

# Ethnicity based variation in expression of E-cadherin in patients with squamous cell carcinoma of the oral tongue

NICOLA SGARAMELLA<sup>1-3</sup>, TORBEN WILMS<sup>4</sup>, LINDA BOLDRUP<sup>1</sup>, LOTTA LOLJUNG<sup>1</sup>, XIAOLIAN GU<sup>1</sup>, PHILIP J. COATES<sup>5</sup>, PETRA HASSELLÖF<sup>1</sup>, LUIGI CALIFANO<sup>3</sup>, LORENZO LO MUZIO<sup>6</sup>, ROBIN FÅHRAEUS<sup>1,5,7</sup>, LENA NORBERG SPAAK<sup>4</sup>, RENATO FRANCO<sup>8</sup>, GIANPAOLO TARTARO<sup>2</sup>, GIUSEPPE COLELLA<sup>2</sup>, MARIO SANTAGATA<sup>2</sup>, GIOVANNI DELL'AVERSANA ORABONA<sup>3</sup>, FABRIZIO CHIRICO<sup>2</sup>, KARIN DANIELSSON<sup>9</sup>, GIUSEPPE TROIANO<sup>6</sup>, FATIMA ARDITO<sup>6</sup> and KARIN NYLANDER<sup>1</sup>

<sup>1</sup>Department of Medical Biosciences, Umeå University, SE-901 85 Umeå, Sweden; <sup>2</sup>Multidisciplinary Department of Medical, Surgical and Dental Specialties, Second University of Naples; <sup>3</sup>Department of Neuroscience Reproductive and Dentistry Sciences, University of Naples Federico II, I-801 38 Naples, Italy; <sup>4</sup>Department of Clinical Sciences/ENT, Umeå University, SE-901 85 Umeå, Sweden; <sup>5</sup>RECAMO, Masaryk Memorial Cancer Institute, 656 53 Brno, Czech Republic; <sup>6</sup>Department of Clinical and Experimental Medicine, University of Foggia, I-71122 Foggia, Italy; <sup>7</sup>University Paris Diderot, INSERM UMRS1162, Paris 75010, France; <sup>8</sup>Dipartimento Universitario di Anatomia Patologica, Second University of Naples, Piazza Miraglia, I-801 38 Naples, Italy; <sup>9</sup>Department of Odontology, Umeå University, SE-901 85 Umeå, Sweden

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**Abstract.** The oral tongue is the most common site for tumours within the oral cavity. Despite intense research, there has been no improvement in the survival rate for patients with oral tongue squamous cell carcinoma (OTSCC) during the last decades. Differences between oral cancer patients based on ethno-geographical distribution have been reported. The present study used immunohistochemistry to evaluate commonly used markers of cancer cell phenotypes, E-cadherin,  $\beta$ -catenin and cytokeratins 5 and 19, in 120 patients with OTSCC. To evaluate the impact of ethnicity, patients from Sweden and Italy were included. A higher proportion of Swedish patients exhibited high expression of E-cadherin in their tumours ( $P=0.039$ ), and high levels of E-cadherin in Swedish OTSCC patients that had succumbed to their disease were associated with poor prognosis. These data demonstrated differences in the pathological characteristics of OTSCC between two different European populations. The findings emphasise the need to take ethnicity/geographical location of patients into account when comparing results from different studies of OTSCC.

## Introduction

The oral tongue, comprising the dorsal, lateral and ventral two-thirds anterior to the circumvallate papillae, is the most commonly tumour affected site within the oral cavity and oral tongue squamous cell carcinoma (OTSCC) is increasing in incidence (1). Moreover, amongst all oral subsites, OTSCC shows the most aggressive behaviour and poor prognosis (2,3). Despite intense research, no improvement in survival has been seen for patients with OTSCC in recent years. New knowledge on this tumour is thus of utmost importance. A complicating factor of large multicentric studies is the ethnic difference seen between patients with oral squamous cell carcinoma (OSCC) (4). To explore this in OTSCC, we analysed groups of patients from two different geographical locations; one from Sweden in Northern Europe and another from Italy in Southern Europe. We analysed E-cadherin,  $\beta$ -catenin and cytokeratins 5 and 19 in 120 OTSCCs from the two geographical locations to investigate tumour epithelial phenotypes in correlation to patient outcomes.

