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Comparative E-Cadherin Digital Expression Analysis in HPV and non-HPV Related Squamous Cell Carcinoma of the Oral Cavity

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Abstract. *Background/Aim: Oral squamous cell carcinoma (OSCC) demonstrates aggressive biological behavior in sub-groups of patients with specific molecular characteristics. Concerning metastatic potential, disruption of cell to cell adhesion is a critical event in epithelial malignancies including OSCC. Our aim was to investigate the role of E-Cadherin expression in OSCC patients as a valuable protein marker. Materials and Methods: Fifty (n=50) tissue sections derived from primary OSCCs were analyzed by implementing an immunohistochemistry (IHC) assay based on a proper anti-E-cadherin antibody. Digital image analysis was also implemented for an objective evaluation of the corresponding protein expression levels. Results: E-cadherin altered expression (low to negative) was observed in 34/50 (68%) cases, whereas the rest (16/50-32%) demonstrated normal (high to moderate) expression. E-Cadherin abnormal expressionwas associated with the stage of the examined malignancies (p=0.023), whereas no significant correlations with grade, gender, smoking status or human papilloma virus (HPV) history were observed. Conclusion: E-Cadherin down regulation is frequently observed in OSCC and is correlated to a progressively aggressive phenotype of the malignancy in the corresponding patients (advanced stage), but it seems that the impact of HPV persistent infection on these patients is not a critical parameter.*

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Typically, oral squamous cell carcinoma (OSSC) is characterized by an aggressive phenotype including poor prognosis, moderate response rates to chemo-radiotherapy and targeted therapeutic agents, such as monoclonal antibodies (1, 2). Pre-malignant histology in oral epithelia recognizes different levels of leukoplakia (3). Molecular analyses in solid malignancies -including OSCC- have shown that deregulation and functional loss in critical molecules -such as cadherins and proto-cadherins- that stabilize intra-epithelial cells conjunctions are responsible for the aggressive biological behaviour in these patients (4). Cadherins play a significant role in prohibiting metastatic potential as calcium dependent protein family glycoproteins that secure cell to cell adhesion. Based on their structural nature, they consist of three parts including an extracellular domain, a bridge membrane-cytoplasm region and a cytoplasmic end (5). Concerning their functional status, they are involved in tissue differentiation, whereas a suppressor activity in other genes has been also detected. The e-cadherin/catenin complex affects signal transduction to the nucleus by activating the c-Src factor during epithelial cell adhesion, also modifying actin-filament assembly (6). The E-cadherin gene (CDH1) is located on chromosome 16 (16q22.1) encoding for a calcium-dependent cell-cell adhesion glycoprotein. Mechanisms of CDH1 gene deregulation in carcinomas include somatic point mutations, deletions or promoter methylation leading to a progressive loss of its protein expression (7). In the current study, we focused on evaluating the E-cadherin protein expression levels, by implementing an objective process (digital image analysis) in a series of OSCC tissue sections and correlating them to their corresponding clinico-histological parameters.

Materials and Methods

Study group. In order to explore the role of E-cadherin protein aberrations in OSCC, fifty (n=50) archival, formalin-fixed, and paraffin-embedded tissue specimens of histologically confirmed primary OSCCs were used. It should be mentioned that all the corresponding histopathological material was derived from surgical operations at the beginning of the diagnosis of the disease as a primary malignancy and not from a local recurrence. The hospital ethics committee consented to the use of these tissues in Hippocration Hospital, University of Athens, Athens, Greece, for research purposes (Reference ID research protocol: 2226/09.09.2018), according to World Medical Association Declaration of Helsinki guidelines (2008, revised 2014). The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of the histopathological diagnoses. All lesions were classified according to the histological typing and staging criteria of the World Health Organization (WHO) Pathology Series (8). The majority of the examined lesions were derived from the tongue (n=46), whereas the rest of them were derived from the retromolar trigone (1), the floor of the mouth (n=2), and the buccal mucosa (n=1). Concerning HPV DNA status (positivity or not), the corresponding information was derived from the patients' medical records. Among them, eighteen (n=18) cases were recognized as HPV DNA-positive. HPV 16/31/53 high-risk (HR) subtypes were detected mainly by analyzing the corresponding cases. Clinicopathological data of the examined cases are demonstrated in Table I.

