Analysis of the distribution and expression of some tumor invasiveness markers in palate squamous cell carcinomas

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Abstract
Oral cancer remains an important global public health issue, and despite recent diagnostic and therapeutic advances, it continues to have an unfavorable prognostic and decreased survival. Although palatal tumors represent one of the rarest locations of oral squamous cell carcinomas (SCCs), they are among the most aggressive local tumors, leaving behind important morpho-functional disabilities. In order to explain such local aggressiveness, the present study aims to investigate the immunohistochemical expression in palate SCCs of some markers known to be involved in the process of tumor invasiveness, such as Wiskott–Aldrich syndrome like (WASL), Claudin-1 (CLDN1), Integrin beta-6 (ITGB6) and c-Mesenchymal to epithelial transition protein (c-Met). We have found here a higher tumor WASL and CLDN1 reactivity in well-differentiated (G1) palate SCCs, and regardless the histological type, degree of differentiation or tumor topography, an overexpression at the invasion front, and in those palate SCC cases with muscular invasiveness and with lymph node (LN) dissemination. ITGB6 and c-Met had a higher reactivity in moderately differentiated (G2) palate SCCs, especially at the periphery of tumor proliferations, at the invasion front and in those high invasive cases and as well as in those that associated LN dissemination. All four investigated markers were also positive at the level of LN metastatic proliferations. None of the markers could statistically stratify on age group and pain, and on bone and perineural invasion while all of them statistically stratified on survival and grading. We concluded that these markers have a prognostic role allowing the identification of those cases with an unfavorable clinical evolution and decreased survival.

Keywords: immunohistochemistry, oral cavity, palate, squamous cell carcinomas, tumor invasiveness markers.

Introduction
Oral cancer is an important global public health issue, being the 16th most common neoplasm worldwide, with an estimated incidence in 2018 of about 355,000 new diagnoses from which more than 177,000 were expected to die [1]. Worldwide, there are differences in incidence and mortality, with the highest rates in developing countries, especially in India and other South/Eastern Asia regions [2, 3]. Despite the progress in research and therapy, survival has not improved significantly in the last years, continuing to be late clinically detected with poor prognosis and important effects on the quality of life of these patients [4, 5]. The most important prognostic factor in oral squamous cell carcinomas (SCCs) is the presence of lymph node (LN) metastasis, which decreases the survival rate with about 50% [6]. This aspect suggests an aggressive behavior of these tumors characterized by a propensity for rapid local invasion and spread. Invasion of cancer cells into the underlying stroma is the first step during cancer progression and metastasis. In oral SCCs, the pattern of invasion at the tumor invasive front has been shown to have predictive value for such patients [7]. Thus, in the cases with individual tumor cells in the invasive front, an increased rate of lymph LN metastases and a worsening of the prognosis were recorded [8, 9]. Although palatal tumors represent one of the rarest locations of oral SCCs, they are among the most aggressive local tumors, leaving behind important morpho-functional disabilities [10, 11].

Aim
In order to explain such local aggressiveness further thorough studies are needed, this even more as in tumor processes of invasiveness and metastasis are involved a large number of factors and signaling pathways. In this context, the present study aims to investigate the immunohistochemical (IHC) expression in palate SCCs of some markers known to be involved in the process of tumor invasiveness, such as Wiskott–Aldrich syndrome like (WASL), Claudin-1 (CLDN1), Integrin beta-6 (ITGB6) and c-Mesenchymal to epithelial transition protein (c-Met).
Materials and Methods

Palate SCC archival cases from the Laboratory of Pathology, Emergency County Hospital of Craiova, Romania, between 2010 and 2019, were retrieved. In our study were enrolled 45 cases of palate SCC, that had sufficient paraffin-embedded tissue specimens. All microscopic slides were reviewed by two oral pathologists, both to confirm the diagnosis and grading of tumor according to the World Health Organization (WHO) Classification (2005) [12]. After confirmation of the respective histopathology, 4 μm-thick seriate sections were cut and processed for immunohistochemistry. Briefly, the tissue sections were further deparaffinized, and hydrated in decreasing ethanol series. Antigen retrieval was performed by incubating the slides with 0.1 M pH 6 citrate buffer, and microwaving at 650 W for 20 minutes, and then the slides were allowed to cool to room temperature (RT) for further 30 minutes. The nonspecific binding was prevented by incubation for one hour, at RT, in 2% bovine serum albumin (BSA) solution. Then, we incubate the slides, overnight, at 4°C, with the primary antibodies, as detailed in Table 1.

Table 1 – The antibodies and immunostaining protocol

| Antibody | Clone / Manufacturer, Catalog No. | Dilution | External positive control |
|----------|---------------------------------|----------|--------------------------|
| WASL     | Rabbit, polyclonal / Sigma-Aldrich, HPA005750 | 1:100 | Colon |
| CLDN1    | Rabbit, polyclonal / Thermo Scientific, RB-9209-P | 1:100 | Breast carcinoma |
| ITGB6    | Rabbit, polyclonal / Sigma-Aldrich, HPA023626 | 1:200 | Colon |
| c-Met    | Rabbit, monoclonal / BiosB, EP1454Y | 1:20 | Breast carcinoma |

c-Met: c-Mesenchymal to epithelial transition protein; CLDN1: Claudin-1; ITGB6: Integrin beta-6; WASL: Wiskott–Aldrich syndrome like.

A three-step indirect immunoperoxidase technique was performed using the Labeled Streptavidin–Biotin 2 (LSAB2) enzyme detection system and the correspondent Dako kit (Redox, Romania – K06753). The reactions were visualized using the 3,3’-Diaminobenzidine (DAB, Dako, K3468) and for counterstaining, the Mayer’s Hematoxylin (Thermo Scientific, RB-9209-P) kit (Bio-Optica, Romania – M06002) was used. Negative control staining was carried out by substituting the primary antibodies with phosphate-buffered saline (PBS) solution. The IHC assessment was performed and agreed upon by two pathologists who were blinded to the patient data and using the semiquantitative IHC score (immunoreactive score – IRS), as described by Remmele & Stegner [13].

