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Research article

The expression of AIB1 correlates with cellular proliferation in human prolactinomas

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SUMMARY

Estrogens as well as certain growth factors strongly influence the development and growth of prolactinomas. However, the molecular mechanisms by which extracellular factors trigger prolactinomas are not well known. Amplified in breast cancer 1 (AIB1), also known as steroid receptor co-activator 3 (SRC-3), belongs to the p160/SRC family of nuclear receptor co-activators and is a major co-activator of the estrogen receptor. Here, we report that the estrogen receptor coactivator AIB1 is overexpressed in human prolactinomas and correlates with the detection of aromatase and estrogen receptor α (ERα). Of the 87 pituitary tumors evaluated in women, 56%, corresponding to hyperoprolactinemic women, contained an enriched population of prolactin-positive cells and hence were further classified as prolactinomas. All prolactinomas stained positive for both ERα and AIB1. Moreover, AIB1 sub-cellular distribution was indicative of the cell-cycle status of tumors; the nuclear expression of AIB1 was correlated with proliferative markers whereas the cytoplasmic localization of AIB1 coincided with active caspase-3. Thus, our results demonstrate for the first time that AIB1 is expressed in prolactinomas and suggest its participation in the regulation of proliferation and apoptosis of tumoral cells. Because aromatase expression is also enhanced in these prolactinomas and it is involved in the local production of estradiol, both mechanisms, ER-AIB1 and aromatase could be related.

1. Introduction

Several co-activator proteins have been identified and implicated in the mechanisms of the transcriptional activation of estrogen receptors and are therefore now considered important modulators of hormonal action (Leo and Chen, 2000; Misiti et al., 1998; Nagy et al., 1999; Shang et al., 2000). Consequently, differential responses of hormone-dependent tumors to sex steroids could be strongly influenced by the relative amounts of co-activator proteins such as AIB1 (Hudelist et al., 2003). Amplified in breast cancer 1 (AIB1), also known as steroid receptor co-activator 3 (SRC-3), is a member of the p106 coactivator family and plays an important role in cell growth, reproduction, metabolism, and cytokine signaling (Wang et al., 2000; Xu et al., 2000; Zhou et al., 2003). It is a major co-activator of the estrogen receptor (ER) in human breast cancer cell lines (Tikkanen et al., 2000), is also overexpressed in several types of cancers, including breast and ovarian (Anzick et al., 1997; Kurebayashi et al., 2000), prostate (Gnanapragasam et al., 2001), gastric (Sakakura et al., 2000), pancreas (Ghadimi et al., 1999; Henke et al., 2004), liver (Wang et al., 2002) and colon cancers (Xie et al., 2005). When overexpressed in mammary epithelial cells, AIB1 can function as an oncogene in mouse models (Torres-Arzayus et al., 2004), producing adenocarcinomas of various subtypes.

Prolactinomas are hormone-dependent tumors that originate in the lactotrophs of the pituitary gland and constitute the most common endocrine tumors. Chronic treatment with estradiol is known to induce pituitary hyperplasia and prolactinomas (Gooren et al., 1988; Heaney et al., 2002; Molitch, 2001; Phelps and Hymer, 1983; Wingrave et al., 1980). Moreover, estradiol stimulates the proliferation of prolactin-producing cells (Lloyd et al., 1975; Perez et al., 1986) and, interestingly, ER expression in human prolactinomas correlates with prolactin synthesis and tumor growth (Stefaneanu

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et al., 1994). Therefore, AIB1 could represent a key modulator in the growth of normal as well as prolactinoma cells since it regulates estrogen-induced cyclin D1 expression (Planas-Silva et al., 2001) and promotes cell-cycle progression by coactivating E2F (Louie et al., 2004). Although the expression of aromatase has been described in prolactinomas (Carretero et al., 2002; Akinci et al., 2012); however, the expression of AIB1 and its relationship to aromatase (the enzyme responsible for the local aromatization of testosterone to estradiol) in prolactinomas has not yet been characterized. To investigate the role of coactivators in human pituitary tumors, we evaluated the expression of AIB1, aromatase, and the estrogen receptor in human adenomas and correlated the expression of these molecules with the proliferative status of the prolactinomas.

