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# Pleomorphic adenoma and adenoid cystic carcinoma of salivary glands: E-cadherin immunoexpression and analysis of the *CDH1* -160C/A polymorphism

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ABSTRACT

*Objective:* Despite their similar cellular origin, pleomorphic adenomas (PA) and adenoid cystic carcinomas (ACC) present distinct behaviors. This study aimed to analyze the immunoexpression of E-cadherin in PA and ACC of salivary glands, and to investigate differences in its expression in relation to E-cadherin gene (*CDH1*) -160C/A polymorphism.
*Design:* Twenty-four PA (15 cell-rich and 9 cell-poor tumors) and 24 ACC (10 tubular, 8 cribriform and 6 solid tumors) were selected for the analysis of pattern of distribution, and cellular localization of E-cadherin. In addition, E-cadherin expression was evaluated using the H-score scoring system. The *CDH1* -160C/A polymorphism was investigated by PCR-RFLP.
*Results:* No significant differences in pattern of distribution ($p = 0.181$) and cellular localization ($p = 0.192$) of E-cadherin were observed between PA and ACC. Comparison of PA and ACC cases revealed a higher median H-score in the latter ($p = 0.036$). Cell-rich PA presented a higher H-score than cell-poor tumors ($p = 0.013$), whereas no significant differences in E-cadherin expression were observed between ACC subtypes ($p = 0.254$). The heterozygous genotype of the *CDH1* -160C/A polymorphism was detected in only one PA and one ACC. H-scores for tumors carrying the polymorphism were below the lower quartile of their respective groups.
*Conclusions:* The results suggest that E-cadherin expression in PA and ACC is mainly related to cellular composition (epithelial cells versus myoepithelial cells) and degree of differentiation of myoepithelial cells in these tumors. The *CDH1* -160C/A polymorphism does not seem to significantly influence the expression of E-cadherin in PA and ACC of salivary glands.

## 1. Introduction

Salivary gland tumors are a fairly heterogeneous group of neoplasms that are characterized by marked variations in their clinical and histological features. Pleomorphic adenomas (PA) are the most common benign salivary gland tumors, whereas adenoid cystic carcinomas (ACC) are the second most frequent malignant salivary gland neoplasms (Jones, Craig, Speight, & Franklin, 2008). Despite their similar origin and cell components, these tumors present distinct behaviors (Lourenço & Lima, 2007). ACC is a relatively slow-growing malignancy, but is highly invasive (Westernoff, Jordan, Regezi, Ramos, & Schmidt, 2005). Frequent local recurrences and late distant metastases account for the poor prognosis of ACC (van Weert, van der Waal, Witte, Leemans, & Bloemena, 2015). Conversely, PA is a benign neoplasm that grows and expands circumferentially, causing compression of surrounding tissues, and therefore has an excellent prognosis (Westernoff et al., 2005).

Cadherins comprise a large family of transmembrane glycoproteins that mediate $Ca^{2+}$-dependent cell-cell adhesion and play pivotal roles in cell adhesion, tissue architecture, and development

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(Stemmler, 2008; van Roy & Berx, 2008). E-cadherin, a 120-kDa glycoprotein encoded by the *CDH1* gene located on chromosome 16q22.1, is a classical cadherin that forms the key functional component of adherens junctions between epithelial cells (Stemmler, 2008; van Roy & Berx, 2008). Several studies have demonstrated an association between reduced E-cadherin expression and tumor invasion, metastasis, recurrence, and poor prognosis (Cowin, Rowlands, & Hatsell, 2005; Franchi et al., 1999; Liu et al., 2006; Liu et al., 2010; Park et al., 2008). Therefore, a tumor suppressor role has been assigned to the *CDH1* gene (Nakamura et al., 2002; Qian et al., 2007).

Aberrant expression of E-cadherin may be due to gene mutation, loss of heterozygosity, and methylation of the *CDH1* promoter region (Caldeira et al., 2006; Liu et al., 2006; Park et al., 2003). Additionally, several single nucleotide polymorphisms (SNPs) have been identified in the *CDH1* gene (Nakamura et al., 2002). Particularly, the SNP -160C/A has been widely studied and its two alleles confer the E-cadherin promoter different transcriptional activities (Li, Pan, Guo, & Li, 2014). A study using a human prostate cancer cell line showed that the A allele of the -160C/A polymorphism in the promoter region decreased transcriptional activity of the *CDH1* gene by approximately 68% when compared with the C allele (Li et al., 2000).

Despite these important findings, to the best of our knowledge, there have been no studies investigating the -160C/A polymorphism in the promoter region of the *CDH1* gene in PA and ACC of salivary glands (PubMed database). Therefore, the aim of the present study was to analyze the immunoexpression of E-cadherin in PA and ACC, and to investigate possible differences in its expression in relation to the *CDH1* -160C/A polymorphism.

