Loss of 5-Hydroxymethylcytosine and TET2 in Oral Squamous Cell Carcinoma

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Abstract. *Aim: Epigenetic modifications, such as DNA methylation, are considered important in the regulation of target genes in cancer development. 5-Hydroxymethylcytosine (5hmC) was recently discovered to be related to the process of malignant transformation. The influence of DNA methylation in oral squamous cell carcinomas (OSCC) is not fully- understood. Therefore, the aim of the present study was to investigate the DNA methylation pattern in OSCC compared to healthy oral epithelium. Materials and Methods: Oral mucosal samples from patients with OSCC (n=15) and healthy mucosa (n=12) were analyzed using immunohistochemistry with antibodies against 5hmC, 5mC and ten-eleven-translocation-2 (TET2). Results: A significant decrease in 5hmC and TET2 expression was found in OSCC compared to healthy oral epithelium. In contrast, there was a significant increase in 5mC expression in OSCC compared to healthy epithelium. Conclusion: Our results indicate that loss of 5hmC is an epigenetic event of OSCC.*

Oral cancer constitutes approximately 2% of all cancers worldwide (1). In 2008, 263,900 new cases of oral cancer were estimated and the mortality rate was estimated at 128,000 (2). The most common histopathological type of cancer in the oral cavity is squamous cell carcinoma, representing more than 90% of all oral cancer (3).

Carcinogenesis is a multi-step process that requires several different changes in the genome (4). These changes are not only caused by changes in DNA sequences but are also due to epigenetic alterations resulting in abnormal gene expression (5-7). The definition of epigenetics is heritable changes in gene expression that are not accompanied by changes in DNA sequence (7). One of the main epigenetic mechanisms is DNA methylation of cytosine (5mC) (5, 6). This modification alters the configuration of the DNA, resulting in an alteration of gene expression. Dysregulation in the DNA methylation pattern leads to abnormal gene expression and is an epigenetic hallmark of cancer (8).

Recent studies have shown that the ten-eleven-translocation (TET) family of enzymes catalyzes the conversion of 5mC into 5-hydroxymethylcytosine (5hmC) (9). 5hmC is known as the sixth base of the genome and is related to the process of malignant transformation and suggested to be an intermediate in active DNA de-methylation (9,10). In addition, TET enzymes also have a central role in the downstream process of de-methylation of 5hmC into unmethylated cytosine, resulting in DNA hypomethylation (11).

A significant loss of 5hmC has been reported in several types of cancer, such as melanoma, colorectal, breast, liver, lung and pancreatic cancer (8, 10, 12-15). 5hmC has been suggested to be a biomarker for recognition of and progression in melanoma. In addition, it has been reported that down-regulation of the TET2 enzyme could be a mechanism causing the loss of 5hmC seen in melanoma (12).

At present, there is a lack of knowledge regarding the distribution and importance of 5hmC and its related enzyme in OSCC. Therefore, the aim of this study was to investigate the alterations in 5mC, 5hmC and TET2 expression in OSCC compared to healthy oral epithelium.

Materials and Methods

Samples. Paraffin-embedded biopsies from 15 patients diagnosed with OSCC were obtained from the archives of the Department of Oral Medicine and Pathology at Gothenburg University and Department of Oral and Maxillofacial Surgery at the Uppsala

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University. In addition, 12 healthy oral mucosa samples were also collected from the archives of the Department of Oral Medicine and Pathology at Gothenburg University. The study was approved by the Ethical Board at the University of Gothenburg, Sweden, with approval number Dnr: T427-13.

