Int J Clin Exp Pathol 2016;9(8):7854-7866
www.ijcep.com /ISSN:1936-2625/IJCEP0031638

Original Article

N-cadherin and vimentin expression in small rounded-shaped cells of non-functioning human pituitary adenomas

Dulce Ávila-Rodríguez[1], Alma Ortiz-Plata[2], Elsa Aguirre-Benitez[3], Juan Gomez-Amador[4], Daniel Rembao-Bojorquez[5], Martha Tena-Suck[5], Carmen Solano-Agama[1], Maria Eugenia Mendoza-Garrido[1]

[1]Department of Physiology, Biophysics and Neurosciences, Center of Research and Advanced Studies-IPN, Mexico, Mexico; [2]Laboratory of Experimental Neuropathology, National Institute of Neurology and Neurosurgery, Manuel Velasco Suarez, Mexico, Mexico; [3]Department of Embryology, Medicine Faculty, Mexican Autonomous National University, Mexico, Mexico; [4]Department of Neurosurgery, National Institute of Neurology and Neurosurgery, Manuel Velasco Suarez, Mexico, Mexico; [5]Department of Neuropathology, National Institute of Neurology and Neurosurgery, Manuel Velasco Suarez, Mexico, Mexico

Received May 3, 2016; Accepted June 22, 2016; Epub August 1, 2016; Published August 15, 2016

Abstract: It has been suggested that pituitary macroadenomas present an epithelial-mesenchymal transition (EMT) related with the invasive behavior. This process is characterized by the expression of vimentin and N-cadherin. Accordingly, in a genomic study of non-functioning pituitary macroadenomas (NFPM), an increase in the expression of *CDH2* gene was observed. The aim of the present study was to analyze the presence of N-cadherin and vimentin in tissues and cultured cells from NFPM. In order to correlate the cytoskeleton organization with a cell invasion type, the shape and cytoskeletal organization of the cultured cells were determined. In ten NFPM, we analyzed the presence of vimentin and N-cadherin by immunohistochemistry. In the corresponding primary cultures, the distribution of vimentin, β4 integrin subunit, N-cadherin, tubulin, and actin filaments were also analyzed. We found that all the NFPM expressed vimentin and N-cadherin. Analyzing the cell size, a significant decrease with respect to normal tissue was observed. *In vitro* the cells exhibited a small, rounded shape with actin cortical rings and also disorganized microtubules, with different organization patterns of vimentin and N-cadherin. These observations sustain an aggressive comportment and suggest they acquired an amoeboid behavior. These cells showed different organization patterns of vimentin and N-cadherin. Considering these pituitary adenoma cells have lost their secretory phenotype, our observations support the overall change in the physiology of these cells. The NFPM studied expressed the EMT markers vimentin and N-cadherin, suggesting they acquire mesenchymal characteristics, and the small size coincided with that observed in small aggressive cancer cells.

Keywords: Non-functioning human pituitary adenomas, vimentin, N-cadherin, cytoskeleton arrangement, cultured cells

Introduction

Clinically non-functioning pituitary adenomas are among the most common type of pituitary adenomas, comprising 25-30% of all pituitary adenomas [1-3]. Non-functioning pituitary adenomas: clinically are characterized by the absence of hormone hypersecretion and usually are macroadenomas, frequently they invade the cavernous sinus, with compression of adjacent structures [4-6]. The invasive mechanisms of NFPM are not yet known. It has been proposed that a behavior like the EMT occurs initially, and then the tumor cells can invade [7-9]. It is important to consider that pituitary secretory cells have a parenchymal phenotype more than a classical epithelial phenotype. In typical epithelial cells, during the EMT, a change from E-cadherin to N-cadherin and from cytokeratin to vimentin occurs [10]. E-cadherin is an adhesion protein that has been widely analyzed [11, 12]. With regard to N-cadherin expression, in NFPM, Moreno et al. [13] identified an increase in the expression of the *CDH2* (N-cadherin gene). However, according to Ezzat et al. [14], the expression of a deleted isoform of fibroblast growth factor receptor-4 correlates with the loss of membrane N-cadherin and with

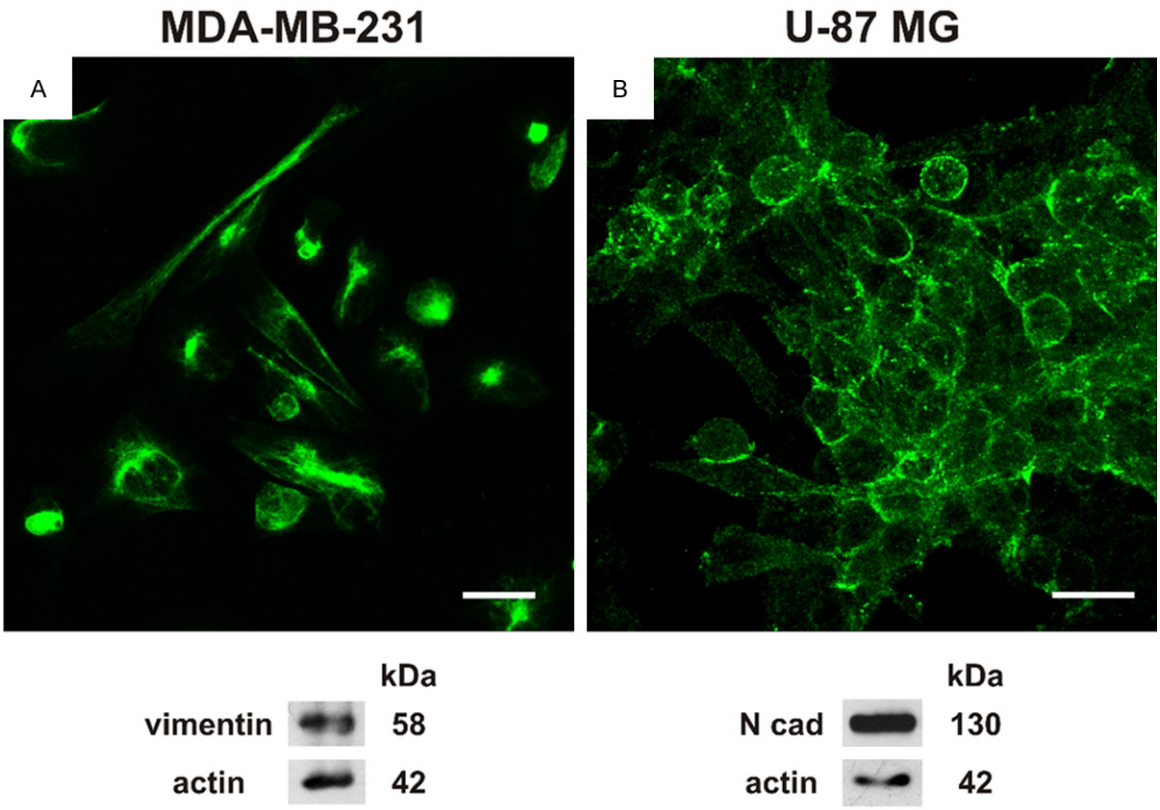

Figure 1. Vimentin expression in human breast cancer cells (MDA-MB-231) and N-cadherin expression in human glioblastoma cells (U-87MG). The positive controls of the immunocytochemistry assays and western blot assays performed for vimentin (A) and N-cadherin (B) were obtained using MDA-MB-231 cells and U-87 MG cells, respectively. In MDA-MB-231 Vimentin was observed fibrillary throughout the cell body and in U87MG N-cadherin was observed at the cell-cell boundary and cytoplasmic. Bar =25 μm.

tumor invasiveness. Changes in intermediate filaments are also observed in pituitary adenomas, which express cytokeratins, vimentin and neurofilaments [15, 16], whereas normal secretory cells only express cytokeratins 8-18 [17, 18], suggesting the occurrence of a transition to a mesenchymal phenotype. It is well known that cell migration and invasion require changes in adhesive interactions among cells, regulated interactions with the extracellular matrix and coordinated communication between elements of the cytoskeleton [19, 20]. Cells have two well-defined patterns of migration: mesenchymal, characterized by an elongated morphology with abundant cellular protrusions; and amoeboid, characterized by a rounded morphology with no obvious polarity [20]. Each invasion pattern has been associated with a variety of patterns of actin and tubulin cytoskeletal organization [20]. In invasive, NFPM, little is known about the cytoskeletal arrangement of actin filaments and tubulin. In tissue, it is difficult to performed actin filaments and microtubules observations because of the disarrangements of these structures after tissue fixation, while in cultured cells it is possible preserved them. The purpose of this study was to characterize the cytoskeleton arrangement of this type of pituitary adenoma cells using the primary cell culture method. Then, cells were obtained from NFPM and was evaluated the capacity of this type of tumor cell to organize the cytoskeleton, adapting them to a specific migration/invasion behavior. The expression of N-cadherin and vimentin, both present during the EMT in classical epithelial tissues, was analyzed in these adenomas.