2. Materials and methods

2.1. Sample processing

For the present study, a series of 87 pituitary adenomas detected in women were obtained upon surgical removal in the Neurosurgery Department of the University Hospital of Salamanca, Spain. Informed consent was obtained from all patients. Moreover, 5 normal pituitary glands were collected from medical autopsies and used as controls in our experiments. Immediately after surgery, a portion of the tissue was fixed by immersion in 4% paraformaldehyde in phosphate buffered saline (PBS: 0.1 M, pH 7.4, +0.8% NaCl) for 24 h at 4 °C and then washed in PBS for 24 h. The other portion of each non-tumoral gland or each pituitary tumor was processed for Western blot.

The fixed tumors were dehydrated in ethanol and embedded in paraffin to obtain 5 μm serial sections for immunohistochemistry.

2.2. Immunohistochemistry

Immunohistochemical studies were performed using the strepto-avidin–peroxidase method. The sources and working dilutions of primary anti-sera were as follows: monoclonal antibody against human AIB1 (BD Transduction Laboratories, diluted 1:200), anti-Prolactin rabbit polyclonal serum (Dako®, diluted 1:800), anti human aromatase P450 rabbit polyclonal serum (Rb-SG 1230 Sigma®, diluted 1:500), monoclonal antibody against ER α(Alexis®, diluted 1:400), anti-PCNA monoclonal antibody (Dako®, diluted 1:3000) and polyclonal antibody anti active caspase 3 (Alexis®, diluted 1:250). Antibodies were incubated with tissue sections at 4 °C overnight. After washing, the slides were then incubated for 45 min at RT with biotinylated-goat anti-rabbit or mouse IgG (Caltag®, diluted at 1:100 in TBS) and then for 45 min at room temperature with streptavidin–biotin–peroxidase complex (Caltag®, diluted at 1:150). The reaction was developed with freshly prepared 3,3′-diaminobenzidine (Sigma®, 0.024% in TRIS buffer +0.03% H_2O_2). The washes and antibody dilutions were made in TBS: HCl-Trizma, 0.05 M, pH 7.4, +0.8% NaCl.

The specificity of the anti-aromatase P450 antibody used in the present study was examined by western blotting, in which the primary anti-serum was replaced with non-immune rabbit serum or pre-absorption of the antiserum. For the pre-absorption test of anti-aromatase Rb-SG 1230 polyclonal antibody, antiserum was diluted (1:500) and pre-absorbed (50 μg peptide/ml antibody solution, 24 h at 4 °C) with the antigen peptide sequence NMLEMIFTPRNSDRCLEH, corresponding to residues 486–503 of human cytochrome P450 (Chen et al., 1986; Harada, 1988; PubMed accession number: P11511). No specific reactivity was detected using either specificity test.

2.3. In situ hybridization

The study was performed using a non-isotopic method involving the immunohistochemical detection of biotin by means of the streptavidin–biotin–peroxidase method. To perform *in situ* hybridization, the biotinylated oligonucleotide 5′BIO-gcg cat gac caa gtc cac gac agg ctg 3′, 100% specific to human aromatase P450 according to GenBank data base (sequence 847–873 bp of the aromatase gene, Harada et al., 1999; BLASTN2 searching, emb: HSARP450, Y07508; emb: HSAROMAT, X13589) was used as probe. Slides were prehybridized in Omnibuffer™ for 30 min at 37 °C; hybridization with the biotinylated-probe (100 pg/ml in Omnibuffer) was carried out overnight at 37 °C using a Hybaid thermocycler. The reaction was stopped by washes in 1× SSC at 54 °C for 20 min, 1× SSC at room temperature for 20 min and for 20 min in PBS (0.01 M, pH 7.4, +0.8% NaCl). Biotin was detected using monoclonal anti-biotin antibodies (Roche™, diluted 1:250 in TBS) over night at 4 °C in a humidity chamber, followed by biotinylated goat anti-mouse (Caltag™, 1:250 in TBS). The reaction was amplified using the tyramide amplification kit (Dako™) according to the manufacturer's instructions. The final reaction was developed with 3,3′-diaminobenzidine (0.025 M, Sigma™, in 0.05 M Tris–HCl buffer, pH 7.4) to which 0.03% H_2O_2 had been added. The slides were counterstained using Mayer's acid hematoxylin. As controls, omission of the probe, pretreatment with Rnase and *in situ* hybridization with a sense probe were performed: no reaction was found in any case.