Immunohistochemistry. Four-micrometer thick sections were de-paraffinized and incubated in DIVA Decloaker antigen retrieval solution (Biocare Medical, Concord, CA, USA) at 60˚C overnight. The sections were incubated with a primary antibody for 30 min, followed by incubation with Envision horseradish peroxidase (HRP)-labeled polymer (DakoCytomation A/S, Glostrup, Denmark) for 30 min. Positively-stained cells were detected using 3,3'-Diaminobenzidine (DAB) substrate (DakoCytomation). The sections were counterstained using hematoxylin. The antibodies used were against TET2 (ab127416, 1:100; Abcam, Cambridge, UK), 5mC (clone 33D3, 39649; 1:50) and 5hmC (39769; 1:500; Active Motif, Carlsbad, CA, USA). Omission of primary antibodies served as a negative control.

Histological analysis. Quantitative analysis was performed in three areas for each biopsy. The areas were randomly selected within the tumor tissue in OSCC and in the epithelium in the healthy mucosa. Each selected area varied between approximately 50,000-240,000 μm^2, depending on size of epithelium and tumor. Digitalized images were obtained using a light microscope (Leitz Wetzler; Leica Microsystems, Wetzlar, Germany) with a Leica DC100 camera (Leica Microsystems). Counting of positively-stained cells was carried out with use of CellSense computer software (Olympus, Hamburg, Germany) at ×100 magnification. Results are expressed as the number of positively stained cells/mm^2.

Statistical analysis. Median values and ranges were calculated for each variable. Differences between groups were analyzed with the Mann–Whitney *U*-test, using the statistical software SPSS v17 (SPSS Inc., Chicago, IL, USA). *p*-Values <0.05 were considered significant.

Results

The result of the immunohistochemical analysis revealed a high number of cells positively staining for 5hmC in healthy oral epithelium. The same was found for TET2, but with slightly weaker staining. However, in the OSCC samples, the number of 5hmC- and TET2-positive cells was significantly reduced (Figure 1).

A significant decrease in 5hmC and TET2 expression was found in OSCC compared to healthy oral epithelium ($p<0.001$) (Figure 2). In contrast, the expression of 5mC was increased in OSCC compared to healthy oral epithelium ($p<0.05$).

Discussion

Changes in methylation and hydroxymethylation pattern have been suggested to be epigenetic hallmarks of cancer. At present, there is lack of knowledge about these processes in tumor development, and particularly in the field of OSCC. In the present study, we investigated the presence of 5mC and 5hmC, as well as of the TET2 enzyme, in OSCC and healthy oral epithelium. Using immunohistochemistry, we showed that 5hmC and TET2 expression in OSCC is significantly lower compared to those in healthy epithelium. In contrast, the expression of 5mC was higher in OSCC than in healthy epithelium, although showing large variation between the samples.

Our results indicate that loss of 5hmC is an epigenetic event in OSCC. Interestingly, the distribution and expression of TET2 correspond to the expression pattern of 5hmC, indicating a possible role for TET2 in the loss of 5hmC expression during carcinogenesis in oral squamous epithelial cells. This is in line with the findings by Lian and co-workers, who reported that down regulation of TET2 is one mechanism contributing to the loss of 5hmC in melanoma (12). They also showed that increasing TET2 resulted in re-establishing in the 5hmC level in melanoma cells *in vitro* and in a less aggressive tumor phenotype in an animal model (12).

Our study shows that the loss of 5hmC in OSCC is not a result of decreased levels of 5mC, the substrate of 5hmC. In a study on melanoma, it was suggested that a decrease of 5hmC results in an accumulation of 5mC in melanoma (12). These findings were further supported by Gambichler *et al*. who showed significantly reduced levels of 5hmC but no significant reduction of 5mC in melanoma compared to benign nevi (16). The increased level of 5mC seen in OSCC in the present study may not only be caused by accumulation of 5mC due to non-conversion into 5hmC, but also a result of loss of function of 5hmC, since 5hmC is suggested to play a role as an intermediate in the process of DNA de-methylation (10).

The significant loss of 5hmC found in several different types of cancer, now also including OSCC, indicate a general epigenetic event in malignant transformation rather than a specific event in a specific type of cancer (16). Loss of 5hmC has been suggested as a diagnostic biomarker for melanoma (12). The findings in the present study indicate that this event may also have a diagnostic value in OSSC.

