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Inhibin-alpha subunit is an independent prognostic parameter in human endometrial carcinomas: Analysis of inhibin/activin-alpha, -betaA and -betaB subunits in 302 cases

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ABSTRACT

Inhibins are dimeric glycoproteins, composed of an alpha-subunit (inhibin-α) and one of two possible beta-subunits (βA or βB), with substantial roles in human reproduction and in endocrine-responsive tumours. The aims of this study were to determine the distribution of inhibin-α, -βA and -βB subunits in malignant human endometrial tissue and the assessment of an association with specific clinicopathologic tumour features and clinical outcome. A series of 302 endometrial cancer tissue samples were immunohistochemically analysed with monoclonal antibodies against inhibin subunits. The inhibin-α subunit showed a significant association with histological grading, surgical staging, lymph node status and diabetes in patients with endometrial cancer. Interestingly, loss of inhibin-α expression resulted in a poorer survival of endometrial cancer patients. Additionally, survival analysis demonstrated that inhibin-α immunoreactivity was an independent prognostic factor for progression-free survival, cause-specific survival as well as for overall survival. In contrast, although inhibin-βA- and -βB subunits showed a significant association between endometrial histological subtypes and histological grading, both subunits were not found to be associated with survival in endometrial cancer patients. Therefore, inhibin-α immunostaining might be used as a simple and efficient marker to identify high-risk patients leading to the selection of patients for an aggressive adjuvant therapy.

1. Introduction

Endometrial cancer is the most frequent gynaecologic genital malignancy in the western world, with an increasing incidence in the industrial nations.[1,2] Traditional prognostic factors are histological type, tumour grade and depth of myometrial invasion. Although endogenous and exogenous sources of unopposed oestrogen increase the risk of endometrial adenocarcinoma, the molecular pathogenesis of endometrial carcinoma remains unclear.[1] However, the currently applied diagnostic technology is insufficient to identify endometrial cancer patients with poor prognosis. Therefore, additional immunohistochemistry of specific markers might be a useful alternative to select high-risk patients.[3]

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Inhibins and activins are homologous proteins, sharing common β-subunits, comprising a nine-cysteine distribution pattern similar to the transforming growth factor-beta (TGF-β) family of proteins.[4,5] Inhibins, in contrast to activins, consist of an α-subunit and one of two possible β-subunits (βA or βB). The α-subunit is able to form heterodimers with either βA- or βB-subunit, resulting in the formation of either inhibin A (α-βA) or inhibin B (α-βB), respectively. Activins are homodimers of β-subunits linked by a disulphide bond. Thus, depending on the subunit combination, there are three isoforms of activin, namely activin A (βA–βA), activin B (βB–βB) and activin AB (βA–βB), which were found to be expressed.[4,5]

Inhibin has been demonstrated in normal, hyperplastic and malignant human endometrium[6–11], although the precise roles of inhibin subunits remain still unknown. Interestingly, TGF-β has been recognised as a tumour suppressor in premalignant stages of carcinogenesis with an additional dual role as a pro-oncogene in the later stages of disease, leading to metastasis.[12] The tumour suppressor activity of the inhibin α-subunit was first identified after functional deletion of the inhibin-α gene in male and female mice, resulting in primary gonadal sex cord-stromal tumours.[13] We recently demonstrated a higher expression of the inhibin-α, -βA and -βB subunits in hyperplastic endometrial tissue than in adenocarcinomas, suggesting an involvement of these subunits in endometrial pathogenesis.[11,14,15] Furthermore, determination of the expression pattern of the inhibin-α subunit in human endometrial tissue might be of predominant importance, since the preferred secretion form of inhibin or activin is determined by this subunit.

Therefore, the aims of this study were

(a) the determination of the expression and tissue distribution pattern of the inhibin-α, -βB and -βB subunits in malignant endometrial tissues;
(b) the assessment of these subunits as immunohistochemical markers for malignant endometrial adenocarcinoma;
(c) the assessment of an association of inhibin/activin subunit expression with specific clinicopathologic tumour features and clinical outcome in a large number of endometrial cancer tissues.

2. Materials and methods

2.1. Tissue samples

Three hundred and two hysterectomy specimens containing endometrial carcinoma tissue were obtained from the pathological archives of the 1st Department of Obstetrics and Gynaecology – Ludwig-Maximilians-University Munich between the years 1990 and 2002. In a previous study, endometrial specimens until the year 2001 were analysed for the expression of oestrogen receptor alpha (ERα) and beta (ERβ), progesterone receptor A (PR-A) and B (PR-B).[41] All haematoxylin and eosin-stained slides were re-reviewed by a gynaecological pathologist (N. S.) to verify the diagnosis, histological grade, histological type, surgical stage, lymphangiosis and haemangiosis.[16] Women with sarcoma of the uterus were excluded from this study. Pathological stage and histological subtype were determined according to International Federation of Gynaecology and Obstetrics (FIGOs) criteria.[17] Histological classification was performed according to the World Health Organisation (WHO) system in well-differentiated (G1; $n = 165$), moderately differentiated (G2; $n = 83$) and poorly differentiated (G3; $n = 54$) carcinomas.

Patients with endometrial carcinoma received modified radical hysterectomy, salpingo-oophorectomy or selective pelvic lymphadenectomy, with or without para-aortic lymphadenectomy. Lymph node dissection was generally performed in patients having tumours with deep myometrial invasion and/or high-grade or aggressive histological features. Obesity, advanced age and excessive comorbidity were factors preventing full surgical staging.

