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Inhibin beta B: a useful tumor marker in uterine endometrioid adenocarcinomas?

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Summary. Inhibins, dimeric peptide hormones composed of an alpha-subunit and one of two possible beta-subunits (betaA or betaB), exhibit substantial roles in human reproduction and in endocrine-responsive tumours. However, the prognostic significance and clinical implications of the inhibin-betaB subunit in uterine endometrioid adenocarcinomas is still not defined yet. A series of 227 uterine endometrioid adenocarcinomas of a previous well-characterized cohort were re-evaluated for the expression of the inhibin-betaB subunit and correlated with several clinicopathological characteristics and the clinical outcome. In this re-analysis, the betaB-subunit expression demonstrated a significant association with the patients' age and cervical involvement. However, inhibin-betaB did not significantly affect the patients survival in this large cohort group. However, patients with a higher intensity of betaB-subunit immunolabelling had a slightly worse survival expectation, although without any significant association, suggesting that this subunit might have a substantial role in the carcinogenesis and pathology of endometrioid adenocarcinomas. Thus, the inhibin-betaB subunit appears not to be a useful prognostic marker regarding endometrioid adenocarcinomas. However, further research is warranted in elucidating the possible implications of inhibin-ßB and endometrial carcinogenesis.

Key words: Endometrial cancer, Endometrioid adenocarcinoma, Immunohistochemistry, Inhibin-betaB, Prognosis, Survival

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Introduction

Endometrial cancer has become the most frequent gynaecologic malignancy in the Western world with an estimated incidence of 15-20 to 100.000 women per year (Jereczek-Fossa et al., 1999; Prat, 2004; Amant et al., 2005). Although several prognostic factors like histological type, histological grade, surgical stage, pelvic lymph node involvement and myometrial invasion have been established (Prat, 2004; Amant et al., 2005), as many as 20% die of their disease (Jereczek-Fossa et al., 1999). This is an unusual situation, compared to other solid tumours being diagnosed in early stage, and may reflect the failure of current diagnostic methods for identifying endometrial cancer patients with a poor prognosis.

Inhibins and activins are secreted polypeptides, representing a subgroup of the TGF-ß superfamily of growth and differentiation factors (Vale et al., 1988, 2004; Xia and Schneyer, 2009). Inhibins are heterodimers that consist of an α-subunit and one of two possible ß-subunits (A or ,B), resulting in the formation of either inhibin A (α-ßA) or B (α-ßB), respectively. On the contrary, activins are homodimers of ß-subunits linked by a disulphide bond, leading to the formation of activin A (ßA-ßA), activin B (ßB-ßB) or activin AB (ßA-ßB) (Vale et al., 1988, 2004; Xia and Schneyer, 2009). Recently, two additional ß-subunits have been identified in humans, determined as ßC and ßE (Xia and Schneyer, 2009), although their precise function remains still unclear.

Several autocrine and paracrine actions of inhibins and activins have been reported, including modulation of ovarian and placental hormone secretion and local regulation of macrophage function (de Kretser et al., 2002; Welt et al., 2002; Xuan et al., 2007; Florio et al., 2010), regulation of gonadotropins (Gregory and Kaiser, 2004), local steroidogenesis (Ni et al., 2000),

hematopoesis (Shav-Tal and Zipori, 2002; Ramos-Mejia et al., 2010), embryogenesis (Smith et al., 1990; He et al., 1999), inflammation (Phillips et al., 2009), apoptosis (Chen et al., 2002; Denkova et al., 2004; Chen et al., 2007), dentritic cell function and regulation (Salogni et al., 2009) as well as stem cell differentiation (Watabe and Miyazono, 2009; Djouad et al., 2010; Tsai et al., 2010).