In order to improve the prognosis of OSCC, an early diagnosis is of most importance (17). The majority of the OSCCs are preceded by pre-malignant lesions in the oral mucosa (18, 19). These lesions are clinically visible and may develop into OSCC (20, 21). There is a lack of well-established clinical and histopathological criteria identifying lesions at high risk of malignant transformation (22). Today, the level of dysplasia in pre-malignant lesions is used to assess the risk of malignant transformation. However, the level of dysplasia is a very subjective histological assessment and it is, therefore, of great importance to identify markers specifically associated with high malignant transformation. Additional studies are needed to investigate if pre-malignant lesions express low levels of 5hmC, and if they do whether they may have a higher risk of malignant transformation.

The present study indicates that loss of 5hmC is an epigenetic event in OSCC, and that the expression of TET2 enzyme corresponds to the expression of its product, 5hmC.

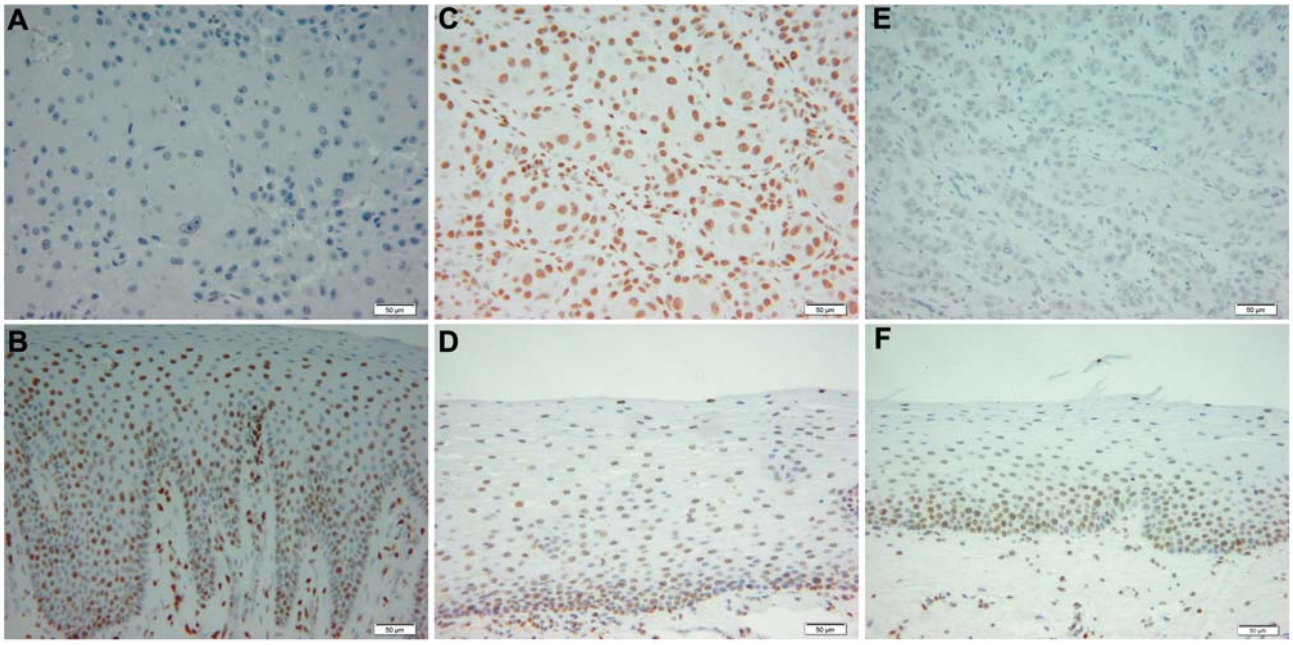

Figure 1. *5-hydroxymethylcytosine-positive cells in (A) oral squamous cell carcinoma (OSCC) and (B) healthy mucosa. 5-methylcytosine-positive cells in (C) OSCC and (D) healthy mucosa. Ten-eleven-translocation-2-positive cells in (E) OSCC and (F) healthy mucosa. Positive cells stain brown. The bars in the lower right corners correspond to a distance of 50 μm. Magnification ×100.*

