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Original Article

Expression of Eag1 K+ channel and ErbBs in human pituitary adenomas: cytoskeleton arrangement patterns in cultured cells

Margarita González del Pliego[1], Elsa Aguirre-Benítez[1], Karina Paisano-Cerón[2], Irene Valdovinos-Ramírez[2], Carlos Rangel-Morales[3], Verónica Rodríguez-Mata[4], Carmen Solano-Agama[2], Dolores Martín-Tapia[2], María Teresa de la Vega[2], Miguel Saldoval-Balanzario[3], Javier Camacho[5], María Eugenia Mendoza-Garrido[2]

[1]Department of Embryology and [4]Department of Histology, Medical Faculty, Autonomous National University of Mexico, [2]Department of Physiology, Biophysics and Neurosciences and [5]Department of Pharmacology, Center for Research and Advanced Studies, I.P.N., [3]Neurosurgery Service, Medical Center "La Raza", Social Security Medical Institute, México D.F., México

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Abstract: Pituitary adenomas can invade surrounded tissue, but the mechanism remains elusive. Ether à go-go-1 (Eag1) potassium channel and epidermal growth factor receptors (ErbB1 and ErbB2) have been associated to invasive phenotypes or poor prognosis in cancer patients. However, cells arrange their cytoskeleton in order to acquire a successful migration pattern. We have studied ErbBs and Eag1 expression, and cytoskeleton arrangements in 11 human pituitary adenomas. Eag1, ErbB1 and ErbB2 expression were studied by immunochemistry in tissue and cultured cells. The cytoskeleton arrangement was analyzed in cultured cells by immunofluorescence. Normal pituitary tissue showed ErbB2 expression and Eag1 only in few cells. However, Eag1 and ErbB2 were expressed in all the tumors analyzed. ErbB1 expression was observed variable and did not show specificity for a tumor characteristic. Cultured cells from micro- and macro-adenomas clinically functional organize their cytoskeleton suggesting a mesenchymal pattern, and a round leucocyte/amoeboid pattern from invasive clinically silent adenoma. Pituitary tumors over-express EGF receptors and the ErbB2 repeated expression suggests is a characteristic of adenomas. Eag 1 was express, in different extent, and could be a therapeutic target. The cytoskeleton arrangements observed suggest that pituitary tumor cells acquire different patterns: mesenchymal, and leucocyte/amoeboid, the last observed in the invasive adenomas. Amoeboid migration pattern has been associated with high invasion capacity.

Keywords: Pituitary adenomas, ErbBs, Eag1, cytoskeleton, invasion, cell culture

Introduction

Pituitary adenomas are typically benign tumors that have low proliferation rates. Moreover, they typically arise from the clonal expansion of a transformed secretory cell [1-4]. The disorders that they produce are generally related to the hormone secreted by the parent cell and/or the physical expansion of the tumor itself. However, some adenomas, referred to as endocrinologically silent tumors, do not induce high hormone serum levels. These tumors can be classified based on their size, being termed micro- or macro-adenomas, and are classified as invasive if they penetrate surrounding structures [1, 3, 5]. The mechanisms by which pituitary adenomas gain an invasive phenotype are little understood. It is well known that cytoskeletal and adhesion molecules are crucial participants in migratory and invasive cell behaviors [6-8]. Moreover, cells employ different invasive strategies that are correlated with a variety of cytoskeletal organizations [7, 9]. During the transition to a tumor phenotype, cells change their actin and tubulin organization and the expression of intermediate filaments and adhesion molecules, effectively modifying their capacity for motility. With regard to tumor pituitary cells, their cytoskeletal arrangement of is poorly understood. Most cytoskeletal analyses have investigated changes in the expression of intermediate filaments. Tumor cells express

cytokeratins, vimentin and neurofilaments, whereas normal secretory cells express only cytokeratins [11-13]. Furthermore, alterations in epidermal growth factor receptor (EGFR) expression in tumor pituitary cells have been associated with cell transformation to an invasive phenotype [14-17], as is seen in other tumor cells [18]. Inhibitors of EGFRs have been used in different cancer therapies [18]. Additionally, the levels of several ion channels, including ether à-go-go potassium channel 1 (Eag1) [19], are also deregulated in many tumor types, making them potential tools for the diagnosis and therapy of numerous cancers [20, 21]. Because the electrophysiological properties of Eag1 are determined by cytoskeletal interactions [22] and its current density is modulated by extracellular matrix components [23], Eag1 modulation and activity may be involved in cellular events in which cytoskeletal rearrangement occurs, namely the migration of cancer cells and epithelial cell transformation [24]. The purpose of this study was to examine in pituitary adenomas the expression of molecules associated with an aggressive tumor phenotype, particularly the growth factor receptors ErbB1 and ErbB2, and the potassium channel Eag1. We also characterized, in a more detailed manner, the cytoskeleton organization in cultured tumor cells. We used a cell culture model because it allows for the fixation and visualization of the cytoskeleton with minimal disorganization. From the cytoskeletal organization observed in these particular pituitary tumor cells, we suggest that they have adopted an amoeboid invasive pattern, and their aggressive behavior is correlated with a high expression of ErbB2 and Eag1. Our data shows that Eag 1 potassium channel and ErbB2 were expressed in all of the adenomas studied, while ErbB1 expression was variable. The cytoskeleton organization observed was a mesenchymal pattern and a leucocyte/amoeboid pattern. The leucocyte/amoeboid cytoskeleton arrangement was observed in invasive tumors suggesting that pituitary adenoma cells acquire this actin filaments organization as the successfully pattern for invasion.

