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# Immunolabelling of the inhibin/activin-ßC subunit in normal and malignant human uterine cervical tissue and cervical cancer cell lines

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**Abstract.** Inhibins are dimeric glycoproteins, composed of an α-subunit and one of two possible ß-subunits (ßA or ßB), with substantial roles in human reproduction and in endocrine-responsive tumours. Recently a novel ß subunit named ßC was described, although it is still unclear if normal or cancerous cervical epithelial cells as well as cervical cancer cell lines can synthesise the inhibin-ßC subunit. Four normal cervical tissue samples together with specimens of well-differentiated squamous cervical cancer and adenocarcinoma of the cervix were immunohistochemically analyzed. Additionally, two cervical carcinoma cell lines (HeLa and CaSKi) were analyzed by immunofluorescence for the expression of this novel subunit. We demonstrated for the first time an immunolabelling of the inhibin-ßC subunit in normal and malignant cervical tissue, as well as cervical cancer cells. Although the physiological role is still unclear in cervical tissue, the inhibin-ßC subunit might play important roles in carcinogenesis. Moreover, the synthesis of this subunit in cervical carcinoma cell lines of squamous and epithelial origins allows the use of these cell lines in elucidating its functions in cervical pathogenesis.

## Introduction

Since the implementation of screening programs with the objective to prevent invasive cervical cancer by detecting its precursor cervical lesions, the incidence of this cancer has declined in the more developed countries. However, cervical cancer is the second most common malignant disease among women worldwide (1,2). Approximately 80% of cervical cancers are arising from squamous cell dysplasia, while 15% are adenocarcinomas and 5% clear cell adenocarcinomas (2). However, although the Papanicolaou-smear is the most cost-effective cancer screening test ever developed, it still can be non-diagnostic or falsely negative in the presence of invasive cancer. Although several risk factors for the development of cervical cancer are meanwhile recognized, including HPV infection (3-5), the precise carcinogenesis is still quite unclear and no effective tumour markers are yet available.

Inhibins and activins are secreted polypeptides, representing a subgroup of the TGF-ß superfamily of growth and differentiation factors (6,7). Within the inhibin/activin subgroup, one α-subunit and four ß-subunit isoforms (ßA, ßB, ßC and ßE) have been identified in mammals (6-10). These ß-subunits can either form activins by dimerization with a second ß-subunit, or alternatively inhibins by dimerizing with an α-subunit. Thus, depending on the subunit combination, there are two forms of inhibin [namely inhibin A (α-ßA) and inhibin B (α-ßB)] and three isoforms of activin [namely activin A (ßA-ßA), activin B (ßB-ßB) and activin AB (ßA-ßB)]. Additionally, two additional ß-subunits have been identified in humans, determined as ßC and ßE (7). Although these novel subunits are synthesised in wide range of normal and malignant tissues (11-15), their precise function remains unclear. Moreover, the ßC-isoform is involved in the formation of homodimeric activin C (ßC-ßC) as well as heterodimeric activins AC (ßA-ßC), BC (ßB-ßC), CE (ßC-ßE), as well as inhibin C (α-ßC) have been demonstrated (16,17).

The inhibin/activin-subunits have been detected in normal female reproductive tissue and endocrine tumours (18), including normal and pathological endometrial and placental tissue (19-28), suggesting possible roles in cancer proliferation and growth (18,29). While inhibin A, inhibin B and activin A were detected in normal and neoplastic human uterine tissues, including cervical cancer (25), it is still unclear if normal or cancerous cervical epithelial cells as well as cervical cancer cell lines can synthesise the novel inhibin-ßC subunit. We have demonstrated the expression of the novel ßE subunit in cervical cancer and cervical cancer cell lines, suggesting a

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substantial function in cervical pathogenesis (11). The inhibin-ßA and -ßB subunits demonstrated a differential expression in CIN and squamous cancer, suggesting important roles in cervical carcinogenesis (22). Inhibin-ßA might be important during progression of cervical intraepithelial neoplasia, while the inhibin-ßB subunit could exert a substantial function during differentiation of cervical carcinomas (22).