The epithelial calcium dependent adhesion molecule E-cadherin is associated with squamous differentiation in squamous cell carcinoma (SCC) (5) and oral SCC (OSCC), where low levels associate with poor prognosis (6), metastasis (7) and local recurrence (8). E-cadherin is a commonly used marker of epithelial cell differentiation and is expressed at different levels in individual SCCs. E-cadherin is involved in cell adhesion, being anchored to the cytoskeleton via  $\beta$ -catenin, a cytoplasmic plaque protein that maintains cell-cell adhesion in the normal oral squamous epithelium. Cytoplasmic  $\beta$ -catenin correlates with advanced stages and poor differentiation in OTSCC (9). Cytokeratins (CK) are also used as markers of epithelial differentiation and are variably

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*Correspondence to:* Professor Karin Nylander or Dr Nicola Sgaramella, Department of Medical Biosciences, Umeå University, Building 6M, 2nd floor, SE-901 85 Umeå, Sweden  
E-mail: karin.nylander@umu.se  
E-mail: sgaramellanicola12@gmail.com

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expressed in SCC. CKs are intermediate filament proteins that act in specific pair-wise combinations depending on epithelial type and degree of differentiation (10). CK5 and CK19 are expressed by basal epithelial cells (11). CK5 is paired with CK14 in squamous epithelium and CK19 can be seen both in basal squamous cells and simple epithelial cells (10). CK5 is helpful in detection of cervical micro-metastases in head and neck cancer tissue (12). CK5 is almost ubiquitously expressed in head and neck SCC (HNSCC), whereas CK19 is more frequently expressed in tumours from pharynx and larynx (10) and also correlates with poor prognosis in OTSCC (13).

## Materials and methods

**Patients.** Formalin fixed and paraffin-embedded (FFPE) biopsies from 87 consecutive patients with primary OTSCC available at Clinical Pathology, Umeå University Hospital, Sweden, and 33 patients at Dipartimento Universitario di Anatomia Patologica, Second University of Naples, Italy were included in the study. All tumours were derived from the mobile tongue. All Swedish patients belonged to the Scandinavian ethno-geographical area and all Italian patients to the South-Italian ethno-geographical area. Of the 120 patients, 60 were men and 60 women with a mean age of 63.3 years, ranging from 19-93 years. Of all tumours, 68% were localised on the lateral border of the oral tongue, 19% on the ventral side and 3% on the dorsal side. Lesions were too widespread to state the location in 10% of the patients. Most of the Swedish patients (54%) were treated with radiotherapy followed by surgery, whereas 64% Italian patients were treated by surgery only (Table I). The majority of tumours (109) had previously been analysed for HPV16, p16 and podoplanin (14,15). The mean follow-up time was 47 months (range 1-179 months). Data on survival and cause of death were obtained from the clinical files. The study was performed retrospectively on surplus tissues after diagnosis. The use of redundant tissues for this study was approved by the local Ethical Committee (dnr 01-057 and 03-201). All patient data were anonymised and the study was performed in accordance with European Union regulations and the Declaration of Helsinki. For clinical information and hospital location see Table I.

At the end of the study, 54% of patients were cancer free, either alive disease free (ADF) or disease free but dead from another cause (DDF). The remaining 46% were still affected by cancer, dead of disease (DOD), alive with disease (AWD) or dead with disease (DWD) but from a cause other than their OTSCC. Of these latter patients, 44% showed tumour relapse. Two years after treatment (available for 113 patients, 94%) 70 were alive and 43 dead, and after five years (available for 93 patients, 82%) 46 were alive and 47 dead (Table I).

**Immunohistochemistry.** Sections were pretreated in CCI-buffer (Cell Conditioner 1; Ventana Medical Systems, Inc., Tucson, AZ, USA) at 95°C for 36 min (E-cadherin,  $\beta$ -catenin), at 95°C for 64 min (CK19) and at 100°C for 36 min (CK5). Slides were then incubated with primary antibodies diluted in Ventana antibody diluent for 32 min at 36°C and detected using Ultra View Universal DAB Detection kit using a Bench Mark Ultra (Ventana Medical Systems,

Inc.). For slides stained with CK5 an extra step adding an Opti View HQ Linker (Ventana Medical Systems, Inc.) was added before detection. Slides were counterstained with Hematoxylin and Bluing Reagent (Ventana Medical Systems, Inc.). The antibody against E-cadherin (M3612, DAKO; Agilent Technologies, Inc., Santa Clara, CA, USA) was diluted 1:25, anti- $\beta$ -catenin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) 1:1,500, anti-CK-5 (Novocastra; Leica Microsystems, Inc., Buffalo Grove, IL, USA) 1:100 and anti-CK-19 (M0888, DAKO; Agilent Technologies, Inc.) 1:50.