The inhibin-subunits have been detected in endocrine tumours (Risbridger et al., 2001) and their differential expression has suggested an important role in malignant cell transformation in human endometrium (Petraglia et al., 1998a; Worbs et al., 2007; Mylonas et al., 2009). Interestingly, TGF-ß has been recognized as a tumour suppressor in premalignant stages of carcinogenesis with an additional dual role as a pro-oncogene in later stages of disease, leading to metastasis (Risbridger et al., 2004). Interestingly, the inhibin-α subunit was an independent prognostic parameter in a large cohort analysis of human endometrial carcinomas (Mylonas et al., 2009), suggesting a putative tumour suppressive function in human endometrial cancer as suggested in knock-out mouse model (Matzuk et al., 1992, 1994). However, the prognostic significance and clinical implications of the inhibin-ßB subunit in endometrioid adenocarcinomas has not been complete elucidated yet. Although the ßB-subunit did not constitute an independent prognostic parameter in a large cohort analysis of human endometrial carcinomas (Mylonas et al., 2009), a reevaluation of the inhibin-ßB subunit in uterine non-endometrioid carcinomas revealed a better cause-specific survival in patients with a higher immunohistochemical expression of this subunit (Mylonas, 2010a). However, it is quite unclear if this subunit does exert import roles in endometrioid adenocarcinomas and can be used as prognostic parameter in this type of cancer. Therefore, the aim of this analysis was the re-evaluation of inhibin-ßB expression in a large, well-characterized cohort group (Shabani et al., 2007; Mylonas et al., 2009, 2011; Bassarak et al., 2010; Brüning et al., 2010; Mylonas, 2010b, 2011a,b; Käufl et al., 2011) with respect to endometrioid adenocarcinomas.

Materials and methods

Tissue samples

Pathological and surgical records of 227 patients who have been operated in the 1st Department of Obstetrics and Gynecology, Ludwig-Maximilians-University Munich between 1990 and 2002 were reviewed for this retrospective analysis. The evaluated patient group has been previously well-characterized (Shabani et al., 2007; Mylonas et al., 2009, 2011; Bassarak et al., 2010; Brüning et al., 2010; Mylonas, 2010b, 2011a,b; Käufl et al., 2011). In this study, women with other histological types than endometrioid adenocarcinomas (mucinous adeno-carcinoma, serous adenocarcinoma, clear-cell adenocarcinoma, mixed adenocarcinoma, squamous-cell carcinoma, transitional-cell carcinoma, small-cell carcinoma and undifferentiated carcinoma) were excluded from this study as previously described (Brüning et al., 2010; Mylonas, 2010b, 2011a,b; Mylonas et al., 2011). Additionally, patients with variants of endometrioid adenocarcinoma (including the variant with squamous differentiation, villoglandular variant, secretory variant and ciliated cell variant) were also excluded from this study. Pathological stage and histological subtype were determined for each surgical specimen according to 1988 International Federation of Gynecology and Obstetrics

Table 1. Clinicopathological characteristics of the analyzed uterine endometrioid carcinomas.

Paramater	Definition	N (%)
Age (years)	≤65	115 (50,66%)
	>65	112 (49,34%)
Grading	grade 1	139 (61,23%)
	grade 2	62 (27,31%)
	grade 3	26 (11,45%)
FIGO stage	FIGO I	184 (81.06%)
	FIGO IA	135 (59,47%)
	FIGO IB	49 (21,59%)
	FIGO II	14 (6,17%)
	FIGO III	21 (9,25%)
	FIGO 3A	7 (3,08%)
	FIGO 3B	3 (1,32%)
	FIGO 3C1	11 (4,85%)
	FIGO 3C2	0 (0.00%)
	FIGO IV	8 (3,52%)
Myometrial invasion	only endometrial invasion	31 (13,66%)
	< 50% myometrium	121 (53,3%)
	> 50% myometrium	75 (33,04%)
Cervical Invasion	Negative	201 (88,55%)
	Positive	26 (11,45%)
Ovarial invasion	Negative	211 (92,95%)
	Positive	16 (7,05%)
LN status	Negative	145 (63,88%)
	positive	13 (5,73%)
	unknown	69 (30,4%)
Lymphangiosis	negative	208 (91,63%)
	positive	19 (8,37%)
Adipositas	Negative	145 (63,88%)
	Positive	82 (36,12%)
Diabetes	Negative	199 (87,67%)
	Positive	28 (12,33%)
Hypertension	Negative	136 (59,91%)
	positive	91 (40,09%)
Chemotherapy	Negative	221 (97,36%)
	Positive	5 (2,2%)
	Denial	1 (0,44%)
Radiotherapy	Negative	141 (62,11%)
	Positive	80 (35,24%)
	Denial	6 (2,64%)
Anti-hormonal therapy	Negative	220 (96,92%)
	Positive	7 (3,08%)

