Original article:

Evaluating Xenobiotic Metabolizing Enzymes, Cell Proliferation and Apoptosis Markers in Epithelial Ovarian Cancer patients.

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Abstract:

Introduction: Adequate screening methods are not available for early stage ovarian cancer detection. We hypothesised that correlating the levels of phase I and phase II biotransformation enzymes involved in estrogen metabolism and correlating it with cell proliferation and apoptosis evasion would provide key hallmark traits for ovarian malignancy. The aim of the study was to evaluate the expression levels of phase I (cytochrome P450 total and the CYP isoforms CYP1A1, CYP1A2 and CYP2B) and phase II (GST and QR) xenobiotic-metabolizing enzymes, as well as expression of markers reflecting cell proliferation (PCNA and cyclin D1) and apoptosis (Bcl-2 and Bax) in patients with epithelial ovarian cancer.

Material & Methods: Hundred and fifteen ovarian tumor patients were chosen for the study. Out of 115 ovarian tissues of patients, 18 were histologically non-malignant and rest were malignant with grade III epithelial ovarian tumors.

Results : The results of the present study demonstrated that the increase in phase I enzymes in ovarian tumour tissues were not compensated by a corresponding increase in phase II enzymes, indicating insufficient detoxification in the face of increased carcinogenic activation.

Conclusion: The present study results provided evidence that an imbalance in phase I and phase II xenobiotic-metabolizing enzymes, coupled with an imbalance in cell proliferation and apoptosis, are important contributors for the development of epithelial ovarian cancer.

Key words: Epithelial ovarian cancer; PCNA; cyclin D1; Bax/Bcl2

Introduction:

Worldwide, ovarian cancer is the seventh most common cause of cancer death (4.2%) with an incidence rate of 224,747 cases [1]. The incidence of ovarian cancer is high in Western and Northern European countries as well as in Northern America and is least in China and Central Africa [2]. In India, ovarian cancer is the third most common cancer among women, with 28,080 cases and 19,558 deaths [1]. Based on the tissue and anatomic structures from which the tumour originates, ovarian cancer is divided into two major groups namely, sex cord stromal tumours and germ cell tumours, with various subtypes [3]. The prevalence of epithelial stromal tumours is about 80-85% of all occurring ovarian carcinomas [4].

In most ovarian cancer cases the cause is unknown. Various factors affecting ovarian cancer are identified, which include family history, age, use of hormone replacement therapy (HRT), parity,

infertility, hormonal factors, incessant ovulation, inflammation, late menopause, early menarche, BMI diabetes, diet, medication, and environment [5].

There are no adequate screening methods for early stage ovarian cancers. The most widely used serum biomarker for ovarian cancer is CA-125. It is increased in ~80% of patients with advanced stages of ovarian cancer. The normal ovarian surface epithelium does not express CA-125 [6]. Due to poor sensitivity and specificity of CA-125 for early detection of ovarian cancer, various other promising serum biomarkers are being screened for their specificity alone or in combination with CA-125 serum levels [7].

Phase I enzymes, cytochrome P450 (CYP 450), metabolically activate various procarcinogens and initiate tumorigenesis [8]. A major risk factor in hormone dependent cancers like ovarian and breast cancer is increased lifetime exposure levels of estrogen (E2) [9,10]. An increase in the transcriptional activity of CYP1B1 in hormone dependent ovarian cancers was reported by McFadyen et al., [11]. Microarray studies by Downie et al., [12] reported higher levels of various CYP450 genes in ovarian cancer tissues, whereas absence of CYP1B1 expression in control ovarian tissues. Phase II enzymes protect cells against estrogen mediated DNA damage. Ansell et al., [13] had reported that E2 downregulates phase II enzyme activities and increase oxidative DNA damage in cells. Estrogen independently regulates the expression of cyclin D1 in hormone dependent cancers like breast cancer [14]. A number of studies have reported increased levels of cyclin D1 and PCNA in ovarian cancer cells [15-17]. Apoptosis evasion plays a significant role during tumorigenesis. In ovarian cancer patients, Bcl-2 and Bax levels have been reported to be important markers for predicting chemoresistance [18]. Spillman et al., [19] reported that E2 promote growth in size and lymph node metastasis in ER+ ovarian tumours in Intra-peritoneal mouse xenograft model. E2 also downregulates negative regulators of Bcl-2 such as Bad or Nip-2 [20].

