Annals of Diagnostic Pathology 38 (2019) 59–61

Contents lists available at ScienceDirect

Annals of Diagnostic Pathology

journal homepage: www.elsevier.com/locate/anndiagpath

Original Contributions

Gene and protein expression of E-cadherin and NCAM markers in non-functioning pituitary adenomas☆

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ARTICLE INFO

Keywords:
E-cadherin
NCAM
Non-functioning pituitary adenomas

ABSTRACT

Non-functioning pituitary adenomas (NFPA) are classified as benign tumors of slow growth, but 40% of them present local invasion, a characteristic of behavior still unpredictable with the use of current tumor markers. This work aims to evaluate the tissue markers E-cadherin and NCAM, which act on cell adhesion, in tumor tissue samples of NFPA and its relationship with the degree of local invasiveness. Gene expression of E-cadherin (*CDH1*) and NCAM (*NCAM1*) was assessed by real-time PCR and tissue expression by immunohistochemistry. Fifty-three patients with macroadenomas were submitted to transsphenoidal surgery, presented grade II invasive adenomas in 16 cases (30.2%), grade III in 7 (13.2%) and grade IV in 30 (56.6%). In the immunohistochemistry, one case was negative for E-cadherin, 7 showed weak immunostaining, 17 moderate and 28 strong, whereas for NCAM, 5 showed negative, 28 weakly, 14 moderate and 6 strong. Regarding gene expression, 43.3% showed expression for *CDH1* (mean of 2.12) and 50% for *NCAM1* (mean of 1.86). There was no significant correlation between the immunohistochemical expression of the markers, as well as the gene expression, the degree of invasiveness and clinical data. The results suggest that E-cadherin and NCAM markers are not directly related to the invasiveness in NFPA.

1. Introduction

Non-functioning pituitary adenomas (NFPA) represent 30–35% of pituitary adenomas and are diagnosed due to symptoms associated with mass compression or possibly incidentally [1,2]. Although classified as benign slow-growing tumors, 40% of them present local invasion characteristics, which may cause erosion of the sella turcica, sphenoid and cavernous sinus invasion and compromise other adjacent tissues. After surgical resection, adenomas may show tumor recurrence or re-growth. The potential for proliferation and invasion may be independent factors [3]. The potential for predicting invasiveness and aggressiveness in NFPA was evaluated in markers related to cell adhesion, such as Ep-CAM [4], N-Caderin [5], SLUG [6], among others.

The E-cadherin (E-CAD) and the neural cell adhesion molecule (NCAM) are proteins involved in cell adhesion and, when their expression is altered, cellular mobility and, consequently, invasiveness can be observed [7-9]. However, the role of these markers in the process of invasiveness in pituitary adenomas is still controversial [10,11]. With the aim to explore the study of this topic, in order to aid therapeutic decisions in cases of more aggressive tumor behavior, the present study evaluated the expression of E-CAD and NCAM in a series with a significant number of NFPA, and related the expression with the degree of invasiveness.

2. Materials and methods

Free and informed consent term was obtained from all patients, and the study was approved by the Institutional Research Ethics Committee and was conducted in accordance with the Helsinki Declaration. Fifty-three patients with NFPA underwent transsphenoidal tumor resection by an experienced neurosurgeon at a referral hospital in southern Brazil. The sample of the study was for convenience. The diagnosis of NFPA was made based on clinical and biochemical, as well as histological and immunohistochemical evaluation (IHC). Clinical data were

☆ Declarations of interest: none.

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https://doi.org/10.1016/j.anndiagpath.2018.10.003

obtained from medical records.

Tumor grade was defined on the basis of magnetic resonance imaging (MRI 1.5 T) and classified according to Hardy [12]: grade I (microadenomas, < 1 cm in diameter), grade II (≥1 cm in diameter, intrasellar or with suprasellar extension without causing bone erosion), grade III (locally invasive tumors that may be associated with diffuse sellar enlargement and bone erosion of the sella turcica), and grade IV (invasive tumors that involve extrasellar structures including bone, hypothalamus, and the cavernous sinus). Grade I and II pituitary adenomas were considered non-invasive tumors, while grade III and IV were considered invasive [13].