Clinical and histopathological (HP) data were stored in an Excel 2016 (Microsoft, USA) file. Each patient was described by several fields: gender, age group, pain, survival, tumor localization, grading, bone invasion, muscular invasion, perineural invasion, stage, tumor T score (TNM), nodule N score (TNM), and IRS for WASL, CLDN1, ITGB6, and c-Met. The IRS were computed by multiplying the qualitative score (0, 1, 2, 3 – quantifies the intensity of the reactions) with the semi-quantitative one (0, 1, 2, 3, 4 – quantifies the number of positive cells, the thresholds being 25%, 50% and 75%), thus obtaining a final score ranging from 0 to 12. Each score was independently given, then agreed on by two pathologists. For better visualization and for inferential statistics, due to a small dataset, the IRS were resampled, values ranging from 1 to 6 were considered Class A, and values higher than 6 were considered Class B. In this manner, seen as categorical data, the IRS could be stratified and viewed as contingency tables, thus, χ² (chi-squared) test or Fisher’s exact tests were used to assess the differences, a p-value <0.05 was considered statistically significant.

Seen as continuous numerical data, different stratification of the IRS permitted the computation of average scores. Thus, continuous statistics could be applied. Normal data distribution was assessed through the Shapiro–Wilk test, and since all the analyzed subgroups had a normal distribution, in order to compare the means of two or more groups Student’s t-test and analysis of variance (ANOVA), parametric tests were used; a p-value <0.05 was considered statistically significant. Correlation between numerical data was assessed through Pearson’s r correlation coefficient. The resulted r value of the test is the covariance of two variables, divided by the product of their standard deviations, a number between -1 and 1, where 1 means positive correlation, -1 negative correlation and zero means no correlation. Empirically set, the thresholds for r are: [0; 0.2] – very weak, lack of correlation, [0.2; 0.4] – weak correlation, [0.4; 0.6] – reasonable correlation, [0.6; 0.8] – high correlation, [0.8; 1] – very high correlation. Descriptive and inference statistical assessment were done in part in Excel, and in part in GraphPad Prism 9 (GraphPad, USA).

The study protocol was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania.

Results

The clinico-morphological data of our casuistry were published in a previous article [14]. Briefly, the average age was of 60.17±10.04 years, minimum 46 years, maximum 76 years, most patients were men (5.43:1) and most of the tumors developed from hard palate (24 cases). Histopathologically, at the hard palate, SCC prevailed the conventional well-differentiated tumor, and the papillary and verrucous SCC variant, while for the soft palate SCC, we noticed the predominance of moderately and poorly differentiated conventional SCC and the basaloid and acantholytic SCC forms. For hard palate lesions, 64% of the cases were diagnosed in stages II and III pTNM, while 90.47% of soft palate SCC presented in stages III and IV pTNM. Overall, about 69% of the patients did not survive more than 12 months after surgery, the number of deaths being almost double for those with tumors of the soft palate compared with those with hard palate tumors.

WASL immunoexpression

Positive immunostaining for WASL was observed in the cytoplasm of basal and suprabasal keratinocytes of normal palatine epithelium adjacent to the tumors (Figure 1A). However, in the hyperplastic and dysplastic epithelium, the reaction was much more extended towards the upper layers but decreasing progressively in intensity towards them (Figure 1B). In addition to cytoplasmatic reactivity, a nuclear reactivity was also observed, which was more evident in hyperplastic and dysplastic epithelia. WASL cytoplasmic reactivity was also observed in the minor salivary glandular tissue (reactions were much more intense in the excretory ducts compared with glandular...
acini, and among the latter the reactivity was higher in serous acini and serous part of acini mixed compared to mucous acini) (Figure 1C), in the in adipose tissue (Figure 1D), smooth muscle tissue, striated muscle tissue (Figure 1E), lymphoid tissue (more evident in the germinal centers) (Figure 1D), in vascular endothelial cells (Figure 1F), fibroblasts and macrophages.

![Image of distribution and expression of some tumor invasiveness markers in palate squamous cell cancer](image-url)

Figure 1 – WASL reactivity: (A) In cytoplasm of basal and suprabasal keratinocytes of normal palatine epithelium adjacent to the tumors; (B) Cytoplasmic and even nuclear reactivity extended towards the upper layers in the hyperplastic and dysplastic epithelium; (C) Cytoplasmic reactivity in the minor salivary glands with higher intensity in the excretory ducts compared with glandular acini; (D) Reactivity at the level of adipose tissue and in the lymphoid tissue especially in the germinal centers; (E) Reactivity in the striated muscle tissue; (F) Reactivity at the level of vascular endothelial cells, fibroblasts and macrophages. Anti-WASL antibody immunolabeling: (A–C and F) ×200; (D and E) ×100. WASL: Wiskott–Aldrich syndrome like.

The WASL reactivity was more obvious in the tumor samples, but this reactivity was heterogeneous and varying depending on the histological subtype and the status of the loco-regional tumor progression. The highest IRS were recorded in the verrucous palate SCC (IRS=10) (Figure 2A), WASL staining being more obvious at the invasive front compared with superficial part of the tumor (Figure 2B). Also, we noticed a high reactivity in the neoplastic cells from the periphery of proliferations compared to those inside the tumor, this reactivity progressively decreasing...
with the degree of neoplastic cells keratinization. This aspect was recorded in all investigated cases regardless of histological subtype. On the second place as reactivity were the well-differentiated (G1) palate SCCs (with the median IRS=8), with WASL reactivity more obvious at the periphery of tumor proliferations (Figure 2C). A slightly lower reactivity was recorded in the papillary palate SCC cases (the IRS scores were 6 and 8) but with similar pattern (Figure 2D). Then, there were the moderately differentiated (G2) palate SCCs, with the median IRS=4. The WASL reactivity was more obvious at the invasive front compared with superficial tumor part (Figure 2, E and F).

A slightly lower reactivity was recorded in those two cases of acantholytic SCC, respective IRS=4 and IRS=6. The WASL reactivity was more obvious at the level of the tumor acantholysis zones (Figure 3A), the discohesive tumor cells being both cytoplasmic and nuclear positive (Figure 3B). For the basaloid palate SCC cases, we noticed

Figure 2 – WASL reactivity in tumor samples of palate SCC: (A) The most reactive specimens were those from the patients with verrucous palate SCC subtype; (B) The reactivity was more obvious at the invasive front compared with superficial part of the tumor; (C) A slightly lower reactivity was recorded in well-differentiated (G1) palate SCC, especially at the periphery of tumor proliferations; (D) These cases were followed as reactivity by the papillary palate SCC subtype; (E and F) In these cases, reactivity was more obvious at the invasive front compared with superficial tumor part. Anti-WASL antibody immunolabeling: (A) ×50; (B, C, E and F) ×200; (D) ×100. SCC: Squamous cell carcinoma; WASL: Wiskott–Aldrich syndrome like.

