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Serum inhibin, activin and follistatin in postmenopausal women with epithelial ovarian carcinoma

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Objective To investigate the role of serum inhibin A, inhibin pro-αC immunoreactivity, activin A, and follistatin in postmenopausal women with epithelial ovarian cancer.

Design Case–control study.

Sample Serum samples from 27 postmenopausal women with epithelial ovarian cancer and 54 controls from the general population participating in an ovarian cancer screening trial.

Results Women with epithelial ovarian cancer had significantly higher serum levels of pro-αC immunoreactivity ($P = 0.03$), activin A ($P = 0.004$) and follistatin ($P = 0.04$), but not inhibin A ($P = 0.13$). Using the 90th centile in the control group as the cut off, pro-αC levels were elevated in 41% of women with epithelial ovarian cancer, while inhibin A was elevated in only 15%. Using the 95th centile as the cut off, serum pro-αC was elevated in only 11% of women with epithelial ovarian cancer (3/27), while activin A was elevated in 48% (11/23). Follicle stimulating hormone levels were significantly lower in women with epithelial ovarian cancer ($P = 0.01$). Although, inhibin-related peptides can modulate follicle stimulating hormone levels, there was no correlation between inhibin A, pro-αC immunoreactivity, activin A or follistatin and follicle stimulating hormone.

Conclusion These data demonstrate that though there is preferential secretion of precursor forms of the α subunit rather than dimeric inhibin A by epithelial ovarian cancer, pro-αC is unlikely to be a useful tumour marker. Activin A is more commonly elevated in postmenopausal women with epithelial ovarian cancer and its role as a tumour marker in the diagnosis and screening of epithelial ovarian cancer warrants further evaluation.

INTRODUCTION

Ovarian cancer is the fifth most common malignancy among women in the UK with 5318 cases registered in England and Wales in 1991[1], the majority of which are epithelial in origin. Inhibin-related peptides are useful in the differential diagnosis and surveillance of granulosa cell tumours[2], but their role in epithelial ovarian cancers remains to be defined. Inhibins and activins are structurally related dimeric proteins of the transforming growth factor-β superfamily. Inhibin is a heterodimeric glycoprotein composed of a common α subunit and one of two β subunits (βA and βB), resulting in inhibin A (αβA) and inhibin B (αβB). Activin is a dimer of the two β subunits and exists as activin A (βAβA), activin B (βBβB) and activin AB (βAβB). Follistatin is a structurally distinct, single chain glycoprotein, which is the major binding protein for activin and inhibin.

Using a nonselective assay, Healy *et al.*[3] and Blaakaer *et al.*[4] found that 25%–60% of women with epithelial ovarian cancer had elevated serum inhibin levels. More recently, using specific immunoassays measuring inhibin A, elevated levels were found in 5–31% of women with epithelial ovarian cancer[5–7]. Serum inhibin B was detected in these women, but levels were within the normal range[5,8]. The serum also contained immunoreactive forms of the α subunit which were not linked to the β subunit. Some of these precursor forms of inhibin (e.g. pro-αC and pro-αN-αC) could be measured using the pro-αC assay[9]. It has been stated that among the inhibin assays, measurement of pro-αC immunoreactivity may be of most value in women with epithelial ovarian cancer[2,5]. Welt *et al.*[10] found that activin A and follistatin were secreted by epithelial ovarian cancer *in vitro*, and that serum activin A levels were significantly raised in women with epithelial ovarian

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cancer compared with controls. No studies have looked at serum follistatin levels in these women.

The present study was undertaken to investigate the role of serum inhibin A, pro-αC, activin A and follistatin in postmenopausal women with epithelial ovarian cancer.