Patient data were obtained from three sources: hospital tumour registry, automated database and chart review. The Munich tumour registry systematically collects baseline data, including demographic data, diagnosis, additionally diseases (i.e. obesity, diabetes and hypertension) and treatment information on all the cancer patients, who are diagnosed or treated at the 1st Department of Obstetrics and Gynaecology. Automated records and, when available, charts for each patient were reviewed to verify the diagnosis and the presence or the absence of radiologic or pathological evidence of disease recurrence. Patients' data were analysed anonymously. All cases of recurrence had radiologic evidence or biopsy-proven progression of disease. Only the records of patients who died of disease were considered to be uncensored; the records of all patients who were alive at follow-up or who did not die of disease (or a related cause) were considered to be censored. Additionally, those cases where the exact cause of death was unknown but died within two years after the diagnosis of a metastatic lesion have been censored as previously described.[41]

2.2. Immunohistochemistry

Immunohistochemistry with specific antibodies against inhibin subunits was performed using a combination of pressure cooker heating and the standard streptavidin-biotin-peroxidase complex with the use of the mouse-IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, California, United States) as previously described.[8] Briefly, paraffin-fixed tissue sections were dewaxed using xylol for 15 min, rehydrated in an ascending series of alcohols (70%, 96% and 100%) and subjected to antigen retrieval on a high setting for 10min in a pressure cooker in sodium citrate buffer (pH 6.0), containing citric acid 0.1M and sodium citrate 0.1 M in distilled water. After cooling, the slides were washed twice in PBS. Endogenous peroxidase activity was quenched by immersion in a 3% hydrogen peroxide solution (Merck, Darmstadt, Germany) dissolved in methanol for 20 min. Non-specific binding of the primary antibodies was blocked by incubating the sections with diluted normal serum (10 ml PBS containing 150 μl horse serum) for 20 min at room temperature. Sections were incubated at room temperature for 120 min with the monoclonal primary antibodies (*inhibin-α*, clone R1, diluted in PBS 1:50; *inhibin-βA*, E4, diluted in PBS 1:50; *inhibin-βB*, C5, diluted in PBS 1:10; obtained from Serotec – Oxford – United Kingdom). After

Table 1 – Clinicopathological characteristics of the analysed endometrial carcinomas.

		Total (n = 302)	Endometrioid adenocarcinomas			Non-endometrioid carcinomas		
			Endometrioid (n = 221)	Mucinous (n = 14)	Mixed (n = 34)	Serous (n = 22)	Clear cell (n = 6)	Undifferentiated (n = 5)
Age (years)	< 65	156 (51.7%)	114 (51.6%)	7 (50%)	21 (61.8%)	7 (31.8%)	4 (66.7%)	3 (60%)
	> 65	146 (48.3%)	107 (48.4%)	7 (50%)	13 (38.2%)	15 (68.2%)	2 (33.3%)	2 (40%)
WHO grading	Grade 1	165 (54.6%)	138 (62.4%)	10 (71.4%)	11 (32.4%)	5 (22.7%)	1 (16.7%)	0 (0%)
	Grade 2	83 (27.5%)	58 (26.2%)	4 (28.5%)	14 (41.2%)	5 (22.7%)	2 (33.3%)	0 (0%)
	Grade 3	54 (17.9%)	25 (11.3%)	0 (0%)	9 (26.5%)	12 (54.5%)	3 (50%)	5 (100%)
FIGO stage	FIGO I	225 (74.5%)	176 (79.6%)	12 (85.7%)	19 (55.9%)	13 (59.1%)	2 (33.3%)	3 (60%)
	FIGO II	22 (7.3%)	15 (6.8%)	0 (0%)	4 (11.8%)	0 (0%)	3 (50%)	0 (0%)
	FIGO III	46 (15.2%)	22 (10%)	2 (14.3%)	11 (32.3%)	8 (36.4%)	1 (16.7%)	2 (40%)
	FIGO IV	9 (3.0%)	8 (3.6%)	0 (0%)	0 (0%)	1 (4.5%)	0 (0%)	0 (0%)
LN status	Negative	193 (63.9%)	145 (65.6%)	9 (64.3%)	17 (50%)	15 (68.2%)	5 (83.3%)	2 (40%)
	Positive	25 (8.3%)	13 (5.9%)	1 (7.1%)	6 (17.6%)	3 (13.6%)	1 (16.7%)	1 (20%)
	Unknown	84 (27.8%)	63 (28.5%)	4 (28.6%)	11 (32.4%)	4 (18.2%)	0 (0%)	2 (40%)
Lymphangiosis	Negative	272 (90.1%)	202 (91.4%)	13 (92.9%)	29 (85.3%)	19 (86.4%)	6 (100%)	3 (60%)
	Positive	30 (9.9%)	19 (8.6%)	1 (7.1%)	5 (14.7%)	3 (13.6%)	0 (0%)	2 (40%)
Haemangiosis	Negative	294 (97.4%)	215 (97.3%)	14 (100%)	33 (97.1%)	22 (100%)	6 (100%)	4 (80%)
	Positive	8 (2.8%)	6 (2.7%)	0 (0%)	1 (2.9%)	0 (0%)	0 (0%)	1 (20%)
Diabetes	Negative	268 (88.7%)	193 (87.3%)	14 (100%)	32 (94.1%)	20 (90.9%)	6 (100%)	3 (60%)
	Positive	34 (11.3%)	28 (12.7%)	0 (0%)	2 (5.9%)	2 (9.1%)	0 (0%)	2 (40%)
Adipositas	Negative	198 (65.6%)	139 (62.9%)	8 (57.1%)	27 (79.4%)	15 (68.2%)	6 (100%)	3 (60%)
	Positive	104 (34.4%)	82 (37.1%)	6 (42.9%)	7 (20.6%)	7 (31.8%)	0 (0%)	2 (40%)
Hypertension	Negative	183 (60.6%)	129 (58.4%)	5 (35.7%)	26 (76.5%)	16 (72.7%)	5 (83.3%)	2 (40%)
	Positive	119 (39.4%)	92 (41.6%)	9 (64.3%)	8 (23.5%)	6 (27.3%)	1 (16.7%)	3 (60%)
Radiotherapy	Negative	177 (58.6%)	136 (61.5%)	9 (64.3%)	13 (38.2%)	14 (61.5%)	4 (66.7%)	1 (20%)
	Positive	119 (39.4%)	79 (35.7%)	5 (35.7%)	21 (61.8%)	8 (36.4%)	2 (33.3%)	4 (80%)
	Denial	6 (2.0%)	6 (2.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Anti-hormone therapy	Negative	293 (97.0)	216 (97.7%)	14 (100%)	32 (94.1%)	20 (90.9%)	6 (100%)	5 (100%)
	Positive	9 (3.0%)	5 (2.3%)	0 (0%)	2 (11.3%)	2 (9.1%)	0 (0%)	0 (0%)
	Denial	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