**Scoring.** The Quick Score (QS) method was used to assess the overall levels of staining for each antibody. Staining was evaluated by combining the proportion of positive tumour cells (1=0-4%, 2=5-19%, 3=20-39%, 4=40-59%, 5=60-79% and 6=80-100%) with intensity of staining (0=negative, 1=weak, 2=intermediate and 3=strong). The final QS was achieved by multiplying these two scores, ranging between 0-18 (16). LB and KN scored E-cadherin and  $\beta$ -catenin, 88 samples stained for CK5 and CK19 were scored by LL, PH and KN and the remaining 32 samples by KN alone.

**Statistical analysis.** Tumours were grouped according to geographical distribution and level of immunostaining, where low/medium and high tumours were defined as a QS of 0-10 and 12-18 respectively. SPSS v.24 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Pearson chi-squared test was used to calculate P-values. Kaplan-Meier curves were plotted to perform survival analysis and differences among groups was explored with Log Rank (Mantel-Cox) test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Clinical data.** Data on two-year survival were available for all 87 Swedish and 26 of the 33 (79%) Italian patients, where the latter group showed a better 2-year survival ( $P=0.0005$ ).

**Immunohistochemistry.** All cases were stained for E-cadherin,  $\beta$ -catenin, CK5 and CK19 (Fig. 1). Positive staining for E-cadherin and  $\beta$ -catenin was seen in 118 of the 120 cases. All 120 cases expressed CK5, whereas only 76 (63%) contained CK19 positive tumour cells.

To study potential differences in markers based on ethnicity, patients were sub-divided into Italian and Swedish origins. Patients in the Swedish cohort showed a higher proportion of high E-cadherin tumours (QS 12-18) than Italian patients ( $P=0.039$ ; Table II).

In Swedish patients that had died of their disease (23 patients) high levels of E-cadherin correlated with poor survival ( $P=0.016$ ), whereas no correlation was seen for the six Italian patients that were dead of their disease ( $P=0.842$ ; Fig. 2). Of the 23 Swedish patients that died of disease, 17 (74%) had received preoperative radiotherapy (RT) and surgery was then performed on 10 of these; two had received post operative RT and four no RT. Of these latter four, two had received surgery and two no treatment at all. For the six Italian patients, five had received RT only and one also post operative surgery. No correlation with survival was seen for  $\beta$ -catenin, CK5 or CK19 in either group of patients.

Table I. Clinical data in relation to ethnicity.

Characteristics	No. of patients (%)		
	Swedish	Italian	Total
Sex			
Male	43 (49)	17 (52)	60 (100)
Female	44 (51)	16 (48)	60 (100)
Age, years			
≤40	14 (16)	2 (6)	16 (13)
41-65	34 (39)	11 (33)	45 (38)
>65	39 (45)	20 (61)	59 (49)
T1/T2	56 (64)	24 (73)	80 (67)
N+	19 (22)	13 (39)	32 (27)
Survival			
2-year	46/87 (53)	24/26 (92)	113 (94)
5-year	35/80 (44)	11/13 (85)	93 (78)
Treatment			
RT followed by surgery	47 (54)	1 (3)	48 (40)
RT only	17 (20)	7 (21)	24 (20)
Surgery followed by RT	8 (9)	4 (12)	12 (10)
Surgery only	12 (14)	21 (64)	33 (28)
None	3 (3)	0 (0)	3 (2)
Total no.	87	33	120

RT, radiotherapy.

## Discussion

Recent advances in diagnosis, surgical management and chemoradiotherapy regimens have only minimally improved the five-year survival for patients with OSCC (17). The present results contribute the important point that E-cadherin levels vary according to ethno-geographical area.

E-cadherin plays a key role in establishing and maintaining intercellular connections and is the main protein of adherens junctions anchoring oral epithelial cells to each other. Dysfunctional E-cadherin-mediated cell adhesion is associated with cancer invasion and metastasis. Many immunohistochemical studies have shown aberrant E-cadherin expression in SCC of the head and neck (HNSCC), and down-regulation of E-cadherin has been reported to indicate poor prognosis in OTSCC (18). Those results contrast with our data showing that higher expression of E-cadherin correlates with poor disease-free survival. However, the results from these two studies are not directly comparable as not only different antibodies were used, which is known to affect results (19), but also different methods to evaluate staining. On the other hand, our results are in concordance with a recent study of laryngeal SCC, using a similar analysis with calculation of percent as well as staining intensity of E-cadherin positive tumour cells (20). Variability in the previously published results of E-cadherin in HNSCC, OSCC and OTSCC probably also depend on sample size, sample types included and their geographical location.

Table II. Expression levels of E-cadherin,  $\beta$ -catenin, CK5 and CK19 in relation to ethnicity.