Figure 3 – WASL antibody reactivity in acantholytic SCC: (A) A clear reactivity is observed at the level of tumor acantholysis; (B) The reactivity is obvious at the level of both cytoplasmic and nuclear tumor cells. Anti-WASL antibody immunolabeling: (A) ×200; (B) ×200. SCC: Squamous cell carcinoma; WASL: Wiskott–Aldrich syndrome like.
for the IRS scores values of 3 and 4 and a peculiar finding, we noticed a nuclear WASL reactivity (Figure 3C). The least reactive proved to be the poorly differentiated palate SCC cases, in which we recorded an IRS=2. In these cases, the WASL reactivity was obvious in those areas with moderately differentiated SCC morphology, while for the undifferentiated tumor zones, we noticed either weak cytoplasmic and nuclear reactivity or no reactivity (Figure 3, D and E). Also, regardless histological variant, the palate SCC cases with LN dissemination proved to be more reactive to WASL. Moreover, this reactivity was also present at the level of tumor proliferations disseminated in the LNs, but with a lower intensity (Figure 3F). In addition, in all tumor specimens that associated inflammatory stroma, more obvious in the acantholytic forms, we also noticed a WASL reactivity at the level of endothelial cells, fibroblasts, lymphocytes and macrophages.

Figure 3 – WASL reactivity in tumor samples of palate SCC: (A) A slightly lower reactivity was recorded in the acantholytic SCC cases and it was more obvious at the level of the tumor acantholysis zones; (B) Both cytoplasmic and nuclear reactivity in the discohesive tumor cells; (C) A nuclear reactivity present in the basuloid palate SCC cases; (D and E) Weak cytoplasmic and nuclear reactivity or no reactivity in the undifferentiated areas of palate G3 SCC cases; (F) Weak reactivity at the level of tumor proliferations disseminated in the lymph nodes. Anti-WASL antibody immunolabeling: (A and F) ×100; (B–E) ×400. G3: Poorly differentiated tumor; SCC: Squamous cell carcinoma; WASL: Wiskott–Aldrich syndrome like.
From the contingency tables, WASL showed significantly different average IRS for tumor localization (the Fisher’s exact test statistical value was 0.0172; the result was significant at \( p < 0.05 \)), hard and soft palate average scores being 6.29±2.22 and 4.43±2.24, respectively, Student’s \( t \)-test \( p = 0.009 \), significant at \( p < 0.05 \). A graphical representation could be seen in Figure 4. Other significant findings from the resulted contingency tables showed WASL stratification with muscle invasion (the Fisher’s exact test statistical value was 0.0005; the result was significant at \( p < 0.05 \)), and nodule N score (the chi-squared statistical value was 7.2009; the \( p \)-value was 0.027311; the result was significant at \( p < 0.05 \)).

**Figure 4 – WASL distribution on localization: Class A groups the IRS scores ranging from 1 to 6; Class B groups the IRS scores higher than 6. IRS: Immuno-reactive score; WASL: Wiskott–Aldrich syndrome like.**

Findings from the continuous data analysis showed grading stratification of WASL; average G1 score was 7.61±1.29, while G2 was 5.06±1.25 and G3 was 2.10±0.99, ANOVA \( p = 0.001 \), with individual Student’s \( t \)-test between classes \( p < 0.001 \), WASL partly stratified on gender, stage, tumor T score, with no consistent location differences. WASL did not show any significant stratification with age, pain, bone and perineural invasion.

**CLDN1 immunoexpression**

In the normal palate mucosa adjacent to neoplastic proliferation, we found CLDN1 expression more obvious in the keratinocytes from the lower half of the epithelium with predominantly membranous pattern (Figure 5A). The basal and parabasal layer were not positive to this marker. In the hyperplastic and dysplastic epithelium from the vicinity of neoplastic proliferations, we recorded an increase in CLDN1 reactivity, being observed a cytoplasmic positive reaction in the lower half of the epithelium and even a nuclear pattern in the basal and parabasal epithelial cells (Figure 5B). A strong reaction was noticed in the excretory ducts from minor salivary glands and also a weak staining in their serous acini or serous counterpart of mixed acini (Figure 5C). In addition, weak CLDN1 staining was noticed in endothelial blood vessel cells, the smooth muscle fibers and in the germinal centers of lymphoid follicles.

In tumor tissue, the reactivity was more obvious in the moderate G2 tumors (IRS=9) and acantholytic variant (IRS=9 and IRS=10), where the highest IRS were recorded. In moderate G2 tumors the CLDN1 expression was more obvious inside rather than at the periphery of tumor proliferations, and the dominant pattern was membranous with a peculiar honeycomb appearance (Figure 5D). Also, inside the tumor proliferations we noticed a cytoplasmic reactivity but with a variable intensity from one cell to another. At the invasive tumor front, the CLDN1 reactivity varied depending on the invasion pattern. Thus, in the forms with 1 and 2 invasive pattern, a reduction of the marking intensity was observed especially towards the periphery of proliferations (Figure 5E), while in the tumors with 3 and 4 invasive pattern, an intensification of reactivity was observed in all invasive cells and focally even a faint nuclear staining was noticed (Figure 5F).

In the acantholytic variant, the CLDN1 reactivity decreased progressively until it disappeared towards the lumen proliferations (Figure 6A). Inside these lumens, tumor acantholytic cells temporarily retain a granular cytoplasmic reactivity (Figure 6B). In G1 palate SCCs, the CLDN1 reactivity was a little lower (IRS=8), with more intense staining inside tumor islands than the peripheral basaloïd neoplastic cells. At the level of keratin pearls, the reactivity was either absent or had a faint thin concentric ring pattern (Figure 6C). A somewhat similar reactivity was also present in both cases of papillary palate SCCs, where we record an IRS of 8. The prevalent pattern was membranous, more obvious at the level of the neoplastic keratinocytes from inside proliferations than the basaloïd neoplastic cells from periphery and in the superficial tumor part then invasive front (Figure 6D). A slightly weaker reactivity was observed in the case of palate verrucous SCC (IRS=6) but with very similar pattern as it was noticed in papillary cases (Figure 6E). In the basaloïd palate SCC cases we recorded a weak reactivity (IRS=1 and IRS=2) restricted to center of the tumor islands with a cytoplasmic prevalent pattern (Figure 6F).

The lowest CLDN1 reactivity was noticed in G3 palate SCCs (IRS=1), with a peculiar weak nuclear pattern in the poorly differentiated cells and a higher cytoplasmic reaction in those more differentiated neoplastic cells (Figure 7A). In addition, we observed a tendency to have a higher CLDN1 reactivity in cases with advanced stages and LN dissemination. At the level of LN metastases, the CLDN1 reactivity was present but with a lower intensity then in the primary tumors (Figure 7B).