The main aim and objective of the current work was to understand the key hallmark traits for epithelial ovarian malignancy by evaluating the levels of phase I (cytochrome P450 total and the CYP isoforms CYP1A1, CYP1A2 and CYP2B) and phase II (GST and QR) xenobiotic-metabolizing enzymes, as well as expression of markers reflecting cell proliferation (PCNA and cyclin D1) and apoptosis (Bcl-2, Bax) in patients with epithelial ovarian cancer and histologically non-malignant ovarian tissues.

Materials & Methods:

Chemicals

Acrylamide, bisacrylamide, bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), ethidium bromide, ethylene diamine tetraacetic acid (EDTA), glutathione reductase, methoxyresorufin, oxidized glutathione (GSSG), pentoxyresorufin, resorufin, reduced nicotinamide adenine dinucleotide phosphate (NADPH), sodium dithionite, trichloroacetic acid (TCA), trizol, and primers were purchased from Sigma Chemical Company, St. Louis, MO, USA. Primary and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. All other reagents used were of analytical grade.

Patient samples

Hundred and fifteen newly diagnosed ovarian tumor patient tissues and blood samples, mean age 55 ± 5.34 years, who had not undergone any previous treatment for their tumors, collected by NIN Hyderabad, India, were chosen for the study. Based on the histological reports of the tissue samples, the samples were categorized as

malignant and non-malignant ovarian tissues. Out of 115 ovarian tissues of patients, 18 were histologically non-malignant and rest were malignant with grade III epithelial ovarian tumors. Ovarian tissues which were histologically non-malignant and their corresponding blood samples were used as control for the current study. The Human Ethics Committee, India approved the study.

METHODS

Biochemical estimations

Tissue samples for biochemical analysis were weighed and homogenized using an appropriate buffer in an all glass homogeniser with Teflon pestle. The S9 fraction was prepared at 4 °C as described by Ames et al. [21]. Cytochrome P450 and was assayed by the method of Omura and Sato [22]. Ethoxyresorufin O-decarboxylase (EROD), methoxyresorufin O-decarboxylase (MROD), and pentoxyresorufin O-decarboxylase (PROD) activities were determined by measuring the formation of resorufin spectrofluorimetrically at excitation and emission wavelength of 536 nm and 585 nm respectively, according to the method of Burke and Mayer [23]. The activities of GST and QR were assayed by the methods of Habig et al. [24] and Ernster [25] respectively.

Protein extraction for western blot analysis

For extracting the total protein, approximately, 50 mg of each tissue sample was subjected to lysis in a sample buffer containing 62.5 mM Tris (pH 6.8), 10% SDS, 5% 2-mercaptoethanol, 10% glycerol and bromophenol blue. The protein concentration of lysates was determined by Lowry et al., method [26]. Nuclear and cytoplasmic extracts were prepared as described by Legrand-Poels et al. [27]. Tissue samples were homogenized with 1 ml of a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 10 µl protease inhibitor cocktail. The lysate was then centrifuged at 8,000×g for 2 min at 4°C, the cytoplasmic supernatant was removed, aliquoted, and frozen at −80°C. The nuclear pellet was reconstituted in 1 ml of buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 10 µl protease inhibitor cocktail, followed by vigorous vortexing for 20 min at 4°C. The nuclear lysate was then centrifuged at 14,000×g for 5 min, and nuclear extracts were aliquoted and stored at −80°C for further analysis. Nuclear and cytoplasmic proteins were quantitated by Lowry et al., method [26].