(FIGO) criteria (FIGO stages (announcements), 1989) and updated to the novel FIGO classification of the year 2009 (Pecorelli, 2009).

Patient data were obtained from three sources: hospital tumour registry, automated database and chart review as previously described (Brüning et al., 2010; Mylonas, 2010b, 2011a,b; Mylonas et al., 2011). All cases of recurrence had radiographic evidence of disease 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, censored cases were also considered those cases where the exact cause of death was unknown but died within two years after the diagnosis of a metastatic lesion (Shabani et al., 2007; Mylonas et al., 2009, 2011; Bassarak et al., 2010; Brüning et al., 2010; Mylonas, 2010b, 2011a,b; Käufl et al., 2011).

Immunohistochemistry

Immunohistochemistry was performed using a combination of pressure cooker heating and the standard streptavidin-biotin-peroxidase complex by using the mouse-IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, California, USA) as previously described (Mylonas et al., 2004, 2009). Mouse monoclonal antibodies used for the experiments was inhibin-ßB (clone C5, diluted in PBS 1:10; Serotec - Oxford - United Kingdom) as previously described (Mylonas et al., 2004, 2009; Mylonas 2010a).

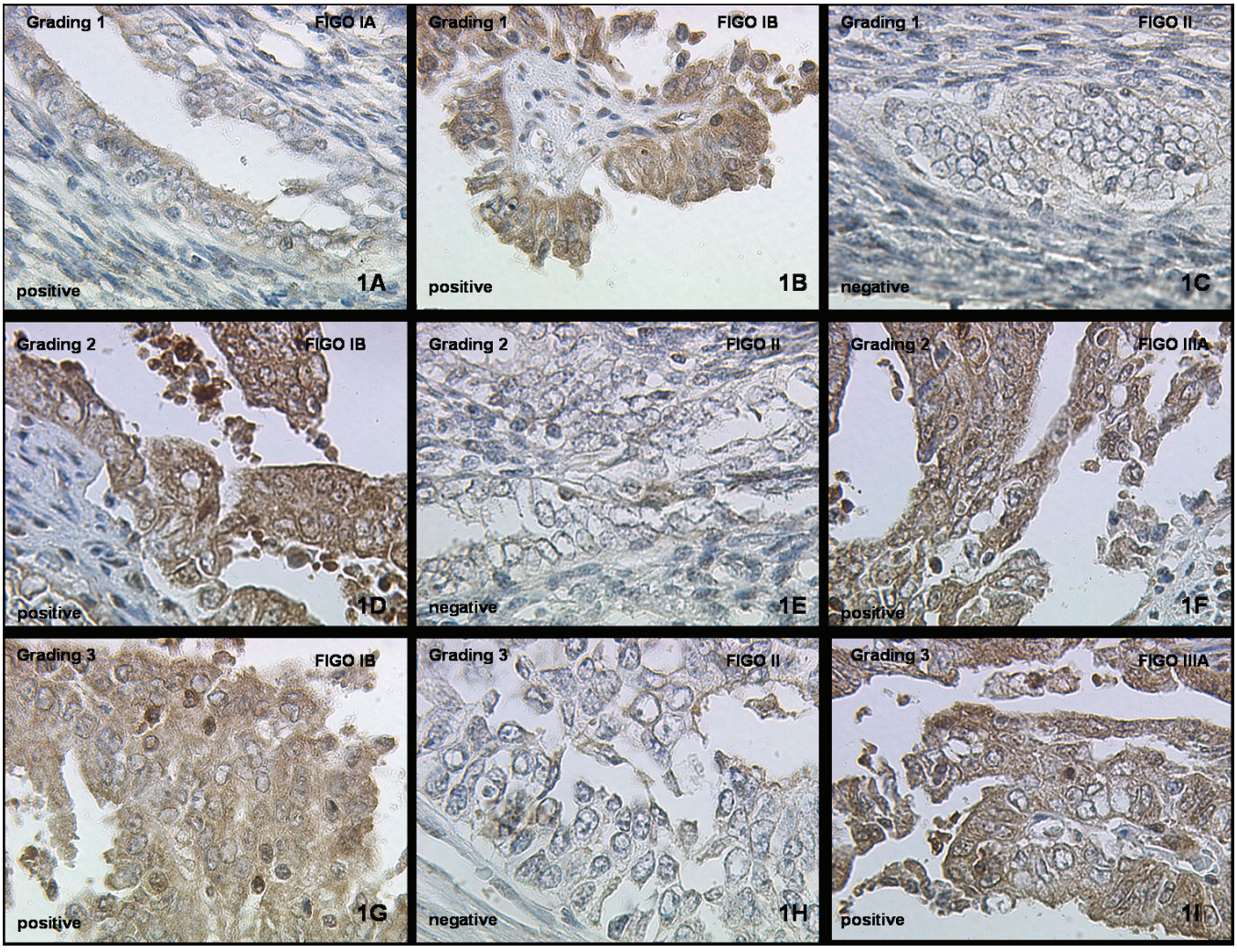

Fig. 1. Expression of inhibin-ßB subunit in human uterine endometrioid adenocarcinomas. Inhibin-ßB reacted with a minimal **(C, E, F, H)** to moderate staining intensity **(A, B, D, F, G, I)** in uterine endometrioid adenocarcinomas. However, no significant differences were of the staining intensities between the different histological gradings were observed. A, C, E, F, x 250; B, D, F, G-I, x 400.

Statistical analysis