Materials and methods

Sample procurement

Ten NFPM tissues were obtained via transsphenoidal surgical procedures after informed written consent (ethical approval: IRB0004785-CINVESTAV IRB#1-COBISH). The surgeries were performed in the National Institute of Neurology and Neurosurgery “Manuel Velasco Suarez”.

Table 1. Pituitary hormone expression and invasiveness of adenoma tissues. Patient clinical features and immunohistochemistry results

Case	Age	Gender	Clinical simptoms	Grade	PRL	GH	FSH	LH	TSH	ACTH
1	52	M	Visual disturbances	IV-D	-	-	-	-	-	-
2	61	M	Visual disturbances	II-C	-	-	+	-	-	-
3	38	M	Visual disturbances, cephalea	IV-D	-	-	+	-	-	-
4	49	F	Visual disturbances, cephalea	IV-C	-	-	-	-	-	-
5	71	M	Visual disturbances, cephalea	II-A	-	-	++	++	-	-
6	46	F	Visual disturbances, cephalea	II-B	-	-	++	-	-	-
7	83	F	Visual disturbances, cephalea	III-B	-	-	+	-	-	++
8	49	M	Visual disturbances	III-C	-	-	+	+++	-	-
9	37	F	Visual disturbances, cephalea	II-C	-	-	-	-	-	-
10	58	M	Visual disturbances, cephalea	IV-D	-	-	+++	-	-	-

F, female; M, male; H-V, Hardy-Vezina; II, intact sella turcica, enlarged fossa; III, localized sellar destruction; IV, diffuse sellar destruction; tumor suprasellar, A, suprasellar cistern only, B, recess of the third ventricle; C, whole anterior third ventricle; D, intracranial extradural. Semi-quantitative immunoreactivity scores for pituitary hormones: +, 1-25%; ++, 26-60%; +++, 65-100%; -, negative cells stained in tissue sections. PRL, prolactin; GH, growth hormone; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropin hormone.

NFPM were collected at the time of surgery, and samples of each NFPM were used for histopathological and immunohistochemical analysis and to obtain a primary culture. Normal pituitary glands were obtained by autopsy with previous consent of the families. All the experimental procedures were made in double blinded. The human breast cancer cell line MDA-MB-231 (HTB-26) was used as a positive control for vimentin expression [21], and the human glioblastoma cell line U-87 MG (HTB-14) was used as a positive control for N-cadherin expression [22] (**Figure 1**); both cell lines were obtained from ATCC (Manassas VA, USA) and grown according to the provider's instructions.

Immunohistochemistry

The tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Four-micrometer-thick sections were deparaffinized and stained for immunohistochemistry. Sections were pretreated in an antigen retrieval solution (Biocare Medical, Tempe, AZ, USA). The primary antibodies used were: prolactin, growth hormone, follicle-stimulating hormone, luteinizing hormone, and thyroid-stimulating hormone (BioGenex; San Ramon, CA, USA); adrenocorticotropin (1:100, DAKO; Glostrup, Denmark) mouse monoclonal anti-vimentin (1:800, Sigma; Saint Louis, MO, USA), mouse monoclonal anti-N-cadherin (1:100, Invitrogen; Carlsbad, CA, USA) and mouse monoclonal anti-steroidogenic factor 1 (SF-1, 1:100, Santa Cruz Biotech; Dallas, TX, USA). Immunohistochemistry was performed using a peroxidase-conjugated streptavidin complex (BioGenex) and counterstained with hematoxylin. When double immunostainings were performed, first was stained the N-cadherin with the procedure described previously and then the tissue was incubated stained for LH using the kit NovaRed (Vector, Burlingame, CA, USA). The sections were analyzed using a microscope (Olympus H2; Tokyo, Japan) with a 40x and 100x objectives. Tumor cell immunoreactivity for the different hormones, vimentin and N-cadherin was semi-quantitatively scored using four classes: negative, "-", 0% cells stained; weak positive, "+", 1-25% cells stained; intermediate, "++", 26-60% cells stained; strong, "+++", 61-100% cells stained in tissue sections. Normal pituitary gland sections were used as a control.

Primary culture

NFPM samples were collected at the time of the surgery under sterile conditions and immediately placed in 199 culture medium

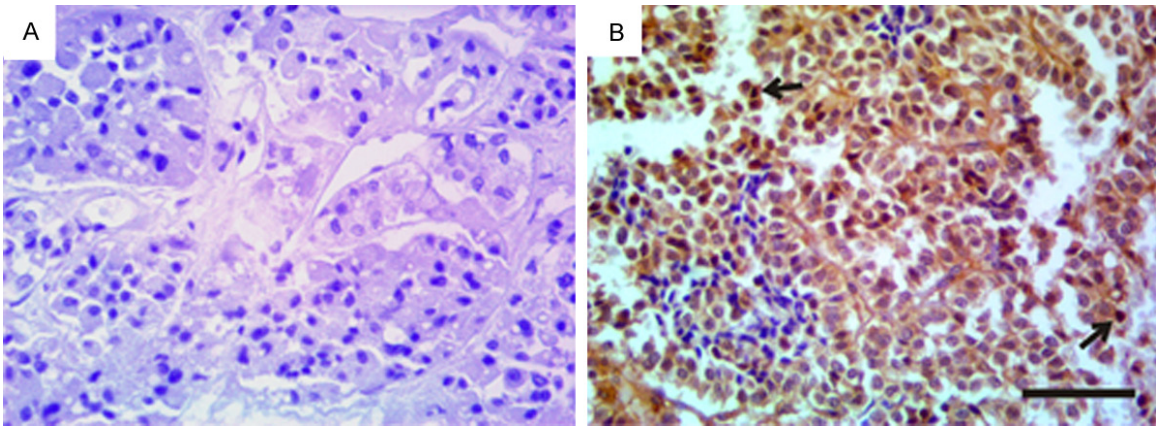

Figure 2. Immunolocalizacion of the transcription factor SF-1 in a hormone immunonegative adenoma. The Case No. 1 was immunonegative for all hormones (A) and immunopositive for SF-1 at the cell nuclei (B, arrow). Bar =50 μm.