Table I. *Clinicopathological parameters and total E-cadherin IHC results*.

Clinicopathological parameters OSCC (n=50)		E-cadherin		*p*-Value
		LE/N	OE	
n (%)		34/50 (68%)	16/50 (32%)	
Gender				0.564
Male	44 (88%)	32/50 (68%)	12/50 (24%)	
Female	6 (12%)	2/50 (6%)	4/50 (8%)	
HPV history				0.227
Positive	18 (36%)	11/50 (22%)	7/50 (14%)	
Negative	32 (64%)	23/50 (46%)	9/50 (18%)	
Grade				
1	9 (18%)	5/50 (10%)	4/50 (8%)	0.421
2	26 (52%)	19/50 (38%)	7/50 (14%)	
3	15 (30%)	10/50 (20%)	5/50 (10%)	
Stage				**0.023**
I	18 (18%)	14/50 (28%)	4/50 (8%)	
II	21 (58%)	12/50 (24%)	9/50 (18%)	
III	11 (24%)	8/50 (16%)	3/50 (6%)	
Smoking status				
Current	38 (76%)	27/50 (54%)	11/50 (22%)	0.522
Former	12 (24%)	7/50 (14%)	5/50 (10%)	

OSCC: Oral squamous cell carcinomas; OE: Over expression (Moderate to high expression): staining intensity values ≤141 (spectrum between 81 and 141); LE/N: Low expression/Negative (loss of expression): staining intensity values ≥151 (spectrum between 151 and 189). Bold values indicate statistical significance.

Antibodies and immunohistochemistry assay (IHC). For protein anlyses we selected and applied in the corresponding OSCC tissue sections ready-to-use anti-E-cadherin (Clone NCH-38 Dako, Glostrup, Denmark) mouse monoclonal antibody. The IHC protocol for the antigen was based on 3 μm serial tissue sections. The slides were deparaffinized and rehydrated. The NBA kit (Zymed-InVitrogen, San Fransisco, CA, USA) was used. Blocking solution was applied to all slides for 10min, followed by incubation for 1 h using the monoclonal antibody at room temperature (25˚C). Following incubation with the secondary antibody for 10 min, diaminobenzidine-tetrahydrocloride-DAB (0.03%) containing 0.1% hydrogen peroxide was applied as a chromogen and incubated for 5 min. Immunostained tissue sections were counterstained, dehydrated and cover-slipped. For negative control slides, the primary antibody was omitted. IHC protocol was performed by the use of an automated staining system (I 6000 Biogenex, CA, USA). Predominantly membranous and cytoplasmic staining was considered acceptable for the marker, according to manufacturers' data sheet (Figure 1a-b). Normal appearing oral epithelia expressing the protein were used as the control group.

Digital image analysis assay (DIA). E-cadherin protein expression levels were evaluated quantitatively by calculating the corresponding staining intensity levels (densitometry evaluation) in the stained malignant tissues. We performed DIA using a semi-automated system (Microscope: CX-31, Olympus, Melville, NY, USA; Digital camera: Sony, Tokyo, Japan; Software: NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Areas of interest per tissue section were identified (5 optical fields at ×400 magnification) and filed in a digital database as snapshots. Measurements were performed by implementing a specific macro (mainly membranous and sub-membrane cytoplasmic expression for tumor cells, according to the manufacturer's datasheet for mouse monoclonal anti-E-cadherin, Clone NCH-38 Dako). Based on an algorithm, normal tissue sections (controls) were measured independently and compared to the corresponding values in malignant tissue sections. A broad spectrum of continuous grey scale values (0-255) at the RedGreenBlue (RGB) analysis was available for discriminating different protein expression levels (Figure 1c-e). Immunostaining intensity values decreasing to 0 represent a progressive over expression of the marker, whereas values increasing to 255 show a progressive loss of its staining intensity. Total results and DIA values are demonstrated in Table I.