washing with PBS, the slides were incubated in diluted biotinylated mouse anti-serum (10 ml PBS containing 50 μl horse serum) for another 30 min at room temperature. After incubation with the avidin-biotin peroxidase complex (ABC) for another 30 min and after repeated washing steps with PBS, visualisation was performed with substrate and chromogenic 3,3′-diaminobenzidine (DAB; Dako, Glostrup, Denmark) for 8–10 min. The slides were counterstained with Mayer's acidic haematoxylin, and washed in an ascending series of alcohols (50–98%). After xylol treatment, the slides were covered. Negative controls were performed by replacing the primary antibody with normal mouse IgG in the same dilution used for inhibin detection as previously described.[8]

2.3. *Immunohistochemical evaluation*

The intensity and distribution patterns of specific inhibin-subunit immunohistochemical cytoplasmatic staining reaction were evaluated by two blinded, independent observers, including a gynaecological pathologist, using a semi-quantitative score (IRS) as previously described[18], and were previously used to asses inhibin expression in endometrial and placental tissues.[8,19,20]

The IRS was calculated by multiplication of the optical staining intensity (graded as 0 = no, 1 = weak, 2 = moderate and 3 = strong staining) and the percentage of positively stained cells (0 = no staining, 1 = <10% of the cells,

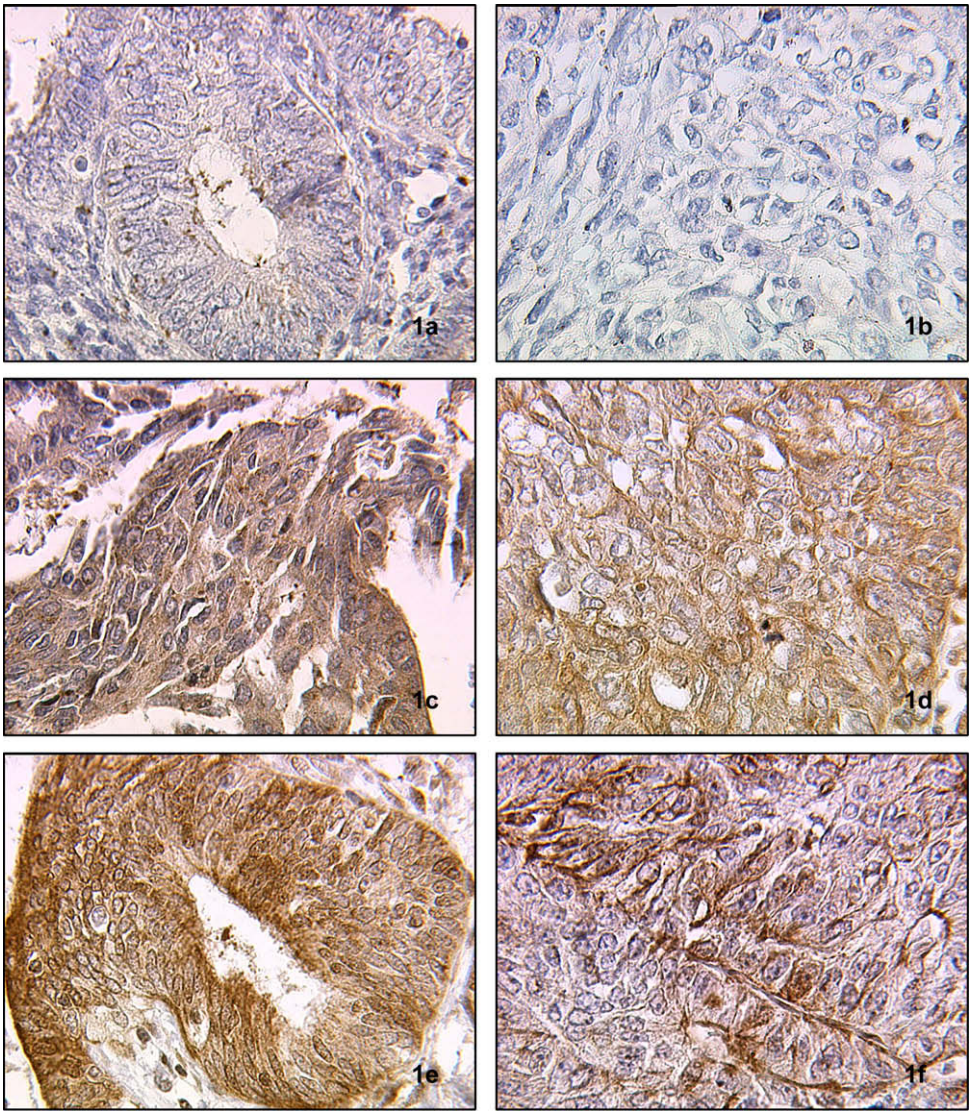

Fig. 1 – (a–c) Expression of inhibin-α, -βA and -βB subunits in malignant human endometrial tissue. Inhibin-α showed minimal to moderate expression in well-differentiated endometrial cancer tissue (Fig. 1a, ×250), whereas poorly differentiated carcinomas did not demonstrate an inhibin-α immunoreactivity (Fig. 1b, ×400). The staining intensity of the inhibin-βA and -βB subunits was higher compared to the α-subunit. The staining reaction against inhibin-βA was low in well-differentiated endometrial cancer (Fig. 1c, ×250) than in poorly differentiated carcinomas (Fig. 1d, ×400). Similar to the inhibin-βA expression, the inhibin-βB immunoreactivity was lower in well-differentiated endometrial cancer (Fig. 1e, ×250) than in poorly differentiated carcinomas (Fig. 1f, ×400).

2 = 11–50% of the cells, 3 = 51–80% of the cells and 4 = >81% of the cells). Sections were examined using a Leica (Solms, Germany) photomicroscope. The IRS of the inhibin-α, -βα and -βB immunohistochemical expression levels was compared using the Kruskal–Wallis one-way analysis of variance by ranks.

2.4. Statistical analysis

For the purposes of statistical survival analysis, inhibin/activin subunits in tumour samples were considered to be elevated if the immunohistochemical staining score of a tumour sample was higher than the median as previously described.[22,41] Increased/positive versus not increased/negative immunostaining in tumour samples was compared using the *chi-square* test and the exact Fisher's test wherever applicable.