Materials and methods

Tumor biopsies

Eleven pituitary adenomas were obtained through a transsphenoidal surgical procedure. The surgeries were performed in the Neurosurgery Service, Medical Center "La Raza", Mexican Institute of Social Security in México City. The study was approved for the use of human material by local ethical committees of the Medical Institution and of the Center of Research and Advance Studies of the National Polytechnic Institute.

Immunohistochemistry

Tissues were cut into 1-2 mm pieces and fixed in Karnovsky´s fixative (1.25% glutaraldehyde, 0.3% picric acid) in 0.15 M sodium cacodylate buffer, pH 7.4. The Karnovsky-fixed pieces were embedded in paraffin and 5-10 μm sections were stained in order to analyze the expression of EGF receptors and the Eag 1 potassium channel. The paraffin was extracted from the paraffin-embedded sections with xilol and ethanol followed by re-hydration. Previous to the immunoassay, the sections were treated with citrate buffer 0.1 M pH 6 at 90°C for 2 min. After an incubation period with 3% hydrogen peroxide for 10 min the immune assays were performed using the primary antibodies for human ErbB1 (1:200, mouse mono-clonal from UPSTATE, USA, and rabbit poly-clonal from Cell Signaling, USA), human ErbB2 (1:200, rabbit poly-clonal from Santa Cruz Biotech, USA) and human Eag 1 potassium channel (1:150 mouse monoclonal from Max Planck Ins. Exp. Med, Göttingen, Germany) and an immuno-peroxidase kit (Vectastain Elite ABC kit, Vector, USA). Negative controls were performed excluding the primary antibody. The tissue sections were mounted with Entellan resin and viewed with a microscope (Axiolab, Zeiss, Germany) with a 10x, 40x and 100x objectives. The procedure of immunohistochemistry and evaluation were performed by researchers blinded for clinical data. The adenomas immuno-reactivity was semi quantitatively scored in four classes: "++++" > 50% of positive cells section; "+++" ≥ 25% positive cells; "++" < 25% but > 10% positive cells; "+" < 10% positive cells in the tissue section.

Cell culture

After surgical removal, tumor tissues were maintained at 4°C in sterilized 199 culture medium (Gibco, USA) enriched with 0.1% bovine serum albumin (Research Organics, USA), 0.6 mg/ml sodium bicarbonate (Sigma, USA), 2.4

mg/ml N-(2-Hydroxyethyl) piperazine-N´-2ethanesulfonic acid (HEPES, Sigma, USA), 1% antibiotics (penicillin 10,000 UI/ml and streptomycin 10,000 µg/ml, Sigma, USA), at pH 7.4. A cell suspension was obtained using an enzymatic-mechanical method [25]. Briefly, the culture medium was removed and substituted with Ca^{2+}- and Mg^{2+}- free phosphate-buffered saline (PBS). Then, the tissue was cut into small pieces and incubated in a 0.25% trypsin solution for 10 min at 37°C, followed by the addition of 1 mg/ml soybean trypsin inhibitor (Gibco, USA) and 50 µg/ml DNase (Sigma, USA). Tissue fragments were passed through a Pasteur pipette to separate the cells. Cell aliquots were seeded on poly-D-lysine-coated cover glasses and cultured in 199 medium enriched with 20% fetal bovine serum (PAA, Austria) and antibiotics under standard culture conditions. The rest of the suspended cells were cultured in a three-dimensional system of alginate gel pearls (Sigma, USA) [26]. Because alginate gel pearls maintain their polymerization structure via Ca^{2+} bridges, the cells were liberated from the gel when the pearls were washed with sodium citrate (55 mM). Then, the cells were cultured on cover glass for immunocytochemical analysis. HuH-7 hepatocellular carcinoma cells were supplied by Dr. María Rivas (UANL, Mexico) and CHO cells stable transfected with human Eag 1 were kindly provided by Walter Stühmer (Max-Planck Institute for Experimental Medicine, Göttingen, Germany), and cultured following standard recommendations. Cells were cultured on cover glasses for immunocytochemical analysis.