Statistical analysis. Descriptive statistics were performed. In order to identify statistical significance, correlations between variables such as protein expression levels and clinicopathological parameters including gender, tumor grade and stage were performed using the Pearson Chi square test (χ^2), estimated along with its 99%CI and Spearman coefficient [SPSS v20 (SPSS Inc, Chicago, IL, USA)]. Two-tailed *p*-values ≤0.05 were considered statistically significant. Results and *p*-Values are described in Table I.

Results

According to IHC evaluation guidelines, the examined cases demonstrated different E-cadherin expression levels with a prominent membrane and also sub-membrane cytoplasmic pattern. Stratification regarding the marker's expression was

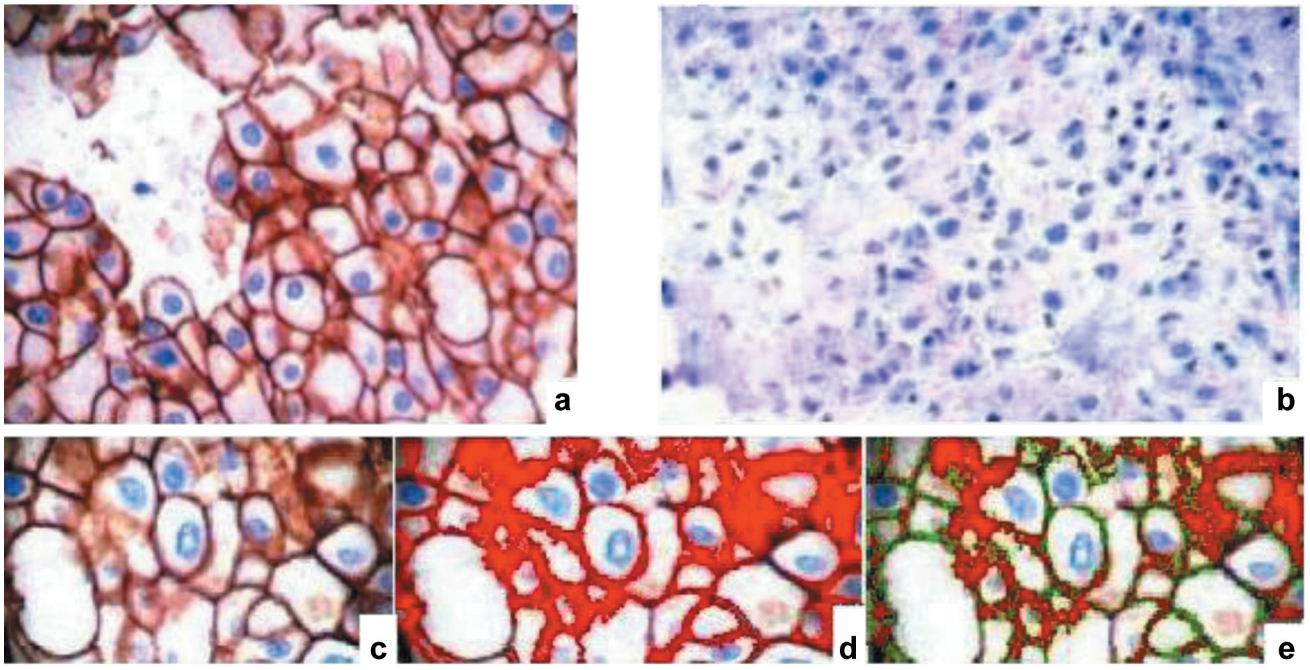

Figure 1. *E-cadherin differential expression patterns in OSCC using IHC (a) Note the membranous ring-like dense dark staining pattern (high expression). (b) Loss of protein expression (negative). (c-e) Digital image analysis procedure for evaluation of protein expression. (DAB chromogen, original magnification 100×).*