The intensity and distribution patterns of specific inhibin-ßB-subunit immunohistochemical cytoplasmatic staining reaction was evaluated by two independent observers as previously described (Mylonas et al., 2004, 2009). For the purposes of statistical survival analysis, the inhibin-ßB staining intensity the median for all tumour samples was used (median for inhibin-ßB=6) as previously described (Mylonas et al., 2009). However, ROC analysis revealed that the area under the curve was higher by using specific immunohistochemical staining intensity with a cut-off value of 1 for inhibin-ßB (Mylonas, 2010a), instead of the IRS with the previously described cut-off value of 6 (Mylonas et al., 2009). Therefore, staining intensity with the value ≤1 for inhibin -ßB was considered to be a negative expression. For the evaluation of increased/positive versus not increased/negative immunostaining in tumour samples was compared using the χ^2 test and the exact Fisher's test where applicable (Shabani et al., 2007; Mylonas et al., 2009, 2011; Bassarak et al., 2010; Brüning et al., 2010; Mylonas, 2010b, 2011a,b; Käufl et al., 2011).

The outcomes analyzed were progression-free survival, cause-specific survival and overall survival. Univariate analysis was performed with Kaplan-Meier life-table curves to estimate survival (Kaplan and Meier, 1958) and were compared using the log-rank test. Significance of differences was assumed at $p \leq 0.05$ (SPSS version 16.0; SPSS Inc., Chicago, IL).

Results

Clinicopathological characterization

The median patient's age at the time of diagnosis was 65.73 years (range, 36.18-89.35 years). Histological classification was performed according to the World Health Organization system in well-differentiated (G1; n=139), moderate differentiated (G2; n=62) and poor-differentiated (G3; n=26) (Table 1). 180 (79.3%) and 18 (7.93%) patients were diagnosed in FIGO stage I and II respectively, while 21 (9.25%) patients had FIGO stage III and 8 patient (3.52%) presented with metastatic disease (FIGO IV). Pelvic and/or para-aortic lymph node sampling was performed for 158 patients (69.6%). 13 patients (5.73%) demonstrated lymph node metastasis (Table 1). A low FIGO stage (FIGO Ia), obesity, advanced age and excessive comorbidity were factors against a full surgical staging in 69 patients (30.4%).