From the contingency tables, CLDN1 showed statistical stratification with muscle invasion (the Fisher’s exact test statistical value was 0.0272; the result was significant at \( p < 0.05 \)), survival (the Fisher’s exact test statistical value was 0.0001; the result was significant at \( p < 0.05 \)), and nodule N score (the chi-squared statistical value was 7.2009; the \( p \)-value was 0.027311; the result was significant at \( p < 0.05 \)). Findings from the continuous data analysis showed grading stratification of CLDN1, average G1 score was 7.68±1.25, while G2 was 5.06±1.25 and G3 was 2.10±0.99, ANOVA \( p = 0.001 \), with individual Student’s \( t \)-test between classes \( p < 0.001 \), WASL partly stratified on gender, stage, tumor T score, with no consistent location differences. WASL did not show any significant stratification with age, pain, bone and perineural invasion.

**Figure 5 – CLDN1 immunoexpression in the normal palate mucosa:**

(A) Membranous pattern; (B) Cytoplasmic staining; (C) Membranous pattern in the excretory ducts; (D) Reducing intensity from superficial to invasive front; (E) Concentric ring pattern in keratin pearls; (F) Faint nuclear staining.
Figure 5 – CLDN1 reactivity: (A) Keratinocytes reactivity from the lower half of the normal adjacent epithelium with predominantly membranous pattern; (B) A cytoplasmic positive reaction in the lower half of the hyperplastic and dysplastic epithelium and even a nuclear pattern in the basal and parabasal epithelial cells; (C) A strong reaction in the excretory ducts from minor salivary glands; (D) A strong reaction with membranous prevalent pattern in the palate G2 SCC cases; (E) A reduction in tumor reactivity was noticed in the periphery of neoplastic proliferations, especially in those cases with 1 and 2 invasive pattern; (F) A strong reaction was noticed in all invasive cells from those palate SCC with 3 and 4 invasive pattern. Anti-CLDN1 antibody immunolabeling: (A–C, E and F) ×200; (D) ×100. CLDN1: Claudin-1; G2: Moderately differentiated tumor; SCC: Squamous cell carcinoma.
Figure 6 – CLDN1 reactivity: (A) A progressive reactivity decreasing in the acantholytic variant with no reactivity towards the lumen proliferations; (B) Inside these lumens, tumor acantholytic cells temporarily retain a granular cytoplasmic reactivity; (C) A lower reactivity was noticed in the G1 SCC cases with either absent or a faint thin concentric ring pattern at the level of keratin pearls; (D) In the papillary palate SCC cases was observed a membranous reactivity pattern more obvious at the level of the neoplastic keratinocytes from inside proliferations than the basaloid neoplastic cells from periphery; (E) A slightly weaker reactivity was observed in the case of palate verrucous SCC; (F) In the basaloid palate SCC cases, we recorded a weak reactivity restricted to center of the tumor islands, with a cytoplasmic prevalent pattern. Anti-CLDN1 antibody immunolabeling: (A and C) ×100; (B and F) ×400; (D and E) ×50. CLDN1: Claudin-1; G1: Well-differentiated tumor; SCC: Squamous cell carcinoma.
Figure 7 – CLDN1 reactivity: (A) In G3 palate SCCs, we noticed a peculiar weak nuclear pattern in the poorly differentiated cells and a higher cytoplasmic reaction in those more differentiated neoplastic cells; (B) The reactivity was also present at the level of lymph node metastases but with a lower intensity than in the primary tumors. Anti-CLDN1 antibody immunolabeling: (A) ×200; (B) ×50. CLDN1: Claudin-1; G3: Poorly differentiated tumor; SCC: Squamous cell carcinoma. ITGB6 reactivity: (C) Reactivity was present in the lower part of the tumor adjacent epithelium, with membranous and cytoplasmic pattern; (D) A nuclear pattern was observed in the atypical cells from hyperplastic and dysplastic epithelium; (E) Reactivity at the level of minor salivary glands, with serous acinar cells as the most reactive, followed by the ductal epithelial cells and as the least reactive were the mucous acinar cells; (F) An intense positive reaction was observed at the level of smooth muscle fibers and at the level of endothelial cells from intra- and peritumoral blood vessels. Anti-ITGB6 antibody immunolabeling: (C and F) ×200; (D and E) ×400. ITGB6: Integrin beta-6.
ITGB6 immunostaining

In the resection margins, the normal palate epithelium far away from the tumor tissue was not positive to ITGB6, but the epithelium adjacent to tumor tissue and the hyperplastic and dysplastic epithelium presented ITGB6 reactivity. This reactivity was present in the lower part of the epithelium, with membranous and cytoplasmic pattern (Figure 7C) and even with a nuclear pattern in atypical cells (Figure 7D). We also noticed an ITGB6 reactivity at the level of minor salivary glands, with serous acinar cells as the most reactive, followed by the ductal epithelial cells and the least reactive were the mucous acinar cells (Figure 7E). An intense positive ITGB6 reaction was observed at the level of smooth (Figure 7F) and striated muscle fibers and at the level of endothelial cells from intra- and peritumoral blood vessels (Figure 7C).

The highest ITGB6 reactivity was recorded in the G2 palate SCCs (IRS=9), with a cytoplasmic and nuclear dominant pattern (Figure 8A). This reactivity was more obvious at the invasion front than inside of neoplastic proliferation and also at the invasive front compared with tumor superficial part (Figure 8B). A slightly lower reactivity was noticed in those two cases of palate papillary SCCs, in both being recorded an IRS score of 8. The reaction was more intense in the neoplastic epithelium that bordered the papillae cores and at the invasion front with a cytoplasmic prevalent pattern (Figure 8C). In the single case of verrucous palate SCC, the IRS score was about 4 and the reactivity was most cytoplasmic and more intense in the basal and parabasal neoplastic cells (Figure 8D). A decrease in reactivity has also been recorded in G3 palate SCCs (the median recorded IRS=3) where the dominant pattern was the nuclear one (Figure 8E). A similar reactivity was observed in those two basaloid palate SCCs (both cases had IRS=3), with the nuclear ITGB6 staining more prevalent at the invasive front and at the periphery of neoplastic proliferations (Figure 8F).

The ITGB6 does not seem to statistically stratify with any of the described parameters as resulted from the contingency tables. However, findings from the continuous data analysis showed survival stratification of ITGB6, survival group having an average score of 5.35±3.10, while group B of 3.57±2.38, Student’s t-test p=0.043, significant at p=0.05. Another significant stratification of the ITGB6 was on grading, average G2 score being 8.06±1.85, while for G1 and G3 2.83±1.65, and 2.80±0.42, respectively. Student’s t-test showed significant differences between G2 and G1 (Student’s t-test p=0.001, not significant at p=0.05), between G2 and G3 (Student’s t-test p=0.001, not significant at p=0.05), and no differences between G1 and G2 (Student’s t-test p=0.936, not significant at p=0.05). ITGB6 did not show any significant stratification with location, gender, age, pain, bone, muscle and perineural invasion, stage, tumor T score, nodule N score.