Western blot analysis

Proteins separated by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride membranes. The blots were incubated in 1X PBS containing 5% non-fat dry milk for 2 h to block nonspecific binding sites. The blot was incubated with primary antibody (diluted according to the manufacturer's instructions) overnight at 4°C. After washing, the blots were incubated with 1:1,000 dilution of horse radish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 45 min at room temperature. After extensive washes with high and low salt buffers, the immunoreactive proteins were visualized using enhanced chemiluminescence detection reagents (Sigma). Densitometry was performed on IISP scanner and quantitated with Total Lab 1.11 software.

Reverse transcriptase (RT) reaction and PCR amplification

Reverse-transcription of isolated RNA (1 µg) to cDNA and further PCR amplification was done as described previously [28]. Table 1 provides details of primer sequences for the PCR. Amplification products were analysed by electrophoresis in a 2% agarose gel containing ethidium bromide with 100 bp DNA ladder. The PCR products were visualized

as bands with a UV-transilluminator and photographs taken using gel documentation system (GelDocMega™, United Kingdom).

Statistical analysis

The data are expressed as mean ± Standard Deviation SD. Statistical comparisons were performed by paired sample 't' test and the results were considered statistically significant if the p value was <0.05.

Observations & results:

The activities of phase I enzymes CYP450 and its isoforms, EROD, MROD, and PROD were significantly increased in the S9 fraction of the ovarian cancer tissues compared to non-malignant control ovarian tissues (Fig. 1). RT-PCR and western blot analysis revealed a significant increase in the expression of CYP1A1 and CYP1B1 in ovarian cancer tissues compared to control ovarian tissues. However, the expression of CYP1B1 was higher than CYP1A1 expression in ovarian cancer tissues (Fig. 2 & 3). Western blot analysis showed a significant increase in the expression of CYP1A1 and CYP1B1 in the blood of ovarian cancer patients. However, there was no expression of CYP1B1 in blood from control subjects (Fig. 4). There was no significant difference in the activities of phase II enzymes, GST and QR between tumour and control tissues (Fig. 5). Western blot analysis also revealed no significant difference in the expression of GST between tumour and control tissues (Fig. 6). RT-PCR and western blot analysis showed a significant increase in the expression of cyclin D1 and PCNA in ovarian tumours compared to control tissues (Fig. 7 & 8). RT-PCR and western blot analysis also showed a significant decrease in the expression of Bax and increase in the expression of Bcl-2 in ovarian tumours compared to control tissues (Fig. 9 & 10).

Discussion:

Cytochrome P450 (CYP 450) belongs to the family of phase I enzymes, which metabolically activate various procarcinogens and initiate tumorigenesis [8]. An increased lifetime exposure level of estrogen (E2) is one of the major risk factors in hormone dependent cancers like ovarian and breast cancer [9,10]. The increased level of estrogen results in transcriptional activation of CYP genes and conversion of estrogen into genotoxicants [29]. McFadyen et al., [11] reported increased transcriptional activity of CYP1B1 in hormone dependent ovarian cancers. Further studies on microarray gene expression profile of ovarian cancer tissues by Downie et al., [12] demonstrated higher levels of various levels of CYP450 genes including CYP1B1, CYP2A/2B, CYP2F1, CYP2R1, etc. Downie et al., [12] also reported absence of gene expression of CYP1B1 in control ovarian tissues. In the present study, increased activity of CYP450 and its isoforms with increased expression levels of CYP1B1 relative to CYP1A1 in ovarian tumours, provide evidence of tumour initiation by metabolic conversion of E2 to 4-hydroxy estradiol (4-OHE2) by CYP1B1. 4-OHE2 methylation by catechol-O-methyltransferase (COMT) occurs at a slower rate than 2-hydroxy estradiol (2-OHE2). 4-OHE2 undergoes peroxidation and produces superoxide and reactive semiquinone/quinine intermediates, which cause DNA damage and genotoxicity [30,31]. Saini et al., [32] demonstrated that CYP1B1 knockdown in endometrial carcinoma cell line resulted in decreased cell proliferation and invasion, and promoted cell cycle arrest and apoptosis. Genetic polymorphism of CYP1B1 gene can further alter E2 metabolism, resulting in higher risk of estrogen-dependent cancers [33,34]. A higher level of CYP1B1 in blood of ovarian cancer patients and lack of expression in control blood samples was