(Invitrogen) enriched with 0.1% bovine serum albumin and 1% antibiotics (10,000 UI/ml penicillin and 10,000 μg/ml streptomycin, Sigma), at pH 7.4. A cell suspension was obtained using an enzymatic-mechanical method. Briefly, the tumor tissue was cut into small pieces and incubated with 1 mg/ml collagenase type 2 (Worthington; Lakewood, NJ, USA) for 30 min at 37°C. The cells were suspended by passing the tissue fragments through a Pasteur pipette. Tumor cells were cultured in a three-dimensional system of alginate beads [23]. The tumor cell suspension was mixed with 1.2% sodium alginate (Sigma) and maintained with 199 culture medium enriched with 20% FBS (PAA; Pasching, Austria) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Immunocytochemistry of cultured cells

The cells were liberated from the alginate by washing the beads with 55 mM sodium citrate and seeded on glass coverslips covered with poly-D-Lysine for further immunocytochemical analysis after a 48 h culture period. Different fixation procedures were used. For vimentin, cells were fixed in 3.5% paraformaldehyde and 5% sucrose in PBS for 30 min at room temperature. For the detection of actin and tubulin, cells were initially fixed and permeabilized in a fixation buffer (0.1 M PIPES, pH 6.75, 4% PEG-6000, 1 mM EGTA, 1 mM $MgSO_4$, 0.5% Triton X-100 and 2% formaldehyde) for 10 min at 37°C to avoid tubulin de-polymerization [24], followed by 30 min in 3.5% paraformaldehyde in PBS, 3 mM MgCl, and 5% sucrose, pH 7.4. To visualize N-cadherin, cells were fixed in ethanol at -20°C for 20 min. The primary antibodies used were the same as for the immunohistochemistry and FITC conjugated-mouse monoclonal anti-tubulin (1:25, Sigma), and anti-β4 integrin subunit (1:400, Chemicon; Temecula, CA, USA). Secondary antibodies were: donkey anti-mouse Alexa 488-conjugated antibodies (1:350, Invitrogen), and goat anti-rabbit rhodamine-conjugated (1:350, Jackson; West Grove, PA, USA). To stain filamentous actin, rhodamine-conjugated phalloidin (Sigma, USA) was used at a concentration of 1 μM. The cells were coverslipped and observed with a confocal microscope (Leica TCS SP2; Solms, Germany) with a 63x objective.

Nucleus/cytoplasm ratio measurement

The nuclear and cytoplasmic areas were measured using SigmaScan Pro 4 (Systat Software; San Jose, CA, USA). The nucleus/cytoplasmic ratio (N/C ratio) was calculated by dividing the nuclear area by the cytoplasmic area for each tumor tissue and cultured cells, and normal pituitary tissue. Ten random fields were analyzed in each sample.

Western blot

One part of the cell suspension obtained in the primary culture, was lysed with RIPA lysis buffer and analyzed by Western blot. The membranes were incubated with antibodies against vimentin (1:1000), N-cadherin (1:350) and actin (1:2000). The secondary antibody was a HRP-conjugated anti-mouse (1:15000, invitrogen). The immune complexes were developed by chemoluminescence. The densitometry analysis was performed using Image Quant TL

Table 2. Relationship between the nucleus and cytoplasmic areas in non-functioning pituitary adenomas

Case	Tissue	Cell culture
1	0.39 ± 0.09	0.44 ± 0.10
2	0.40 ± 0.09	0.32 ± 0.10
3	0.44 ± 0.09	0.50 ± 0.11
4	0.51 ± 0.07	0.51 ± 0.17
5	0.69 ± 0.10	0.60 ± 0.11
6	0.41 ± 0.09	0.48 ± 0.13
7	0.57 ± 0.10	0.56 ± 0.11
8	0.58 ± 0.08	0.66 ± 0.18
9	0.42 ± 0.09	0.49 ± 0.16
10	0.40 ± 0.12	0.48 ± 0.11
N	0.19 ± 0.04	n.d.
N-G	0.18 ± 0.06	n.d.

The nucleus and cytoplasmic areas were measured in tissue samples and in cultured cells from the ten adenomas studied; the ratio was obtained by dividing the cytoplasmic area by the nucleus area. N corresponds to the normal human pituitary tissue; N-G corresponds to normal human gonadotrope cells; n.d., not determined.

v.2003 (GE Healthcare; Buckinghamshire, England). The vimentin- and N-cadherin-to-actin ratios were calculated for each adenoma.

Statistical analysis

Data correspond to the mean ± SD. Differences were assessed using Student's t-test, and correlations were analyzed using Pearson correlation (GraphPad Prism version 4.0, 2003; La Jolla CA, USA).

Results

Clinical characteristics

Ten patients were included in this study, 6 males and 4 females. The diagnoses of NFPM were made according to clinical and histopathological analysis provided by histological assessment and immunohistochemical staining for all anterior pituitary hormones. In accordance with the immunohistochemistry assessment, three were hormonally inactive adenomas and seven showed immunoreactivity to gonadotropins (**Table 1**). One case was a plurihormonal adenoma because it was also positive for ACTH. The three hormonally inactive adenomas were positive for SF-1 showing they belong to the gonadotrope adenoma family (**Figure 2**) [25]. Invasiveness was determined by magnetic resonance imaging using Hardy's criteria [26]. Seven non-functioning pituitary adenomas were invasive (Grade III and IV) and three non-invasive (Grade II), and all were macroadenomas. An evaluation of suprasellar extensions in these adenomas showed that all of them exhibited portions of tumor outside these region [27, 28] (**Table 1**).

Adenoma tissues

Cell size determination: Nucleus/Cytoplasm ratio: The nucleus/cytoplasm (N/C) ratio was analyzed in tumors and in normal pituitary tissue (**Table 2**). The mean N/C ratio was 0.47 ± 0.13 in pituitary adenomas, 0.19 ± 0.04 in normal pituitary tissue and 0.18 ± 0.06 in normal gonadotropin cells. The N/C ratios in pituitary adenomas were significantly higher compared with those obtained for normal tissue ($P<0.001$).

Immunohistochemistry results

Vimentin expression: We analyzed the expression of the intermediate filament vimentin. All NFPM expressed vimentin. The tissue staining for vimentin showed that three of the pituitary adenomas exhibited strong positive staining, four presented intermediate staining, and three exhibited weak staining (**Figure 3A-C**). Vimentin was organized throughout the cell bodies of the adenoma cells and in the blood vessels. By western blot, we observed that vimentin expression was higher in invasive NFPM compared with non-invasive NFPM. Case number 3 was not analyzed by western blot due the limited tissue sample (**Figure 3D**). In normal human pituitary tissue (**Figure 4**), vimentin was observed in pituicytes of the posterior pituitary (**Figure 4A**) and at the tunica intima and media of blood vessels that enter to the hypophysial system (**Figure 4B**). At the anterior pituitary tissue, vimentin positive cells were observed forming a follicle filled with colloid material, suggesting they are follicle-stellate cells (**Figure 4C**). Interestingly, the endothelial cells of pituitary capillaries were negative for vimentin (**Figure 4D**) in contrast with those observed in the adenoma tissues.

N-cadherin adhesion protein

N-cadherin is a cell-to-cell adhesion protein. The loss or down-regulation of this protein in pituitary adenomas is related to tumor invasion