METHODS

Pre-operative serum samples were obtained from 27 postmenopausal women with epithelial ovarian cancer who were not on hormone replacement therapy and had given informed consent as approved by the local ethics committee. Histology reports were reviewed to confirm the diagnosis. Controls were chosen from healthy, postmenopausal women over the age of 50 who had enrolled in an ongoing general population screening trial for sporadic epithelial ovarian cancer[11] based on CA125 measurement. The control population had no significant family history of ovarian cancer, was medically fit with no hepatic or renal disorders and had been followed up for a minimum of two years without developing ovarian cancer. The women with epithelial ovarian cancer were matched for menopausal status (12 months amenorrhoea following a natural menopause), lack of hormone replacement therapy and age (within five years), with the first two women on the screening trial who fulfilled these criteria. Serum inhibin, activin and follistatin levels do not alter with age in postmenopausal women[12–14]. The age matching was only done as follicle stimulating hormone and luteinising hormone were measured. All blood samples were allowed to clot and centrifuged within 24 hours of venepuncture, then stored at –20°C until aliquots were required for the assay. Follicle stimulating hormone and luteinising hormone levels were assayed in all cases and controls.

Immunoassays for inhibins, activins and follistatin

Dimeric inhibin A, inhibin forms containing pro-αC and αC immunoreactivity, activin A and follistatin were measured using two-site enzyme linked immunosorbent assays described previously[9,15–17] with some modifications[18,19].

All serum samples and standards were treated similarly. Dilutions were made using fetal calf serum as diluent for the inhibin A assay and assay buffer for the pro-αC assay. For the activin assay, samples were diluted in phosphate buffered saline containing 5% bovine serum albumin (Sigma-Aldrich Ltd, Poole, UK). Sodium dodecyl sulphate (2% final volume) was added and the tubes mixed. To eliminate false positive results, dissociate complexes and modify the β subunits to improve antibody interactions, samples were placed in boiling water for 3 min, then allowed to cool before addition of H_2O_2 (2% final volume; 30 min at 23°C)[20]. For the follistatin assay, samples were diluted using dissociating solution: 84 mM sodium deoxycholate, 3·4% Tween 20 (v/v), 1% bovine serum albumine (w/v), 5% mouse serum (v/v)[21]. For all assays, duplicate samples were dispensed onto plates (Nunc Maxisorb, Life Technologies Ltd, Paisley, UK), passively adsorbed with the appropriate capture antibody and stored dry utilising dry coating reagent (Bionostics Ltd, Wyboston, UK).

The dimeric inhibin A assay uses as standard a partially immunopurified follicular fluid preparation calibrated against 32 kD recombinant human inhibin A (Genentech, San Francisco, California, USA). A mouse monoclonal antibody (E_4) raised against the βA subunit was used as the capture antibody, with a mouse monoclonal antibody (R_1) raised against the inhibin αC subunit conjugated to alkaline phosphatase as the detection antibody, as described previously[15,18,20]. Cross-reactivities of this assay with recombinant activin A, activin B, inhibin B, follistatin and purified human pro-αC are all < 0·1%[15]. The detection level of the assay was < 7 pg/mL and intra- and inter-plate coefficients of variation were 5% and 9%, respectively.

A highly purified preparation of inhibin α subunit pro-αC was used as the standard in the assay for inhibin forms containing pro and αC immunoreactivity[9]. A mouse monoclonal capture antibody raised against a sequence of the pro- portion of the α subunit was used as the capture antibody with the same detection antibody and detection system as for the dimeric inhibin A assay[9,18]. Inhibin A, inhibin B and follistatin all cross-react less than 0·02%, although this antibody may cross-react with the larger dimeric inhibin isoforms containing the α subunit prosequences, as demonstrated by immunoblot[9]. The detection limit was < 3 pg/mL and intra- and inter-plate coefficients of variation were 4% and 7%, respectively.