observed for the first time in our study. These findings provide evidence that CYP1B1 and E2 are the key players in initiation of ovarian carcinogenesis, and are potent therapeutic targets for ovarian cancer.

Phase II enzymes like GST and QR protect cells against estrogen mediated DNA damage. Both in vitro and in vivo studies by Ansell et al., [13] provide evidence that E2 downregulates phase II enzyme activities and increase oxidative DNA damage in cells. No significant difference in GST and QR levels were found between the control and tumour tissues in our study. In the face of increased CYP isoform activity that generates toxic electrophiles and reactive oxygen species, absence of a corresponding increase in phase II enzymes in ovarian cancer tissues implies relatively decreased detoxification that could result in DNA damage, genotoxicity, and malignant transformation.

Proliferation is controlled by cyclin/CDK complexes in normal cells. In hormone dependent cancers like breast cancer, estrogen independently regulates the expression of cyclin D1, which further mediates the estrogen effect on cell cycle progression [14]. Cyclin D1 forms complex with estrogen receptor (ERα/β) and a co-activator. This complex then translocates to the nucleus, binds to estrogen response elements (EREs), and promotes transcription of various genes. Cyclin D1 may also act by forming complexes with CDK4 and/or CDK6, and phosphorylate retinoblastoma gene product pRB. Phosphorylated pRB cannot restrain cell cycle progression by binding to transcription factor E2F, thus allowing cells to enter S phase [35]. Over-expression of cyclin D1 makes the growing normal cells less dependent on growth factors and induces cell passage through G1 phase of cell cycle. In transgenic mice model, overexpression of cyclin D1 was shown to stimulate aberrant mammary epithelial cell proliferation, promoting tumorigenesis [36].

Various in vitro and human case studies have reported increased levels of cyclin D1 and PCNA in ovarian cancer cells [15-17]. A higher level of cyclin D1 is an indicator of poor prognosis and reduces life expectancy of ovarian cancer patients [15]. OVCA1 that down-regulates cyclin D1 and inhibits cell proliferation in ovarian cancer cells, is found down-regulated or deleted in various ovarian cancers [37]. Cell cycle related kinases (CCRK) also influence ovarian tumour cell proliferation by inducing cyclin D1 expression [15]. The present study reports an increase in cyclin D1 in ovarian tumours. Cyclin D1 thus plays a pivotal role in ovarian carcinogenesis, and is an important prognostic marker for predicting ovarian cancer patient's life expectancy.

Cyclin D1/ER complex can trans-criptionally activate cell proliferation genes like PCNA. PCNA plays a very important role in DNA replication and initiation of cell proliferation. PCNA along with cyclin/CDK complex promotes DNA replication. PCNA is found elevated in hormone mediated breast and ovarian cancers, and in various other human tumours. PCNA gene contains EREs and is thus upregulated in cells treated with E2 [7,38,39]. An increased expression level of PCNA in ovarian tumours was observed in the present study. Thus, PCNA may be used as an important target for ovarian cancer treatment and prognosis.