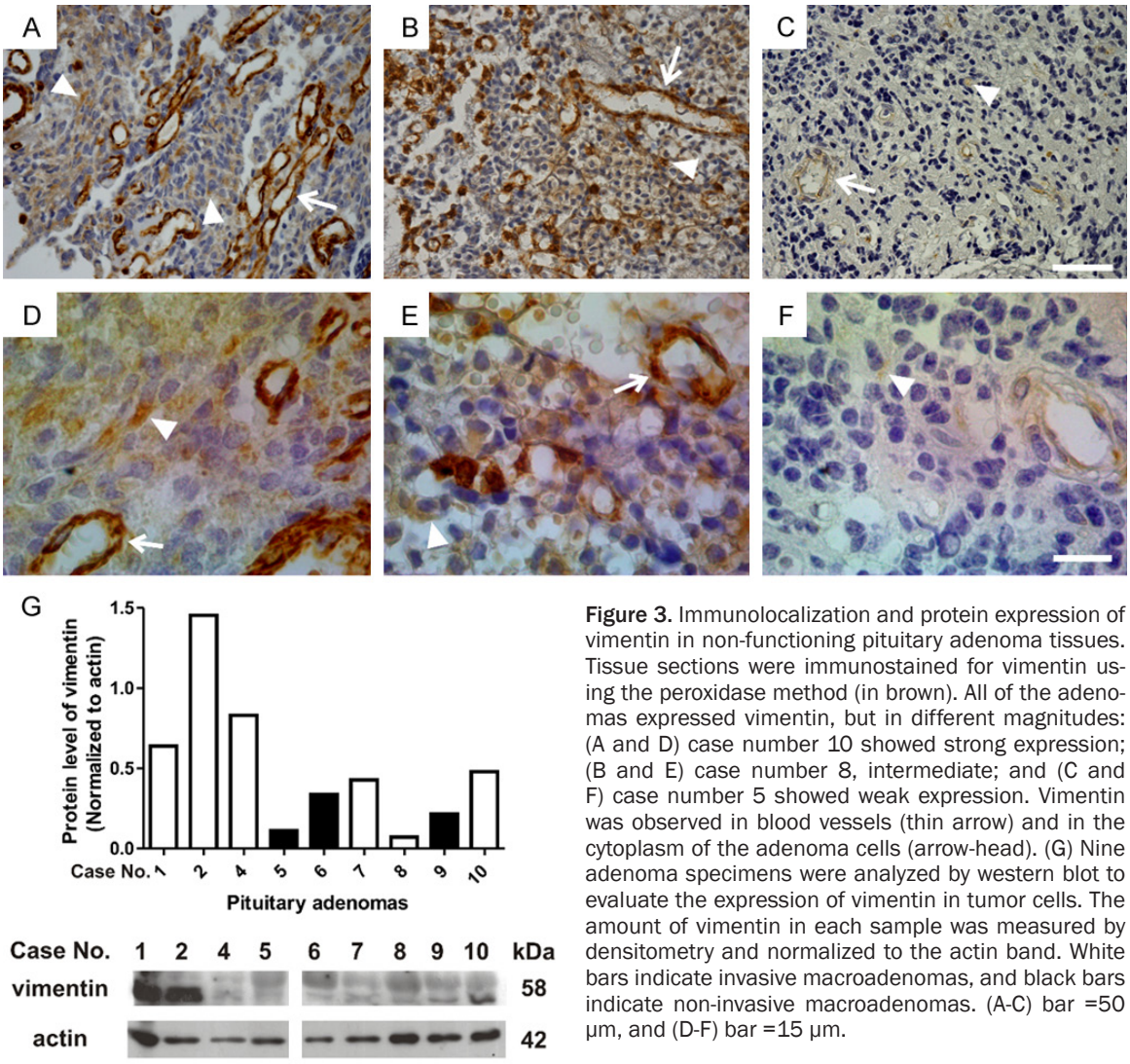

Figure 3. Immunolocalization and protein expression of vimentin in non-functioning pituitary adenoma tissues. Tissue sections were immunostained for vimentin using the peroxidase method (in brown). All of the adenomas expressed vimentin, but in different magnitudes: (A and D) case number 10 showed strong expression; (B and E) case number 8, intermediate; and (C and F) case number 5 showed weak expression. Vimentin was observed in blood vessels (thin arrow) and in the cytoplasm of the adenoma cells (arrow-head). (G) Nine adenoma specimens were analyzed by western blot to evaluate the expression of vimentin in tumor cells. The amount of vimentin in each sample was measured by densitometry and normalized to the actin band. White bars indicate invasive macroadenomas, and black bars indicate non-invasive macroadenomas. (A-C) bar =50 μm, and (D-F) bar =15 μm.

[14]. We looked for this cadherin in the NFPM, and observed that all of the adenoma samples expressed N-cadherin at different levels. The different sample tissues showed different N-cadherin immunostaining: two of the NFPM showed strong positive staining; three, intermediate positive staining; and five weak staining, corroborating the data obtained by western blot (**Figure 5**). The staining pattern was predominantly cytoplasmic in 5 out of the 10 NFPM; in the cell boundaries and cytoplasm in 4 out of the 10; and in only one adenoma, case number 3, N-cadherin was observed in few cells at the cell border.

Cultured adenoma cells

Cell size determination: Nucleus/Cytoplasm ratio: The N/C ratio of the cultured cells was obtained. In culture, the NFPM cells exhibited a rounded shape (**Table 2**). In general, the cells exhibited scant amounts of cytoplasm. The N/C ratio of the cultured cells was 0.51 ± 0.15. The correlation between N/C ratios of each adenoma in tissue sections and cultured cells were positive ($r=0.61$, $P<0.01$).

Immunocytochemistry results

Actin and tubulin cytoskeleton organization: To analyze the cytoskeleton arrangement in detail, a primary culture approach was used, which allowed us to visualize the actin and tubulin cytoskeleton with minimal disorganization. Based on their cytoskeleton arrangement, the NFPM cells were classified in two groups. Group I: Rounded cells with discontinuous cortical F-actin, with a fine mesh of F-actin throughout

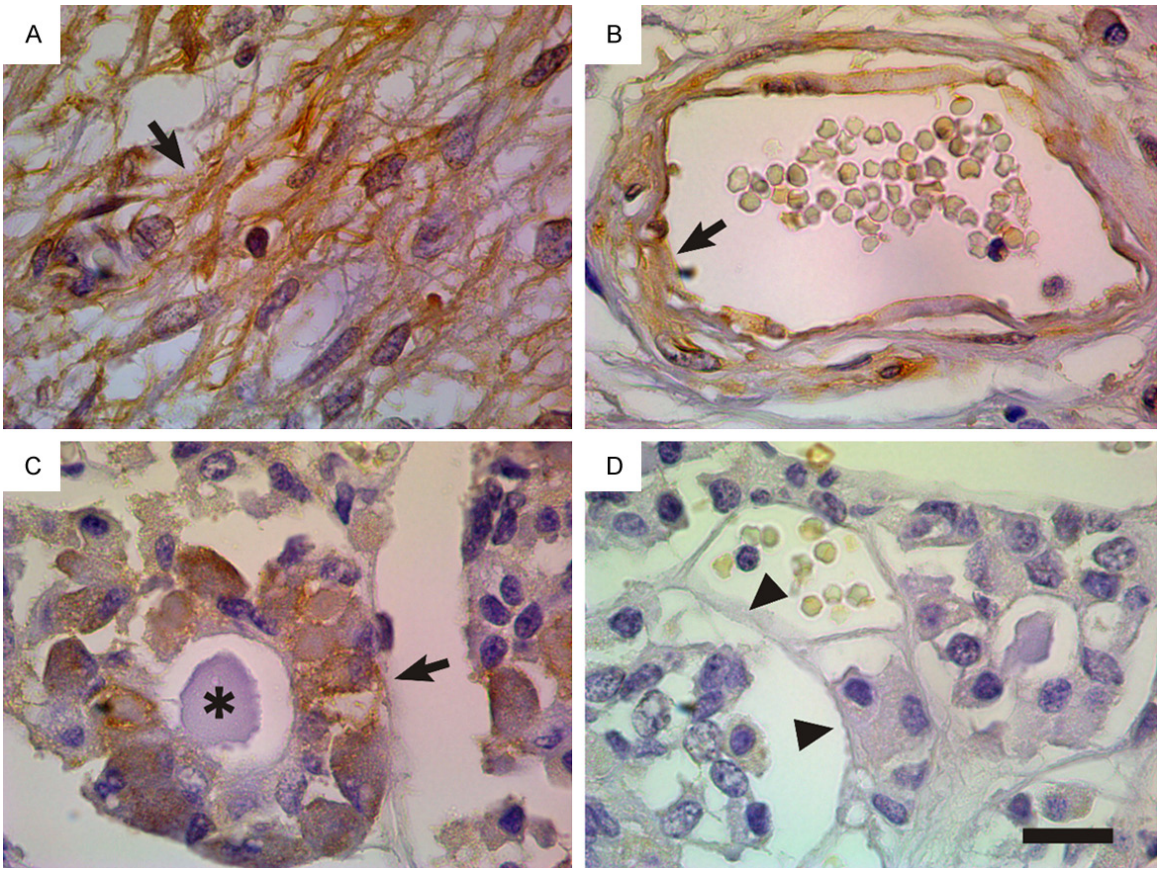

Figure 4. Vimentin expression in normal human pituitary tissue. Posterior pituitary pituicytes (A) and blood vessels (B) and, in anterior pituitary tissue, follicle-stellate cells (C) showed immunopositive staining for vimentin (arrow). (D) Capillaries from anterior pituitary tissue were negative for vimentin (arrow head). Colloid material (asterisk). Bar =15 μm.

the cell body. At the substrate adhesion level, small F-actin plaques were observed, suggesting small adhesion complexes and low substrate attachment (**Figure 6A** and **6B**). Microtubules were observed to be arranged in a diffuse network and condensed at the cell periphery (**Figure 6D**). Group II: Rounded cells showing a flatter morphology with membrane protrusions where F-actin was organized in laxly interwoven filaments and in bundles at the cell periphery (**Figure 6C**). At the substrate adhesion level, these cells showed small F-actin plaques, like the other type of cells. These cells had a tubulin cytoskeleton organized in a loose network (**Figure 6E**); in only one NFPM, the microtubules were observed in an extended filament network, showing that they had come from a condensed zone adjacent to the nucleus until the cell periphery (**Figure 6F**, arrowhead). Half of the NFPM cells were classified in group I; the other half, in group II.

