Original Article

Pattern of invasion in squamous cell carcinomas of the lower lip and oral cavity

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ABSTRACT

Background: A number of factors may be responsible for the differences in the biologic behaviors of oral and lower lip squamous cell carcinomas (SCCs). Immunohistochemical invasion profiles have been used to detect invasion patterns like epithelial-mesenchymal-transition (EMT) and collective-cell-invasion (CCI), which have not been investigated in lower lip neoplasms. The aim of the present study was to compare the invasive phenotypes of SCCs of the lower lip and oral cavity.

Method: A total of 44 OSCCs and 37 lower lip SCCs were immunostained with E-Cadherin, N-Cadherin, and podoplanin. Based on their expression patterns, tumors were allocated to EMT, CCI or non-EMT/non-CCI categories.

Results: None of the oral SCCs showed EMT; while 5 lower lip SCCs demonstrated this phenotype. CCI was observed in 12 oral SCCs and 4 lower lip SCCs. The third group included 32 and 28 cases of oral and lower lip tumors, respectively. A significant difference in invasive phenotype was found between the two locations ($P=0.009$).

Conclusion: Oral cavity and lip tumors differ in various aspects and according to our results; the pattern of invasion may be added to these features. Between the two major invasion patterns, EMT was more prevalent in lip tumors while CCI was observed more commonly in oral neoplasms. The significance of the different expression patterns of the non-EMT/non-CCI category requires further investigation.

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1. Introduction

The ability of neoplastic cells to infiltrate, invade and penetrate adjacent tissues can ultimately result in distant and local metastases, which is a distinctive feature of cancers and a determinant of prognosis in malignancies.^{1,2} Evaluation of recognized immunohistochemical invasion profiles has been suggested for determining the infiltrative pattern of tumors.^{1,3}

Studies have shown that various invasion patterns differ in the cellular/molecular events that take place between migration initiation and the final penetration of neoplastic cells into normal surrounding tissues. When solitary cells invade the underlying stroma, they undergo epithelial-mesenchymal transition (EMT) which disrupts the expression and function of adhesion proteins so that tumor cells lose their epithelial molecules like E-Cadherins (ECads) and acquire mesenchymal markers such as N-Cadherins (NCads), also known as the cadherin switch.^{4,5} This replacement results in fundamental changes in cellular behavior: the cell loses

its ability to adhere to neighboring epithelial cells and by acquiring NCad, its capacity for migration and invasion, increases.⁶ It is noteworthy that these exact changes may not always be discernible in the invasive margin and despite the absence of ECad in tumor cells, NCad might not be expressed, leading to the suggestion of incomplete EMT in such cases.² Yet another scenario could be the preservation of cell-to-cell contacts without the development of mesenchymal markers, which puts forward the possibility of collective cell invasion (CCI).^{2,3} Podoplanin (Pod) has been suggested to play an essential role in CCI and its expression in the invasive front of tumors, may be associated with this type of invasion.⁴

Cadherins have been previously investigated in oral cavity squamous cell carcinomas (OSCCs), reporting expression rates of 37–92% for NCad and a decreased expression of ECad in the invasive margin, leading to the speculation of EMT in these tumors.^{7,8} On the contrary, a number of studies have detected limited expression of NCad in oral and oropharyngeal SCCs with 90% negativity for this marker. These investigations suggested that the probability of complete EMT in OSCC is very low.^{9,10} In line with these reports, Pod was used alongside E- and N-Cads to evaluate invasive patterns of OSCC and the results indicated the probability

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of CCI in these cancers.^{3,11} A plethora of molecular pathways and markers is involved in the invasion of cancer cells, however the most popular proteins to define EMT and CCI have been the cadherin family, mesenchymal markers like vimentin and the trans-membrane glycoprotein Pod due to its possible role in CCI.^{3,4,5,8,12}

There are limited studies on the invasion pattern of OSCC^{3,12}; however, similar investigations in lip SCCs have not been performed. OSCC typically has a worse prognosis than lower lip SCC^{13,14} which might be related to a number of factors including their different molecular expression profiles. Neoplastic invasion that is known to occur in two main patterns of EMT and CCI with specific immunohistochemical profiles^{3,4,12} is an important factor in metastasis which has a major effect on prognosis. Due to the differences in the biologic and clinical behaviors of oral and lip SCCs, various neoplastic aspects have been compared between these sites.^{13,14} Conforming to these studies we aimed to compare the invasion modes of lower lip and OSCCs, using the immunohistochemical expression of ECad, NCad, and Pod proteins. We were not able to find previous research in this regard.