Table 2. Univariate statistical analysis for positive inhibin-ßB subunit staining intensity (cut-off=1) according to various clinicopathological features.

			Inhibin betaB		
		n	Negative	Positive	Significance
age (years)	≤65	115 (50,66%)	45 (39,13%)	70 (60,87%)	0.007
	>65	112 (49,34%)	64 (57,14%)	48 (42,86%)	
Grading	Grade 1 + 2	201 (88,55%)	96 (47,76%)	105 (52,24%)	N.S.
	Grade 3	26 (11,45%)	13 (50%)	13 (50%)	
FIGO stage	FIGO I + II	198 (87,22%)	97 (48,99%)	101 (51,01%)	N.S.
	FIGO III + IV	29 (12,78%)	12 (41,38%)	17 (58,62%)	
Myometrial invasion	<50%	152 (66,96%)	67 (44,08%)	85 (55,92%)	0.091
	>50%	75 (33,04%)	42 (56%)	33 (44%)	
Cervical Invasion	negative	201 (88,55%)	92 (45,77%)	109 (54,23%)	0.047 (1-sighted)
	positive	26 (11,45%)	17 (65,38%)	9 (34,62%)	
Ovarial invasion	negative	211 (92,95%)	103 (48,82%)	108 (51,18%)	N.S.
	positive	16 (7,05%)	6 (37,5%)	10 (62,5%)	
LN status	negative	145 (63,88%)	70 (48,28%)	75 (51,72%)	N.S.
	positive	13 (5,73%)	6 (46,15%)	7 (53,85%)	
	unknown	69 (30,4%)	33 (47,83%)	36 (52,17%)	
LVSI	negative	208 (91,63%)	103 (49,52%)	105 (50,48%)	N.S.
	positive	19 (8,37%)	6 (31,58%)	13 (68,42%)	
Adipositas	negative	145 (63,88%)	70 (48,28%)	75 (51,72%)	N.S.
	positive	82 (36,12%)	39 (47,56%)	43 (52,44%)	
Diabetes	negative	199 (87,67%)	97 (48,74%)	102 (51,26%)	N.S.
	positive	28 (12,33%)	12 (42,86%)	16 (57,14%)	
Hypertension	negative	136 (59,91%)	66 (48,53%)	70 (51,47%)	N.S.
	positive	91 (40,09%)	43 (47,25%)	48 (52,75%)	

LN: lymph node; LVSI: Lymphovascular space invasion; N.S.: not significant.

