EXPERIMENTAL STUDY

Gonadal malignant germ cell tumors express immunoreactive inhibin/activin subunits

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Abstract

Objective: Inhibin and activin are proteins produced by ovarian granulosa cells and testicular Sertoli cells and are members of the transforming growth factor- β superfamily. Since increased circulating levels of immunoreactive inhibin were detected in women with malignant ovarian tumors, they were proposed as tumor markers for ovarian carcinoma. Immunohistochemical studies later confirmed the presence of inhibin and activin subunits in granulosa cell tumors and epithelial ovarian cancer, as well as in Sertoli and Leydig cell testicular cancer. However, there is discrepant information on the detection of inhibin and activin in malignant germ cell tumors (MGCT). The aim of the present study was to evaluate the immunohistochemical expression of the inhibin/activin α , β A and β B subunits in ovarian and testicular MGCT specimens using polyclonal antisera.

Methods: The ovarian tissue samples were composed of 19 MGCT, including dysgerminoma ($n = 18$) and yolk sac tumor ($n = 1$). The testis specimens included classic seminomas ($n = 20$), embryonal carcinomas ($n = 7$), choriocarcinomas ($n = 2$), and yolk sac tumor ($n = 1$).

Results: Ovarian and testicular malignant germ cell tumors expressed positive staining for inhibin/activin α , β A and β B subunits, with some variations between and within individual tumors: while ovarian dysgerminomas were diffusely positive for α , β A and β B, testicular tumors expressed α and β B subunits, whereas β A staining was weak.

Conclusions: The present results show positive staining for inhibin/activin subunits in ovarian and testicular MGCT, suggesting a possible role in tumorigenesis with the resultant clinical implication.

European Journal of Endocrinology 145 779–784

Introduction

Inhibin and activin are members of the transforming growth factor- β (TGF- β) superfamily. Inhibin is composed of a common α subunit and one of two β -subunits (β A and β B), resulting in inhibin A ($\alpha\beta$ A) and inhibin B ($\alpha\beta$ B). Activin is a dimer composed of two inhibin β subunits, and activin A (β A β A), activin B (β B β B) and activin AB (β A β B) are recognized (1). First isolated from the gonads, they were recognized as hormones and named inhibins and activins because of their opposing effects on pituitary follicle-stimulating hormone (FSH) secretion. Further investigations elucidated their sequence homology with TGF- β and their effect on cell differentiation and proliferation in various organs, suggesting a role as growth factors (1–3).

Serum inhibin was proposed as a tumor marker since high serum immunoreactive inhibin levels were detected in women with malignant ovarian tumors (4). This hypothesis has been intensively investigated

and high circulating inhibin A and activin A levels have been shown in women with epithelial ovarian tumors (5, 6) while inhibin B is increased in patients with granulosa cell tumors (7, 8). Immunohistochemical studies have confirmed the presence of inhibin/activin subunits in granulosa cell tumors and epithelial ovarian cancer (9–15). In the meantime, the evidence that ovarian tumors developed in inhibin α knock-out mice strongly supported the possible role of these proteins in ovarian tumorigenesis (16). On the other hand, no evidence of increased inhibin levels in men with testicular tumors has been shown, but immunohistochemical localization of inhibin subunits in Sertoli cell tumors and Leydig cell tumors was shown (17, 18).

Ovarian and testicular malignant germ cell tumors (MGCT) derive from primitive germ cells of the embryonic gonad and represent 5–6% of ovarian neoplasms (19) and 95% of testicular neoplasms (20). Dysgerminoma is the most common ovarian MGCT form, whereas seminoma is the commonest histological type

in the testis. Conflicting data are available on the immunohistochemical detection of inhibin α subunit in ovarian MGCT (9, 13, 17, 18), with no information on inhibin/activin β A and β B subunits, and little information on testicular MGCT (14). The aim of the present study was to evaluate the immunohistochemical expression of inhibin/activin α , β A and β B subunits in ovarian and testicular MGCT.