Expression	No. of patients (%)		
	Swedish, (%)	Italian, (%)	P-value
E-cadherin			
QS 0-10	54 (62)	27 (82)	0.039
QS 12-18	33 (38)	6 (18)	
$\beta$ -catenin			
QS 0-10	70 (80)	31 (94)	0.071
QS 12-18	17 (20)	2 (6)	
CK5			
QS 0-10	48 (55)	22 (67)	0.254
QS 12-18	39 (45)	11 (33)	
CK19			
QS 0-10	78 (90)	31 (94)	0.468
QS 12-18	9 (10)	2 (6)	
Total no.	87	33	120

CK, cytokeratin.

In the present study, we investigated patients from Sweden and Italy to examine the potential geographic variation in phenotype and phenotype-related clinical outcome in OTSCC patients. Better survival was seen in Italian patients, even though more of the Italian patients had nodal metastasis at diagnosis (39% vs. 22% of the Swedish patients). As survival is influenced by treatment and most Swedish patients had received neo-adjuvant radiotherapy in contrast to the Italian group, no conclusions can be drawn from the difference in survival data seen in this study. Nonetheless, differences in protein expression between Swedish and Italian patients show biological variation between tumours in different patient populations. Whilst we have not studied the underlying reasons for these variations, there are some obvious potential factors that may influence tumour phenotypes between Sweden and Italy, including the effects of different diets, where the Mediterranean diet typical for our Italian cohort is known to influence the incidence and nature of oral cancer (21,22). An alternative and non-exclusive factor would be the use of different tobacco products, where snus usage is common in Sweden and influences the oral microbiome (23,24), known to be important for OTSCC (25).

In summary, the present study shows that levels of E-cadherin vary between patients based on ethno-geographical distribution. This finding can help explain the inconsistencies seen in studies from different parts of the World that often use the same markers as surrogates for cancer cell phenotypes and their association with clinical outcome. Further studies are required to explain the reasons for the different phenotypes of OTSCC in Northern and Southern Europe, but, similar to other worldwide geographical cancer variations, factors including diet and lifestyle such as smoking habits are prime candidates to account for the differences we have observed (4).