2.4. Western blotting

For Western blotting, prolactinomas were frozen immediately. Tissues were later disrupted by homogenization in lysis buffer (137 mM NaCl, 10 mM Tris pH 7.4, 10% glycerol and 1% Tx-100 containing a cocktail of protease inhibitors). Insoluble material was removed from lysates by centrifugation at 10,000 rpm for 10 min. Protein concentrations were determined using a standard Bradford assay. A 50 μg aliquot of total protein from each rat sample was separated by 10% SDS-PAGE. Following electrophoresis, proteins were transferred to nitrocellulose and then blocked for 1 h with 5% non-fat dry milk in PBS. Nitrocellulose membranes were then incubated overnight with either pre-immune serum, anti-aromatase antibody diluted 1:500, anti-AIB1 antibody diluted 1:200 or monoclonal anti-β-actin (Sigma®). Blots were subjected to 3× 15 min washes with PBS and then incubated for 1 h with HRP-labeled secondary antibodies (1:10,000 in PBS). Following extensive washing, blots were revealed by ECL (Amersham®). Average exposure time was 2 min. Densitometric analysis was performed (molecular dynamics) to assess relative levels of aromatase expression.

3. Results

3.1. Human prolactinomas express aromatase

Based on immunostaining, 56% of the eighty-seven human pituitary adenomas in our study group were classified as prolactinomas (Fig. 1a and d). Hyperprolactinaemia – serum prolactin levels higher than 300 μg/ml – was confirmed by serum prolactin levels as measured by ELISA (data not shown). We next evaluated aromatase expression and found that all the prolactinomas stained positive for this hormone-converting enzyme (Fig. 1b and d), observing, by means of western blotting, approximately a two-fold increase in aromatase expression in comparison with normal pituitary controls (Fig. 1e). By means of *in situ* hybridization, a positive expression of mRNA for aromatase was found in the immunohistochemical aromatase-positive tumors (Fig. 1c). Aromatase-positive