Immunocytochemistry

After 48 h in culture, cells were washed with PBS with Ca^{2+} and fixed. Different fixation procedures were performed. For EGF receptors and Eag 1 potassium channel were used 3.5% paraformaldehyde and 5% sucrose in PBS for 30 min at room temperature. For actin and tubulin cells were fixed at 37°C to avoid tubulin de-polymerization. First, cells were fixed and permeabilized with 2% formaldehyde, 0.1 M piperazine-N,N´-bis(2-ethanesulfonic acid), 4% polyethylene glycol-6000, 1 mM ethylene glycol-bis(β-aminoethyl ether (EGTA), 1 mM $MgSO_4$, 1% Triton X-100 pH 6.75 during 10 min [27], followed by a second fixation period of 30 min with 3.5% paraformaldehyde in PBS Ca^{2+}-free, 3 mM Mg^{2+}, 5% sucrose, pH 7.4 [25]. The primary antibodies used for EGR receptors and Eag 1 potassium channel were the same as for the immunohistochemistry, for tubulin a mouse mono-clonal fluorescein conjugated antibody (1:25, Sigma, USA) was used, and secondary antibodies used were goat anti-mouse Alexa 488 conjugated and goat anti-rabbit Alexa 488 conjugated (1:300, Molecular Probes, USA). To stain filamentous actin, rhodamine-conjugated phalloidin (Sigma, USA) was used at a concentration of 50 µM for 7 min. All primary antibody incubations were performed overnight, with the exception of anti-tubulin, which was performed for 30 min. All secondary antibody incubations were performed for 60 min. In order to verify primary antibodies against ErbB1 and ErbB2 receptors we used human hepatocellular carcinoma cell line (HuH-7). The specificity of Eag 1 antibody was verified in CHO cells stable transfected with hEag 1 potassium channel [22]. Tests for non-specific secondary antibody binding were performed in the absence of the primary antibody. Cell preparations were mounted with Vectashield mounting media (Vector, USA) and were observed with a confocal laser scanning microscope (Leica TCS SP2, Germany) with a 63x objective. **Figure 1** shows control immunoassays for ErbB1, ErbB2 and Eag 1 potassium channel. HuH-7 hepatocellular carcinoma cells were used as positive controls for ErbB1 and ErbB2 receptor proteins and CHO cells stable transfected with human Eag 1 as control positive for the ion channel (**Figure 1A**) [22, 28], and A 431 cell homogenates (Millipore, USA) processed by Western blot [25] (**Figure 1B**).

Results

ErbB1, ErbB2 and Eag 1 potassium channel expression in tumor pituitary adenoma tissues

We examined the expression of EGF receptors, ErbB1 and ErbB2, and Eag 1 in normal pituitary glands and nine pituitary adenoma tissues. In **Figure 2**, normal pituitary tissue was smoothly stained for ErbB2 and Eag 1 proteins, specifically certain cells were positive. We could not obtain significant staining for ErbB1 receptor. **Figure 3** shows the expression of the two receptors and the ion channel in three different tumor tissues. The expression of ErbB2 receptor and Eag 1 potassium channel were observed in the three tumor specimens analyzed. An intense immuno-reactivity was obtained for