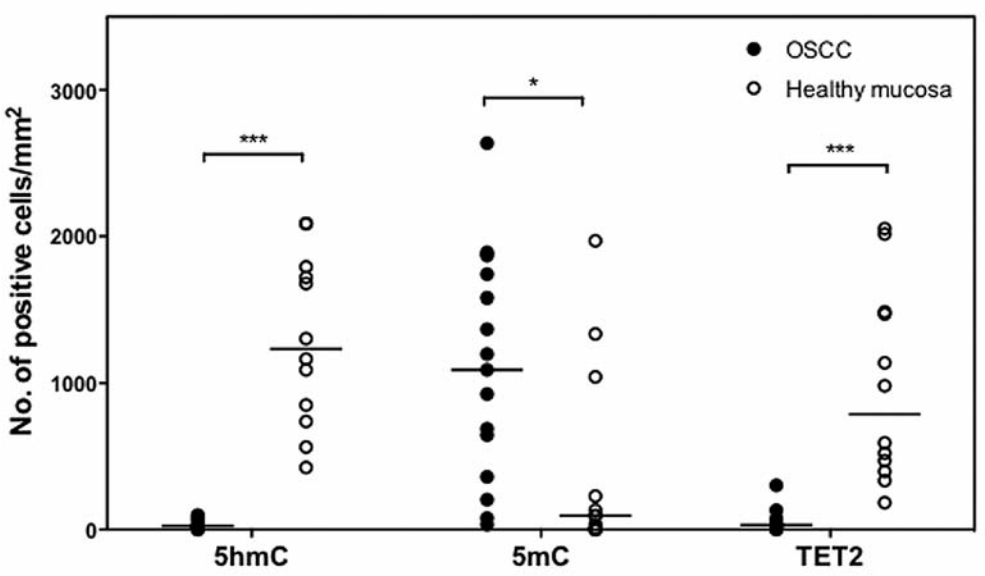

Figure 2. *Number of 5-hydroxymethylcytosine (5hmC)-, 5-methylcytosine (5mC)- and ten-eleven-translocation 2 (TET2)-positive cells/mm^2 in oral squamous cell carcinomas (OSCC) and in healthy oral mucosa. The graph shows the distribution and median values. *p<0.05, ***p<0.001.*

In summary, 5hmC and TET2 may have an important function in the epigenetic regulation of OSCC development.

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References

1 Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer *127*: 2893-917, 2010.

2 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin *61*: 69-90, 2011.