2. Materials and methods

2.1. Samples

This retrospective study was performed on tissue blocks from primary, completely excised oral and lower lip SCCs retrieved from the pathology archive of our Institution from 2011 to 2015. None of the patients involved with cancer, had concomitant tumors or metastasis elsewhere and they had not received chemotherapy, radiotherapy, or any other treatment prior to surgery. Samples with significant necrosis and inadequate tissue were excluded from the investigation. The Ethics Committee of our University approved the protocol for this research.

2.2. Immunohistochemical staining procedures

Staining was performed as explained previously.³ In brief, positively charged slides were used to mount 4 μ m sections and were subsequently subjected to xylene deparaffinization and rehydration. All specimens were washed in tap water, submerged in phosphate buffered saline (PBS), immersed in 3% H₂O₂ for hydrogen peroxide blocking and rinsed in PBS. Following treatment with 10 mM citrate buffer (pH=6), antigen retrieval was performed in a microwave oven for 5 and 15 min at high and low power, respectively. After 20 min cooling at room temperature, the slides were washed with PBS and incubated with primary monoclonal mouse antihuman antibodies against Podoplanin (D2-40; Dako, Glostrup, Denmark; 1:100 dilution), NCad (6G11; Dako, Glostrup, Denmark; 1:50 dilution) and ECad (NCH-38; Dako, Glostrup, Denmark; Ready-to-Use). The EnVision System (Dako Cytomation, Glostrup, Denmark) was used for detection of bound antibody by incubation for 30 min at room temperature. Positive controls consisted of a tissue known to contain abundant lymph vessels and internal lymph vessels for Pod, normal oral mucosa for ECad and stomach tissue for NCad. These were run simultaneously with the negative controls (omission of primary antibodies) and sample slides.

2.3. Analysis of immunostained sections

According to our previous study,³ both cadherins were scored at the invasive front by assessment of staining intensity and proportion of positive tumor cells. For the former, lack of immunostaining was assigned a score of 0, while weak, moderate and strong positivity were given scores of 1–3, respectively. The

latter was classified into 0 where <1% of tumor cells stained positive; 1, if ≥ 1 –<40% were positive; 2, when ≥ 40 –<80% showed immunoreactivity and 3 in cases with ≥ 80 % staining of neoplastic cells. The multiplication of these values was rated as negative (0), weak (1–2), moderate (3–4) and strong (6–9). Negative and weak tumors were considered E- and N-cadherin negative, while moderate and strong cases were regarded positive for these markers.

A similar method was used for podoplanin. Staining intensity was classified as negative (0), weak (1), moderate (2) and strong (3). These values were multiplied by the percentage of cells with cytoplasmic and/or membranous immunostaining, which was calculated as follows: no immunoreactive tumor cells: 0; staining of 1%–10% neoplastic cells: 1; observation of 11%–30% positivity: 2; immunostaining of 51%–50% neoplastic cells: 3; immunoreactivity in 51%–80% tumor cells: 4 and staining of 81%–100% of neoplastic cells: 5. The final scores ranged from 0 to 15 with 0–3, 4–7 and >8 representing weak, moderate and strong staining, respectively. All neoplasms with strong immunoreactivity (final scores >8), were classified as Pod-positive.

A combination of these three markers was used for dividing the SCC specimens into 3 invasion phenotypes as described previously.³ ECad+/NCad–/Pod+ tumors were considered as having CCI, ECad–/NCad+/Pod– cases were regarded as demonstrating EMT and a combination of all other staining patterns was grouped together as non-EMT/non-CCI.