2.1. Immunohistochemistry (IHC)

Tumor samples (n = 53) were fixed in 10% formalin for 24 h and embedded in paraffin. The blocks were sectioned at 4 μm and placed on organosilane treated slides. To detect protein expression, the slices were incubated with the anti-E-CAD G-10 monoclonal antibody (sc-8426; Santa Cruz Biotechnology, Dallas, TX, USA) at a 1:50 dilution and the anti-NCAM ([EPR2566] [ab133345], Abcam, Cambridge, UK) at a dilution of 1:300. The streptavidin-biotin method (LSAB kit + Peroxidase; Dako, Carpinteria, CA, USA) was used for detection. Endogenous peroxidase activity was blocked using 5% hydrogen peroxide in methanol. Blocking of non-specific proteins was done with 1% BSA. Incubation with the primary antibody was done overnight at 4 °C and that of the secondary and tertiary antibodies was done at room temperature for 40 min each. For the negative control, the primary antibody was replaced with saline. Human tonsil was used as a positive control for E-cadherin and glioma for NCAM. The antigen-antibody complex was visualized by the DAB (diaminobenzidine) chromogen method.

Positive expression was defined by labeling of the plasma membrane. The immunostaining intensity was rated 0 (unmarked), 1 (weak), 2 (moderate) and 3 (strong). The percentage of labeled cells was classified as 0 (0–5%), 1 (6–10%), 2 (11–50%), 3 (51–80%) and 4 (> 80%). The final score was obtained by multiplying the intensity score by the percentage of labeled cells, resulting in: 0 (−, negative expression), 1–3 (+, weak expression), 4–6 (++, moderate expression) and > 6 (+++, strong expression) [9]. The slides were read by two independent observers under an optical microscope.

2.2. Quantitative real time PCR (qRT-PCR)

Tumor fragments were stored in liquid nitrogen and stored in a biofreezer at −80 °C. Thirty-two samples were tested for the *NCAM1* marker and 30 for the *CDH1* marker. Total RNA was extracted using TriReagent (Ludwig Biotec, Alvorada, RS, Brazil), according to the manufacturer's instructions. The cDNA was obtained by reverse transcription from the RNA with a final volume of 21 μl using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The samples were diluted and a final concentration of 250 ng/μL was obtained for E-CAD (*CDH1*) and 100 ng/μL for NCAM. The samples were amplified using Sybr Green (Applied Biosystems, Foster City, CA, USA) with a total reaction volume of 15 μl under the following conditions: initial denaturation at 50 °C for 2 min and at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The following primers were used for *CDH1* (Forward 5′-GCCGAGAGCTACACGTTCAC-3′ Reverse 5′-ACT TTGAATCGG GTGTCGAG-3′) and *NCAM-1* (Forward 5′-AACAAAGCA TGATGGGTGAA-3′ Reverse 5′-GTCTGTGGTGTTGGAAATGC-3′). All reactions were performed in duplicate using StepOnePlus system (Applied Biosystems, Foster City, CA, USA). Samples without cDNA were used as a negative control. As an endogenous control, *GAPDH* was used as the reference standardization gene (Forward 5′-GGAAGGTGA AGGTCGGAGTCA-3′ Reverse 5′-GTCATTGATGGCAACAATATCC ACT-3′). *GAPDH* was amplified by qRT-PCR for each sample and also for the negative control under the same conditions specified for the analysis of the genes. Cycle threshold (CT) < 40 demonstrated good cDNA quality. For the calibration of the analysis, a commercial pituitary RNA pool (Human Pituitary Gland Pool of RNA-636157, Clontech Laboratories, Palo Alto, CA, USA) was used consisting of 39 healthy pituitary gland samples from adult men and women. Data were converted to normalize the expression ratios using the method recommended by Applied Biosystems 2 (−ΔΔCT), where: ΔΔCT = [(CT target gene − CT *GAPDH* gene in the sample) − (CT target gene in normal pituitary − CT gene *GAPDH* in normal pituitary)]. Data for tumor tissues are expressed as 1 (reference level). A level of expression < 1 was defined as absent expression and ≥1 as presence of expression [14].

2.3. Statistical analysis

Data were analyzed using SPSS, version 23.0. The age, according to the degrees of symmetry found, was expressed as mean ± standard deviation. Gender and degree of invasiveness were expressed by frequency. The immunohistochemical expression was presented as frequency and percentage and the gene expression of E-CAD and NCAM was presented as the median and interquartile range. Invasive and non-invasive adenomas were compared using the chi-square and Mann-Whitney tests. The level of significance was 5%.

3. Results

Of the 53 patients, 33 were men (62.3%). The age ranged from 24 to 79 years, with a mean of 55.8 ± 13.3 years. All tumors were macroadenomas. Regarding the degree of invasiveness, 16 cases were grade II (30.2%), 7 grade III (13.2%) and 30 grade IV (56.6%). The samples were submitted to immunohistochemistry for E-CAD and NCAM proteins. Of these, one case was negative for ECAD, 7 showed weak staining (+), 17 moderate (++) and 28 strong (+++). For NCAM, 5 were negative, 28 weak staining (+), 14 moderate (++) and 6 strong (+++). There was no significant correlation between the degree of invasiveness and the immunohistochemical expression of the markers (Table 1), age and gender.