Materials and methods

Tissues samples

Specimens from ovarian and testicular MGCT ($n = 49$) were evaluated: they were composed of ovarian MGCT ($n = 19$) and testicular MGCT ($n = 30$).

Ovarian MGCT included dysgerminomas ($n = 18$) (patient age range 9–24 years, median 17.2) and yolk sac tumor ($n = 1$) (patient aged 40) according to the World Health Organisation (WHO) classification (21) and were retrieved from the files of the Istituto Tumori of Milan. The tissues were fixed in Bouin's fluid and embedded in paraffin.

Testicular MGCT included 20 classic seminomas ($n = 20$) (patient age range 18–65 years, median 31.6), embryonal carcinomas ($n = 7$) (patient age range 18–42, median 27.7), choriocarcinomas ($n = 2$) (patients aged 25 and 35) and yolk sac tumor ($n = 1$) (patient aged 41) according to the WHO classification (22) and were selected from the files of the Department of Pathology of the University of Udine. The tissues were fixed in 10% buffered formalin and embedded in paraffin.

The expression of inhibin/activin α , β A and β B subunits was studied by using an immunoperoxidase technique. All hematoxylin and eosin-stained slides were reviewed and representative sections of each case were selected for immunohistochemical staining. One paraffin block from dysgerminoma included residual normal ovary; in 18 cases of testicular tumors the selected sections showed residual atrophic/subatrophic testis.

Immunohistochemistry

Immunohistochemical analysis was performed on 5 μ m sections using the streptavidin-biotin-peroxidase technique. The sections were routinely deparaffinized, rehydrated and antigen retrieval was carried out by microwaving the slides at 800 W for 5 min and 300 W for 3 min in 0.01 mol/l citrate buffer, pH 7.3. After endogenous peroxidase blocking, the sections were incubated overnight at 4 °C with the following specific antibodies: rabbit anti α (1–26)-Gly-Tyr (diluted 1:100); mouse anti- β A (81–113)-NH₂ and mouse anti- β B (81–112)-NH₂ (diluted 1:10). These were affinity purified polyclonal antisera raised against synthetic peptide fragments (Salk Institute, La Jolla, CA, USA). A monoclonal antibody raised against the α -inhibin subunit, clone R1 (diluted 1:100) was also used (Serotec,

Oxford, Oxon, UK) for comparison with previous studies (23).

The sections were then incubated with biotinylated link antibodies followed by peroxidase-conjugated streptavidin (LSABTM, Dako, Glostrup, Denmark) at room temperature for 30 min each. After peroxidase developing with 3,3'-diaminobenzidine/hydrogen peroxide, the slides were counterstained with hematoxylin, dehydrated and mounted.

Negative and positive controls were included in each staining series: first trimester placental tissue was used as a positive control and the same biopsy incubated with normal rabbit immunoglobulin or mouse ascites fluid instead of primary antibodies was used as a negative control.

The immunostaining was evaluated by estimating semiquantitatively the distribution and the intensity of positive cells. A positive reaction was characterized by the presence of granular brown staining. The staining was scored as diffuse ($\geq 75\%$ of tumor cells positive) or focal (partial) ($< 75\%$ of tumor cells positive). The intensity of staining was graded semiquantitatively as follows: negative (–); weak (+); moderate (++) ; strong (+++).

Results

Ovarian and testicular MGCT expressed positive immunoreactivity for inhibin/activin α , β A and β B subunits with some variations between and within individual tumors (Table 1). The antibodies detected cytoplasmic proteins in neoplastic cells, and in some sections a nuclear stain was observed. Since previous studies had shown no staining of inhibin α subunit, the same monoclonal antibody (23) was tested on our specimens and no positive signal was observed in any ovarian and testicular tumoral form; positive controls stained as expected.