The outcome variables analysed were progression-free survival, cause-specific survival and overall survival. Univariate analysis was performed with Kaplan–Meier life-table curves to estimate survival, and was compared using the log rank test.[23] Prognostic models used multivariate Cox regression analysis for multivariate analyses of survival in a forward stepwise manner.[24] Data were adjusted for age, FIGO stage, WHO grade, lymph node involvement, lymphangiosis, haemangiosis, unfavourable histology (endometrioid versus papillary serous/clear cell), hypertension, obesity, diabetes, anti-hormone therapy and radiation therapy. Lymph node involvement was entered as categorical variable defined as no lymph node involvement, positive lymph nodes and unknown status. Significance of differences was assumed at $p \leqslant 0.05$ as the two-sided tests (SPSS, version 14.0; SPSS Inc., Munich, Germany) were used.

3. Results

3.1. Clinicopathological characterisation

Of the total amount of 302 endometrial samples, 293 endometrial carcinomas were analysed for the expression of oestrogen receptor alpha (ERα) and beta (ERβ), progesterone receptor A (PR-A) and B (PR-B) in a recent study.[41] The median patients' age of this analysed population at the time of diagnosis was 64.5 years (range 35.5–87.9 years). 225 (74.5%) and 22 (7.3%) patients were diagnosed in FIGO stages I and II, respectively, while 46 (15.2%) patients had FIGO stage III and 9 patients (3%) presented with metastatic disease (FIGO IV). Of the 302 patients analysed, 265 had an endometrioid histology (87.7%), while 37 (12.3%) patients presented with a serous/clear cell or undifferentiated carcinoma (Table 1). Of the endometrioid carcinomas, 221 patients demonstrated an endometrioid adenocarcinoma (83.4%), 5.3% showed a mucinous carcinoma and 12.8% showed a mixed adenocarcinoma. Lymph node sampling or dissection was generally performed in patients having tumours with deep myometrial invasion and/or high-grade or aggressive histological features. Pelvic and/or para-aortic lymph node sampling was performed for 218 patients (72.2%) with 25 patients (11.5%) demonstrating lymph node metastasis. A low FIGO stage (FIGO Ia), obesity, advanced age and excessive comorbidity were factors prohibiting full

Table 2 – Summary of immunohistochemical analysis of endometrial carcinomas.

		Total (n = 302)	Endometrioid adenocarcinoma (n = 265)			Non-endometrioid carcinomas (n = 37)		
			Endometrioid (n = 221)	Mucinous (n = 14)	Mixed (n = 34)	Serous (n = 22)	Clear cell (n = 6)	Undifferentiated (n = 5)
Inhibin-alpha	Median	0	0	0	0	0	0	0
	Mean ± SEM	0.47 ± 0.06	0.48 ± 0.67	0.43 ± 0.25	0.41 ± 0.18	0.55 ± 0.24	0.67 ± 0.67	0 ± 0
	Negative	231 (76.5%)	166 (75.1%)	11 (78.6%)	28 (82.4%)	16 (72.7%)	5 (83.3%)	5 (100%)
	Positive	71 (23.5%)	55 (24.9%)	3 (21.4%)	6 (17.6%)	6 (27.3%)	1 (16.7%)	0 (0%)
	Kruskal-Wallis	0.793						
	Chi-square	0.733						
Inhibin-βA	Median	4	4	4	8	8	4	8
	Mean ± SEM	6.43 ± 0.18	6.29 ± 0.21	4.79 ± 0.63	6.76 ± 0.43	8.36 ± 0.69	5.17 ± 1.72	8 ± 1.265
	Negative	154 (51.0%)	120 (54.3%)	10 (71.4%)	13 (38.2%)	6 (27.3%)	4 (66.7%)	1 (80%)
	Positive	148 (49.0%)	101 (45.7%)	4 (28.6%)	21 (61.8%)	16 (72.7%)	2 (33.3%)	4 (20%)
	Kruskal-Wallis	0.007						
	Chi-square	0.024						
Inhibin-βB	Median	6	4	5	4	8	2	8
	Mean ± SEM	6.31/-0.2	6.26 ± 0.23	5,14 ± 0,69	6,56 ± 0,56	9,23 ± 0,62	3.17 ± 1,05	8 ± 0
	Negative	161 (53,3%)	120 (54,3%)	10 (71.4%)	22 (64,7%)	4 (18,2%)	5 (83.3%)	0 (0%)
	Positive	141 (46,7%)	101 (45,7%)	4 (28.6%)	12 (35.3%)	18 (81.8%)	1 (16.7%)	5 (100%)
	Kruskal-Wallis	<0.001						
	Chi-square	<0.001						