Vimentin organization

In the cultured cells, a detailed analysis of vimentin organization was performed. To analyze if this intermediate filament was anchored to hemidesmosomes, we looked for co-localization of the β4 integrin subunit with vimentin at the cell substrate level (**Figure 7**). Vimentin was observed arranged around the nucleus. In seven of the adenoma cell cultures, vimentin was observed at the cell periphery and in close proximity to the β4 integrin subunit in a pattern of patches, suggesting that vimentin was part of hemidesmosomes.

N-cadherin protein

N-cadherin is a cell-to-cell adhesion protein. When the data obtained from tissues was compared with that obtained from cultured cells, a positive correlation was found between them with respect to the magnitude of expression. In

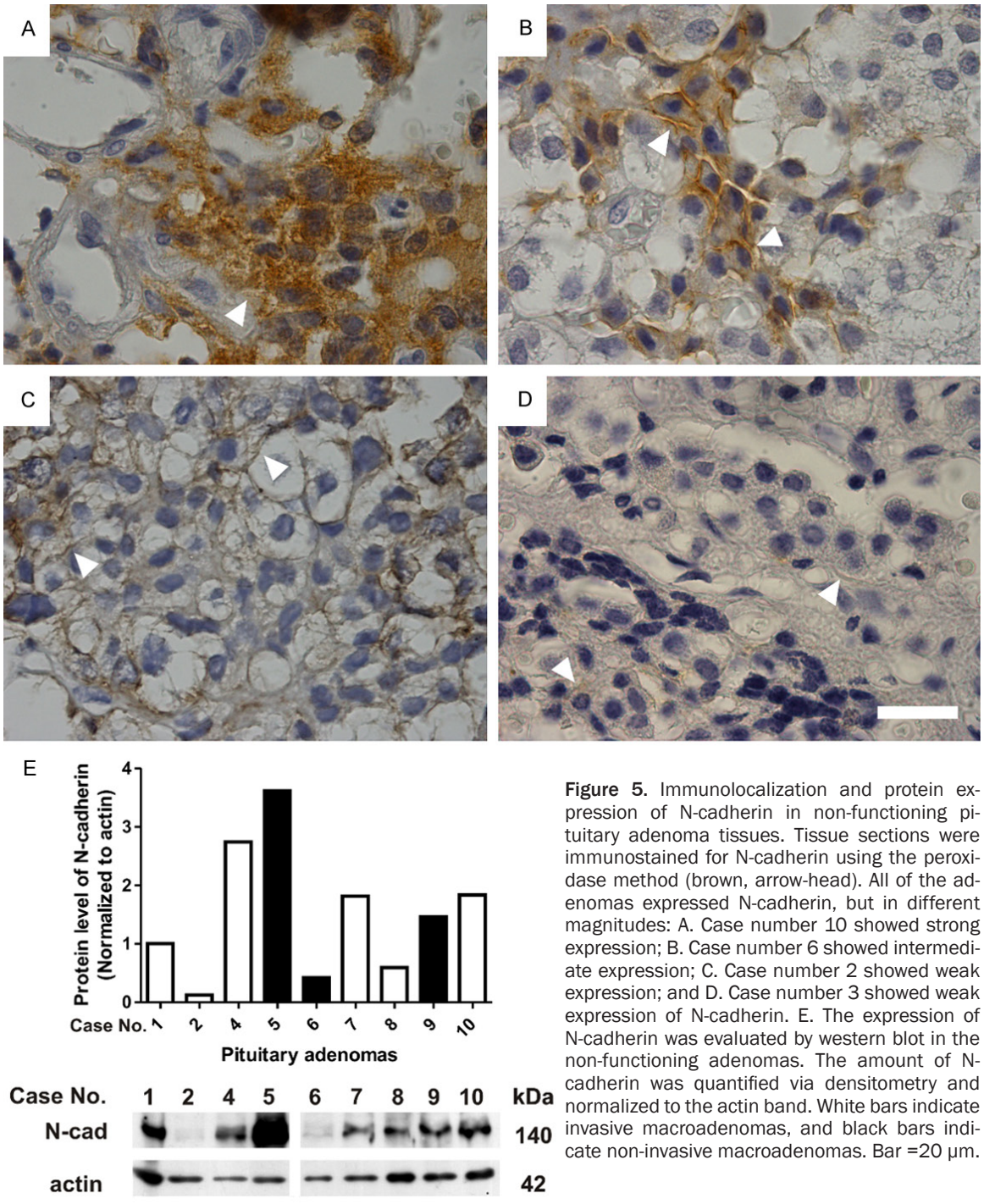

Figure 5. Immunolocalization and protein expression of N-cadherin in non-functioning pituitary adenoma tissues. Tissue sections were immunostained for N-cadherin using the peroxidase method (brown, arrow-head). All of the adenomas expressed N-cadherin, but in different magnitudes: A. Case number 10 showed strong expression; B. Case number 6 showed intermediate expression; C. Case number 2 showed weak expression; and D. Case number 3 showed weak expression of N-cadherin. E. The expression of N-cadherin was evaluated by western blot in the non-functioning adenomas. The amount of N-cadherin was quantified via densitometry and normalized to the actin band. White bars indicate invasive macroadenomas, and black bars indicate non-invasive macroadenomas. Bar =20 μm.

the cultured cells, N-cadherin was distributed at sites of cell-to-cell contact and in the cytoplasm in six NFPM and was diffusely distributed in the cytoplasm in three NFPM; in case number 3, only a low N-cadherin signal was obtained (**Figure 8**).

Discussion

Non-functioning pituitary adenomas are usually macroadenomas that frequently invade cavernous sinus structures [29, 30]. The mechanism underlying this invasive behavior is not known.