Apoptosis is a very important pathway targeted during tumorigenesis. Cell proliferation of mutated cells results in the accumulation of various genetic mutations [40]. Gene expression profiling of breast cancer cell lines (MCF-7) by microarray analysis, showed up-regulation of positive proliferation regulators, various growth promoting factors, genes involved in cell cycle progression,

and the downregulation of transcriptional repressors, anti-proliferative and pro-apoptotic genes [41]. Intra-peritoneal mouse xenograft model of various cancers by Spillman et al., [19] provided evidence of E2 promoting growth in size and lymph node metastasis in ER+ ovarian tumours. E2 downregulates negative regulators of Bcl-2 such as Bad or Nip-2 [20]. Bcl-2 and Bax levels have been reported to be important markers for predicting chemoresistance in ovarian cancer patients [18]. Anderson et al., [42] reported an increase in urinary Bcl-2 levels in ovarian cancer patients without being affected by tumour grade, stage, histological subtypes, levels of creatinine or age of patient, and the results also showed co-relation with serum CA125 levels of the patients. Studies by Camlica et al., [43] also reported high levels of serum Bcl-2 and CA125 in late stage ovarian cancer patients. Bcl-2 gene contain ERE, which is up-regulated by E2/ER complex [44]. Present study demonstrated an increase in Bcl-2 and a decrease in Bax levels in ovarian tumour samples that reflects poor prognosis. Increased Bcl-2/Bax ratio suggest E2 mediated up-regulation of Bcl-2 gene through ERE, mediated by ER [45,46]. Thus Bcl-2 in combination with Bax and other biomarkers such as CA125 can be useful as a prognostic, chemoresistant or diagnostic factor for ovarian cancer patients.

Conclusion:

In conclusion, the findings of the present study exhibit imbalance in phase I and phase II xenobiotic-metabolizing enzymes, together with an imbalance in cell proliferation and apoptosis in epithelial ovarian cancer tissues, which may be the key contributors for the development of epithelial ovarian cancer. This work provides an insight in the understanding of the mechanisms of epithelial ovarian cancer and opens new avenues of research in this field.

Acknowledgements:

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Conflicts Of Interest:

The author declares that there is no conflict of interests.

Table 1 Sequence of Oligonucleotide Primers Used for RT-PCR

Gene Product	Primers	Oligonucleotide Sequences	Fragment Size
CYP1B1	Sense Antisense	5'-CAGTGACACGACTACTA-3' 5'-CAAGGTGACGATGACTT-3'	260 bp
CYP1A1	Sense Antisense	5'-TGGTCAGACGACAGTGTC-3' 5'-TCGGATGACATAGCTCA-3'	416 bp
Bax	Sense Antisense	5'-TAGCTATGCGAGTGTC-3' 5'-CATATATTGGTTTGAC ATGC-3'	415 bp
Bcl-2	Sense Antisense	5'-GCACTTAAGCCGTGTC-3' 5'-ACAAATAGCTAGGATTCG ACGC-3'	293 bp

Cont....

cyclinD1	Sense Antisense	5'-CGGAGCACAACAGATC-3' 5'-GGTGTCA GCCAGTCCA-3'	331 bp
PCNA	Sense Antisense	5'-GCCGTAATGAGCT CAAA-3' 5'-GCACGGACTCAGAAC-3'	472 bp
GAPDH	Sense Antisense	5'-TCAAGAAGGTGGTGAAGCAG-3' 5'-AGGTGGAAGAATGGGAGTTG-3'	420 bp