Since specific antibodies against this inhibin-ßC subunit have been available for just a short period of time, systematic investigations on cervical tissue has not been performed yet. Although the used polyclonal inhibin-ßC antibody is not suitable for immunoblot analysis, it has been successfully used in human placental and endometrial tissue as well as cancer cell lines with a good specificity as confirmed by RT-PCR analysis (12,13,15,30). Therefore, the aim of this study was to analyse the expression of the ßC-subunits in normal and pathological cervical tissue as well as cervical carcinoma cell lines.

## Materials and methods

*Tissue samples*. Samples of human uterine cervical tissue were obtained from 4 premenopausal, non-pregnant patients undergoing hysterectomy for uterine leiomyomata of a well-characterized group (11). Additionally, 10 specimens of well-differentiated (G1) squamous cervical cancer and 10 tissue samples of well-differentiated (G1) adenocarcinoma of the cervix were obtained from the pathological archives of the First Department of Obstetrics and Gynaecology, Ludwig-Maximilians-University Munich as previously described (11).

*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, CA, USA) as previously described (12,13,15,30) with slightly modifications.

Briefly, paraffin-fixed tissue sections were dewaxed using xylol for 15 min and rehydrated in 100% of ethanol twice. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 20 min. After washing slides were subjected to antigen retrieval for 5 min in a pressure cooker using sodium citrate buffer (pH 6.0), containing 0.1 M citric acid and 0.1 M sodium citrate in distilled water. After cooling to room temperature, sections were washed twice in phosphate-buffered saline (PBS). Non-specific binding was blocked by incubating the sections with Ultra-V-Block (Lab Vision, Fremont, USA) for 45 min at room temperature. Sections were then incubated at 4˚C overnight with the inhibin-ßA mouse antibody (mouse IgG2b, clone E4, Serotec, Oxford, UK), at a dilution of 1:50 in Ultra-V-Block (Lab Vision) or inhibin-ßB mouse antibody (mouse IgG2a, clone C5, Serotec), at a dilution of 1:70 in Ultra-V-Block (Lab Vision). After washing with PBS, sections were incubated with biotinylated secondary anti-mouse antibody (provided by Vector Laboratories) for 30 min at room temperature. After incubation with the avidin-biotin peroxidase complex (diluted in 10 ml PBS; Vector Laboratories) for 30 min and repeated washing steps with PBS, visualization was performed with ABC substrate buffer (Vectastain Elite ABC kit, Vector Laboratories) and chromagen 3,3'-diaminobenzidine (DAB; Dako, Glostrup, Denmark) at 1 mg/ml concentration for 4 min. Sections were then counterstained with Mayer's acidic hematoxylin and dehydrated in an ascending series of alcohol (50-98%). After xylol treatment, sections were mounted. Negative controls were performed by replacing the primary antibody with normal rabbit IgG as isotype control in the same dilution compared to the primary antibody, respectively. Immunohistochemical staining was performed using an appropriate positive control comprising ovaries containing follicular cysts (26). Sections were examined using a Leica (Solms, Germany) photomicroscope and saved on computer. Positive cells showed a brownish colour and negative controls as well as unstained cells were blue.

*Cells and cell culture*. The cervical adenocarcinoma cell line HeLa (ATCC, CCL2) and the cervical epidermoid carcinoma cell line CaSki (ATCC, CRL-1550) are ATCC-available cell lines (ATCC, LGC Promochem GmbH, Wesel, Germany). Cells were cultured in Quantum 263 medium (PAA, Pasching, Austria) supplemented with antibiotics at 37˚C in a humidified atmosphere with 5% $CO_2$ as previously described (11-13,15,31).

*Immunofluorescence analysis*. Cells grown on glass cover slips were fixed with acetone for 10 min at room temperature and washed twice with PBS as previously described (12,13,15). Non-specific binding was blocked by incubating the sections with Ultra-V-Block (Lab Vision) for 15 min at room temperature. Thereafter, slides were incubated with inhibin-ßC antibody (1:50 in dilution medium provided by Dako) over night at 4˚C, followed by a 1:500 diluted Cy3-conjugated donkey anti-goat antibody (Dianova, Hamburg, Germany). The slides were finally embedded in mounting buffer containing 4,6-diamino-2-phenylindole (DAPI) resulting in blue staining of the nuclei. Slides were embedded with Vectashield mounting medium (Axxora, Lörrach, Germany) and examined with a Zeiss (Jena, Germany) Axiophot photomicroscope. Digital images were obtained with a digital camera system (Axiocam, Zeiss) and saved on a computer.