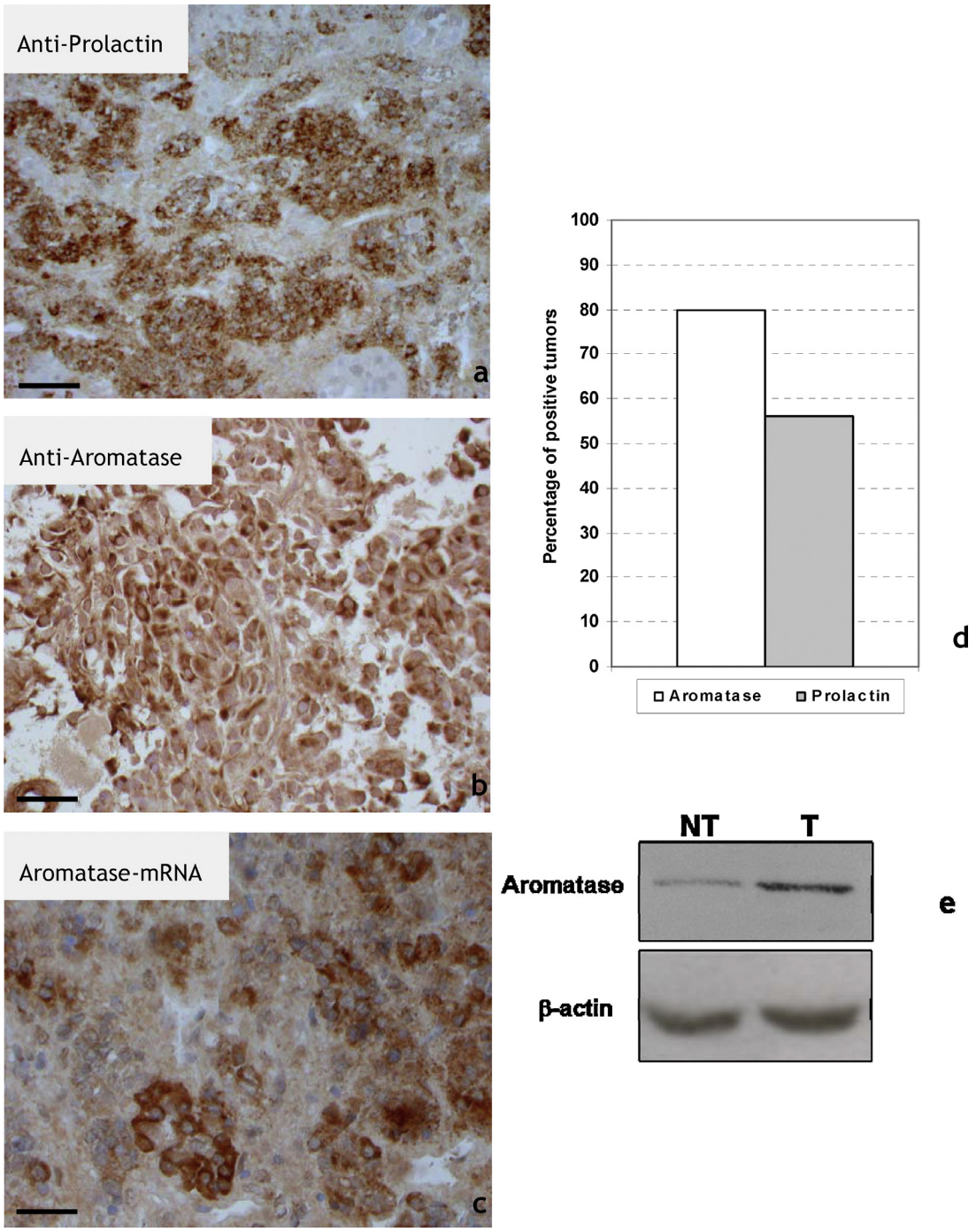

Fig. 1. Immunohistochemical analysis of human pituitary tumors. (a) Paraffin-embedded sections were stained with anti-prolactin antibodies. A representative prolactinoma is shown. (b) Pituitary tumors were incubated with anti-human aromatase antibodies. A representative prolactinoma is shown that is positive for aromatase. (c) Micrograph showing positive mRNA of aromatase cells after *in situ* hybridization. (d) Graph showing the percentages of total pituitary tumors (87) that were positive according to immunostaining for either prolactin or aromatase. (e) Western blotting for aromatase in tumoral (T) and non tumoral (NT) pituitary gland, the blots are representative of 5 samples analyzed, β-actin was used as control. Scale bar: (a) 40 μm; (b) and (c) 20 μm.

cells were distributed over the tumor surface and were detected in tumors with varying morphological features, including solid as well as hemorrhagic tumors.

3.2. Both ERα and AIB1 are expressed in human prolactinomas

Interestingly, 100% of tumors positive for both prolactin and aromatase also expressed ERα (Fig. 2a and d). Since AIB1 is co-expressed with ERα in breast and ovarian tumors, we examined the expression of this coactivator in our group of prolactinomas by immunohistochemistry. Surprisingly, all prolactinomas in our study were AIB1-positive tumors (Fig. 2b–e). However, by immunohistochemistry, two different immuno-reactive patterns were observed: nuclear staining (arrowheads, Fig. 2b) and cytoplasmic reactivity (arrows, Fig. 2b). Although individual cells with either nuclear or cytoplasmic staining for AIB1 could be detected within any given prolactinoma, each tumor displayed an overall predominant pattern with respect to the sub-cellular localization