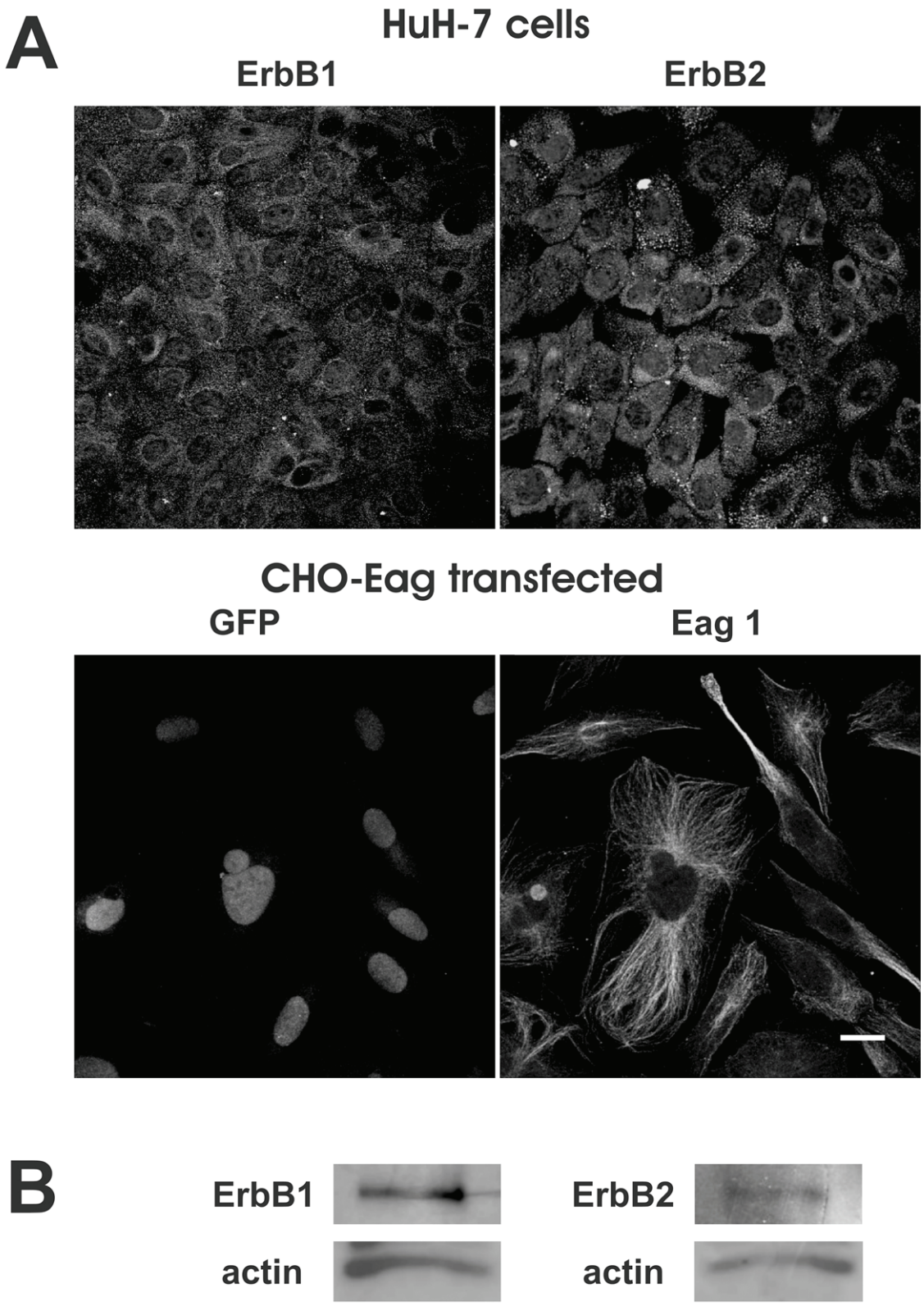

Figure 1. ErbB1, ErbB2 and Eag 1 positive controls. HuH-7 cells and Eag 1 transfected CHO cells were cultured on cover glasses, fixed and immunoassayed for ErbB1, ErbB2 and Eag 1. A: ErbB1 and ErbB2 receptor expression in HuH-7 cells, and Green fluorescent protein expression (GFP) and Eag 1 expression in transfected CHO cells. Bar = 25 μm. B: Western blot of A 431 cell homogenates.

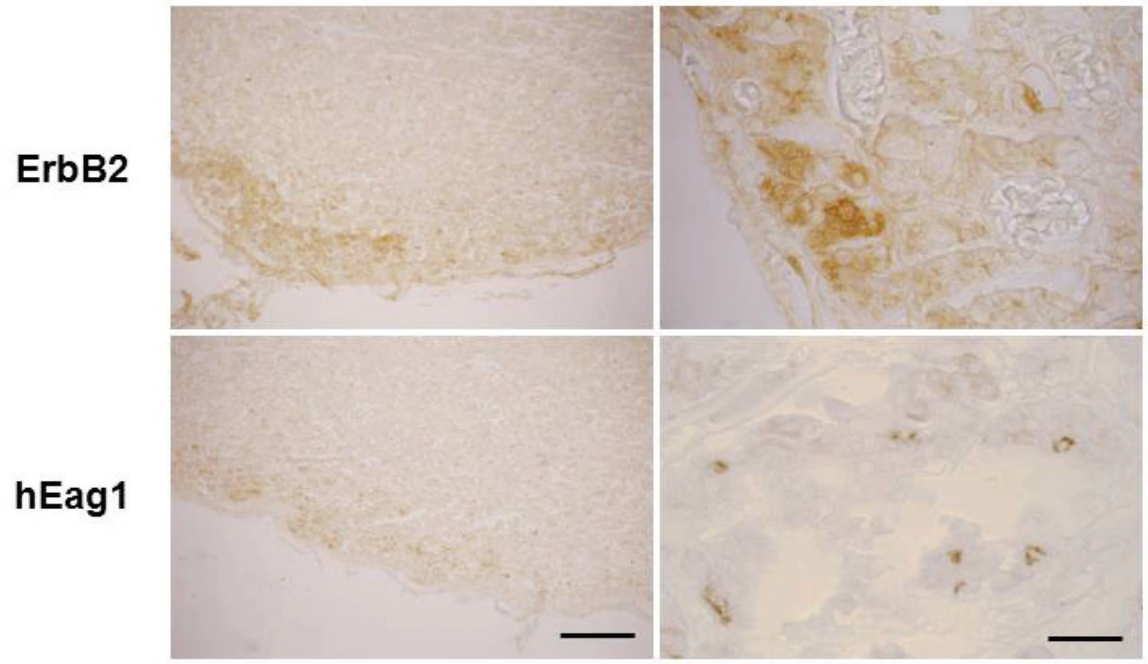

Figure 2. Normal pituitary tissue express ErbB2 receptor and Eag 1 potassium channel. Left panels bar = 300 μm, right panels bar = 30 μm.