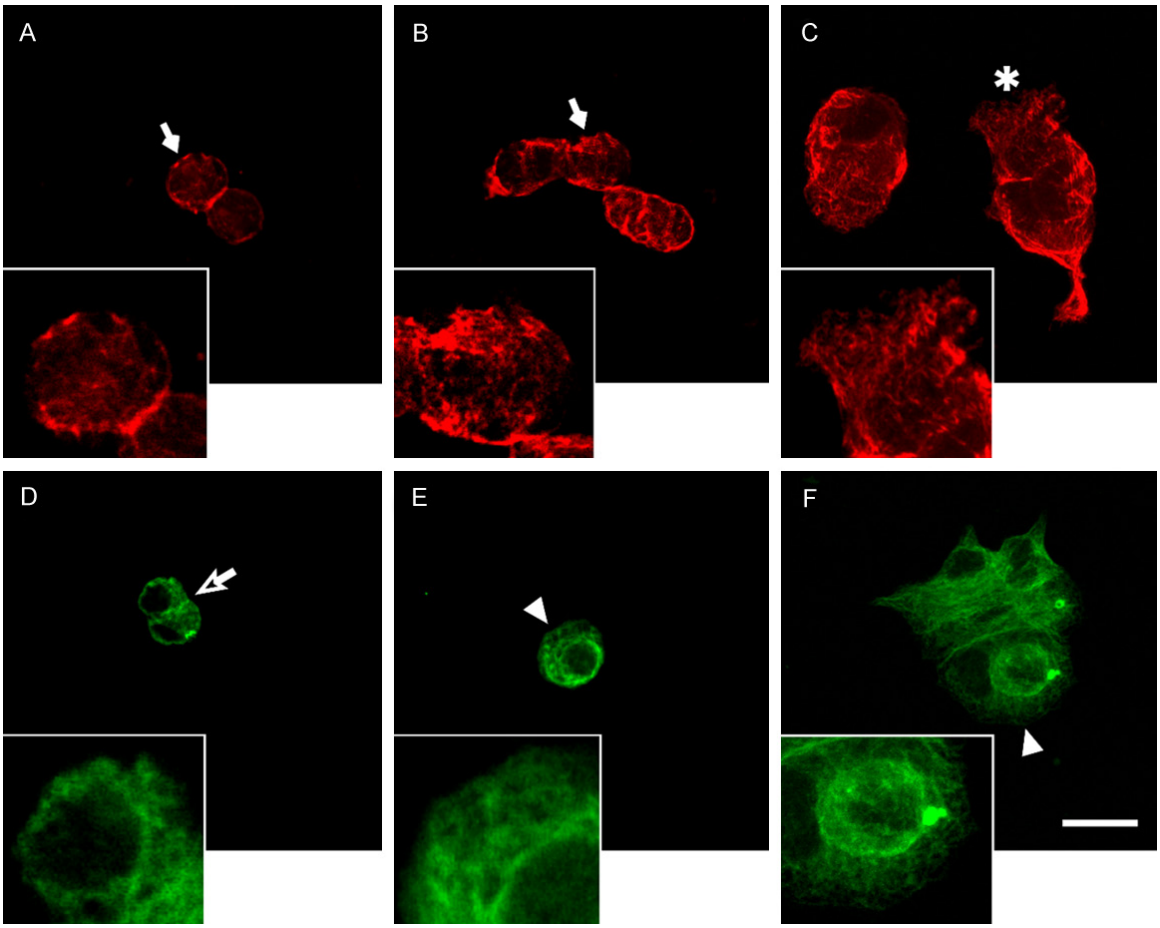

Figure 6. Cytoskeleton arrangement of cultured cells obtained from non-functioning pituitary adenomas. Cultured cells were fixed and stained for actin filaments with TRITC-phalloidin (red) or for microtubules with a FITC-conjugated monoclonal antibody (green). Cells classified in group I (A, B and D) showed discontinuous actin cortex (filled arrow) and condensed microtubules (empty arrow), and cells in group II (C, E and F) were more extended over the substrate, showing a lax mesh of actin filaments (asterisk) and a network of microtubules (arrow-head). Inserts showed higher magnifications of the cell areas pointed by the characters. Bar =15 μm.

It has been proposed that an EMT is involved in the invasive behavior of non-functioning and secretory adenomas [7-9, 11, 31]. The EMT is characterized by the loss of E-cadherin expression and the expression of vimentin and N-cadherin. E-cadherin has been more extensively analyzed in pituitary adenomas [8, 11, 12]. Increased N-cadherin expression is a key event of the EMT. This was observed in all the NFPM studied, in both tissues and cultured cells: all of them expressed N-cadherin at different levels. In these tumors, N-cadherin was observed localized in the membrane and cytoplasm or only in the cytoplasm, suggesting it was delocalized. These results are in accordance with those of Ezzat et al. [14]. Although N-cadherin is present in normal pituitary tissue [14, 32, 33], N-cadherin gene expression is increased in NFPM [13]. Secretory cell express N-cadherin, as do most corticotropes and a smaller proportion of lactotropes, as Tsuchiya et al. [32] have shown. Another protein associated with the EMT is vimentin, which is normally present in folliculo-stellate cells [18]. We found that all of the NFPM expressed this intermediate filament, and this *de novo* expression correlated with invasiveness, as evaluated by Hardy's criteria. *In Vitro*, we observed different patterns of vimentin distribution; most of the adenoma cells exhibited vimentin arranged at the cell periphery, in close proximity to the β4 integrin subunit, suggesting it was part of hemidesmosomes. The role of vimentin in normal tissues is related to different processes, such as cell attachment, migration and signaling mediated by 14-3-3 proteins [34, 35]. In

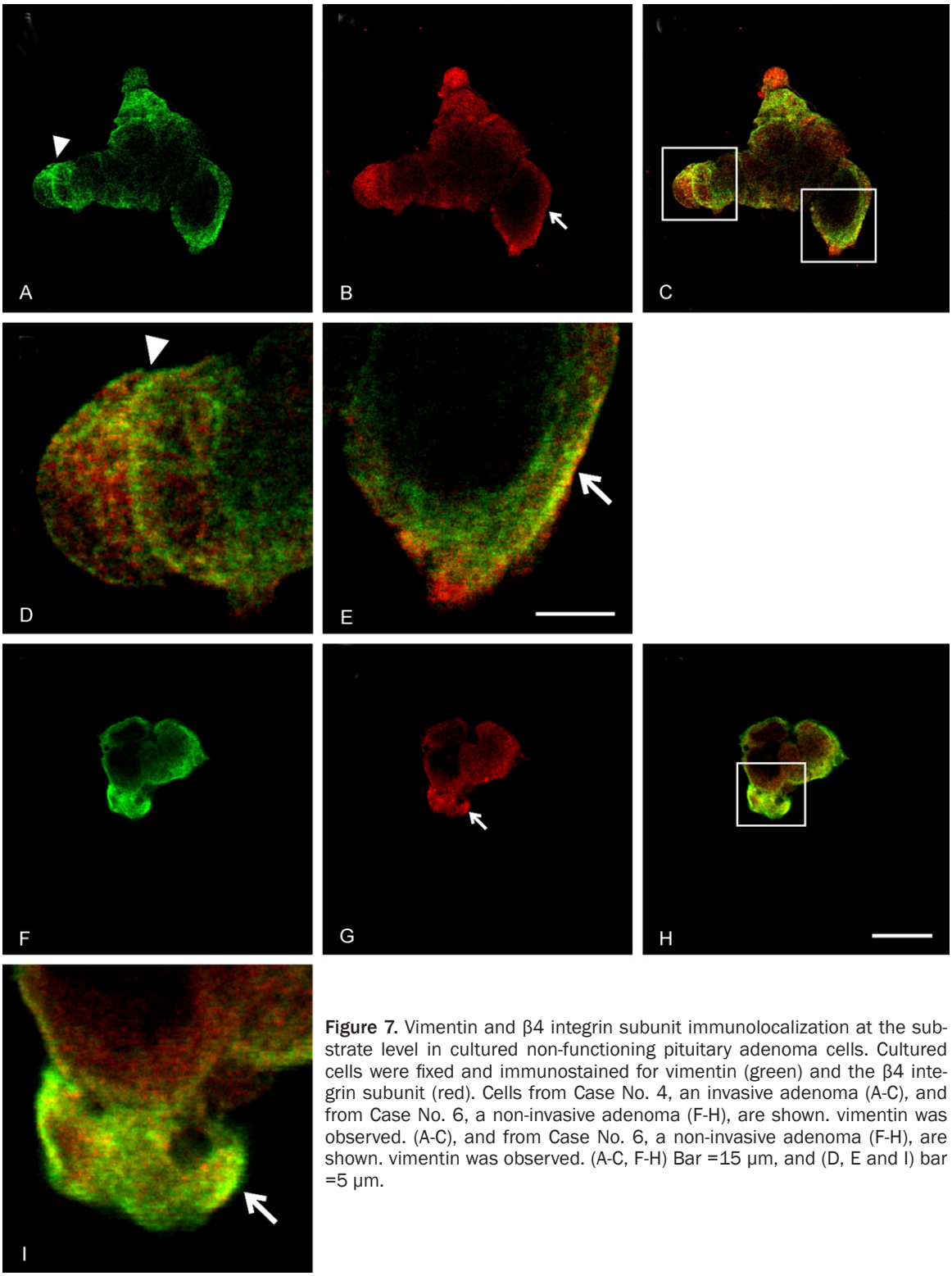

Figure 7. Vimentin and β4 integrin subunit immunolocalization at the substrate level in cultured non-functioning pituitary adenoma cells. Cultured cells were fixed and immunostained for vimentin (green) and the β4 integrin subunit (red). Cells from Case No. 4, an invasive adenoma (A-C), and from Case No. 6, a non-invasive adenoma (F-H), are shown. vimentin was observed. (A-C), and from Case No. 6, a non-invasive adenoma (F-H), are shown. vimentin was observed. (A-C, F-H) Bar =15 μm, and (D, E and I) bar =5 μm.