surgical staging in 84 patients (27.8%). Obesity was observed in 104 (34.4%) cases, while 119 (39.4%) and 34 (11.3%) patients presented with hypertension and diabetes, respectively. Of the 302 patients analysed, 119 patients (39.4%) received a radiation therapy, while 6 (2%) denied a recommended radiation therapy. Nine patients (3%) received an anti-hormone treatment. During the follow-up interval, tumour recurrence was observed in 47 patients (15.6%), and 40 patients (13.2%) died of the disease.

3.2. *Endometrial carcinoma samples*

3.2.1. *Inhibin-α*

Positive inhibin-α immunostaining was observed in 71 out of 302 endometrial carcinoma samples (23.5%) (Fig. 1a–b). No significant difference in the inhibin-α expression was found among the various subtypes of endometrial carcinomas ($p = 0.793$) (Table 2). Inhibin-α expression in endometrial carcinoma samples revealed a significant association with patient age ($p < 0.01$), grading ($p < 0.001$), FIGO stage ($p < 0.001$) lymph node status ($p < 0.05$) and diabetes ($p < 0.01$) (Table 3).

3.2.2. *Inhibin-βA and –βB*

A positive immunohistochemical staining reaction for inhibin-βA (Fig. 1c–d) and -βB (Fig. 1e–f) was observed in 296 cases. Significant differences in the IRS of inhibin-βA and -βB were demonstrated among the various subtypes of endometrial carcinomas ($p = 0.007$ and $p < 0.001$, respectively). By analysing positive and negative expressions, we could also observe a significant difference between the different histological subtypes ($p < 0.05$ and $p < 0.01$, respectively). Additionally, we demonstrated a correlation between inhibin-βA and -βB expression and grading ($p < 0.05$ each, respectively) (Table 3). A significant correlation between the βA and βB-subunits was also observed ($p < 0.001$).

3.3. *Survival analysis*

The median overall survival time for the uncensored subgroup was 39.1 months (range 0.7–145.7 months), whereas the median follow-up of censored patients was 89.7 months (range 0.3–176.8 months). The follow-up time until the occurrence of progression was 33.8 months (range 0.01–145.7

Table 3 – Univariate statistical analysis for inhibin-α, -βA and -βB subunits according to various clinicopathologic features. N.S. = not significant. LN = lymph node.

		Total ($n = 302$)	Inhibin-alpha	Inhibin-βA	Inhibin-βB
Age (years)	<65	156 (51.7%)	47 (30.1%)	71 (45.5%)	75 (48.1%)
	>65	146 (48.3%)	24 (16.4%)	77 (52.8%)	66 (45.2%)
	Chi-square		0.006	N.S.	N.S.
WHO grading	Grade 1 + 2	248 (82.1%)	68 (27.4%)	114 (46.0%)	108 (43.5%)
	Grade 3	54 (17.9%)	3 (5.6%)	34 (63.0%)	33 (61.3%)
	Chi-square		< 0.001	0.025	0.024
FIGO stage	FIGO I + II	247 (81.8%)	68 (27.5%)	119 (48.2%)	111 (44.9%)
	FIGO III + IV	55 (18.2%)	3 (5.5%)	29 (52.7%)	30 (54.5%)
	Chi-square		< 0.001	N.S.	N.S.
Histology	Endometrioid	265 (87.7%)	63 (23.8%)	123 (48.0%)	116 (42.8%)
	Non-endometrioid	37 (12.3%)	8 (21.5%)	25 (67.6%)	25 (67.6%)
	Chi-square		N.S.	0.022	0.008
LN status	Negative	193 (63.9%)	52 (26.4%)	93 (48.2%)	89 (46.1%)
	Positive	25 (8.3%)	1 (0.04%)	13 (52%)	13 (52%)
	Unknown	84 (27.8%)	18 (21.4%)	42 (50%)	39 (46.4%)
	Chi-square		0.034	N.S.	N.S.
Lymphangiosis	Negative	272 (90.1%)	68 (25%)	135 (50.7%)	123 (45.2%)
	Positive	30 (9.9%)	3 (10%)	13 (43.3%)	18 (60%)
	Chi-square		N.S.	N.S.	N.S.
Haemangiosis	Negative	294 (97.4%)	71 (24.1%)	144 (49%)	137 (46.6%)
	Positive	8 (2.6%)	0 (0%)	4 (50%)	4 (50%)
	Chi-square		N.S.	N.S.	N.S.
Diabetes	Negative	268 (88.7%)	69 (25.4%)	134 (50%)	124 (46.3%)
	Positive	34 (11.3%)	2 (5.9%)	14 (41.2%)	17 (50%)
	Chi-square		0.009	N.S.	N.S.
Adipositas	Negative	198 (65.5%)	49 (24.7%)	98 (49.5%)	98 (49.5%)
	Positive	104 (34.4%)	22 (21.5%)	50 (48.1%)	43 (41.3%)
	Chi-square		N.S.	N.S.	N.S.
Hypertension	Negative	183 (60.6%)	50 (27.3%)	96 (52.4%)	93 (50.8%)
	Positive	119 (39.4%)	21 (17.6%)	52 (43.7%)	48 (40.3%)
	Chi-square		N.S.	N.S.	N.S.
Radiotherapy	Negative	183 (60.6%)	46 (25.1%)	92 (50.3%)	84 (45.9%)
	Positive	119 (39.4%)	25 (219%)	56 (47.1%)	57 (47.9%)
	Chi-square		N.S.	N.S.	N.S.
Anti-hormone therapy	Negative	293 (97.0)	68 (23.2%)	144 (49.1%)	136 (46.4%)
	Positive	9 (3.0%)	5 (55.6%)	4 (44.4%)	5 (55.6%)
	Chi-square		0.036	N.S.	N.S.