*Statistical analysis*. The intensity and distribution patterns of specific inhibin/activin-subunit immunohistochemical cytoplasmatic staining reaction was evaluated by two, independent observers, including a gynaecological pathologist (N.S.), in a blinded manner using a semi-quantitative score as previously described (21,26,27,32,33). The IRS score was calculated by multiplication of optical staining intensity (graded as 0, no; 1, weak; 2, moderate; and 3, strong staining) and the percentage of positive stained cells (0, no staining; 1, <10% of the cells; 2, 11-50% of the cells; 3, 51-80% of the cells; and 4, >81% of the cells). Sections were examined using a Leica photomicroscope. The IRS-scores of inhibin-ßC immunohistochemical expression levels were compared using the non-parametric Mann-Whitney U test. Significance of differences was set at $p \leq 0.05$ at the two-sided test.

## Results

*Immunohistochemical analysis of inhibin-ßC expression*. As an appropriate positive control normal liver specimens were

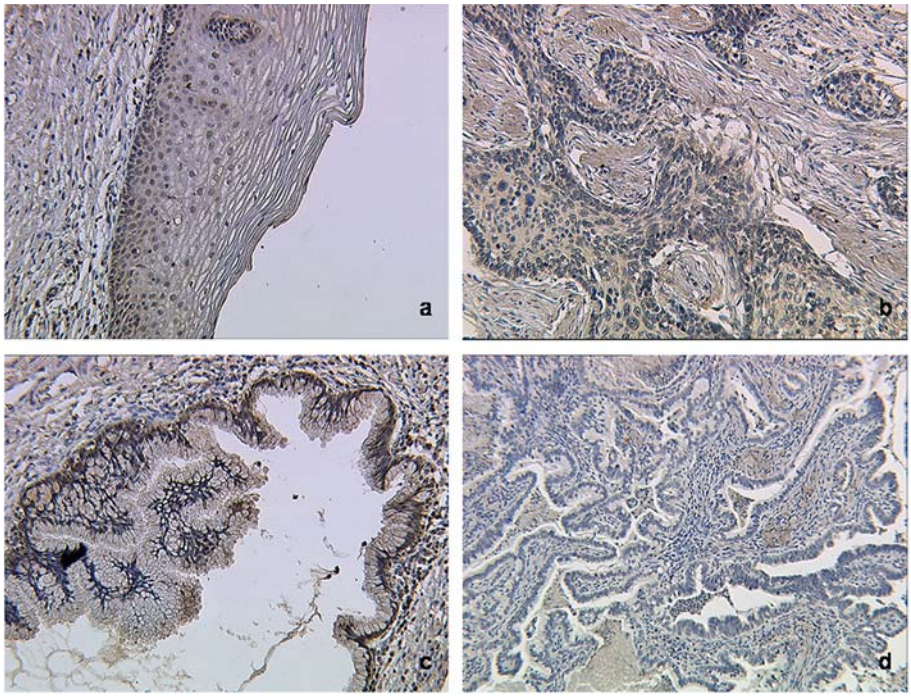


Figure 1. Immunohistochemical staining reaction of inhibin-ßC in normal and malignant cervical tissue. Normal squamous epithelial cells reacted with the inhibin-ßC antibody, being primarily positive in the basal membrane and the underlining stromal compartment (a, x100). Squamous carcinomas also demonstrated a stronger positive immunohistochemical reaction (b, x75). Normal cervical glandular epithelium demonstrated also a strong reaction (c, x250), while cervical adenocarcinomas also reacted, but to a lesser extent, with the inhibin-ßC antibody (d, x250).