cancer cells, vimentin has been associated with highly invasive phenotypes and has been proposed to be a potential molecular therapeutic target [36, 37]. The observation of vimentin expression in the non-functioning adenomas suggests that this intermediate filament participates in the acquisition of a more motile phenotype. Moreover, tumor tissue endothelial cells showed vimentin staining. According to Dave and Bayless [38] vimentin is expressed in

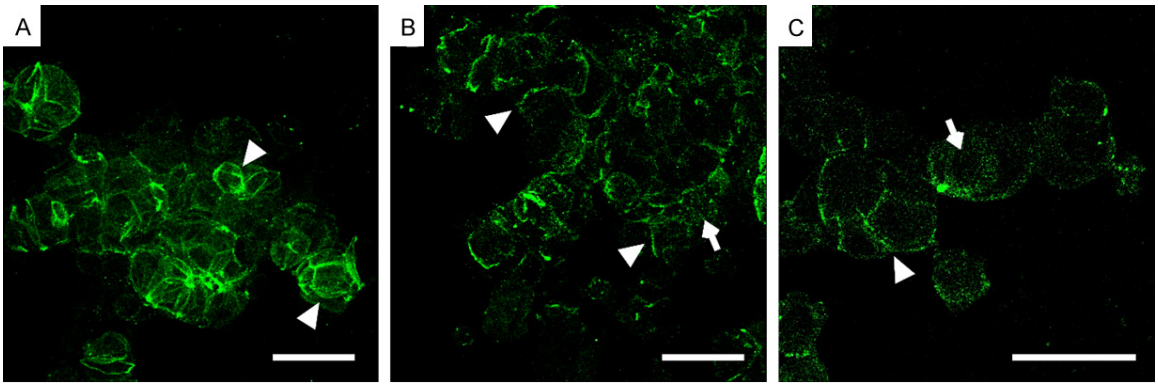

Figure 8. Different N-cadherin immunolocalization in cultured non-functioning pituitary adenoma cells. Cultured cells were fixed and immunostained for N-cadherin (green). N-cadherin was observed at the cell-cell boundary (arrow-head) or diffuses in the cytoplasm (arrow). Bar =25 μm.

processes of neovascularization, as occurred in pituitary adenomas. However, normal endothelial tissues were negative for vimentin, which is in accordance with Halliday et al. [39]. The cytoskeleton organization is crucial for migration behavior, and the way cells arrange the cytoskeleton enables the development of a migratory phenotype. Thereby, cell migration has been characterized by two main phenotypes: a mesenchymal and an amoeboid [20]. We were interested in characterizing the cytoskeleton organization present in non-functioning adenoma cells. To this end, we used a primary culture approach. First of all, these cells were small in size and showed a high nucleus/cytoplasm ratio, either in tissue or in culture. Dolfi et al. [40] have shown that the shape and size of cancer cells correlates with metabolic demands and proliferation: a higher N/C ratio has been positively correlated with malignancy. Secondly, the cells exhibited a rounded shape, even when they were cultured with 20% fetal bovine serum. The cultured cells showed small actin filament plaques at the substrate level, suggesting low substrate attachment. They showed poor spreading capacity, exhibiting few membrane protrusions, but with a discontinuous cortical actin. Microtubules were compressed, probably as a result of the scant cytoplasm present in the cell, which was also correlated with the absence of or poor hormone expression. Interestingly, a micro-prolactinoma previously studied showed spreading cells with a well-developed microtubule system [41]. To summarize, the NFPM cells studied showed a rounded shape with small actin filament plaques and few membrane extensions, resembling a cytoskeleton arrangement of cell which exhibits an amoeboid migration. In contrast, Gonzalez-del-Pliego et al. [41] showed cells of invasive secretory adenomas with a mesenchymal phenotype. Thus, adenoma cells can acquire different shapes and cytoskeleton arrangements to exhibit a specific phenotype. In conclusion, we observed that NFPM cells express the mesenchymal markers N-cadherin and vimentin and acquired a small shape, suggesting they have a behavior similar to cancer cells and organize their cytoskeleton as a cell with an amoeboid migration pattern. Our observations in these NFPM showed that the cells have performed changes in microtubules organization that allows understanding in part, the loss of the hormone secretion. Moreover, the association of the small size and the disappearance of the secretory phenotype show a more aggressive behavior of these NFPM cells. Further studies have to be performed to elucidate, at the molecular level, the intracellular pathways that maintain this behavior.

Acknowledgements

We thank Dr. M. Hernandez (Cell Biology Department, Center of Research and Advanced Studies, Mexico) for his generous gift of the actin antibody. We also wish to thank Mr. O. Rios for his technical assistance, and Mrs. C. Arceo for secretarial assistance. D. Avila-Rodriguez was supported by a fellowship from the National Council of Science and Technology of Mexico (244955).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Maria Eugenia Mendoza-Garrido, Department of Physiology, Biophysics and Neuroscience, Center of Research and Advance Studies-IPN 2508 Instituto Politecnico Nacional Avenue, Mexico, Mexico. Tel: (52) 55 5747 3800 Ext. 5122; Fax: (52) 55 5747 3754; E-mail: mmendoza@fisio.cinvestav.mx

References

[1] Asa SL, Kovacs K. Clinically non-functioning human pituitary adenomas. Can J Neurol Sci 1992; 19: 228-235.

[2] Al-Shraim M, Asa SL. The 2004 World Health Organization classification of pituitary tumors: What is new? Acta Neuropathol 2006; 111: 1-7.

[3] Kovacs K, Horvath E, Vidal S. Classification of pituitary adenomas. J Neurooncol 2001; 54: 121-127.

[4] Katznelson L Alexander JM, Klibanski A. Clinical review 45: Clinically nonfunctioning pituitary adenomas. J Clin Endocrinol Metab 1993; 76: 1086-1094.

[5] Scheithauer BW, Horvath E, Lloyd RV, Kovacs K. Pathology of pituitary adenomas and pituitary hyperplasia. In: Thapar K, Kovacs K, Scheithauer BW, Lloyd RV, editors. Pituitary Tumors. Totowa, The Human Press Inc; 2001. pp. 91-163.

[6] Wichers-Rother M, Hoven S, Kristof RA, Bliesener N, Stoffel-Wagner B. Non-functioning pituitary adenomas: endocrinological and clinical outcome after transsphenoidal and transcranial surgery. Exp Clin Endocrinol Diabetes 2004; 112: 323-327.

[7] Sano T, Rong QZ, Kagawa N, Yamada S. Downregulation of E-cadherin and catenins in human pituitary growth hormone-producing adenomas. Front Horm Res 2004; 32: 127-132.

[8] Fougner SL, Levka T, Borota OC, Hald JK, Bollerslev J, Berg J. The expression of Ecadherin in somatotroph pituitary adenomas is related to tumor size, invasiveness, and somatostatin analog response. J Clin Endocrinol Metab 2010; 95: 2334-2342.

[9] Zhou K, Jin H, Luo Y. Expression and significance of E-cadherin and β-catenins in pituitary adenomas. Int J Sur Pathol 2013; 21: 363-367.

[10] Lamouille S, Xu J, Derynck R. Molecular mechanisms of epitelial-mesenchymal transition. Nature Rev Mol Cell Biol 2014; 15: 178-196.

[11] Qian ZR, Sano T, Yoshimoto K, Asa SL, Yamada S, Mizusawa N, Kudo E. Tumor-specific downregulation and methylation of the CDH13 (H-cadherin) and CDH1 (E-cadherin) genes correlate with aggressiveness of human pituitary adenomas. Mod Pathol 2007; 20: 1269-1277.