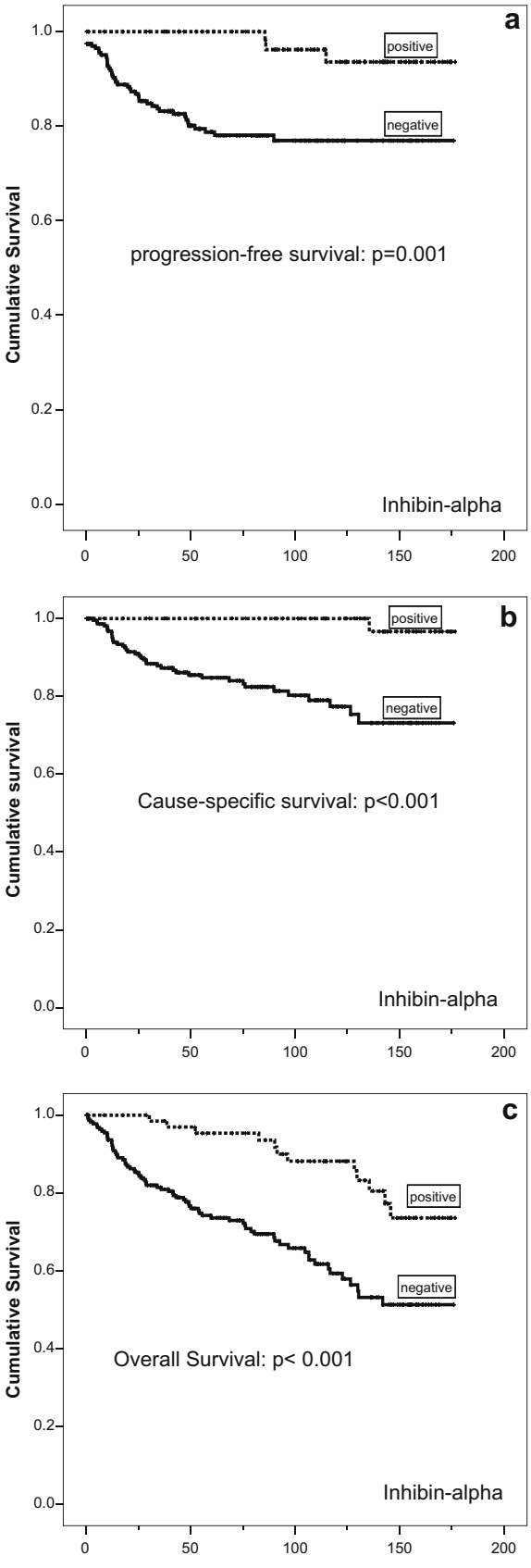

Fig. 2 – (a–c) Kaplan–Meier curves of clinical outcome regarding inhibin-α expression for progression-free survival (Fig. 2a), cause-specific survival (Fig. 2b) and overall survival (Fig. 2b).

months). Univariate survival analysis demonstrated a significantly better progression-free survival of patients who expressed the inhibin-α subunit (Fig. 2a) ($p = 0.04$, log rank test). Additionally, patients without inhibin-α expression demonstrated a significantly poorer cause-specific survival (Fig. 2b) and overall survival (Fig. 2c). In contrast, both inhibin-βA and inhibin-βB subunits did not demonstrate any significant differences in progression-free survival, cause-specific survival and overall survival (Fig. 3a–f). Other clinical parameters, including advanced surgical stage (stage I/II versus stage III/IV) and advanced histological grade (G3 versus G1 or G2), known prognostic factors of endometrial cancer, significantly affected the survival rates in the evaluated patients, demonstrating the validity of the patient group enroled in our study (p in log rank was <0.001 for both analysed parameters for progression-free, cause-specific and overall survival, respectively).

The Cox regression led to a model containing four independent terms that were predictive of progression-free survival: WHO grading ($p = 0.002$), FIGO stage ($p < 0.001$), lymph node involvement ($p = 0.016$) and inhibin-α expression ($p = 0.026$) (Table 4). Independent prognostic factors for cause-specific survival were age ($p = 0.004$), FIGO stage ($p < 0.001$), grade ($p = 0.003$) and inhibin-α expression ($p = 0.025$). The overall survival was influenced by age ($p < 0.001$), FIGO stage ($p < 0.001$), tumour grade ($p = 0.008$), lymph node involvement ($p < 0.001$), haemangiosis ($p = 0.018$), high blood pressure ($p = 0.042$) and the immunohistochemical inhibin-α expression ($p = 0.021$) (Table 4).