Total activin A was measured using as standard a partially immunopurified human follicular fluid preparation titrated and expressed in terms of recombinant human activin A by calibration with the appropriate recombinant preparation (Genentech). An antibody (E_4) raised against the αA subunit was used as the capture antibody with specificity conferred using a biotinylated form of the same antibody for detection as described previously[16,19]. Cross-reactivities of this assay have been reported[16]. The detection limit was < 0·156 ng/mL. Intra- and inter-plate coefficients of variation were 5% and 11%, respectively.

Total follistatin immunoreactivity was measured with recombinant follistatin 288 as standard and mouse monoclonal capture and detection antibodies raised against recombinant follistatin 288 as described previously[17,19]. Cross-reactivities with activins and dimeric inhibin isoforms have been previously reported as < 0·3%, with significant cross-reaction with follistatin 315 (9·9%)[17]. The limit of detection

was < 19 pg/mL and intra- and inter-plate coefficients of variation were 7% and 12%, respectively.

Data analysis

The median and range for each peptide assayed was calculated for cases and controls. For each parameter (inhibin A, pro-αC, activin and follistatin), no significant difference was found between the values for the two matched controls by Student's paired *t* test. Hence the mean value of the two controls was used in the comparison of cases and controls. Cases and controls were compared using the Wilcoxon matched pairs signed rank test. Results were considered significant if $P < 0.05$. Spearman's co-efficient (r_s) was used to investigate if any association could be established between inhibins, activin, follistatin and follicle stimulating hormone and luteinising hormone.

RESULTS

The epithelial ovarian carcinomas included in the study were mucinous ($n = 4$), serous ($n = 10$), endometroid/clear cell ($n = 4$), undifferentiated ($n = 5$), transitional cell ($n = 1$) and borderline ($n = 3$) (Table 1). Five patients were Stage I, one Stage II, 14 Stage III and seven Stage IV according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria. The median age of the cases was 68 years (range 47–85) and that of the controls was 67·5 years (range 51–80).

There was no significant difference between inhibin A levels in women with epithelial ovarian cancer and controls ($P = 0.13$). In contrast, women with epithelial ovarian cancer had significantly higher serum levels of inhibin forms containing pro-αC immunoreactivity ($P = 0.03$), activin A ($P = 0.004$) and follistatin ($P = 0.04$) than healthy controls (Fig. 1). An analysis of the sensitivity of the markers for epithelial ovarian cancer at cut off points defined by the 90th and 95th centiles in the control group is summarised in Table 2. The most sensitive single marker was activin A which achieved a sensitivity of 48%. Follicle stimulating hormone levels were significantly lower in women with epithelial ovarian cancer ($P = 0.01$) while there was no significant difference in the luteinising hormone levels ($P = 0.2$). There was no correlation between serum inhibin A, pro-

Table 1. Results of the assays for women with epithelial ovarian cancer. Values are given as *n*. NS = not sufficient serum sample for assay; LH = luteinising hormone; FSH = follicle stimulating hormone.

Histology	Case no.	Age	Stage	LH (U/L)	FSH (U/L)	Pro-αC (pg/mL)	Inhibin A (pg/mL)	Activin A (pg/mL)	Follistatin (pg/mL)
Mucinous	1	83	3	0·1	3·2	131	7	1356	635
	2	72	3	NS	NS	95	7	1047	436
	3	71	3	NS	NS	146	7	546	537
	4	82	1	NS	NS	4294	7	NS	517
Serous	5	85	4	3·6	18·4	106	7	2472	565
	6	69	3	6	30·6	93	7	1614	736
	7	47	2	42	72·4	65	7	1125	233
	8	81	4	26	32·3	192	7	865	716
	9	54	3	NS	NS	6	7	862	256
	10	79	4	33	35·2	132·7	12	707	452
	11	64	3	40	46·7	48	31	435	344
	12	66	3	42	72·8	118	51	403	942
	13	75	3	27	49·7	219	179	320	917
	14	67	3	51	100	422	78	NS	428
Endometroid	15	74	3	NS	NS	79	7	1706	1225
	16	69	4	46	57·7	223	7	1392	1073
	17	61	3	29	37·2	252	73	NS	376
Clear cell	18	53	4	NS	NS	432	7	1579	719
Undifferentiated	19	52	4	49	109	421	30	4334	704
	20	68	4	19	84·2	355	35	3050	541
	21	69	3	41	50·7	144	10	1634	272
	22	59	3	63	52·9	195	28	1496	354
	23	64	1	21	37·1	29	10	652	925
Transitional cell	24	71	3	NS	NS	1530	7	NS	654
Borderline serous	25	65	1	52	98·2	51	7	1800	480
Borderline mucinous	26	65	1	3·6	7·6	3256	29	834	355
Borderline serous	27	49	1	17	61·4	405	7	415	641