In all cases with dysgerminomas ($n = 18$) a diffuse positive expression of α , β A and β B subunits with

Table 1 Evaluation of the intensity of staining for inhibin/activin subunits (α , β A, β B), according to a subjective semiquantitative analysis.

Malignant germ cells tumor	Alpha	Beta A	Beta B
Ovary			
Dysgerminoma ($n = 18$)	+/++	+/++ ^a	+/++
Yolk sac tumor ($n = 1$)	+++	+++	+++
Testis			
Seminoma ($n = 20$)	+/++	+ ^b	++
Embryonal carcinoma ($n = 7$)	++	+ ^c	++
Choriocarcinoma ($n = 2$)	++/+++	+ ^d	++
Yolk sac tumor ($n = 1$)	++/+++	++	++

+, weak staining; ++, moderate staining; +++, strong staining.

All cases demonstrated diffuse immunostaining unless otherwise specified. ^a, focal staining in 5 cases; ^b, focal staining in 9 cases, diffuse in 11; ^c, focal staining in 4 cases, diffuse in 3; ^d, focal staining in 2 cases.

weak or moderate staining was found; in 5 cases the positivity for β A subunits was focal. A finely distributed labeling of some nuclei was observed with the antibody to β B subunit. In the ovarian yolk sac tumor a diffuse and strong cytoplasmic positive signal for α , β A and β B subunits was shown, with a dot-like pattern for α subunit as well.

Testicular MGCT diffusely expressed α subunit in the cytoplasm. Weak or moderate intensity of α inhibin subunit was shown in seminomatous tumors, while a strong intensity resulted in non seminomatous tumors. A strong positive staining for the α subunit was also detected in syncytiotrophoblast cells present in 4 seminomas. A focal and weak cytoplasmic staining was observed for β A subunit; only the yolk sac tumors showed a moderate staining. All testicular tumors showed a diffuse and moderate cytoplasmic positivity for β B subunit. Positive immunoreactivity for α and β B inhibin/activin subunits in some nuclei of ovarian or testicular MGCT cells was also localized (Fig. 1).

Discussion

The present immunohistochemical results demonstrate for the first time positive staining for inhibin/activin α , β A and β B subunits in ovarian and testicular MGCT, suggesting the possible expression of the different dimeric forms of inhibin/activin in this type of tumor.

Previously, the presence of immunoreactive inhibin α subunit in ovarian neoplasms was identified as a sensitive immunohistochemical marker of granulosa cell tumors (12), sex cord stromal tumor (9, 13) and epithelial tumors (10, 11, 14). Sex cord stromal and epithelial ovarian tumors also showed β A and β B inhibin subunits immunolocalization (15, 24).

Among the testicular tumors, α inhibin subunit was expressed in 91% of the Sertoli cell tumors and in 100% of the Sertoli cell adenomas and Leydig cell tumors (17, 18).

Previous studies investigating the expression of α inhibin subunit using a monoclonal antibody showed conflicting data in ovarian forms of MGCT. Pelkey *et al.* (14) pointed out that the immunostaining of inhibin α in 7 cases of ovarian MGCT was negative, while in the testes α inhibin staining was positive in only one out of 13 seminoma cases in which the syncytiotrophoblast was present. The lack of any α inhibin staining was also shown in another six (25), twenty-four (26) and six (27) ovarian MGCT, except for a positive focus near syncytiotrophoblastic cells. On the other hand, Hussong *et al.* (28) reported positive α inhibin staining in 40% of ovarian MGCT using the same monoclonal antisera.

Our present results which showed diffuse α subunit staining in MGCT using the polyclonal antiserum but not when the R1 monoclonal antibody was tested on the same specimens, suggest that the lack of any α staining in the previous studies may depend upon the

characteristics of the two antibodies. The present polyclonal α inhibin antiserum does not cross react with TGF- β , and has only 1% cross reactivity with β A and β B subunits (29); the question that remains open is the possible detection of free α subunit or pro- α -C- precursor. Therefore, part of our positive staining may be due to the detection of the dimeric forms of inhibins and/or to the precursor/free α subunit.