## 2. Materials and methods

Forty-eight specimens (24 PA and 24 ACC) were randomly selected for this study from the archives of the Department of Oral Pathology, Federal University of Rio Grande do Norte (UFRN), and from the archives of the Department of Pathology and Legal Medicine, Federal University of Ceará (UFC). PA were divided into two categories according to the amount and cellular composition of the stroma: cell-rich tumors (predominance of epithelial cells) and cell-poor tumors (predominance of myxoid and chondroid areas) (Soares, de Araújo, Juliano, & Altemani, 2009). ACC was classified into the following histological subtypes: cribriform (pure cribriform tumors or mixed with $<$30% of solid areas), tubular (tumors with tubular and also cribriform areas without solid components), and solid (predominantly solid pattern) (Ellis & Auclair, 1996). The cases were not matched for age, gender, or type of salivary gland (major or minor). The study was approved by the Research Ethics Committee of UFRN, Natal, Brazil.

### 2.1. Immunohistochemistry

Serial sections (3 μm thick) were obtained from paraffin-embedded tissues and dewaxed sections were processed for antigen retrieval. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. Antigen retrieval was performed in a Pascal pressure cooker with citrate buffer, pH 6.0. After treatment with normal serum, the sections were incubated with the primary anti-E-cadherin antibody diluted 1:50 (clone H108, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min. Immunohistochemistry was performed using the EnVision peroxidase procedure (Dako, Carpinteria, CA, USA). The reaction was developed with diaminobenzidine as chromogen and the sections were counterstained with Mayer's hematoxylin. Healthy salivary glands and oral epithelial lining included in the specimens were used as positive controls. As a negative control, the specimens were treated as described above, except that the primary antibody was replaced with a solution of bovine serum albumin in phosphate-buffered saline.

### 2.2. Analysis of immunostaining

After processing of the histological sections and immunohistochemical treatment, each specimen was analyzed under a light microscope. The pattern of distribution of E-cadherin was evaluated according to the following scores: 0 = no staining, 1 = focal staining, and 2 = diffuse staining. In terms of cellular localization, the immunoexpression of E-cadherin was classified as: cytoplasm or membrane and cytoplasm.

In addition, 1000 neoplastic cells were counted in a series of fields chosen at random in PA and ACC specimens at 400× magnification. Immunostaining intensity was scored as follows: 0 (no staining), 1+ (weak), 2+ (moderate), and 3+ (strong). Using an adaptation of a previously detailed method (McCarty, Miller, Cox, Konrath, & McCarty, 1985), an H-score was calculated as the sum of percentages of cells that stained at each intensity multiplied by the weighted intensity of staining: H-score = $(i + 1)\pi$, where $i = 1, 2, 3$, and $\pi$ ranges from 0 to 100%.

### 2.3. DNA extraction

Genomic DNA was extracted from formalin-fixed paraffin-embedded tissue blocks by preparing six 10-μm thick sections collected into sterile microtubes. The paraffin was removed by incubation in xylene and the specimens were subsequently rehydrated in ethanol. DNA was extracted using proteinase K and Chelex-100 resin solution (BioRad Laboratories, Hercules, CA, USA). The integrity of the extracted DNA was tested by electrophoresis, followed by PCR amplification of the methylenetetrahydrofolate reductase gene.

### 2.4. Genotyping

Genotyping was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. DNA samples were amplified using the following primers: sense 5′-TCCCAGGTCTTAGTGAGCCA-3′ and antisense 5′-GGCCACAGCCAATCAGCA-3′. DNA was amplified in a total volume of 25 μl containing 17 pmol of each primer, 2.5 U Taq polymerase, and 1.5 mM $MgCl_2$ (Illustra PuReTaq Ready-To-Go$^{TM}$ PCR Beads, GE HealthCare, Little Chalfont, GB-BKM, UK). Amplification consisted of 42 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 45 s, and extension at 72 °C for 90 s. The size of the amplified fragment (190 bp) was confirmed by electrophoresis on 1% agarose gel. The PCR products were digested overnight with 5 U of the restriction enzyme (*Hph* I or *Afl III*) and electrophoresed on 6% polyacrylamide gel. The C allele of the SNP creates an *Hph* I site and the A allele creates an *Afl* III site. Successful digestion with *Hph* I and *Afl* III indicated the presence of the C and A alleles, respectively.

### 2.5. Statistical analysis

The results obtained were submitted to statistical analysis using the Statistical Package for the Social Sciences (version 17.0; SPSS Inc., Chicago, IL, USA). Fisher' exact test and Pearson's chi-square test were used for the analysis of pattern of distribution and cellular localization of E-cadherin. For distribution analysis of the H-scores data, the Kolmogorov-Smirnov test was applied which revealed the absence of a normal distribution. Therefore, the median H-scores were compared between PA and ACC by the non-parametric Mann-Whitney test. Comparison of H-scores between the histological subtypes of ACC and PA was done by the non-