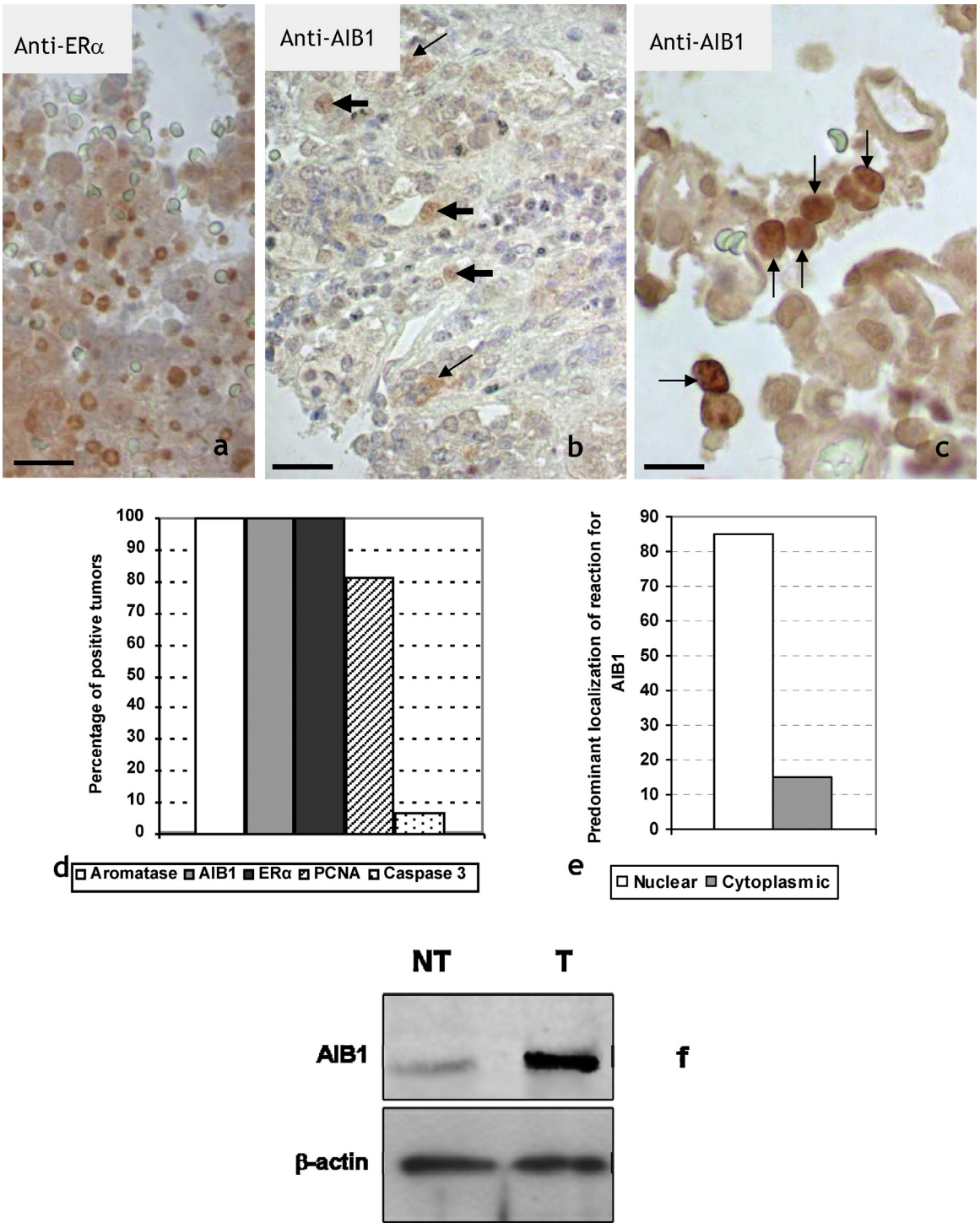

Fig. 2. Characterization of ER and AIB1 expression in human prolactinomas. (a) Representative immunostaining of a human prolactinoma with anti-ER antibodies. A positive reaction yields a brown stain. (b) Immunostaining of prolactinomas with anti-AIB1 antibodies. Arrowheads indicate nuclear stain (brown) and arrows indicate the cytoplasmic detection of AIB1. (c) High-power magnification of a prolactinoma with intense staining for AIB1. (d) Graphic representation of the percentage of prolactinomas scored positive for aromatase, AIB1, PCNA, and active caspase 3. (e) Tabulation of the percentage of prolactinomas displaying nuclear *vs.* cytoplasmic staining for AIB1. (f) western blotting for AIB1 in tumoral (T) and non tumoral (NT) pituitary gland, the blots are representative of 5 samples analyzed, β-actin was used as control. Scale bar: (a) and (b) 20 μm; (c) 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of AIB1 and thus the majority of the prolactinomas were scored as nuclear (85%) (Fig. 2e).

3.3. The nuclear localization of AIB1 correlates with proliferation

To assess proliferation in our human prolactinomas, the tumors were stained with antibodies for PCNA, a nuclear marker of cell growth. A proliferation rate (PCNA-positive nuclei, Fig. 3a) higher than 1.5% was considered to be indicative of a proliferating tumor. Seventy-five percent of all tumors and 81% of prolactinomas were PCNA-positive. Interestingly, all prolactinomas with a predominant nuclear staining for AIB1 were PCNA-positive tumors (Fig. 3c). In contrast, the 7% of prolactinomas that were PCNA-negative displayed mainly cytoplasmic reactivity for AIB1. Moreover, the