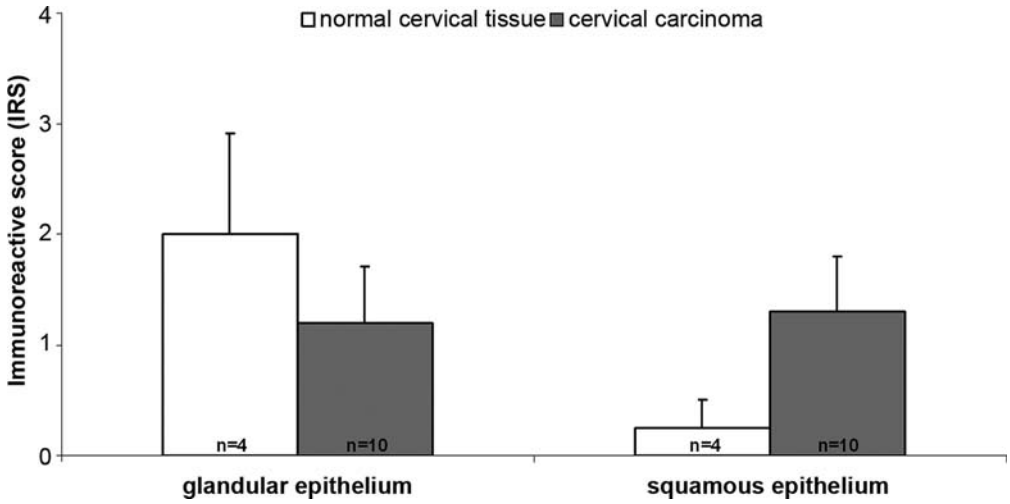


Figure 2. Immunohistochemical analysis for inhibin-ßC subunits. The immunoreactive score (IRS) for inhibin-ßC decreased normal and malignant glandular epithelium, while an increase between normal squamous epithelium and squamous cervical carcinomas could be observed. However, no significant differences could be observed.

used to test the reactivity of the inhibin ßC-antibody. A positive staining reaction for inhibin-ßC subunit was demonstrated on normal human liver tissues, confirming previous results (8,34,35).

The inhibin-ßC subunit demonstrated a positive staining reaction in normal cervical tissue (Fig. 1). Normal squamous epithelial cells reacted with the inhibin-ßC antibody, being primarily positive in the apical part of the epithelium and the underlining stroma (Fig. 1a). In contrast, squamous carcinomas also demonstrated a positive immunohistochemical reaction with a stronger intensity compared to normal cervical tissue (Fig. 1b). Additionally, normal cervical glandular epithelium demonstrated a strong reaction against inhibin-ßC (Fig. 1c), while cervical adenocarcinomas also reacted with the inhibin-ßC antibody albeit with a less staining intensity compared to normal epithelium (Fig. 1d).

The immunoreactive score (IRS) for inhibin-ßC decreased from normal to malignant glandular epithelium, while an increase between normal squamous epithelium and squamous cervical carcinomas could be observed (Fig. 2). However, no

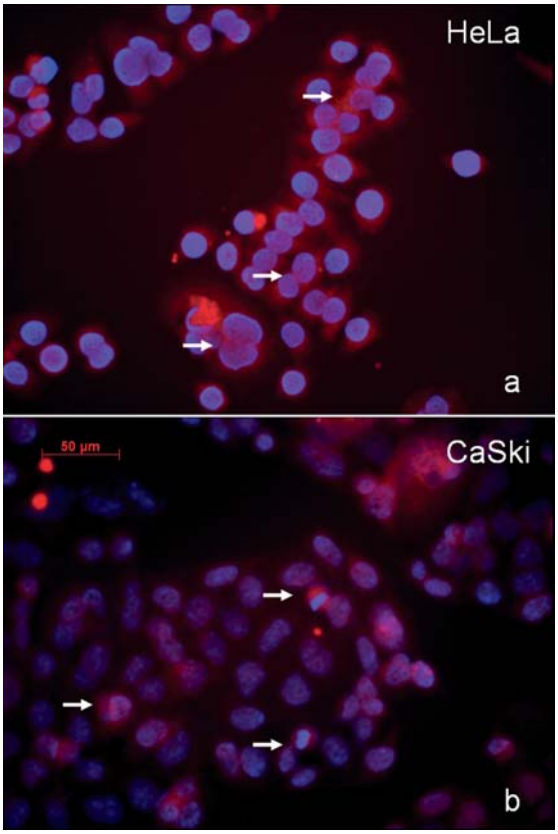


Figure 3. Localization of inhibin-ßC expression in HeLa and CaSKi cells. The cervical carcinoma cell lines HeLa and CaSKi were analyzed by immunofluorescence for the expression of inhibin-ßC, showing a cytoplasmatic positive staining reaction in HeLa (a, x400) and CaSKi (b, x400) cells.

statistically significant differences could be observed, probably due to the small number of analysed cases.