With intermediate reactivity between G2 and G3 palate SCCs were those two cases of acantholytic carcinomas (in both cases was recorded IRS=6) in which the prevalent ITGB6 reactivity was at the membrane level, especially in those neoplastic cells that rim the pseudoglantular lumens (Figure 9A). A membrane and cytoplasmic reactivity were also noticed in some acantholytic tumor cells. The lowest reactivity was observed in G1 palate SCCs (IRS=2) with membranous and cytoplasmic pattern (Figure 9B). This reactivity was more obvious at the periphery of neoplastic proliferation and was absent at the level of keratin pearls. Regardless histological variant, the highest IRS scores were recorded in those cases associated with LN dissemination. The neoplastic proliferation from the LN metastasis kept their ITGB6 reactivity (Figure 9C).

c-Met immunohistochemistry

In the normal epithelium, the c-Met reactivity was either absent or weak cytoplasmic positive in the basal and parabasal layers (Figure 9D). A slightly higher reactivity was observed in the hyperplastic and dysplastic epithelium next to the neoplastic proliferations with a prevalent cytoplasmic pattern extended to the intermediate layer. A slightly more intense reactivity was noticed in the minor salivary glands and especially in the epithelium of the excretory ducts with a dual cytoplasmic and membranous pattern (Figure 9E). We also observed a weak c-Met reactivity, with a predominantly cytoplasmic pattern in smooth and striated muscle fibers, nerve fibers, fibroblasts, cells from the stromal inflammatory infiltrate and endothelial cells form intra- and peritumoral blood vessels (Figure 9F).

In tumor specimens, the reactivity to c-Met was higher than in the normal mucosa or in areas with hyperplastic and dysplastic changes. The highest reactivity was noticed in the G2 palate SCCs (IRS=9). In the superficial part of tumors, the c-Met was with a prevalent membranous pattern, while at the invasion front the prevalent pattern was a cytoplasmic one (Figure 10, A and B). Regardless tumor topography, the c-Met reactivity was higher at neoplastic cells from the periphery with a dual cytoplasmic and membranous pattern, while those inside the proliferations had lower reactivity and predominantly with membranous pattern (Figure 10C). Then followed the papillary palate SCCs cases, both cases presented IRS=6 (Figure 10D). The reactivity was more obvious at the surface of papillary projections, with both cytoplasmic and membranous pattern (Figure 10E) than at the invasive front were the c-Met reactivity was weak and with cytoplasmic pattern (Figure 10F).

A slightly lower reactivity was presented by the only case of verrucous palate SCC, with the IRS=4 (Figure 11A). The reactivity was the same as in the papillary palate SCCs cases, with the tumor superficial part as more reactive than the invasion front (Figure 11, A and B). In the G3 palate SCCs was recorded an IRS=3, the reactivity been weak and with cytoplasmic prevalent pattern (Figure 11C). A higher reactivity was recorded in those areas where were present carcinomatous islands with squamous differentiation. In both cases of basaloid palate SCCs, the c-Met reactivity was also weak, the average IRS score values were about 3 and 4. The prevalent subcellular pattern was the cytoplasmic one and it was more obvious at the neoplastic cells from the periphery of basaloid proliferations (Figure 11D). For the G1 palate SCCs, we recorded an IRS=2 the reactivity being present at the periphery of tumor islands with cytoplasmic prevalent pattern, the keratin pearls being devoid of reactivity (Figure 11E). The least reactive were the two
acantholytic carcinomas, where recorded IRS values of 1 and 2. Positive reaction with cytoplasmic pattern was noticed in the outermost layers of tumor proliferations while in the acantholytic tumor cells no reactivity was recorded (Figure 11F). The reactivity was higher in those cases with LN metastases, the intensity of the reactions being even higher at the level of metastases than compared to the primary tumor. Also, we noticed moderate reactivity in those cases that associated an inflammatory stroma, and also some reactivity was present in the stromal fibroblasts and endothelial cells from intra- and peritumoral vessels.