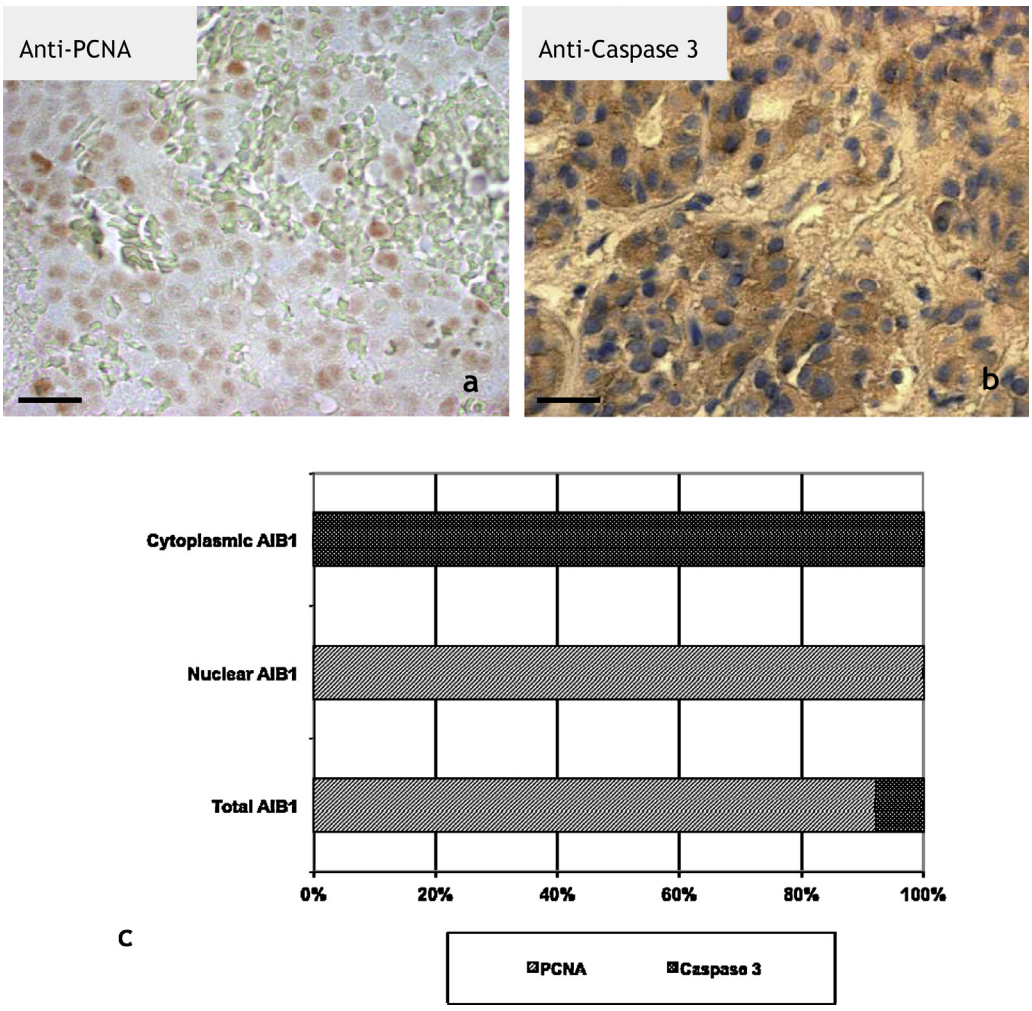

Fig. 3. Analysis of proliferation and apoptosis in human prolactinomas. (a) Representative prolactinoma stained positive for PCNA, a marker of proliferation. Detection of the antibody is revealed by the brown stain. (b) Immunostaining for active caspase 3, affording a brown appearance to the cytoplasm. Sections were counter-stained with hematoxylin to reveal nuclei. (c) Graph of the percentage of AIB1-positive prolactinomas expressing PCNA (proliferation) *vs.* active caspase 3 (apoptosis), according to predominance of the localization, nuclear or cytoplasmic, of AIB1. Scale bar: (a) and (b) 20 μm.

cytoplasmic localization of AIB1 in prolactinomas correlated with the detection of active caspase 3, a hallmark of apoptosis (Fig. 3b and c).

4. Discussion

In the present study, we observed that human prolactinomas express aromatase and also stained positive for the ERα and its co-activator AIB1.

AIB1 plays an important role during puberty, in female reproductive function, and in mammary gland development (Xu et al., 2000) and its overexpression has been correlated with different types of cancers (Anzick et al., 1997; Ghadimi et al., 1999; Gnanapragasam et al., 2001; Henke et al., 2004; Kurebayashi et al., 2000; Sakakura et al., 2000; Wang et al., 2002; Xie et al., 2005).

Interestingly, AIB1 may represent an important diagnostic and therapeutic target since it is a pivotal regulator of steroid-hormone signaling and, in hormone dependent tumors of the breast, high levels of AIB1 and HER-2 may lead to tamoxifen-resistance (Osborne et al., 2003). Moreover, AIB1 integrates signals from growth factors to the estrogen receptor, enhancing its transcriptional activity (Font de Mora and Brown, 2000).