Several studies have investigated inhibin-related proteins in human fetal gonads. Immunohistochemical staining with a polyclonal antibody detected α subunit in interstitial and intratubular cells in midgestation testis, while β A and β B subunits occurred in Leydig cells. The primordial follicles, the most immature ovarian cell population recognizable, were negative for α and β B subunits, and showed very weak staining for β A (30). Using Northern blot analysis, RT-PCR and *in situ* hybridization, fetal ovary during the second trimester of gestation failed to express inhibin subunits, in agreement with the hormonal inactivity of the ovary during intrauterine life (31, 32). On the other hand, positive results were seen in the testis with different staining and gene expression.

During reproductive age human primordial follicles show positive staining for β A and β B subunits, and are negative for α subunit. In the growing follicles all inhibin-related protein subunits are stained and the expression of mRNA shows an increasing intensity (33, 34).

Evidence that activin, together with follistatin and FSH, act and regulate germ cell maturation has recently been provided (35). Rat testis fragments in culture treated with activin show a high proportion of Sertoli cells derived from the germ cell compartment, an effect which is not demonstrable by adding follistatin and FSH in the culture medium. The differentiation of gonocytes in spermatogonia mediated, by activin, that represent the first Sertoli cell product, give reason to a pivotal role of inhibin-related proteins in germ cell differentiation (36). Moreover, activin receptors are expressed by testicular germ cells (37, 38), and are able to bind activin and inhibin (39). Testicular germ cell tumors express activin type I and type II receptors, and the presence of inhibin/activin β A and β B mRNAs in these tumors but not in normal germ cells suggests that testicular germ cell tumor development may, in part, be regulated and modulated by the inhibin/activin system (40).

Recently, serum inhibin B levels have been measured in men with various gonadal dysfunction; the group with germ cell failure, with elevated FSH levels and normal luteinizing hormone and testosterone levels, showed lower amounts of inhibin B compared with the control group (41). These data confirm that inhibin B production in men is regulated by Sertoli cell function, plays a fundamental role in FSH secretion, and is positively correlated with spermatogenesis.

The immunohistochemical localization provides indirect evidence of these proteins; no other data are

Figure 1 Ovarian and testicular germ cell tumors immunostained for inhibin/activin subunits α (left column), βA (centre column) and βB (right column). The following tumors are shown: ovarian dysgerminoma (a,b,c) and testicular classic seminoma (d,e,f). Specific immunoreactivity is indicated by the presence of brown staining. Original magnification was $\times 400$. Abolition of staining was obtained by substituting the primary antibody with non-immune rabbit serum immunoglobulins (not shown).

available for germ cell ovarian neoplasms due to the rarity of these tumors.

MGCT arise from a germinal cell which is totipotent. Inhibin/activin are present during embryonic development and have a role in cellular differentiation; later they may be expressed again when a malignancy occurs. The expression of β A activin mRNA is an event common to various tumor organs (uterus, liver, adrenal, pancreatic, prostate, breast) (42–47) thus suggesting a biological role of activins in tumorigenesis, independent of the tissue of origin.

The positive staining of both β A and β B activin subunits in ovarian and testicular MGCT suggests the possible expression of activin dimers and/or free subunits in these tumors. This is indirect evidence that inhibin-related proteins are present in this type of tumor; a possible role in tumorigenesis is unknown and the putative clinical implication remains to be evaluated.

Acknowledgements

We kindly thank Dr Wylie Vale and Mrs Joan Vaughan, Salk Institute (La Jolla, CA, USA), for providing the antisera against inhibin/activin α , β A and β B subunits, and Dr Nigell Groome (Oxford, UK) for providing the monoclonal antiserum (R1) against inhibin α subunit.

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Received 27 February 2001

Accepted 31 August 2001