Figure 8 – ITGB6 reactivity: (A) The highest ITGB6 reactivity was recorded in the G2 palate SCCs, with a cytoplasmic and nuclear dominant pattern; (B) The reactivity was more obvious at the periphery than inside of neoplastic proliferation and also at the invasive front compared with tumor superficial part; (C) In the palate papillary SCCs, the reaction was more intense in the neoplastic epithelium that rim the papillae cores and at the invasion front, with a cytoplasmic prevalent pattern; (D) In the verrucous palate SCC, the reactivity was most cytoplasmic and more intense in the basal and parabasal neoplastic cells; (E) A decrease in reactivity was recorded in G3 palate SCCs, where the dominant pattern was the nuclear one; (F) A similar reactivity was observed in the basoloid palate SCCs, with the nuclear staining more prevalent at the invasive front and at the periphery of neoplastic proliferations. Anti-ITGB6 antibody immunolabeling: (A and E) ×400; (B and F) ×200; (C and D) ×50. G2: Moderately differentiated tumor; G3: Poorly differentiated tumor; ITGB6: Integrin beta-6; SCC: Squamous cell carcinoma.
Figure 9 – ITGB6 reactivity: (A) In the acantholytic carcinomas, the prevalent reactivity was at the membrane level, especially in those neoplastic cells that rim the pseudoglandular lumens; (B) The slightest reactivity was observed in G1 palate SCCs, with membranous and cytoplasmic pattern; (C) The neoplastic proliferation from the lymph node metastasis kept their reactivity. Anti-ITGB6 antibody immunolabeling: (A) ×200; (B) ×100; (C) ×50. G1: Well-differentiated tumor; ITGB6: Integrin beta-6; SCC: Squamous cell carcinoma. c-Met reactivity: (D) Either absent or weak cytoplasmic positive reaction in the basal and parabasal layers of normal adjacent epithelium; (E) A slightly more intense reactivity was noticed in the minor salivary glands and especially in the epithelium of the excretory ducts, with a dual cytoplasmic and membranous pattern; (F) A weak c-Met reactivity, with a predominantly cytoplasmic pattern was also noticed in fibroblasts, cells from the stromal inflammatory infiltrate and endothelial cells form intra- and peritumoral blood vessels. Anti-c-Met antibody immunolabeling: (D) ×100; (E) ×200; (F) ×400. c-Met: c-Mesenchymal to epithelial transition protein.
Figure 10 – c-Met reactivity: (A and B) The highest reactivity was noticed in the G2 palate SCCs, with a prevalent membranous pattern in the superficial part of tumors, while at the invasion front, the prevalent pattern was a cytoplasmic one; (C) Regardless tumor topography, the reactivity was higher at neoplastic cells from the periphery, with a dual cytoplasmic and membranous pattern, while those inside the proliferations had lower reactivity and predominantly with membranous pattern; (D) A slighter reactivity was noticed in the papillary palate SCCs cases; (E) The reactivity was more obvious at the surface of papillary projections, with both cytoplasmic and membranous pattern; (F) On the contrary, at the invasive front, the reactivity was weak and with cytoplasmic pattern. Anti-c-Met antibody immunolabeling: (A) ×100; (B, E and F) ×200; (C) ×400; (D) ×50. c-Met: c-Mesenchymal to epithelial transition protein; G2: Moderately differentiated tumor; SCC: Squamous cell carcinoma.
Figure 11 – c-Met reactivity: (A) A slightly lower reactivity was presented in the verrucous palate SCC case; (B) The reactivity was the same as in the papillary palate SCCs cases, with the tumor superficial part as more reactive than the invasion front; (C) In the G3 palate SCC cases, the reactivity was weak and with cytoplasmic prevalent pattern; (D) In the basaloid palate SCC cases, the reactivity was also weak, with a prevalent cytoplasmic pattern that was more obvious at the neoplastic cells from the periphery of basaloid proliferations; (E) In the G1 palate SCC cases, the reactivity was present at the periphery of tumor islands, with cytoplasmic prevalent pattern, and the keratin pearls being devoid of reactivity; (F) The least reactive were acantholytic carcinomas, where we noticed a cytoplasmic pattern in the outermost layers of tumor proliferations, while in the acantholytic tumor cells, we did not observe any reactivity. Anti-c-Met antibody immunolabeling: (A) ×50; (B and C) ×100; (D) ×400; (E and F) ×20. c-Met: c-Mesenchymal to epithelial transition protein; G1: Well-differentiated tumor; G3: Poorly differentiated tumor; SCC: Squamous cell carcinoma.
As seen in the ITGB6, c-Met does not seem to statistically stratify with any of the described parameters as resulted from the contingency tables. Findings from the continuous data analysis showed survival stratification of c-Met, survival group a having an average score of 5.13±3.17, while group B of 2.36±1.74, Student’s t-test p=0.001, significant at p<0.05. Another significant stratification of the c-Met was on grading, average G2 score being 7.53±2.48, while for G1 and G3 1.78±0.73, and 3.20±0.36, respectively. Student’s t-test showed significant differences between G2 and G1 (Student’s t-test p=0.001, not significant at p<0.05), between G2 and G3 (Student’s t-test p<0.001, significant at p<0.05), and between G1 and G2 (Student’s t-test p=0.001, significant at p<0.05). Similar to ITGB6, the highest reactivity was at G2, only that, the time, the G1 and G2 averages scores were also different. c-Met did not show any significant stratification with gender, age, pain, location, bone, muscle and perineural invasion, stage, tumor T score, nodule N score.

Regardless of the localization or any other parameters, we have computed the correlation between the four IHC markers. The results can be seen in Table 2.

Table 2 – Immunohistochemistry–IRS correlation

|       | WASL | CLDN1 | ITGB6 | c-Met |
|-------|------|-------|-------|-------|
| WASL  | 1    | 0.47  | -0.20 | -0.30 |
| CLDN1 | 1    | 0.58  | 0.40  |       |
| ITGB6 | 1    | 0.83  | 1     |       |
| c-Met | 1    |       | 0.83  |       |

IRS: Immunoreactive score; ITGB6: Integrin beta-6; WASL: Wiskott–Aldrich syndrome protein; CLDN1: Claudin-1; c-Met: c-Mesenchymal to epithelial transition protein.

WASL has a reasonable correlation with CLDN1, having weak inverse correlations with ITGB6 and c-Met. CLDN1 has a reasonable correlation with ITGB6 and c-Met, while very high correlation is present between c-Met and ITGB6, as we expected from the similar stratification on grading. All four markers statistically stratified on survival and grading. WASL and CLDN1 stratified on muscle invasion, while only WASL stratified on location, and only CLDN1 on gender. None of the described markers stratified on age group, pain, bone and perineural invasion.

**Discussions**

In a Report of the International Agency for Research on Cancer (IARC), who used the resources of GLOBOCAN database, for 2020, it was estimated an incidence of 377,713 new cases of lip and oral cavity cancers (about 2% of all sites cancer) from which 177,757 would die (1.8% of all sites) [15]. Oral SCC is by far the most common SCC of the head and neck, but lately a decrease in its incidence was recorded. Therefore, if in 2012 the oral SCC was considered the 11th most common cancer in the world, in 2018 it became the 18th most common cancer worldwide [16]. This incidence decrease would be due to the lesser chewing habits and geographic heterogeneity, although this type of cancer continues to be the most common cancer in South Asia, South Central Asia, as well as the Pacific Islands. Among oral SCC sites, tumors developed on the tongue and buccal mucosa appear to be the most common, being followed by tumors of the lips and palate [17]. Despite earlier detection and therapeutic progress, the survival rate of patients with oral SCCs has not improved more than 3% to 4% over the last decades [18, 19]. In addition, there were notable differences in the five-year relative survival rate depending on the oral topography of these SCCs. Thus, according to the Surveillance, Epidemiology, and End Result (SEER) program database, during 2010–2016 period, it was about 92% for lip, 67% for tongue, 51% for the floor of mouth and 49% for oropharynx SCCs [20]. In fact, it is well known that SCC of the anterior two-third of the tongue, the floor of the mouth, and the lower alveolar ridge have a high risk of metastasis to loco-regional LNs and showed relatively poor prognosis [21].