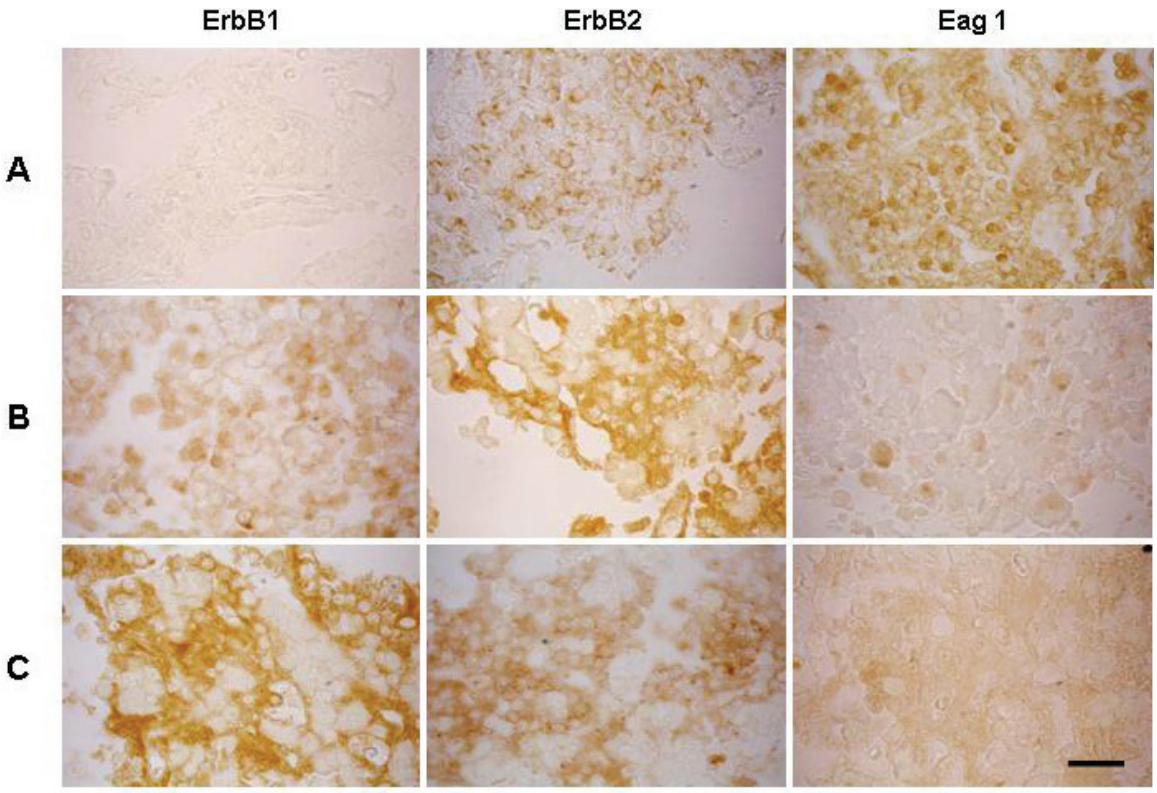

Figure 3. Expression of EGF-receptors, ErbB1 and ErbB2, and the Eag1 potassium channel in tissue pituitary adenomas. Three biopsies of three different adenomas: (A) an ACTH micro-adenoma, and two clinically silent macroadenomas, (B) a non-invasive and (C) an invasive were immunoassayed for ErbB1, ErbB2 and Eag 1 proteins. Bar = 30 μm.

Table 1. Expression of ErbB1, ErbB2 and Eag 1 in tissue of pituitary adenomas

Patient age (years) And sex	Type of adenoma	ErbB1	ErbB2	Eag 1
53, male	microprolactinoma	++++	++++	++++
38, female	microprolactinoma (relapse)	+	+++	++
47, female	micro/ACTH secretion	–	+++	++++
55, male	macro/non func	++++	++++	+++*
33, female	macro/non func	–	++	+
51, female	macro/non func	++++	++++	+++
50, male	macro/non func/inv	–	++++	++++
68, male	macro/non func/inv	++++	++++	+++
50, female	macro/non func/inv	+++	++++	++++*

Semi-quantitative score of immuno-reactivity: ++++ > 50% of positive cells; +++ ≥ 25% positive cells; ++ < 25% but > 10% positive cells; + < 10% positive cells in the tissue section. (*) not strong immunopositivity.

ErbB2 receptor while the ion channel expression was observed with variable intensity. ErbB1 receptor expression was not present in one of the tumor tissues, however was highly expressed in the other two. **Table 1** details the data from the nine tissue adenomas analyzed for immunohistochemistry. The expression of the ErbB2 receptor was observed in all samples, while the ErbB1 receptor was expressed in six of the nine tissue tumors. The Eag 1 potassium channel was expressed in all the nine tissue tumors. However, tumors exhibited variation in between the immuno-reactivity of the ion channel and in cultured cells from a microprolactinoma (not considered in **Table 1**) were absent. The ErbB1 receptor was observed in the three types of adenoma studied, micro-adenomas, macro-adenomas, invasive and non- invasive.

Cytoskeletal arrangement of tumor cells

Figure 4 shows representative examples of actin filament organization in cultured adenoma tumor cells. Thus, we visualized the actin and tubulin arrangement of tumor cells from a micro-prolactinoma and two macro-adenomas invasive and non-invasive. The microprolactinoma cells appeared to be spread out, containing fine arches and stress fibers consisting of actin filaments (**Figure 4A**) and microtubules arranged around a condensed zone adjacent to the nucleus, leading to the cell periphery (**Figure 4B**). The macro-adenoma not invasive showed an actin cytoskeleton arrangement in short stress fibers and different shapes, elongated, rounded and cuboids (**Figure 4C**). These cells exhibit a tubulin cytoskeleton arranged in rings with a polarized condensation neighboring the cell nucleus (**Figure 4D**). Most of the cultured cells from a macro-adenoma invasive exhibited a rounded shape with a thin mesh of actin fibers throughout the cell body. Clusters of rounded cells show an actin belt at cell-cell interaction points, with relatively few filaments crossing the cell basement (**Figure 4E**). Moreover, microtubule condensation was observed at the periphery of the cells, particularly at the level of cell-cell interaction (**Figure 4F**). It is important to note that these cells showed scanty cytoplasm.

