients with malignant ovarian cysts which was absent in other three groups. Since the protein is uniquely expressed in malignant ovarian patients in our study, therefore, we speculate that its expression in patients with ovarian disease could serve as a potential protein biomarker for differential diagnosis of ovarian cancer. However, such proposition can only be made by analyzing samples from a much larger number of patients and controls.

Conflict of interest statement

We declare that we have no conflict of interest.

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