In general, palate cancers are rare, the hard palate lesions represent only 5% of all oral cavity cancers [11], while soft palate SCCs accounts for about 5–12% of oropharyngeal cancers [10] and approximately 15% of head and neck SCCs [22]. Hard palate SCC has a better prognosis than other cancer localizations and is less likely to develop regional and distant metastasis. The T tumor stage has the highest impact on disease-free and overall survival in such cases. Thus, patients with ≥T2 stages, with distant metastasis and positive resection margins were reported to have poor prognosis [23]. The overall five-year survival rates for hard palate SCC were quoted around 62% to 66.3% [24, 25]. The soft palate SCCs are aggressive tumors, with rapid onset and a major negative impact regardless of tumor size at diagnosis [26]. For the T1 and T2 soft palate SCCs was reported a five-year overall survival rate about 91% to 100% and respectively 70% to 75% [27], while for the T3 and T4 lesions despite multimodal treatment the rate values dropped to 33% to 47% [28, 29]. The incidence of LN metastases for soft palate SCCs was estimated to be around 20% for T1–T2 tumors and about 60% to 70% for T3–T4 tumors [30]. In order to a better prognostic and therapeutically stratification of patients with palate SCC, we aimed to investigate the expression in such tumors of a series of biomarkers known to be involved in cancerous loco-regional invasiveness, such as WASP, CLDN1, ITGB6 and c-Met.

**Wiskott–Aldrich syndrome protein**

Wiskott–Aldrich syndrome protein (WASP) belongs to a family of five proteins involved in the remodeling of actin cytoskeleton by which they regulate cell movement, cell signaling, and cell division. More recently, the WASP family members were identified in the nucleus with direct impact on nuclear shape and chromatin organization, but they also are involved in regulating gene expression [31, 32]. Participating in the initiation of invadopodium formation, WASP play a key role in the initiation of invasion, being linked to cancer progression and metastases in some human cancers [33–35].

Our study revealed that the WASL was expressed with variable intensity in normal palate mucosa at the level of basal and suprabasal keratinocytes, in minor salivary glands, smooth and striated muscle tissue, adipocytes, endothelial cells, fibroblasts, lymphocytes and macrophages. A higher reactivity was noticed in hyperplastic and dysplastic areas of palate mucosa adjacent to the tumor.
prognostic and diagnostic markers of esophageal SCC.

In terms of the WASP protein family expression in SCCs with other locations, Wang et al. (2010), proved N-WASP cytoplasmic reactivity in esophageal SCC, and their absence in normal squamous mucosa [36]. Authors revealed significant correlation of N-WASP tumor expression and LN metastasis and pathological staging suggesting that this biomarker may play an important role in the tumorigenesis of such tumors, and it could be served as prognostic and diagnostic markers of esophageal SCC. Kimura et al. (2010), showed that enhancement of N-WASP–actin-related proteins 2/3 (Arp2/3) complex signal in primary SCC of the breast, fact that promotes the lamellipodia formation and subsequently the invasive behavior of the neoplastic cells [37]. The authors suggest a breast cancer classification based on the mechanisms of cell motility in three groups: (i) group with an N-WASP–Arp2/3 complex signal, (ii) group with WASP-family verprolin-homologous protein 2 (WAVE2)–Arp2/3 complex signal, and (iii) group without Arp2/3 complex. The authors concluded that this classification could have prognostic and therapeutic impact for patients with such tumors.

**Claudins**

Claudins, a group of membrane proteins are the major component of the tight junctions which are mainly found in endothelial or epithelial cells. So far, 24 members of this family of proteins have been described, and CLDN1 is the most studied [38]. Its role as either a tumor promoter or suppressor is controversial, both an increase and a decrease in CLDN1 expression have been shown to be associated with tumorigenesis [39]. Thus, in prostate cancer [40], breast cancer [41] and melanocytic neoplasms [42], loss of CLDN1 expression has been associated with cancer progression and invasion while in papillary thyroid carcinoma [43], oral SCC [44], ovarian cancers [45] and other human malignancies [38], CLDN1 overexpression has been associated with aggressiveness and the acquisition of high malignant phenotype.

Our investigation proved a higher reactivity in the G1 and G2 palate SCCs with a membranous prevalent pattern achieving the particular appearance of honeycomb. Also, we noticed a tendency to shift the reactivity pattern from the membranous to a cytoplasmic and even nuclear one as the degree of tumor differentiation decreases. The lowest reactivity being recorded in the basaloid and G3 palate SCCs. In addition, we noticed that the CLDN1 reactivity was more obvious inside the tumor proliferations compared to the periphery as well at the invasion front than in the upper tumor part. Beside G3 cases, the nuclear CLDN1 reactivity was also observed in the basal and parabasal layers from the hyperplastic adjacent epithelia and more extended in atypical cells from the overlying layers from the dysplastic adjacent epithelia and also at the invasion front especially in neoplastic proliferations that have invaded the underlying striated muscle tissue. A higher reactivity was also recorded in cases with advanced stages and LN dissemination, the positive CLDN1 staining being also observed at the level of metastatic neoplastic proliferations.

Regarding the reactivity CLDN1 in the normal oral mucosa, the literature data is controversial. Thus, while some authors did not detect the expression of this marker [46], other found its expression in normal squamous mucosa, especially in its intermediate layer [47–49]. Also, some authors revealed a strong CLDN1 expression in the dysplastic basal cell layer of the in-situ SCCs [44, 47, 49]. It was suggested that in the early stages of oral carcinogenesis, there is an increase in CLDN1 expression and then during progression to invasive disease there is a loss of its expression [49]. Most of authors indicated an enhanced CLDN1 expression in oral SCCs samples with membranous and/or cytoplasmic and nuclear pattern in well-differentiated areas and no reactivity in poorly differentiated regions [46–49]. Ouban & Ahmed reported differential pattern of CLDN1 expression among tumors of different sites within the oral cavity [49]. Thus, most SCCs developed in lip, cheek, and gingiva showed weak or loss CLDN1 expression while most tongue cases presented moderate to strong expression. However, Babkair et al. do not found such different CLDN1 expressions depending on the oral tumor topography [46]. The same authors showed that inhibiting CLDN1 expression will increase the cells dissociation and consequently decreases the invasiveness of SCC cells. In fact, in a previous study, Oku et al. demonstrated that increased expression of CLDN1 was associated with matrix metalloproteinase-2 (MMP-2) activation that led to an increase in invasive-ness of oral SCCs [44]. In another study, Sappayatosok & Phattarataratip found that high CLDN1 expression correlated with perineural and vascular invasion suggesting the involvement of such biomarker in the progression of oral SCCs [50]. At the same time, it was shown that the presence of CLDN1 expression in the invasive front was associated with high risk of recurrences and neck LN dissemination [51]. Given that CLDN1 can be recognized by cytotoxic T-lymphocytes [52], this molecule may serve as novel target for the oral SCCs therapy.

**Integrins**

Integrins are a family of transmembrane-type receptor proteins comprising 24 different integrin receptors, mediating signaling between the intracellular and extracellular environments and playing a major role in the cell–cell and cell–extracellular matrix adhesion achievement [53]. In addition, it was proved that they are involved in cell motility, invasion, proliferation, and migration [54]
and thus they are also involved in tumor growth and metastasis [55]. ITGB6 turned out to be a receptor for fibronectin and cytotactin, and thus this integrin can regulate cancer cell proliferation, apoptosis, migration, epithelial to mesenchymal transition, invasion, metastasis, chemoresistance and cancer cell survival [53, 56–58].