based on grouping the extracted staining intensity levels in negative, low, moderate and high values. E-cadherin altered expression (low to negative) was observed in 34/50 (68%) cases, whereas the rest of them (16/50-32%) demonstrated normal (high to moderate) expression. E-Cadherin abnormal expression was associated with the stage of the examined malignancies (p=0.023). A progressive loss of the protein expression was observed, especially in advanced stage tissue sections. No statistical significance was observed correlating overall E-cadherin expression to the grade (p=0.421), gender (p=0.564), smoking status (p=0.522) or HPV-positive/negative history (p=0.227), respectively.

Discussion

Malignant transformation of normal oral squamous epithelia is a multi-step process based on pro- and neoplastic lesions in different grades of dysplasia under the term oral leukoplakia (9, 10). In these lesions and also progressively in OSCC, a reduction of E-cadherin activity -as a significant adhesion molecule in epithelial tissue homeostasis has been observed (11, 12). Additionally, E-cadherin deregulation seems to be combined with other critical molecules in these early stages of oral carcinogenesis, including β-catenin, Desmoglein-3/γ-catenin, laminin, collagen IV, Twist, and also p53 (13-16). Concerning solid malignancies -such as breast and colorectal adenocarcinomas- E-cadherin down regulation -leading to a progressive loss of expression- is correlated to an aggressive phenotype (advanced stage and poor prognosis) (17, 18). Interestingly, in these malignancies modified expression patterns (membranous or cytoplasmic) have been observed.

In our protein analysis, low level or loss of the marker's expression was observed in a significant number of the examined malignant tissues and correlated to the stage of the examined cases. Another study has shown similar association while focusing on lymph node metastasis (N stage) leading to poor prognosis, but not at the mRNA level in OSCC patients (19). Additionally, another study co-analyzed E-cadherin and vimentin expression levels and patterns in order to find potential correlation in OSCC patients regarding epithelial-mesenchymal transition (EMT). They observed that both were strongly associated with lymph node metastasis (N stage). Interestingly, E-cadherin was correlated to advanced stages of the examined cases and for this reason should be considered a valuable tool for predicting OSCC patient outcome (20). Concerning the impact of HPV-persistent infection in E-cadherin inactivation and modified expression, there are controversial aspects (21). Some studies report E-cadherin reduced activity mediated by E6/E7 HPV oncoproteins that are responsible for PIR gene overexpression in epithelial oral and cervical cell cultures (22). Interestingly, a study group showed that targeted agents –applied for tyrosine-kinase

inhibition of growth factors receptors, such as nilotinib, dasatinib, erlotinib, and gefitinib- led to E-cadherin/ β-catenin complex activation in both HPV16-positive and HPV16-negative cells *in vitro* and this could be a future strategy in HNSCC therapeutic regimens, including OSCCs (23).

In conclusion, our study showed that E-cadherin down regulation is frequently observed in OSCCs and correlated to a progressively aggressive phenotype of the malignancy in the corresponding patients (advanced stage). However, it seems that the impact of HPV persistent infection on these patients is not a critical parameter. As E-cadherin activation and stabilization is a crucial parameter acting as a resistant mechanism to metastases in malignancies, novel targeted agents could provide support in the tumor microenvironment, preventing a potentially aggressive biological phenotype in sub-groups of OSCC patients.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Aristeidis Chrysovergis, Vasileios Papanikolaou: researchers, statistical analysis; Nicholas Mastronikolis: academic advisor, Despoina Spyropoulou, Maria Adamopoulou: academic advisor; Evangelos Tsiambas: researcher, manuscript writing; Dimitrios Peschos, Vasileios Ragos: academic advisor; Efthymios Kyrodimos: researcher, manuscript writing.

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