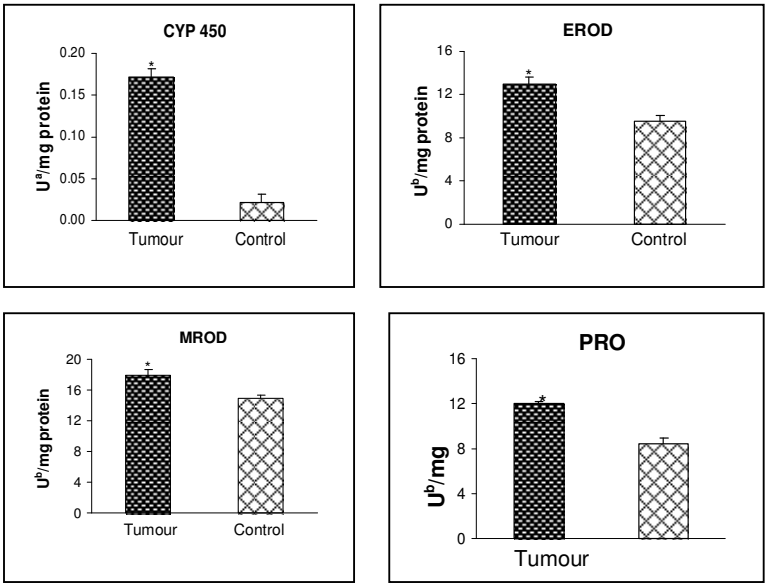

Fig. 1 Activities of total CYP450, EROD, MROD and PROD in the S9 fractions of the tumour and control ovarian tissues (Mean ± SD; n=18)

* Significantly different from control (non-malignant) tissue (p<0.05) Paired sample't' test.

a μmoles of cytochrome P450

b nmoles of resorufin formed per minute

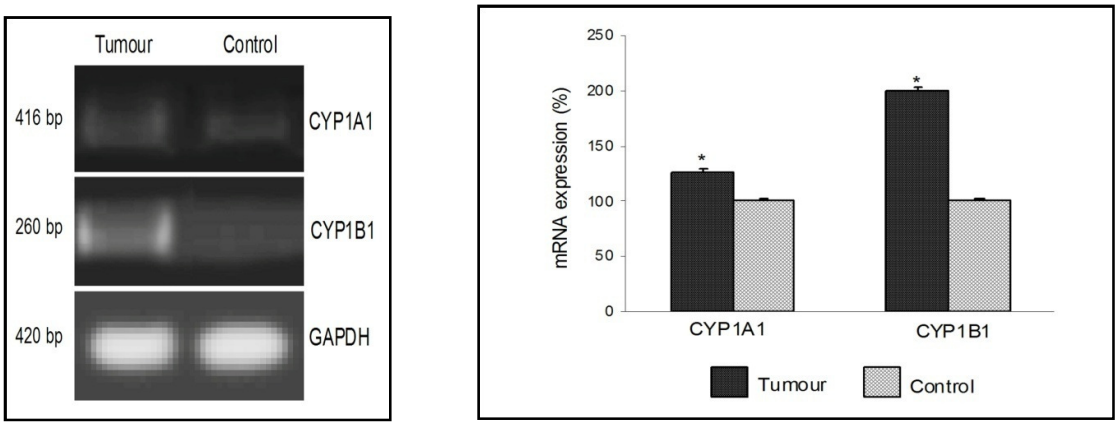

Fig. 2 mRNA expression of CYP1A1 and CYP1B1 in tumour and control ovarian tissues (Mean ± SD;n=18)

A Representative RT-PCR analysis. GAPDH was used as an internal control.

B Densitometric analysis. The mRNA expression from control lysates for eighteen

determinations was designated as 100% in the graph.

* Significantly different from control tissue (p<0.05) Paired sample‘t’ test.

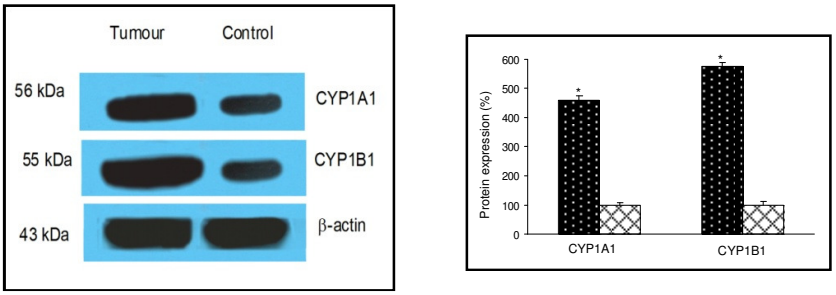

Fig. 3 Protein expression of CYP1A1 and CYP1B1 in tumour and control ovarian tissues (Mean ± SD; n=18)

A Representative immunoblot analysis. Protein samples (50 µg/lane) resolved on SDS-PAGE were probed with corresponding antibodies. β-actin was used as loading control.