4. Discussion

Although more than 50% of the patients with endometrial carcinoma are diagnosed with FIGO stage I, as many as 20% die of their disease.[28] This is an unusual situation, compared to other solid tumours, and may reflect the failure of current diagnostics to identify pre-malignant stages and endometrial cancer patients with a poor prognosis. Several clinical parameters including well-known prognostic factors such as age, advanced surgical stage, advanced histological grading as well as lymph node involvement[2] affected significantly the survival rates in this study, demonstrating the validity of the patient group enroled. Additionally, older age with associated comorbidities, including hypertension or diabetes, is also associated with a poorer survival.[2,29] Moreover, a negative steroid receptor expression (oestrogen and progesterone receptors) also demonstrated a poorer progression-free, cause-specific and overall survival in the analysed endometrial cancer patients[41], confirming previous results[25–27] and contributing also to the validity of this cohort.

In this series of 302 patients, a differential immunohistochemical expression of inhibin subunits was demonstrated in malignant endometrial tissue. The inhibin-α subunit showed a significant association with histological grading, surgical staging, lymph node status and diabetes in patients with endometrial cancer. Additionally, survival analysis demonstrated that inhibin-α immunoreactivity was a significantly independent prognostic factor for progression-free survival and cause-specific survival as well as for overall survival.

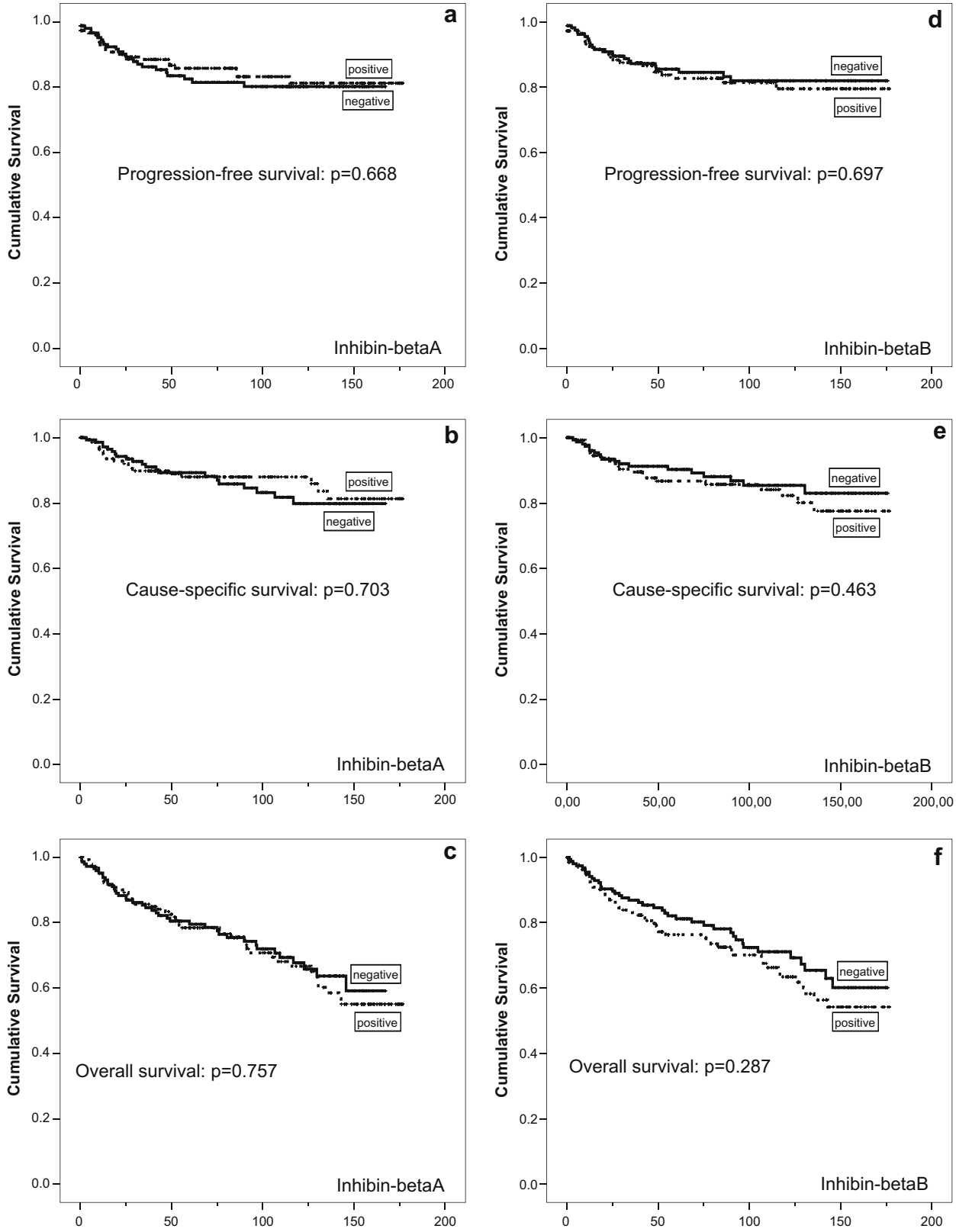

Fig. 3 – (a–f) Kaplan–Meier curves of clinical outcome regarding inhibin-βA expression for progression-free survival (Fig. 3a), cause-specific survival (Fig. 3b) and overall survival (Fig. 3c) and inhibin-βB expression for progression-free survival (Fig. 3d), cause-specific survival (Fig. 3e) and overall survival (Fig. 3f).

Table 4 – Hazard ratios by multivariate Cox regression analysis.