Expression of ErbB1, ErbB2 and Eag 1 potassium channel in cultured pituitary adenomas

We examined the expression of EGF receptors (ErbB1 and ErbB2) and the Eag1 in cultured tumor pituitary cells (**Figure 5**). ErbB1 expression was observed throughout the cells with a condensation around the nucleus of cells from a micro-prolactinoma (**Figure 5A**), and at the cell periphery. ErbB1 expression was observed also at the cell-cell attachment in a cell cluster of an invasive macro-adenoma (**Figure 5B**). Notice that cultured micro-prolactinoma cells exhibited a mesenchymal shape while, in culture, cells of the invasive adenoma acquired a rounded shape in cluster. Invasive macro-adenoma cultured cells showed an arrangement of ErbB2 receptor in rings as the same as actin filaments arrangement (**Figure 4E** and **5C**). With the same tumor cultured cells, we observed Eag 1 at the entire membrane border including cell protrusions (**Figure 5D**).

Discussion

Some pituitary adenomas display unusually invasive behavior. To date, no correlation has been found between mutations in classical

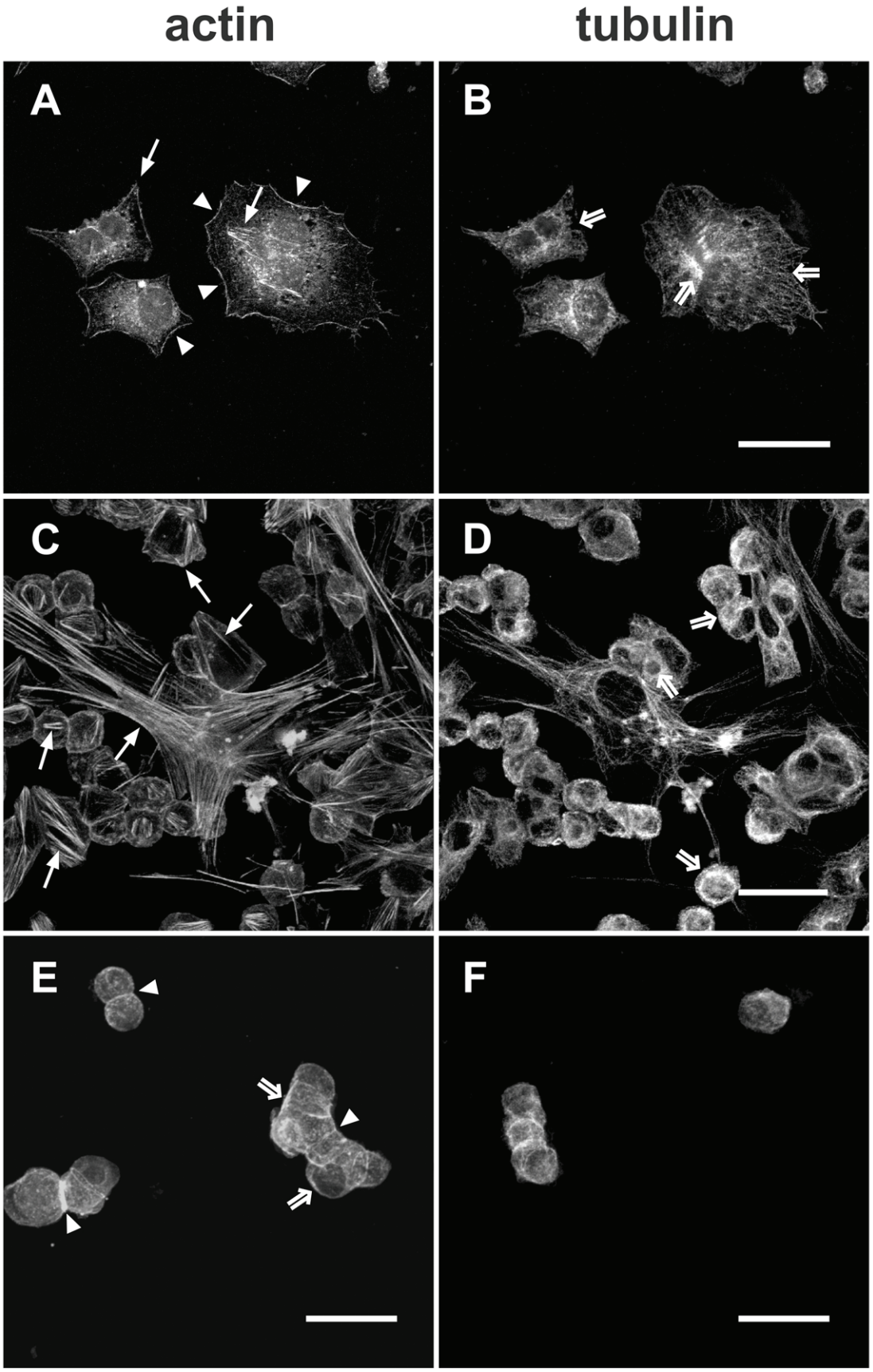
actin
tubulin
A
B
C
D
E
F

Figure 4. Cytoskeleton organization of tumor pituitary cells in culture. (A, C, E) Actin and (B, D, F) tubulin cytoskeleton organization. Cells were obtained from three different adenomas: (A, B) a micro-prolactinoma, (C, D) a macro-adenoma non-invasive, and (E, F) a macro-adenoma invasive, and cultured by 48 h. At the end of the culture period cells were fixed and stained for actin filaments with TRITC-labeled phalloidin and with a FITC-antibody against tubulin. Cells exhibit actin bands which correspond to cell-cell junctions or arcs (arrow heads), actin belts (empty arrows) or stress fibers (thin arrows). Microtubules were clearly observed in the micro-prolactinoma and macro-adenoma non invader cells (empty arrows). Bar = 25 μm.