All analyses were performed by two oral and maxillofacial pathologists under a double-headed microscope and disagreements were resolved by consensus.

2.4. Statistical analysis

Chi-Square was used for statistical analysis and P values of less than 0.05 were considered significant.

3. Results

We retrieved a total of 37 lower lip and 44 oral SCCs from our pathology archive and none of the oral lesions were of the verrucous and exophytic subtypes. According to the immunohistochemical expression profile, the lesions were divided into three groups. The EMT group had a profile of ECad–/NCad+/Pod– that included 5 samples of lower lip SCC (14%) while this pattern was not observed in any of the OSCC cases. The CCI group with an expression pattern of ECad+/NCad–/Pod+, included 12 samples (27%) of OSCC and 4 specimens (11%) of lower lip SCC. The third group consisted of lesions that showed immunohistochemical profiles other than that of EMT and CCI as follows: 15, 3, 12 and 2 oral cavity tumors with ECad–/NCad–/Pod–, ECad–/NCad–/Pod+, ECad+/NCad–/Pod– and ECad+/NCad+/Pod– profiles, respectively. Correspondingly these profiles were found in 14, 5, 6 and 3 lower lip SCCs. In this group there were a total of 32 samples (73%) of OSCC and 28 samples (76%) of lower lip SCC. Figs. 1–3 demonstrate immunoexpression of ECad, NCad and Podoplanin, respectively.

Chi square showed a significant difference in the evaluated invasion patterns between oral cavity and lower lip SCCs (P=0.009).

4. Discussion

Researchers believe that molecular manifestations of tumors can play a major role in their infiltrative behavior. ECad, NCad, and Pod are markers that have been previously evaluated in the context of cell invasion and were selected to compare the invasive phenotypes of oral and lower lip SCC for the first time in the present study. According to our results, the invasion pattern of SCC

Fig. 1. E-Cadherin positive OSCC sample demonstrating membranous immunostaining in large tumor nests at the invasive front (original magnification $\times 200$).

Fig. 2. Representative section of a lower lip SCC with N-Cadherin immunoreactivity in small strands and single cells situated at the tumor-host interface (original magnification $\times 200$).

Fig. 3. Podoplanin immunopositivity in the outer layer of neoplastic cell groups and islands showing muscle infiltration at the invasive front of an OSCC (original magnification $\times 200$).

showed significant differences between the oral cavity and lower lip. Based on former investigations,³ the E-Cad⁺/N-Cad⁺/Pod⁺ profile was selected to represent EMT in cancers. In our samples this pattern was only detected in lower lip SCCs and none of the oral cavity tumors demonstrated this phenotype. Although some studies have put forward the possibility of EMT in OSCCs,⁷ other investigations have reported the opposite, stating that the likelihood of EMT in these tumors is very low.^{9,10} As for lip SCC, considering its anatomic location, a number of investigators have suggested that its biological behavior might be similar to cutaneous SCCs¹⁵ in some aspects and to oral, respiratory or upper aerodigestive tract SCCs in others.^{15,16}

There are reports of EMT in cutaneous SCCs¹⁷ and it seems that the invasive behavior of lower lip SCC might be similar to SCCs of the skin in this regard. In contrast to lip SCC, the CCI pattern with the E-Cad⁺/N-Cad⁻/Pod⁺ profile was more prevalent in OSCC. Other studies have also shown that OSCC is more inclined to invade adjacent structures in the form of cell groups or sheets as opposed to single cells.¹⁸