The normalization reference gene (*GAPDH*) was positive in all cases and in the human pituitary gland pool. Regarding the gene expression for *CDH1*, 43.3% showed expression (mean of 2.12). For *NCAM1*, 50% of the cases showed gene expression (mean 1.86). There was no correlation between gene expression and clinical data.

4. Discussion

One of the main mechanisms involved in tumor progression is the loss of adhesion between cells and between them and the extracellular matrix [15]. The lack of proliferation control is associated with an increase in tumor invasion capacity [16].

E-cadherin, a cell adhesion protein, was few times evaluated in pituitary adenomas [6,9-11,17-19] and rarely in NFPA [8,20]. Detection of IHC expression of E-CAD ranges from 52% [19] to 100% [17] and is

Table 1
Immunohistochemical expression of E-CAD and NCAM according to tumor grade.

Grade	n = 53	E-CAD				NCAM			
		−	+	++	+++	−	+	++	+++
II	16	1	1	5	9	3	9	3	1
III	7	0	0	4	3	0	5	1	1
IV	30	0	6	8	16	2	14	10	4
Total		1	7	17	28	5	28	14	6
p		0.351				0.556			

mostly > 70% [6,10,17,18]. In NFPA, according to Yamada et al. [20], it is present in 70% of the cases. In this study, 52 cases (98.1%) were positive for E-CAD in IHC, a result consonant with the literature.

Few studies have evaluated the gene expression of *CDH1*, the gene responsible for the translation of E-CAD in pituitary adenomas. When evaluated by RT-PCR the detection of *CDH1* mRNA ranges from 36% [18] to 50% [6]. In this study, 43.3% of the evaluated cases presented *CDH1* expression, a result similar to that found by Mendes et al. [6] that, analyzing somatotrophic adenomas, found expression in 50% of the cases.

The relationship between the E-CAD expression, both detected by IHC and by RT-PCR, and the degree of tumor invasiveness is not consistent in the literature. When evaluated the correlation of E-CAD with tumor size in somatotroph adenomas, the results were negative [10], as well as the association with the degree of tumor invasion [6]. In series that investigated various types of pituitary adenomas, no statistical difference was found between invasive and non-invasive [17,19]. On the other hand, Zhou et al. [9], in somatotrophs and lactotrophs adenomas, observed downregulation in invasive and recurrent adenomas in relation to non-invasive and non-recurrent adenomas. Likewise, Chauvet et al. [11] observed, through IHC and RT-PCR, decreased E-CAD expression in invasive somatotrophic adenomas and not in the prolactin secretors. Our findings, in non-functioning adenomas, are in agreement with Yamada et al. [20], who did not find association between E-CAD reduction and invasion of the cavernous sinus, and contrasts with Zhou et al. [8], which related alteration in E-CAD with tumor invasiveness.

The NCAM glycoprotein belongs to the immunoglobulin family and, because it is involved in cell adhesion, plays an important role in growth, differentiation, proliferation and cell survival [21]. The expression of NCAM was evidenced in normal pituitary glands [22] and also in pituitary adenomas [23]. In the current study, NCAM positivity was found in 90% of cases analyzed through IHC, while 50% presented *NCAM1* gene expression. Mendes et al. [6], similarly, found 80% positivity for NCAM with IHC and 53% for *NCAM1* expression with RT-PCR.

Aletsee-Ufrecht et al. [22], in series of 11 pituitary adenomas, detected NCAM in somatotroph adenomas and in NFPA, but not in prolactin secretors. Mendes et al. [6], in somatotrophic adenomas, did not observe a relationship between NCAM and tumor invasiveness both by IHC and RT-PCR. Kleinschmidt-DeMasters et al. [24], in 20 pituitary adenomas of different types, found no correlation between tumor invasiveness and NCAM expression. In our NFPA sample, we also did not observe a relationship between *NCAM1* expression and tumor invasiveness.

5. Conclusion

In conclusion, evaluating a significant sample of non-secretory pituitary tumors, this study did not detect a significant difference in the tissue and gene expression of E-CAD and NCAM between tumors classified as invasive or non-invasive. The search for other markers that aid therapeutic decision making should guide future research in the area.

Acknowledgements

This work was supported by grants from CAPES (Brazilian government research funding agency) PNPD (23038007203201166).

Conflicts of interest

The authors declare that they have no conflict of interest.

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