Our investigation does not find ITGB6 expression in the normal palate epithelium, but this was present in hyperplastic and dysplastic epithelium adjacent to tumor proliferations. Some reactivity was also noticed in the acini and ductal epithelium of minor salivary gland, smooth and striated muscle fibers, and endothelial cells from the intra- and peri-tumoral blood vessels. In tumor tissue, the ITGB6 expression was far higher than in normal or dysplastic adjacent epithelium. The highest ITGB6 reactivity was recorded in the G2 palate SCCs, with a cytoplasmic and nuclear dominant pattern. At the opposite pole were the cases of G1 palate SCCs with cytoplasmic and membranous prevalent pattern. With an intermediate reactivity were the cases of the acantholytic, basoloid and G3 palate SCCs. Regardless histological variant the reactivity was more obvious at the invasion front and in the neoplastic cells from the periphery of tumor proliferations. Also, we noticed a higher reactivity in those high invasive cases and as well as in those that associated LN dissemination. In addition, we recorded an ITGB6 reactivity at the level of intra- and peritumoral vessels and even in the tumor proliferations from the LN metastasis.

Literature date showed that ITGB6 was not detectable in normal oral epithelium but was highly expressed in both dysplastic stratified squamous epithelium and SCC [59, 60]. Many studies had proved that ITGB6 was overexpressed in oral epithelial dysplasia, leukoplasia, in situ carcinomas, verrucous carcinomas and oral SCCs [58, 59, 61]. Most authors were agreeing that the highest tumor reactivity was present at the invasion front [59, 62]. The involvement of this biomarker in the growth and invasiveness of oral SCCs has been demonstrated by a series of in vivo and in vitro transfection studies [63, 64]. Thus, it was found that overexpression of ITGB6 in malignant keratinocytes up-regulates MMP-9 and MMP-2 expression and promotes invasion. Moreover, Dang et al. found that ITGB6 overexpression led to activation of urokinase plasminogen activator, which in turn up-regulates MMP-9 and MMP-2 expression and promotes invasion of h6-transfected oral SCC cells [65]. Li et al., proved that neoplastic cells form oral SCCs overexpressed ITGB6 and MMP3, especially those from the periphery of invasive tumor islands simultaneously with a high rate of collagen fibers degradation in their adjacent stroma [66]. The authors concluded that such reactivity justify the invasiveness behavior of such tumors and the overexpression of these markers correlate with an unfavorable clinical prognostic and decreased survival. More recently, Yu et al. recorded a high ITGB6 reactivity in oral SCCs with LN metastasis compared with those without lymphatic dissemination, therefore, the authors proposed this marker as a predictor of LN metastasis [67].

**c-Met**

c-Met is a receptor tyrosine kinase for the hepatocyte growth factor (HGF) implicated in a series of cellular processes, such as embryogenesis, cell growth, cell differentiation, and angiogenesis [68]. However, is abnormal activation turned out to be involved in development and progression of multiple human cancers, including the head and neck SCCs [69, 70]. It was proved that its activation suppresses E-cadherin expression and increase expression of MMPs, which leads to the acquisition of an invasive phenotype by tumor cells [71, 72]. Also, it seems that c-Met positive cancer cells from head and neck SCCs have the properties of cancer stem cells, which would be responsible for the chemotherapy and radiotherapy resistance of these tumors [73]. Moreover, meta-analysis studies showed that c-Met expression represent a significant adverse prognostic marker in patients with head and neck SCCs [69, 72].

We noticed a weak reactivity in normal samples with a slightly higher reactivity in the hyperplastic and dysplastic epithelium next to the neoplastic proliferations, with a prevalent cytoplasmic pattern extended to the intermediate layer. Also, some reactivity was recorded in tumor stroma especially in those cases that associated an inflammatory infiltrate in their stroma and in the endothelium of the tumor vessels. In tumor specimens, the highest reactivity was observed in G2 palate SCCs, followed by papillary and verrucous palate HP forms. At the opposite pole, the least reactive were the acantholytic and G1 palate SCCs. An intermediate reactivity was recorded in the basoloid and G3 palate SCCs. Regardless HP forms, the tumor reactivity was higher at the cells from the periphery of tumor proliferations, and the dominant pattern was the cytoplasmic one. Also, we noticed that those cases that developed LN metastases were also the most intensely reactive, the c-Met-positive reaction being observed even with higher intensity at the level of metastatic proliferations.

In one of the first studies regarding the expression of this marker in oral SCCs, Lo Muzio et al., showed its expression in the suprabasal layers of normal oral mucosa and in the cells of minor salivary glands [74]. In the tumor specimens, they recorded a 78% positivity and 100% reactivity in the LN metastases. Also, the authors found a statistically significant positive correlation between c-Met expression and patient survival, suggesting that this marker could be used as a prognostic factor in such patients. Some later, Freudlsperger et al., found that in 82.9% of oral SCCs, c-Met was positive in more than 50% of the tumor cells and the only significant correlation recorded was a negative one between its expression and clinical tumor stage [75]. Then, Zhao et al. found a significant correlation between c-Met overexpression and advanced clinical stage, positive LN status, and recurrences [76]. Also, the authors reported that this marker play an important role in oral SCC LN metastasis as a regulator of lymphangiogenesis through an indirect pathway involving vascular endothelial growth factor-C (VEGF-C).

**Conclusions**

We showed a higher tumor WASL reactivity in G1 palate SCCs, and regardless histological type, degree of differentiation or tumor topography an overexpression at the invasion front, and in those palate’ SCC cases with muscular invasiveness and with LN dissemination.
Also, we noticed a tendency to shift the reaction pattern from the membranous to a cytoplasmic and even nuclear one as the degree of tumor differentiation decreases. For CLDN1, ITGB6, and c-Met, we observed a higher reactivity in G2 palate SCCs, especially at the periphery of tumor proliferations, at the invasion front and in those high invasive cases as well as in those that associated LN dissemination. All four investigated markers were also positive at the level of LN metastatic proliferations. From the clinical point of view none of the markers proved to statistically stratify on age group and pain. The only marker with statistical stratification on tumor localization was WAXL. Taken together, our study confirms the prognostic role of these markers allowing the identification of those cases with an unfavorable clinical evolution and decreased survival.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper. All authors read and approved the final manuscript.

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Received: January 23, 2021                      Accepted: June 22, 2021