When comparing these sites, a worse outcome has been suggested for oral tumors.^{13,14} Not many studies have compared EMT and CCI in malignancies and it is not clear which pattern is more aggressive or whether they are fundamentally any different in terms of invasion into adjacent or distant tissues. Basic investigations on CCI, have suggested particular advantages of this pattern over EMT in terms of neoplastic progression. In CCI, higher levels of promigratory elements and matrix proteases exist at the invasive front, because they are secreted by multiple cells that form the groups or sheets, characteristic of CCI. Additionally, the central cells in the collective group are protected against the destructive effects of the host immune response by the outer cells. Moreover, since the migrating cells constituting the functional units are heterogeneous, we basically have a group of cells with various biological capabilities and different clonal origins which can work together to induce the diverse set of characteristics required for invasion. Finally, a group of cells which have metastasized to the vasculature can resist elimination better than single cells and therefore produce metastasis with less difficulty.^{18,19} Superficial cell smoothness/roughness has been shown to impact the pattern of cell invasion and can be different in EMT and CCI. Scanning electron microscopic and atomic force microscopic studies can help clarify the effect of this factor on invasion patterns of tumors.^{20,21} In any case, further studies are required to elucidate the clinical behavior and prognostic relevance of these invasion patterns, especially relative to different anatomic subsites.

It has been suggested that neoplastic microenvironment and the structure/arrangement of stromal elements can affect the pattern of invasion. According to previous studies, tumor stromas that are more organized have been seen to support collective migration. Tissues with higher muscle fiber content and thick collagen bundles tend to promote CCI, while fatty tissues and those with less-organized short collagen fibers are in favor of single-cell migration. The differences between the stroma of the lip and various locations of the oral cavity have not been extensively investigated, but considering that 30 of our 44 oral SCCs were located in the tongue, gingiva and palate which contain lower amounts of fatty tissue and larger numbers of muscle fibers (tongue) compared to labial tissues, maybe the impact of neoplastic microenvironment on invasion pattern resulted in a higher prevalence of CCI in the OSCCs found in the present study.²²

A considerable number of both oral and lip specimens demonstrated molecular phenotypes other than EMT and CCI. Invasion is a complex process which cannot be restricted to two patterns and has been suggested to occur as a spectrum with EMT and CCI at each end and “partial” or “incomplete” EMT in between.³ The various theories that could explain these intermediate

patterns have been thoroughly explained in our previous study.³ Other cases with immunohistochemical profiles different from EMT and CCI have been grouped as non-EMT/non-CCI in the present study. Supporting the existence of intermediate invasion patterns, Klymkowsky and Savagner²³ have described “EMT-like phenotypes” in cancer.

According to their investigation, “the loss of cell-cell adhesion does not make an EMT” and they suggested 4 phenotypes for cell invasion, two of which indicate the possibility that tumor cells lose their attachments but retain their keratin markers and that neoplastic cells demonstrate loss of polarity but maintain both cell adhesion molecules and keratin. These phenotypes could be used to explain the intermediate patterns observed in the current investigation: ECad–/NCad–/Pod– and ECad–/NCad–/Pod+ would be consistent with the former phenotype, while ECad+/NCad–/Pod– and ECad+/NCad+/Pod– could be considered in line with the latter pattern.

It should be noted that these invasive patterns may not necessarily be exclusive of one another; tumor cells have been shown to transit between invasion modes.²⁰ Therefore the immunohistochemical profile of lip and oral tumors reported in the present study, basically reflects the most common expression pattern found at that specific time in the tumor's life cycle and does not indicate that this is the only invasive mode of the examined lip and oral neoplasms.

Due to the diversity of these phenotypes, the number of cases in each group was limited in our study and therefore could not be appropriately compared between lower lip and oral cavity SCCs. One of the drawbacks of the current investigation was that we were not able to perform adequate follow-up for each patient. A larger sample size along with follow-up of each phenotype may help clarify the mechanisms involved in tumor invasion.

An interesting observation in the current investigation was that the ECad negative profile was more prevalent in lip tumors. The higher intensity of inflammation in lower lip SCCs compared to oral cavity neoplasms reported previously²⁴ and the downregulating effect of inflammation on ECad,²⁵ may help explain this finding.

5. Conclusion

Biologic, etiologic, and prognostic features of oral and lip SCCs have been evaluated and compared in many studies during the past years. Accordingly, tumors from these sites have shown considerable differences in many aspects.^{23,24} Based on our results it seems that the neoplastic invasion pattern may also be considered as one of the factors that vary between these locations. The significance of this finding and its particular effect on clinical behavior of lip and oral SCCs needs to be elucidated in future studies.

Conflict of interest statement

The authors have none to declare.

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