[12] Elston MS, Gill AJ, Conaglen JV, Clarkson A, Cook RJ, Little NS, Robinson BG, Clifton-Bligh RJ, McDonald KL. Nuclear accumulation of E-cadherin correlates with loss of cytoplasmic membrane staining and invasion in pituitary adenomas. J Clin Endocrinol Metab 2009; 94: 1436-1442.

[13] Moreno CS, Evans CH-O, Zhan X, Okor M, Desiderio DM, Oyesiku NM. Novel molecular signaling and classification of human clinically nonfunctional pituitary adenomas identified by gene expression profiling and proteomic analyses. Cancer Res 2005; 65: 10214-10222.

[14] Ezzat S, Zheng L, Winer D, Asa SL. Targeting N-cadherin through fibroblast growth factor receptor-4: distinct pathogenetic and therapeutic implications. Mol Endocrinol 2006; 20: 2965-2975.

[15] Höfler H, Denk H, Walter GF. Immunohistochemical demonstration of cytokeratins in endocrine cells of the human pituitary gland and in pituitary adenomas. Virchows Arch A Pathol Anat Histopathol 1984; 404: 359-368.

[16] Ogawa A, Sugihara S, Nakanishi Y, Suzuki S, Sasaki A, Hirato J, Nakazato Y. Intermediate filaments expression in non-neoplastic pituitary cells. Virchows Arch B Cell Pathol Incl Mol Pathol 1990; 58: 331-340.

[17] Ogawa A, Sugihara S, Hasegawa M, Sasaki A, Nakazato Y, Kawada T, Ishiuchi S, Tamura M. Intermediate filaments expression in pituitary adenomas. Virchows Arch B Cell Pathol Incl Mol Pathol 1990; 58: 341-349.

[18] Kasper M. Cytokeratins in intracranial and intraspinal tissues. Adv Anat Embryol Cell Biol 1992; 126: 1-82.

[19] Vicente-Manzanares M, Webb DJ, Horwitz AR. Cell migration at a glance. J Cell Sci 2005; 118: 4917-4919.

[20] Friedl P, Wolf K. Plasticity of cell migration: a multiscale tuning model. J Cell Biol 2009; 188: 11-19.

[21] Sommers CL, Walker-Jones D, Heckford SE, Worland P, Valverius E, Clark R, McCormick F, Stampfer M, Abularach S, Gelmann EP. Vimentin rather than keratin expression in some hormone-independent breast cancer cell lines and in oncogene-transformed mammary epithelial cells. Cancer Res 1989; 49: 4258-4263.

[22] Joo YN, Eun SY, Park SW, Lee JH, Chang KC, Kim HJ. Honokiol inhibits U87MG human glioblastoma cell invasion through endothelial cells by regulating membrane permeability and the epithelial-mesenchymal transition. Int J Oncol 2014; 44: 187-194.

[23] Avila-Rodríguez D, Paisano-Cerón K, Valdovinos-Ramírez I, Solano-Agama C, Ortiz-Plata A, Mendoza-Garrido ME. Tridimentional alginate beads culture of human pituitary adenoma cells. J Vis Exp 2016; 53637.
[24] Caron JM, Jones AL, Kirschner MW. Autoregulation of tubulin synthesis in hepatocytes and fibroblasts. J Cell Biol 1985; 101: 1763-1772.
[25] Asa SL, Bamberger A-M, Kao B, Wong M, Parker KL, Ezzat S. The transcription activator steroidogenic factor-1 is preferentially expressed in the human pituitary gonadotroph. J Clin Endocrinol Metab 1996; 81: 2165-2170.
[26] Hardy J. Transphenoidal microsurgery of the normal and pathological pituitary. Clin Neurosurg 1969; 16: 185-217.
[27] Hardy J, Vezina JL. Transsphenoidal neurosurgery of intracranial neoplasms. Adv Neurol 1976; 15: 261-274.
[28] Di Ieva A, Rotondo S, Syro LV, Cusimano MD, Kovacs K. Aggressive pituitary adenomas-diagnosis and emerging treatments. Nat Rev Endocrinol 2014; 10: 423-435.
[29] Saeger W, Lüdecke DK, Buchfelder M, Fahlbusch R, Quabbe HJ, Petersenn S. Pathohistological classification of pituitary tumors: 10 years of experience with the German Pituitary Tumor Registry. Eur J Endocrinol 2007; 156: 203-216.
[30] Yamada S, Ohyama K, Taguchi M, Takeshita A, Morita K, Takano K, Sano T. A study of the correlation between morphological findings and biological activities in clinically nonfunctioning pituitary adenomas. Neurosurgery 2007; 61: 580-585.
[31] Zhou W, Song Y, Xu H, Zhou K, Zhang W, Chen J, Qin M, Yi H, Gustafsson JA, Yang H, Fan X. In nonfunctional pituitary adenomas, estrogen receptors and slug contribute to development of invasiveness. J Clin Endocrinol Metab 2011; 96: E1237-E1245.
[32] Tsuchiya B, Sato Y, Kameya T, Okayasu I, Mukai K. Differential expression of N-cadherin and E-cadherin in normal human tissues. Arch Histol Cytol 2006; 69: 135-145.
[33] Rubinek T, Yu R, Hadani M, Barkai G, Nass D, Melmed S, Shimon I. The cell adhesion molecules N-cadherin and neural cell adhesion molecule regulate human growth hormone: a novel mechanism for regulating pituitary hormone secretion. J Clin Endocrinol Metab 2003; 88: 3724-3730.
[34] Ivaska J, Pallari HM, Nevo J, Eriksson JE. Novel functions of vimentin in cell adhesion, migration, and signaling. Exp Cell Res 2007; 313: 2050-2062.
[35] Castro-Muñozledo F, Velez-Del-Valle C, Marsh-Moreno M, Hernández-Quintero M, Kuri-Harcuch W. Vimentin is necessary for colony growth of human diploid keratinocytes. Histochem Cell Biol 2015; 143: 45-57.
[36] Satelli A, Li S. Vimentin in cancer therapy and its potential as a molecular target for cancer therapy. Cell Mol Life Sci 2011; 68: 3033-3046.
[37] Yoneyama MS, Hatakeyama S, Habuchi T, Inoue T, Nakamura T, Funyu T, Wiche G, Ohyama C, Tsuboi S. Vimentin intermediate filament and plectin provide a scaffold for invadopodia, facilitating cancer cell invasion and extravasation for metastasis. Eur J Cell Biol 2014; 93: 157-169.
[38] Dave JM, Bayless KJ. Vimentin as an integral regulator of cell adhesion and endothelial sprouting. Microcirculation 2014; 21: 333-44.
[39] Halliday WC, Asa SL, Kovacs K, Scheithauer BW. Intermediate filaments in the human pituitary gland: an immunohistochemical study. Can J Neurol Sci 1990; 17: 131-136.
[40] Dolfi SC, Chan LL Y, Qiu J, Tedeschi PM, Bertino JR, Hirshfield KM, Oltvai ZN, Vazquez A. The metabolic demands of cancer cells are coupled to their size and protein synthesis rates. Cancer Metab 2013; 1: 20. http://www.cancerandmetabolism.com/content/1/1/20.
[41] González-del-Pliego M, Aguirre-Benítez E, Paisano-Cerón K, Valdovinos-Ramírez I, Rangel-Morales C, Rodríguez-Mata V, Solano-Agama C, Martín-Tapia D, de la Vega MT, SaldovalBalanzario M, Camacho J, Mendoza-Garrido ME. Expression of Eag1 K+ channel and ErbBs in human pituitary adenomas: cytoskeleton arrangement patterns in cultured cells. Int J Exp Pathol 2013; 6: 458-468.
[42] Charras G, Paluch E. Blebs lead the way: how to migrate without lamellipodia. Nat Rev Mol Cell Biol 2008; 9: 730-736.
[43] Ridley AJ. Life at the leading edge. Cell 2011; 145: 1012-1022.
[44] Sanz-Moreno V, Marshall CJ. The plasticity of cytoskeletal dynamics underlying neoplastic cell migration. Curr Opin Cell Biol 2010; 22: 690-696.