Undetectable levels of inhibin A have been given the numerical sensitivity limit of 7 pg/mL.

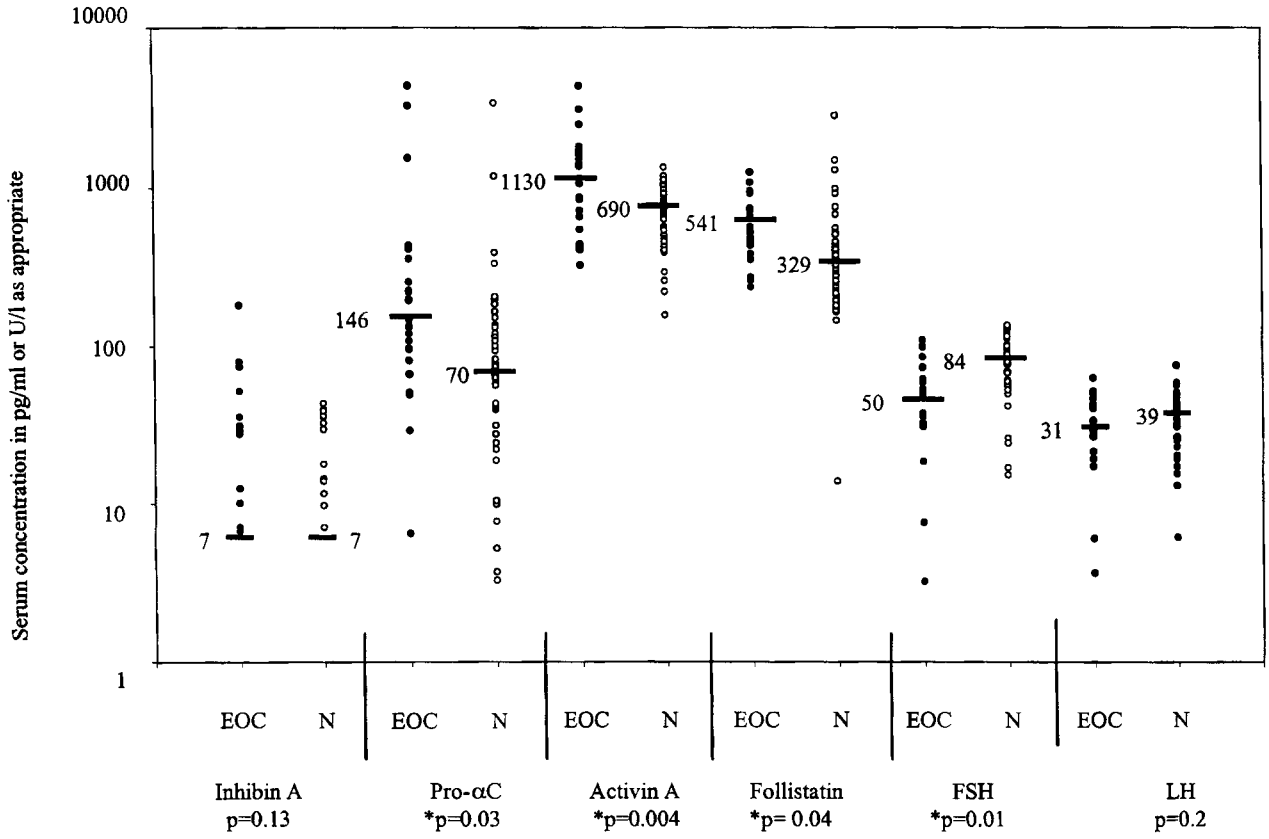

Fig. 1. Serum concentrations (pg/mL) of inhibin A, pro-αC immunoreactivity, activin A, follistatin, FSH and LH for postmenopausal women with epithelial ovarian cancer and healthy age matched postmenopausal controls (N) from the general population. The concentrations are plotted on a logarithmic scale. Median values are highlighted. Undetectable levels have been given the numerical sensitivity limit. 38/54 controls (70%) and 15/27 women (55%) with epithelial ovarian cancer had undetectable inhibin A levels. *There was significant difference between women with epithelial ovarian cancer and controls.

αC immunoreactivity, activin A, follistatin or the ratio of inhibin A/activin A or follistatin/activin A and serum follicle stimulating hormone. As anticipated, follicle stimulating hormone correlated positively with luteinising hormone ($r_S = 0{\cdot}74$) and negatively with age ($r_S = -0{\cdot}5$) in women with ovarian cancer.

A number of other observations in the study were noteworthy (Table 1). First, the few patients with inhibin A elevations had serous ($n = 3$) and endometroid ($n = 1$) cancers, while low or undetectable levels were documented in the five mucinous carcinomas (including the borderline mucinous tumour). Second, activin A was elevated in four of five undifferentiated tumours and all three endometroid cancers. Finally, surprisingly high levels of pro-αC were observed in two postmenopausal women in the control group who have remained apparently healthy in the year they have been followed up as part of the screening trial.