B Densitometric analysis. The protein expression from control lysates for eighteen determinations was designated as 100% in the graph.

* Significantly different from control tissue (p<0.05) Paired sample‘t’ test.

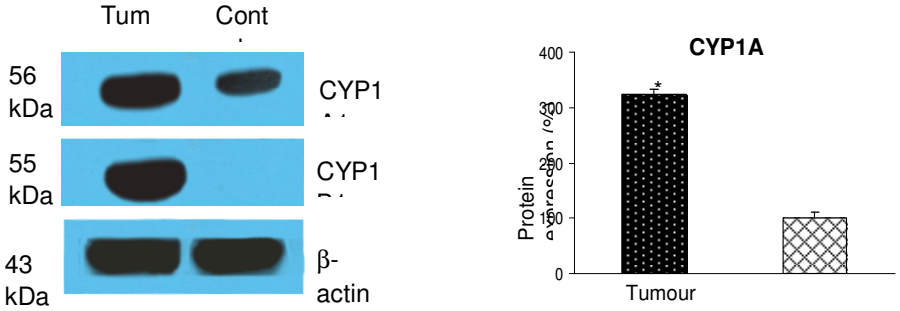

Fig. 4 Protein expression of CYP1A1 and CYP1B1 in tumour and control blood (Mean ± SD; n=18)

A Representative immunoblot analysis. Protein samples (50 µg/lane) resolved on SDS-PAGE were probed with corresponding antibodies. β-actin was used as loading control.

B Densitometric analysis. The protein expression from control blood for eighteen determinations was designated as 100% in the graph.

* Significantly different from non-tumour tissue (p<0.05) Paired sample‘t’ test.

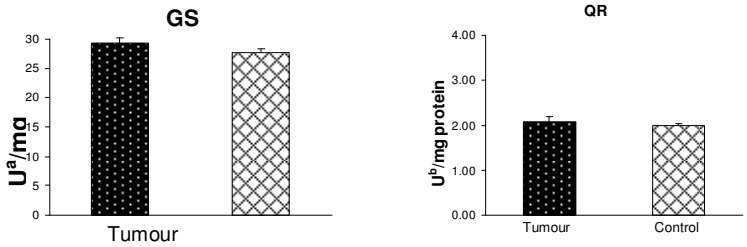

Fig. 5 Activities of GST and QR in tumour and control ovarian tissues (Mean ± SD; n=18)

a µmoles of CDNB conjugated with GSH per min

b µmoles of NADPH oxidized per h

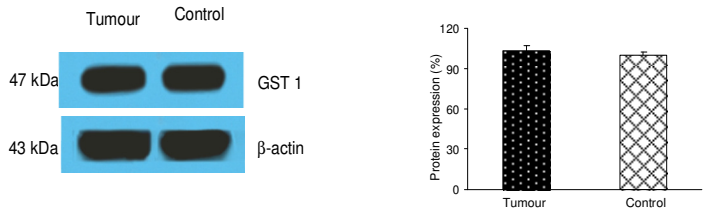

Fig. 6 Protein expression of GST in tumour and control ovarian tissues (Mean ± SD; n=18)
A Representative immunoblot analysis. Protein samples (50 µg/lane) resolved on SDS-PAGE were probed with corresponding antibodies. β-actin was used as loading control.
B Densitometric analysis. The protein expression from control lysates for eighteen determinations was designated as 100% in the graph.