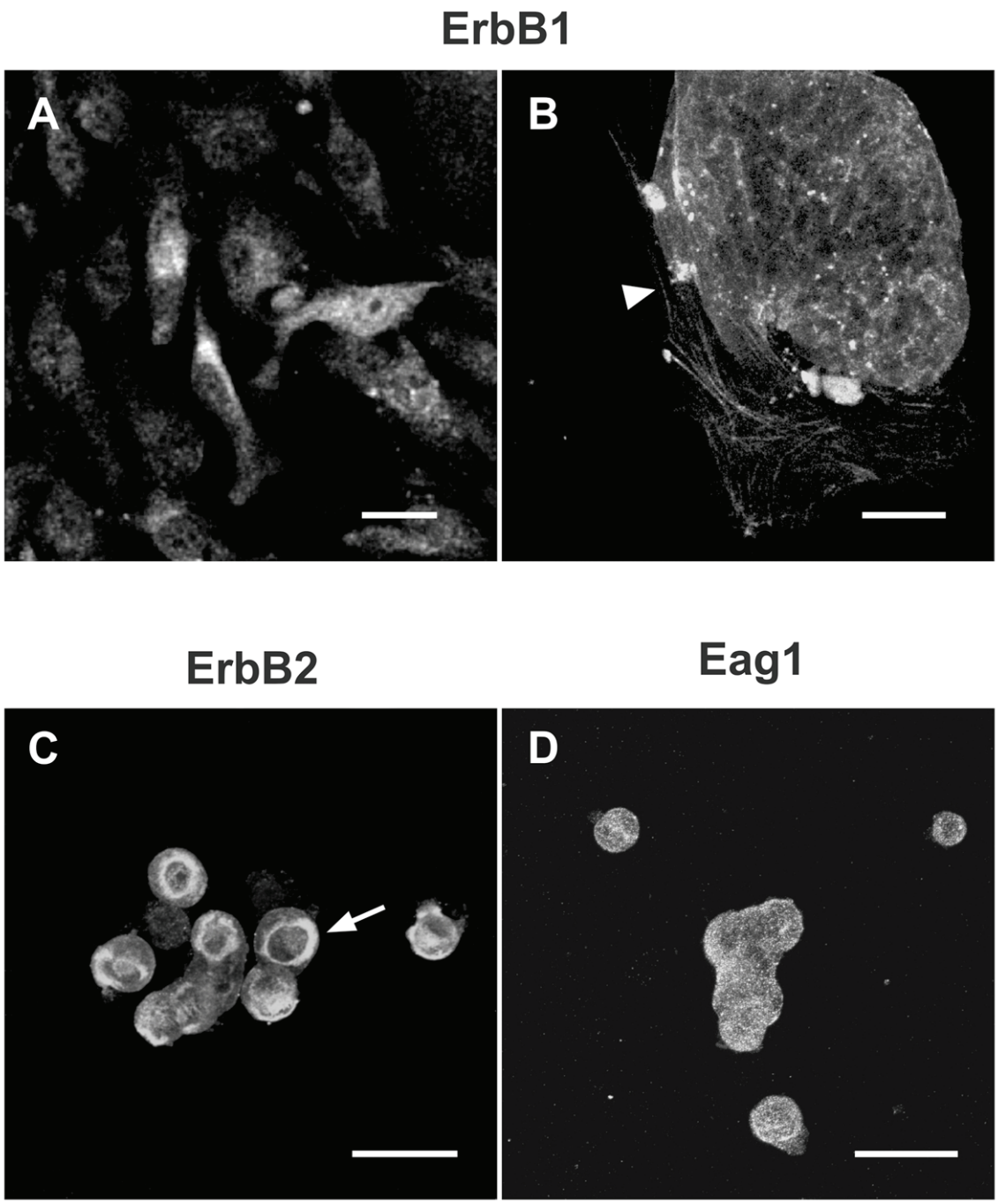

Figure 5. Expression of ErbB1, ErbB2 and Eag 1 in cultured pituitary adenoma cells. ErbB1 receptor expression in: (A) a micro-prolactinoma and (B) a macro-adenoma non-invasive cells. A follicle-stellate cell expressing ErbB1 receptors is shown (B, arrow head). (C) ErbB2 and (D) Eag 1 expression in cultured pituitary cells from an invasive adenoma. ErbB2 receptor is arranged in rings (arrows). Bar = 25 μm.