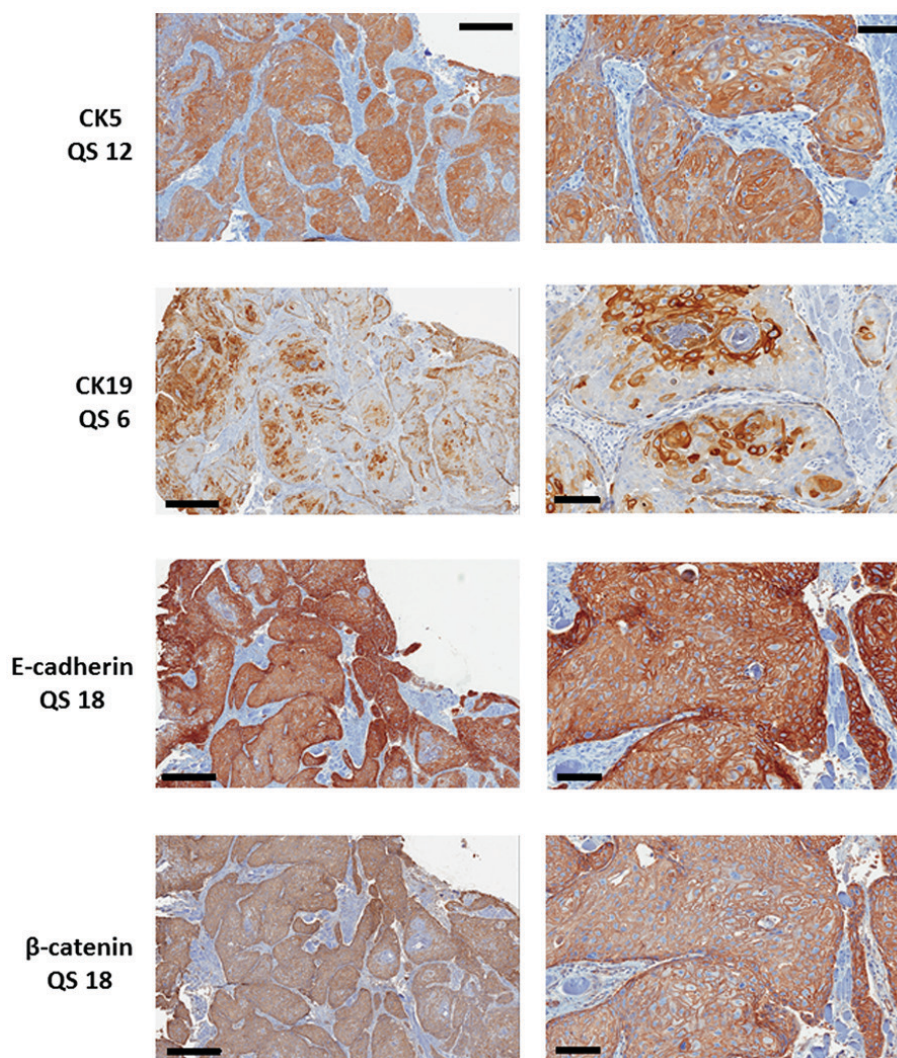


Figure 1. Examples of Quick Score and representative images of immunostaining of OTSCC samples are shown as indicated. Left scale bar, 400  $\mu\text{m}$ ; right scale bar, 100  $\mu\text{m}$ . OTSCC, oral tongue squamous cell carcinoma.

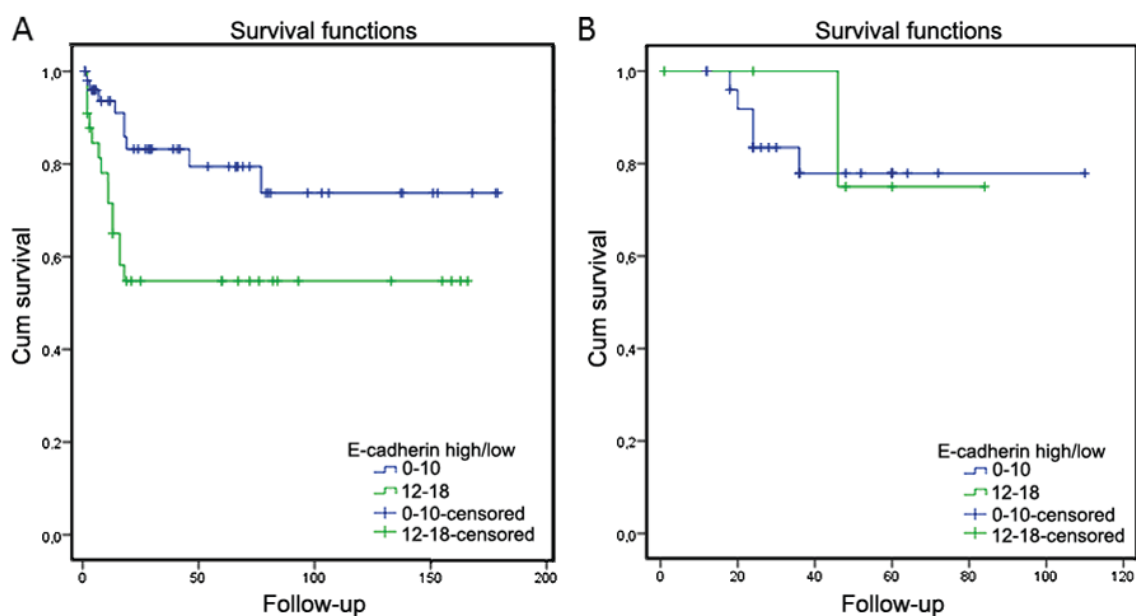


Figure 2. Kaplan-Meier curves for patients that had died of their disease, (A) Swedish (23 patients) and (B) Italian (6 patients), related to E-cadherin expression. Tumours were divided into low/medium (QS 0-10) and high (QS 12-18). Differences were investigated with Log Rank (Mantel-Cox) test showing significantly worse survival for Swedish patients with high E-cadherin tumours ( $P=0.016$ ). No correlation was seen for Italian patients ( $P=0.842$ ).

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## Availability of data and materials

The datasets used during the present study are available from the corresponding author upon request.

## Authors' contributions

NS, TW, PJC, LL, RoF, LC, LLM, LNS, ReF, GC, KD, KN designed the experiments, NS, TW, LB, LL, XG, PH, PJC, RoF, GT, FA, GC, KN performed data analysis. NS, TW, PJC, LB, LL, XG, PH, LC, LLM, RoF, LNS, ReF, GT, GC, MS, GDO, FC, KD, GT, FA, KN interpreted the data, wrote and edited the manuscript. All authors read and approved the manuscript.

## Ethics approval and consent to participate

The project was approved by the local Ethical Committee (dnr 01-057 and 03-201) and the use of surplus archived tissue after diagnosis was granted by the Ethical Committee, waiving the requirement for informed consent.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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