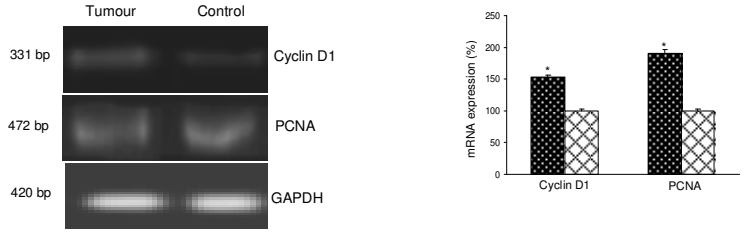

Fig. 7 mRNA expression of cyclin D1 and PCNA in tumour and control ovarian tissues (Mean ± SD; n=18)
A Representative RT-PCR analysis. GAPDH was used as an internal control.
B Densitometric analysis. The mRNA expression from control lysates for eighteen determinations was designated as 100% in the graph.
* Significantly different from control tissue (p<0.05) Paired sample‘t’ test.

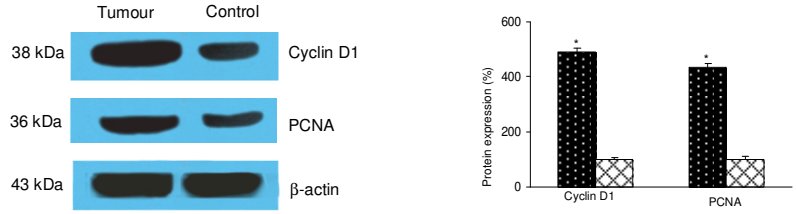

Fig. 8 Protein expression of cyclin D1 and PCNA in tumour and control ovarian tissues (Mean ± SD; n=18)
A Representative immunoblot analysis. Protein samples (50 µg/lane) resolved on SDS-PAGE were probed with corresponding antibodies. β-actin was used as loading control.
B Densitometric analysis. The protein expression from control lysates for eighteen determinations was designated as 100% in the graph.
* Significantly different from control tissue (p<0.05) Paired sample‘t’ test.

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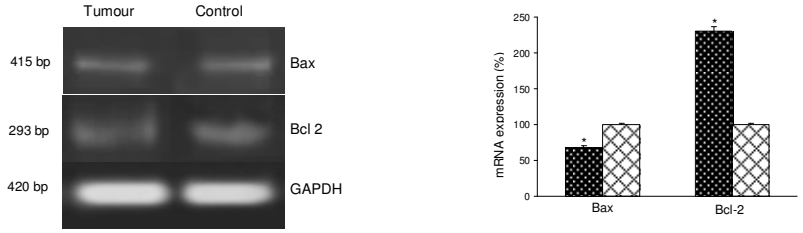

Fig. 9 mRNA expression of Bax and Bcl-2 in tumour and control ovarian tissues (Mean ± SD; n=18)

A Representative RT-PCR analysis. GAPDH was used as an internal control.

B Densitometric analysis. The mRNA expression from control lysates for eighteen determinations was designated as 100% in the graph.

* Significantly different from control tissue (p<0.05) Paired sample't' test.

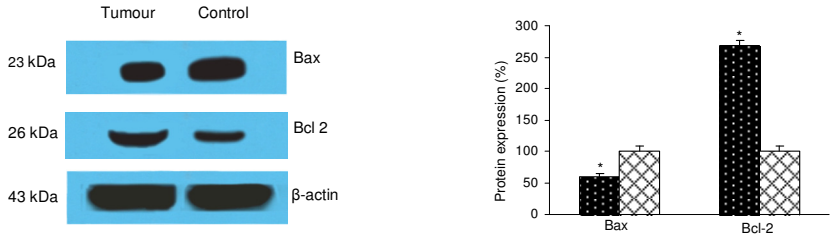

Fig. 10 Protein expression of Bax and Bcl-2 in tumour and control ovarian tissues (Mean ± SD; n=18)

A Representative immunoblot analysis. Protein samples (50 µg/lane) resolved on SDS-PAGE was probed with corresponding antibodies. β-actin was used as loading control.

B Densitometric analysis. The protein expression from control lysates for eighteen determinations was designated as 100% in the graph.

* Significantly different from control tissue (p<0.05) Paired sample't' test.

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