Although ubiquitously expressed, AIB1 is preferentially expressed in the pituitary gland as well as in the uterus, testis, muscle and bone marrow (Suen et al., 1998). AIB3 (also known as ASC-2 or NCOA6), another estrogen receptor coactivator, has been described in the pituitary gland (Zhang et al., 2003) and the overexpression of AIB1 has been reported to increase the incidence of pituitary tumors in mice (Torres-Arzayus et al., 2004).

Accordingly, in the present study we investigated AIB1 expression levels in human prolactinomas. Similar to previous observations made in breast cancers, our analysis revealed that expression of AIB1 in human prolactinomas was strongly correlated with the expression of ERα, suggesting that AIB1 may play a role in the genesis of human prolactinomas.

Aromatase is the enzyme responsible for the local aromatization of testosterone to estradiol. Aromatase is expressed in the pituitary gland of different animal species, including humans (Carretero et al., 1999a; Galmiche et al., 2006a; Kadioglu et al., 2008) and variations in its expression related to age and sex have previously been reported by our laboratory (Carretero et al., 1999b, 2003), as well as the regulation of its expression by gonadal steroids (Galmiche et al., 2006b; Carretero et al., 2011). Moreover, we have previously demonstrated that aromatase is involved in the development of spontaneous prolactinomas in aged rats (Carretero et al., 2002). Given that all the prolactinomas in the present study also expressed aromatase, our findings suggest that the local conversion of testosterone into estradiol by aromatase may also be involved in the

development of these endocrine tumors and, probably, as has been recently described (Akinci et al., 2012), in tumor behavior.