Table 2. Sensitivity of the markers for epithelial ovarian cancer at cut offs defined by the 90th and 95th centiles in the control group. Values are given as $\%(n_{elevated}/n_{total})$.

	90th centile of controls (pg/mL)	Sensitivity using 90th centile as cut off	95th centile of controls (pg/mL)	Sensitivity using 95th centile as cut off
Inhibin A	37	14·8 (4/27)	39	14·8 (4/27)
Pro-αC	201	40·7 (11/27)	582	11·1 (3/27)
Activin A	1082	52·2 (12/23)	1163	47·8 (11/23)
Follistatin	940	11·1 (3/27)	1337	0 (0/27)
Pro-αC + Activin A	—	70·4 (19/27)	—	51·9 (14/27)

DISCUSSION

Our data revealed no significant difference between serum inhibin A concentrations in women with epithelial ovarian cancer and age matched controls, with levels elevated (> 95th centile for controls) in only 15% of cases. Other recent studies, which measured dimeric inhibin A, revealed similar elevations in 5–13% of women with epithelial ovarian cancer[5,6,22]. None of the five mucinous tumours in our study were associated with raised levels. This is in contrast to Burger *et al.*[6] and Robertson *et al.*[22], who noted elevations in three of 12 and four of 20 mucinous carcinomas, respectively. The differences may reflect the small numbers of mucinous tumours in most of these series. Overall the consistent message is that dimeric inhibin A is less informative than other inhibin-related peptides in epithelial ovarian cancer. Frias *et al.*[23] have recently reported that preoperative serum inhibin A may be an independent prognostic factor for survival in postmenopausal women with epithelial ovarian cancer.