oncogenes or tumor suppressors and the phenotype of pituitary tumor cells [1, 3, 29]. However, growth factor receptors are implicated in the acquisition of aggressiveness of pituitary adenomas [29]. In particular, expression and activation of EGF receptors have been associated with aggressive pituitary adenoma behavior [14-16, 29-31]. In the present study, we observed that all the tumors, micro-, macro- and invasive adenomas, were positive for ErbB2 receptor, and a high expression was observed in the three invasive tumors. This data is in agreement with Botelho et al. [31] in considering that this receptor has an important role on the development of pituitary adenomas. The other EGF receptor analyzed was ErbB1 receptor and was observed in the majority of the tissues analyzed. This EGF receptor is the most studied in pituitary adenomas [14-16, 29-32]. Onguru et al. [16] observed in 158 pituitary adenomas a strong ErbB1 expression in 59% and weak in 39% of the adenomas. We observed a high expression of this EGF receptor in 5/9 and only in one adenoma was weak. We also investigate if pituitary adenomas express Eag 1 potassium channel, considered as an oncogene. To our knowledge, has not yet analyzed its expression in pituitary tumors. In adult pituitary gland the potassium channel expression, observed by RT-PCR is low [33] and we observed very few cells strongly stained. Our data showed that Eag 1 was expressed in all tumor tissues analyzed; only cultured cells from a micro-prolactinoma were negative for the ion channel. However, the expression was observed variable and not correlated with a specific tumor characteristic. However, Eag 1 has been found highly expressed in other aggressive tumors and carcinomas [33]. Moreover, the transfection of Eag 1 in tumor cells induces a high proliferation rate and an epithelial-mesenchymal transition, which are characteristics of a tumor aggressive phenotype [24, 33]. We could not found a correlation between Eag 1 expression and the aggressive phenotype in the pituitary adenomas, both a microprolactinoma and an invasive macro-adenoma highly expressed the potassium channel. The data obtained in the present study permits proposing it as tumor target in pituitary adenoma treatments [21]. Interestingly, another member of the EAG family, the human ether à-go-go related gene (HERG) potassium channel, is also present in the pituitary gland and its over-expression in several tumors has been suggested to provide a proliferative advantage for tumor cells [34-36]. The nature of the changes leading to cell motility and the acquisition of an invasive phenotype remain unknown. Considering that the cytoskeleton is a key player in cell motility [6-8], we decided to investigate the cytoskeleton arrangement of pituitary adenoma cells. To this end, we cultured primary cells derived from different pituitary adenomas. It is well known that pituitary tumor cells grow poorly in culture. However, adding 20% fetal bovine serum to the culture medium allows these cells to survive in culture [37]. Fasekas et al. [37] describe cultured cells from null adenomas as having limited adherence and spreading and a small, polygonal shape. Analysis of the actin cytoskeleton of the GH_3 rat tumor pituitary cell line indicated that these cells have few stress fibers with small lamellipodia and a thin actin filament belt [38]. However, a different cytoskeletal organization was observed in cultured cells derived from a prolactin-secreting micro-adenoma, which have a flatter, more spread-out shape, suggesting that these cells attached firmly to the substrate. By comparison, cultured rat pituitary gland cells have a polygonal shape with stress fibers and are more flattened than GH_3 cells, suggesting that during the acquisition of an aggressive phenotype, pituitary tumor cells lose their normal morphology in culture [25]. We observed a similar cytoskeleton arrangement as GH_3 cells in cultured tumor cells from a macro-adenoma non-invasive. On the other hand, tumor cells from an invasive adenoma had a rounded shape. The actin filaments were arranged in a well-developed belt at the cell periphery and a fine mesh throughout the cell. This arrangement showed that the cells have little adhesive surface, suggesting a reduced capacity for cell-substrate adhesion. This was confirmed by the fact that few cells remained attached to the substrate. On this account, it is known that certain tumor cells are highly motile, a process driven by short-lived and relatively weak interactions with the substrate [7, 39]. Moreover, a rounded shape has been correlated with a change in invasion strategy, where instead of using proteases for matrix degradation, tumor cells squeeze through matrix gaps, a process called amoeboid migration [7, 39]. Wang et al. [9], using intra-vital imaging, showed that breast carcinoma cells move along collagen

fibers in an amoeboid pattern. The cells obtained from an invasive adenoma maintained in culture a rounded shape with a belt of actin filaments. Similarly, we hypothesize that the studied pituitary adenoma cells also employ an amoeboid migration pattern. Moreover, we also observed a loss of cell polarity in these cells. It is known that cancer cells fail to maintain their polarity and proper localization of adhesion components [7, 9]. In conclusion, the tumor pituitary adenoma cells studied here display different cytoskeletal organization suggestive of a transition from a mesenchymal pattern to an amoeboid; this last migration behavior allowing cells to invade. We also show for the first time a significant level of expression of the oncogenic Eag1 in adenomas. Taken together, our data provides a novel, broad characterization of pituitary tumor phenotypes that may help to improve tumor diagnosis in the future.

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Conflict of interest statement

We have no financial interest or conflict of interest in association with this work.

Address correspondence to: Dr. María Eugenia Mendoza-Garrido, Department of Physiology, Biophysics and Neuroscience, Center for Research and Advanced Studies, National Polytechnic Institute, Av. IPN No. 2508, Col. San Pedro Zacatenco, Mexico City, Mexico 07360. Tel: (25) 55-5747-3800, Ext. 5122; Fax: (25) 55-5747-3754; E-mail: mmendoza@fisio.cinvestav.mx

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