There are different splice variants of AIB1, such as AIB1-Δ3, AIB1-Δ4, that have been described in several cancers. These variants could have potential effects on cellular proliferation and/or metastatic capability of human cancer cell lines, higher than AIB1 (Tilli et al., 2005). In breast cancer, AIB1-Δ4 is expressed predominantly localized in the cytoplasm, although it can enter and move through the nucleus (Chien et al., 2011).

Although it may be speculated that the different predominant localization of AIB1 into the cellular nucleus or the cytoplasm from one to another prolactinoma, could be related with a splice variant; during normal mammary gland development, as well as in breast cancer, an increase in AIB1 expression and its nuclear localization are correlated with mammary epithelial proliferation, whereas a reduced expression of this coactivator and its cytoplasmic localization are correlated with mammary epithelial quiescence and differentiation (Kuang et al., 2004). Moreover, dysregulation of nuclear shuttling and proteosomal degradation can modulate the oncogenic potential of AIB1 (Ferrero et al., 2008).

In most prolactinomas analyzed in the present study, AIB1 localized to the nuclei of adenomatous cells. The nuclear staining for AIB1 was predominant in tumors with a high percentage of PCNA-positive cells, suggesting that nuclear localization is related to the proliferation status of the tumor. In contrast, we observed that the detection of active caspase 3-positive is coincident with the cytoplasmic distribution of AIB1, implying an activation of apoptotic pathways in these tumors. Thus, our results suggest that alterations to the mechanism(s) that regulates the sub-cellular localization of AIB1 may strongly influence tumor outcome, since it may ultimately define the proliferative capacity of tumoral cells. Perhaps because nuclear AIB1 could be protected from proteosomal degradation in an ERα-dependent manner, and the increased expression of AIB-1 could be related with enhanced estrogen-dependent cellular proliferation (Garabedian and Logan, 2008). It will now be of interest to determine how the role of AIB1 in the cytoplasm differs from that in the nucleus. The presence of AIB1 in the nucleus has been associated to its phosphorylation, playing a role as a gene regulator (Suen et al., 1998) and it has a high-affinity interaction with ERα (Amazit et al., 2007).

Both types of estrogen receptors, ERα and ERβ (Chaidarun et al., 1998; Shupnik et al., 1998), have been described in prolactinomas. In tissues with a predominance of ERβ, the interaction of AIB1 with ERβ and its expression may be helpful to regulate estrogen-mediated gene functions or high tumor grade (Paramanik and Thakur, 2011; Kefalopoulou et al., 2012). However, in prolactinomas, ERα is predominant (Shupnik et al., 1998), and, in tissues in which ERα is predominant, it has been involved in the protection of degradation of AIB1 by atypical protein kinase C-mediated phosphorylation (Garabedian and Logan, 2008), and overexpression of AIB1 and ERα augmented abnormal growth responses (Nakles et al., 2011). In our study all prolactinomas were ERα-positive tumors in concordance with descriptions of other authors (Shupnik et al., 1998).

Taken together, our results demonstrate the expression of AIB1 in human prolactinomas that also express aromatase and ERα. Moreover, the nuclear expression of AIB1 was correlated with increased proliferation in these tumors, suggesting that AIB1 might be directly involved in the growth and survival of human prolactinomas. Thus, AIB1 and the machinery that controls its subcellular localization represent relevant targets for future pharmacological interventions directed at the treatment of human prolactinomas. Because aromatase expression is also enhanced in these prolactinomas and it is involved in the local production of estradiol, both mechanisms, ER-AIB1 and aromatase could be related.

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Supplementary data

Information about the transparent peer review is available as supplementary material in the database Science Direct. The reviewer comments and the authors reply can be viewed by clicking the link: http://dx.doi.org/10.1016/j.aanat.2013.01.009.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aanat.2013.01.009.

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