	Progression			Cause-specific survival			Overall survival		
	RR	CI (5–95%)	*p*	RR	CI (5–95%)	*p*	RR	CI (5–95%)	*p*
Age (>65years)	–	–	–	2.742	1.39–5.42	0.004	3.350	1.96–5.72	<0.001
WHO grading (G3 versus G1/G2)	2.753	1.46–5.14	0.002	2.814	1.43–5.53	0.003	1.978	1.19–3.28	0.008
FIGO stage (III/IV versus I/II)	14.170	7.11–28.25	<0.001	4.905	2.47–9.74	<0.001	3.880	2.06–7.31	<0.001
LN (negative versus positive)	0.441	0.23–0.86	0.016	–	–	–	–	–	–
Hypertension (negative versus positive)	–	–	–	–	–	–	0.617	0.39–0.98	0.042
Haemangiosis (positive versus negative)	–	–	–	–	–	–	3.450	1.24–9.60	0.018
Inhibin-alpha (positive versus negative)	0.259	0.08–0.79	0.026	0.101	0.01–0.76	0.025	0.469	0.25–0.89	0.021

In contrast, although βA- and βB subunits showed a significant association towards the different endometrial histological subtypes and histological grading, both subunits were not associated with survival in endometrial cancer patients.

Meanwhile, it is evident that inhibin subunits are expressed in a wide range of human tissues including placenta[19,20,30], breast tissue[21] and human endometrium[6–11,14,15], suggesting a possible role in endometrial proliferation and growth.[10] However, the most interesting function of inhibin-α might be its action as a possible tumour suppressor gene, according to the results from transgenic mouse models for ovarian cancer.[13,31,32] Recently, a significantly lower inhibin-α expression in well-differentiated adenocarcinomas than in normal and hyperplastic endometrial tissue was demonstrated.[11,33] These results also led to the hypothesis that inhibin-α might be a tumour suppressor with crucial functions in endometrial carcinoma development.[15] In this study, the loss of immunoreactivity of inhibin-α in tumour tissue correlated with advanced surgical stage and histological grading and poor prognosis. Therefore, we conclude that inhibin-α has a tumour suppressive function in endometrial cancer, and influences both the progression process and the disease-associated mortality rate. Moreover, the significant differences in the overall survival suggest further implements of the inhibin- subunit in endometrial cancer patients.

The exact molecular mechanism of an inhibin- subunit downregulation in advanced endometrial carcinomas is still not clear. However, in prostate cancer the inhibin- promoter can be silenced by hypermethylation.[42,43] Promoter hypermethylation, described for cell cycle regulator proteins and E-cadherin expression[44], is a quite often observed gene silencing mechanism during human cancer progression, including that of endometrial cancer.[45] It has further been shown that the inhibin-alpha subunit can be regulated by GATA and CCAAT/enhancer-binding protein-ß transcription factors[46,47], but too little is known about the involvement and the expression of these transcription factors in the human endometrium and endometrial carcinomas. However, generally it is believed that inhibin-alpha subunit is a tumour suppressor gene silenced by mechanisms common to other tumour suppressor genes.[48]

The possible functions of the inhibin-β subunits in human endometrial cancer are controversial and still unclear. Since activin A, the homodimer of the inhibin-βA subunit, is modulated by estradiol[34] and has the ability to enhance proliferation in certain cancer cell lines[35], a role for activin A in endometrial tumourigenesis has been suggested.[36] However, activin may inhibit angiogenesis, probably as its function as a growth inhibitor of vascular endothelial cells.[37] Therefore, expression of inhibin-β subunits during carcinogenesis might play an important role in endometrial angiogenesis.

Interestingly, the loss of the inhibin-α subunit production in mice resulted in marked increase of activin production in the ovary. The inhibin-α sufficient mice presented with cachectic symptoms that were associated with the compensatory excessive secretion of activin.[31] Recently, activin A has been found at significantly higher concentrations in culture media from cell lines, uterine washings and serum from patients with endometrial carcinomas, suggesting a secretion of activin A into the extracellular fluid and into the circulation.[6] Since activin A can modulate cell growth, proliferation and apoptosis[38], a growth inhibitory role for activin A in pre-malignant transformation could be suggested. Activin B has been recently suggested as a marker for patients with ovarian granulosa cell tumours[39], and inhibin B might be a more specific marker than inhibin A.[40] Therefore, inhibin-βB and a possible formation of inhibin B and/or activin B might play important roles in endometrial malignant transformation, although serological data in cancer patients are still missing. Although we could demonstrate an association between these β-subunits and histological grading in this study, no association with other clinicopathological parameters and survival was observed. Therefore, both β-subunits are not useful markers to assess and identify high-risk patients with endometrial carcinomas.

In conclusion, the immunostaining of inhibin-α subunit showed a significant association with histological grading, surgical staging, lymph node status and diabetes in patients with endometrial cancer. The loss of inhibin-α immunoreactivity resulted in a poorer survival of the affected patients. Therefore, inhibin-α might be a tumour suppressor in human endometrium as suggested for ovarian tissue. Additionally, the inhibin-α immunoreactivity was a significantly independent prognostic factor for progression-free, cause-specific and overall survival. In contrast, although βA- and βB subunits showed a significant association between the different endometrial histological subtypes and histological grading, both subunits were not associated with the survival of endometrial cancer patients. Therefore, the inhibin-α immunostaining might be used as an easy, simple and highly efficient marker to identify high-risk patients, and may aid in the selection of patients for a more aggressive adjuvant therapy. However, the prognostic significance of the inhibin-α-subunit and the use of the inhibin subunits as specific

markers in endometrial pathogenesis should be analysed in further studies.

Conflict of interest statement

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