3 Feller LL, Khammissa RR, Kramer BB and Lemmer JJ: Oral squamous cell carcinoma in relation to field precancerisation: Pathobiology. Cancer Cell Int *13*: 31, 2013.
4 Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. Cell *144*: 646-647, 2011.
5 Berdasco M and Esteller M: Aberrant epigenetic landscape in cancer: How cellular identity goes awry. Dev Cell *19*: 698-711, 2010.
6 Jones PA and Liang G: Rethinking how DNA methylation patterns are maintained. Nat Rev Genet *10*: 805-811, 2009.
7 Sharma S, Kelly TK and Jones PA: Epigenetics in cancer. Carcinogenesis *31*: 27-36, 2010.
8 Kudo Y, Tateishi K, Yamamoto K, Yamamoto S, Asaoka Y, Ijichi H, Nagae G, Yoshida H, Aburatani H and Koike K: Loss of 5-hydroxymethylcytosine is accompanied with malignant cellular transformation. Cancer Sci *103*: 670-676, 2012.
9 Münzel M, Globisch D and Carell T: 5-Hydroxymethylcytosine, the sixth base of the genome. Angew Chem Int Ed Engl *50*: 6460-6468, 2011.
10 Kraus TF, Globisch D, Wagner M, Eigenbrod S, Widmann D, Münzel M, Müller M, Pfaffeneder T, Hackner B, Feiden W, Schüller U, Carell T and Kretzschmar HA: Low values of 5-hydroxymethylcytosine (5hmC), the 'sixth base', are associated with anaplasia in human brain tumors. Int J Cancer *131*: 1577-1590, 2012.
11 Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C and Zhang Y: TET proteins can convert 5-metylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science *333*: 1300-1303, 2011.
12 Lian CG, Xu Y, Ceol C, Wu F, Larson A, Dresser K, Xu W, Tan L, Hu Y, Zhan Q, Lee CW, Hu D, Lian BQ, Kleffel S, Yang Y, Neiswender J, Khorasani AJ, Fang R, Lezcano C, Duncan LM, Scolyer RA, Thompson JF, Kakavand H, Houvras Y, Zon LI, Mihm MC Jr, Kaiser UB, Schatton T, Woda BA, Murphy GF and Shi YG: Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. Cell *150*: 1135-1146, 2012.
13 Jin SG, Jiang Y, Qiu R, Rauch TA, Wang Y, Schackert G, Krex D, Lu Q and Pfeifer GP: 5-Hydroxymethylcytosine is strongly depleted in human cancers but its levels do not correlate with IDH1 mutations. Cancer Res *71*: 7360-7365, 2011.
14 Yang H, Liu Y, Bai F, Zhang JY, Ma SH, Liu J, Xu ZD, Zhu HG, Ling ZQ, Ye D, Guan KL and Xiong Y: Tumor development is associated with decrease of TET gene expression and 5-methylcytosine hydroxylation. Oncogene *32*: 663-669, 2013.
15 Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, An J, Lamperti ED, Koh KP, Ganetzky R, Liu XS, Aravind L, Agarwal S, Maciejewski JP and Rao A: Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature *468*: 839-843, 2010.
16 Gambichler T, Sand M and Skrygan M: Loss of 5-hydroxymethylcytosine and ten-eleven translocation 2 protein expression in malignant melanoma. Melanoma Res *23*: 218-220, 2013.
17 Bremmer JF, Brakenhoff RH, Broeckaert MA, Beliën JA, Leemans CR, Bloemena E, van der Waal I and Braakhuis BJ: Prognostic value of DNA ploidy status in patients with oral leukoplakia. Oral Oncol *47*: 956-960, 2011.
18 Pitiyage G, Tilakaratne WM, Tavassoli M and Warnakulasuriya S: Molecular markers in oral epithelial dysplasia: Review. J Oral Pathol Med *38*: 737-752, 2009.
19 World Health Organization Collaborating Centre for Oral Precancerous lesions. Definition of leukoplakia and related lesions: An aid to studies on oral precancer. Oral Surg Oral Med Oral Pathol *46*: 518-539, 1978.
20 Holmstrup P, Vedtofte P, Reibel J and Stoltze K: Long-term treatment outcome of oral premalignant lesions. Oral Oncol *42*: 461-474, 2006.
21 van der Waal I: Potentially malignant disorders of the oral and oropharyngeal mucosa: Terminology, classification and present concepts of management. Oral Oncol *45*: 317-323, 2009.
22 Brennan M, Migliorati CA, Lockhart PB, Wray D, Al-Hashimi I, Axéll T, Bruce AJ, Carpenter W, Eisenberg E, Epstein JB, Holmstrup P, Jontell M, Nair R, Sasser H, Schifter M, Silverman B, Thongprasom K, Thornhill M, Warnakulasuriya S and van der Waal I: Management of oral epithelial dysplasia: A review. Oral Surg Oral Med Oral Pathol Oral Radiol Endod *103*: (Suppl S19) e1-2, 2007.

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