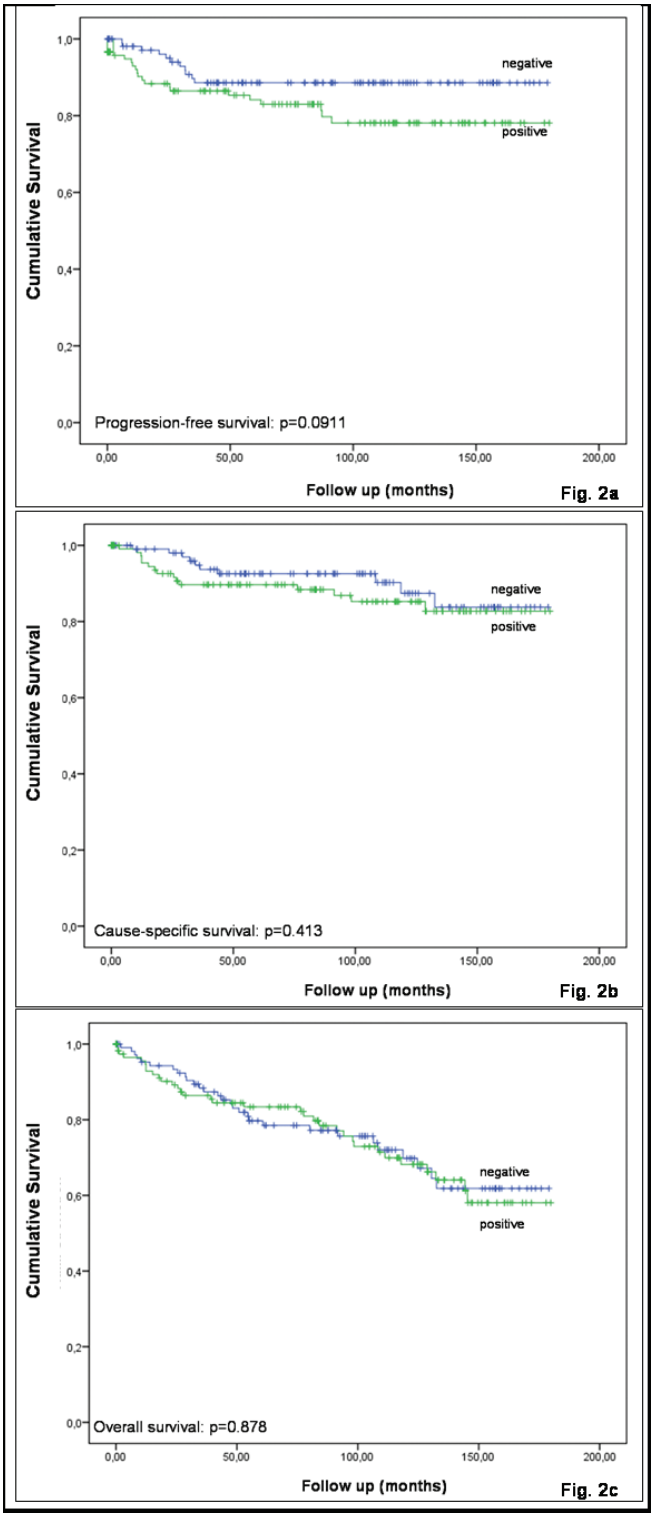

Fig. 2. Kaplan-Meier curves of clinical outcome regarding inhibin-,B expression for progression-free-survival (**Fig. 2a**), cause-specific survival (**Fig. 2b**) and overall survival (**Fig. 2c**). The positive inhibin-,B immunoreaction did not affect survival of patients with endometrioid adenocarcinomas. However, patients with a positive staining intensity of ßB subunit immunolabelling had a slightly worse outcome.

Obesity was observed in 82 (36.12%) cases, while 28 (12.3%) and 91 (40.09%) patients presented with diabetes and hypertension respectively. Of the analyzed 227 patients, 80 patients (35.24%) received a radiation therapy, while seven patients (3.08%) received an anti-hormone therapy (Table 1).

Survival analysis

During the follow-up interval, tumour recurrence was observed in 32 patients (14.1%), and 25 patients (11.0%) died of disease. Overall, 62 patients (27.3%) died during the entire observation period. 222 (97.8%) tumour samples demonstrated a positive immunohistochemical reaction against the used inhibin-ßB antibody. Positive inhibin-ßB immunostaining, as defined of the staining intensity being higher than 1, was observed in 109 out of 227 endometrial carcinoma samples (48.0%) (Fig. 1a,b).

By analyzing positive and negative expression univariate analysis (χ^2 test) revealed a significant association of inhibin-ßB with patient age (p=0.007). Interestingly an association between inhibin-ßB immunolabelling and cervical invasion was demonstrated at the one-sided test (p=0.047) and a tendency to significance was also observed with regard to myometrial invasion (p=0.091) (Table 2). Univariate survival analysis demonstrated no significant differences in the progression-free survival, cause-specific survival and overall survival for inhibin-ßB subunit (Fig. 2a-c). However, patients with a higher intensity of ßB subunit immunolabelling had a slightly worse outcome.