The absolute concentrations of pro-αC containing inhibin forms were approximately tenfold higher than dimeric inhibin A levels in the healthy postmenopausal controls, confirming previous findings[9]. Two healthy postmenopausal women had very high levels of pro-αC immunoreactivity, for which no cause has yet been established. In women with epithelial ovarian cancer, using the 90th centile of controls as the cut off, serum pro-αC immunoreactivity was found to be elevated in 41%, while inhibin A levels were elevated in only 15%. This supports the findings of Lambert-Messerlian *et al.*[5] and Burger *et al.*[2] that there is preferential secretion of precursor forms of the α subunit, rather than dimeric inhibin A, by epithelial ovarian cancer. However, serum pro-αC is unlikely to be a useful tumour marker in epithelial ovarian cancer as levels were elevated in only 11% of women with epithelial ovarian cancer, using the more stringent 95th centile as cut off. A recently published large study of inhibin forms in postmenopausal women with epithelial ovarian cancers concluded that assays detecting all inhibin forms containing the α subunit, and not just those detecting the pro-αC subunit, will probably prove to the most useful screening test[22]. The newly developed αC immunofluorometric assay, which detects all α subunit-containing proteins, has been shown to have increased sensitivity for detection of ovarian cancers, especially in combination with CA125[24].

The most commonly elevated marker was activin A, with undifferentiated and endometroid tumours accounting for the majority of the elevations. Serum activin levels were elevated in 52% of women with epithelial ovarian cancer. Lambert-Messerlian *et al.*[25] found 63% of women with epithelial ovarian cancer had an elevated pre-operative level, using the 90th centile of controls as the cut off. There is increasing evidence both from immunohistochemical analysis and expression studies on cancer cell lines that there is differential production and expression of inhibin/activin subunits with increased production of the inhibin β but not the α subunit in epithelial ovarian cancer[26–28]. Activin A is secreted more frequently than inhibin in tumour explants[10]. Activin receptors have been found in epithelial ovarian cancer cell lines[10,26] and autocrine/paracrine interactions of activin and follistatin are probably involved in signal modulation and tumour cell proliferation[26,29]. The significance of elevated activin levels remains to be elucidated. Although preliminary data from Petraglia *et al.*[8] and Frias *et al.*[23] found no correlation with clinical disease, a recent study[22], using serial assays in women with epithelial ovarian cancer, has found activin A to be a marker of persistent and recurrent disease.

Total serum follistatin was significantly elevated in women with epithelial ovarian cancer, but this was of little clinical value as a marker. No individual women had a serum follistatin concentration that exceeded the normal range. Only one previous study has examined serum follistatin levels in cancer. Elevated serum free follistatin were found in a group of 39 women with solid cancers that included four ovarian malignancies[14]. Ovarian cancer cell lines have been found to express follistatin mRNA and to secrete follistatin[26,30]. However, there is growing evidence that serum follistatin may not be primarily an ovarian product[17,31]. Thus, even if cancers produce follistatin, it may not be reflected in serum measurements. In addition, follistatin is present in a number of isoforms in biological fluids. Recent work suggests that follistatin 315 is the dominant variant in serum[32]. There are no standard assays for measuring follistatin and the lower sensitivity of the current assay for follistatin 315 compared with follistatin 288 may result in an underestimate of the total amount of follistatin[17].

Serum follicle stimulating hormone levels have been found to be significantly lower in women with epithelial ovarian cancer compared with controls[33,34]. Inhibin-related peptides can modulate follicle stimulating hormone levels and an earlier report had shown an inverse correlation between immunoreactive inhibin and follicle stimulating hormone in epithelial ovarian cancer[35]. However, this was not borne out in any of the larger series[3,22]. Our results further confirmed the lack of correlation between dimeric inhibin A, pro-αC, activin A or follistatin and follicle stimulating hormone levels in women with epithelial ovarian cancer.

In summary, the data demonstrates that though there is preferential secretion of precursor forms of the α subunit rather than dimeric inhibin A by epithelial ovarian cancer, pro-αC is unlikely to be a useful

tumour marker in postmenopausal women with epithelial ovarian cancer. Activin A is more commonly elevated and its role as a serum tumour marker in the diagnosis and screening of epithelial ovarian cancer warrants further evaluation.

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