*Expression of inhibin-ßC in human cervical carcinoma cell lines*. Cervical carcinoma cells are malignant cell lines derived from invasive cervical carcinomas of different origin. We therefore tested the expression of inhibin-ßC in the human cervical cancer cell lines HeLa and CaSKi. Immunofluorescence analysis of both cell lines expressed this novel ßC-subunit at the protein level. Expression of inhibin-ßC was found to be located primarily in the cytoplasma (Fig. 3).

## Discussion

The inhibin/activin-subunits have been detected in normal female reproductive tissue and endocrine tumours (18), including breast cancer (36), normal and pathological placental tissue (19,21,32) as well as normal and pathological endometrial tissues (23,24,26,27). Inhibins and activins have been implicated in stem cell biology (37), reproductive biology (38), regulatory role during natural-killer cell regulation (39), systemic inflammation (40) and apoptosis (41).

While inhibin A, inhibin B and activin A were detected in normal and neoplastic human uterine tissues, including cervical cancer (25), it is still unclear if cervical epithelial normal and cancer cells can synthesise the novel inhibin-ßC subunit. We demonstrated for the first time the immunohistochemical expression of this inhibin-ßC subunit in normal and pathological cervical tissue.

The inhibin/activin ßC subunit was demonstrated to be predominantly expressed in hepatocytes (8,42,43), prostate, ovary, testes and pituary (16,44). We have recently also demonstrated this subunit in human endometrial and placental tissue (12,15,30). Interestingly, the formation of homodimeric activin C (ßC-ßC) as well as heterodimeric activins AC (ßA-ßC), BC (ßB-ßC), CE (ßC-ßE), as well as inhibin C (α-ß) has been demonstrated by ectopic expression of the respective subunits in different cell models (17,45). Although the precise role of this subunit is still not elucidated, several possible functions have been suggested, inducing apoptosis (35,46) and increasing the rate of DNA synthesis in primary rat hepatocytes (47). Moreover, the ßC-subunit was identified as an autocrine growth modulator in liver regeneration, leading to mitosis in a subset of hepatocytes (44). Moreover, it was demonstrated that activin C (ßC-ßC) does not activate activin A (ßA-ßA), responsive promoters, and it was suggested that the ßC subunit regulates the levels of bioactive activin A (ßA-ßA) through the formation of signaling incompetent activin AC heterodimers (45,48,49). Therefore, ßC-subunit might function as an antagonist of activin function (16,45,48). If these functions are also applicable in normal human cervical tissue and cervical cancer remains to be clarified.

Expression analysis of inhibin-subunits in cervical tissue is scarce. We recently observed the novel ßE subunit in cervical cancer and cervical cancer cell lines (11). Additionally, we have demonstrated that inhibin-ßA and -ßB are also expressed in cervical squamous epithelial cells (22). Both inhibin-ß subunits showed a differential expression in CIN and squamous cancer, suggesting important roles in cervical carcinogenesis (22). Inhibin-ßA might be important during progression of cervical intraepithelial neoplasia, while the inhibin-ßB subunit could exert a substantial function during differentiation of cervical carcinomas (22). However, the precise function of inhibin-ßC in cervical pathogenesis and carcinogenesis remains still to be elucidated.

In conclusion, we demonstrated the expression of inhibin-ßC subunit in normal and malignant cervical tissue as well as cervical cancer cells. Although the physiological role is still unclear in cervical tissue, it might play important roles in carcinogenesis. Moreover, the synthesis of this sub-unit in cervical carcinoma cell lines allows also the use of these cell lines in elucidating its functions in cervical pathogenesis. The functional role of this inhibin-subunit in normal and pathological human uterine cervical tissue is still unclear and warrants further investigation.

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