Discussion

The inhibin/activin-subunits belong to the TGF-ß superfamily and have been demonstrated in normal female tissue and endocrine tumours (Risbridger et al., 2001), including normal and pathological endometrial tissues (Petraglia et al., 1998a; Mylonas et al., 2004, 2009, 2010; Mylonas 2010a). The precise physiologic roles of endometrial inhibins/activins are still unclear. However, the endometrium is a potential target for inhibin/activin action with substantial functions during endometrial decidualization (Jones et al., 2002, 2006; Florio et al., 2010) and trophoblast differentiation (Caniggia et al., 1997; Stoikos et al., 2010). However, the function of activins in different tissue and cell lines remains still controversial discussed (Risbridger et al., 2001). The role of activins is further complicated since they have been recognized as important cytokines that can regulate cell growth and differentiation (Phillips et al., 2009) and act as a growth inhibitors of vascular endothelial cells (McCarthy and Bicknell, 1993). Moreover, a functional role of inhibin and activins are further being complicated with the identifications of newly ßC- and -ßE subunits that are also synthesized in human placental and endometrial tissue (Kimmich et al.,

2010; Mylonas et al., 2010; Weissenbacher et al., 2010; Gingelmaier et al., 2011; Käufl et al., 2011).

TGF-ß subunits have been implicate in carcinogenesis, tumour progression and metastasis (Risbridger et al., 2001; Buijs et al., 2007; Burdette and Woodruff, 2007; Baselga et al., 2008) been recognized as a tumour suppressor in praemalignant stages of carcinogenesis with an additional dual role as a pro-oncogene in later stages of disease (Risbridger et al., 2004). This superfamily of differentiation factors has evolved into an increased researcher's interest since their signalling might be a promising target for therapeutic interventions in cancer patients (Tsuchida et al., 2009).

Most studies have focused on the inhibin-ßA-subunit and its homodimer activin A, implicating this molecule in cancer cell proliferation in various experimental models in vitro and in vivo (Adkins et al., 2003; Jeruss et al., 2003; Burdette and Woodruff, 2007; Razanajaona et al., 2007; Katik et al., 2009; Ramachandran et al., 2009). Interestingly, inhibin-ßA is overexpressed in lung adenocarcinomas and this overexpression is associated with a poorer survival, probably affecting promoter methylation and histone acetylation (Seder et al., 2009). Whether the inhibin-ßB subunit has similar function as suggested for inhibin-ßA subunit is still not clear yet. When the inhibin-ßB gene is translocated into the inhibin-ßA gene locus, the phenotypes in the inhibin-,A knockout mouse is partially restored, but also results in novel phenotypes (Brown et al., 2000), indicating that the two subunits are not functionally equivalent (Brown et al., 2000; Thompson et al., 2004). Similarly, overlapping and distinct actions for inhibin A and inhibin B have been demonstrated in a mouse adrenocortical cell line (Farnworth et al., 2006). Therefore, the ßB-subunit cannot completely substitute the ßA-subunit (Brown et al., 2000; Farnworth et al., 2006), thus both ß-subunits seem to exert different functions (Thompson et al., 2004; Farnworth et al., 2006; Makanji et al., 2009). Interestingly, from a clinical point of view, activin B and inhibin B has been recently suggested as a better marker for patients with ovarian granulosa cell tumours compared to activin A or inhibin A (Petraglia et al., 1998b; Vihko et al., 2003).

In this analysis, the ßB-subunit demonstrated a significant association with patients' age and cervical involvement. However, inhibin-ßB did not significantly affect patients survival in this analyzed cohort group. Therefore, the inhibin-ßB subunit seems not to be a useful prognostic marker regarding endometrioid adeno-carcinomas. However, patients with a higher intensity of ßB subunit immunolabelling had a slightly worse survival, suggesting that this subunit might have a substantial role in the carcinogenesis and pathology of endometrioid adenocarcinomas. However, such a role remains still to be defined. Additionally, inhibin-ßB and a possible formation of inhibin B and/or activin B might play important roles in endometrial malignant transformation, although serological data in endometrial cancer patients are still missing. Therefore, further research is warranted in elucidating the possible implication of inhibin-ßB and endometrial cancer.

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