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

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Running title:
5hmC and TET2 in Oral Cancer

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Abstract

Background: Epigenetic modifications, such as DNA methylation, are considered important in the regulation of target genes in cancer development. 5-hydroxymethylcytosine (5hmC) is recently discovered and connected 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.

Material and methods: Oral mucosa samples from patients with OSCC (n = 15) and healthy mucosa (n=12) were analyzed using immunohistochemistry with antibodies against 5hmC, 5mC and TET2.

Results: A significant decrease in OSCC compared to healthy oral epithelium was found for 5hmC and TET2. In contrast, there was a significant increase in 5mC in OSCC compared to healthy epithelium.

Conclusion: Our results show that loss of 5hmC is an epigenetic event of OSCC.
Introduction

Oral cancer constituting approximately 2 % of all cancers in the world (Ferlay et al. 2010). In 2008, 263,900 new cases of oral cancer were estimated worldwide and the mortality rate was estimated to 128,000 (Jemal et al. 2011). The most common histopathological type of cancer in the oral cavity is squamous cell carcinoma, representing more than 90 % of all oral cancers (Feller et al. 2013).

Carcinogenesis is a multistep process that requires several different changes in the genome (Hanahan & Weinberg 2011). These changes are not only caused by changes in the DNA sequences but are also due to epigenetic alterations resulting in abnormal gene expression (Berdasco et al. 2010, Jones & Liang 2009, Sharma et al. 2010). The definition of epigenetics is heritable changes in gene expression that are not accompanied by changes in DNA sequence. One of the main epigenetic mechanisms is DNA methylation (5mC) (Berdasco et al. 2010, Jones & Liang 2009). This modification alters the configuration of the DNA resulting in alteration of gene expression. Dysregulation in the DNA methylation pattern leads to abnormal gene expression and is an epigenetic hallmark of cancer (Kudo et al. 2012).

Recent studies have shown that the Ten-Eleven-Translocation (TET) family of enzymes catalyzes the converting of 5mC into 5-hydroxymethylcytosine (5hmC) (Münzel et al. 2011). 5hmC is known as the sixth base of the genome and is connected to the process of malignant transformation and suggested to be an intermediate in active DNA demethylation (Münzel et al. 2011, Kraus et al. 2012, Thahiliani et al. 2009). In addition, TET enzymes also have a central role in the
downstream process of demethylation of 5hmC into unmethylated cytosine resulting in DNA hypomethylation (Ito et al. 2011).

A significant loss of 5hmC has been reported in several types of cancer, i.e. melanoma, colorectal-, breast-, liver-, lung- and pancreas cancer, as well as in myeloid cancers and brain tumors (Lian et al. 2012, Kudo et al. 2012, Jin et al. 2011, Yang et al. 2012, Kraus et al. 2012, Ko et al. 2010). 5hmC has been suggested to be a mark for recognition and progression in melanoma. In addition it was reported that down-regulation of the TET2 enzyme could be a mechanism causing the loss of 5hmC seen in melanoma (Lian et al. 2012).

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.


Material and Methods

Patients

Paraffin embedded biopsies from 15 patients diagnosed with oral squamous cell carcinoma were obtained from the archives of the Department of Oral Medicine and Pathology at Gothenburg University, Sweden, and Department of Oral and Maxillofacial Surgery at Uppsala University, Sweden. In addition, 12 healthy oral mucosa samples were also collected from the archive 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.

Immunohistochemistry

Four µm thick sections were deparaffinized and incubated in DIVA antigen retrieval solution (Biocare Medical, Concord, CA) at 60°C overnight. The sections were incubated with a primary antibody for 30 minutes followed by incubation with Envision HRP labeled polymer (DakoCytomation A/S, Glostrup, Denmark) for 30 minutes. Positive cells were detected using DAB substrate (DakoCytomation). The sections were counterstained using Haematoxylin. The antibodies used were TET2 (ab127416, Abcam, Cambridge, UK) 1:100, 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 in each biopsy. The areas were randomly selected within the tumor tissue in OSCC and in the epithelium in the healthy mucosa. 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 made with use of the CellSense computer software at 100 x magnification. Results are expressed as number of positively stained cells/mm².

**Statistical analysis**

Median values and range were calculated for each variable. Differences in positively stained cells were analyzed using the Mann–Whitney U–test (SPSS). *P*-values < 0.05 were considered significant.
Results

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

Microscopic analysis of OSCC for 5hmC expression resulted in a median value of 2.6 positive stained cells/mm$^2$ (range 0 – 100) (Table 1). The corresponding number for healthy oral epithelium was 1250 (range 427 – 2086) ($P < 0.001$). Staining for 5mC resulted in a median of 1091 positive cells/mm$^2$ (range 48 – 2622) and 100 positive cells/mm$^2$ (range 0 – 1967) for OSCC and healthy epithelium respectively ($P < 0.05$). TET2 positive stained cells showed a distribution similar to the distribution of 5hmC positive cells. The median number of positive stained cells for the TET2 enzyme was 33 cells/mm$^2$ in OSCC (range 0 – 302) and 773 cells/mm$^2$ (range 180 – 2099) in healthy tissue ($P < 0.001$) (Fig. 2).
Discussion

Changes in methylation and hydroxymethylation pattern have been suggested to be epigenetic hallmarks of cancer. At present there is lack of knowledge of 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 the TET2 enzyme, in OSCC and healthy oral epithelium. Using immunohistochemistry we show that there is a significant decrease in 5hmC and TET2 expression in OSCC compared to healthy epithelium. In contrast, the expression of 5mC is higher in OSCC than in healthy epithelium but with 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 correspondance with the findings by Lian and co-workers (2012), who reported that down regulation of TET2 is one mechanism contributing to the loss of 5hmC in melanoma. Lian and co-workers (2012) 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.

The results in the present study shows that the loss of 5hmC in OSCC is not caused by decreased levels of 5mC, which is the substrate of 5hmC. In a similar study on melanoma it was suggested that decreased levels of 5hmC results in an accumulation of 5mC in melanoma (Lian et al 2012). These findings where further supported by
Gambichler et al. (2013) who showed significantly reduced levels of 5hmC but no significant reduction of 5mC in melanoma compared to benign nevi. The increased levels of 5mC seen in OSCC in the present study may not only be caused by accumulation of 5mC due to non-converting to 5hmC, but also a result of loss of function of 5hmC as its suggested role as an intermediate in the process of active DNA de-methylation.

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 (Gamblicher et al 2013). Loss of 5hmC as a diagnostic biomarker for melanoma has been suggested (Lian et al. 2012), and the results in the present study indicate that this event also may have a diagnostic value in OSSC.

In order to improve the prognosis of OSCC an early diagnosis is of most importance. The majority of the OSCC are preceded by premalignant lesions in the oral mucosa (Gayani Pitiyage). These premalignant lesions are clinically visible and may develop into OSCC (Holmstrup et al. 2006, van der Waal 2009). There is a lack of well-established clinical and histopathological criteria identifying lesions with high risk for malignant transformation (Brennan et al. 2007). Today, the level of dysplasia in premalignant lesions is used to assess the risk for malignant transformation. However, the level of dysplasia is a very subjective histological assessment and it is therefore of great importance to identify markers associated with high malignant transformation. Further studies are needed to investigate if premalignant lesions express low levels of
5hmC, and if premalignant lesions that express low 5hmC levels have a higher risk of malignant transformation.

The results of the present study show that loss of 5hmC is an epigenetic event in OSCC as well as that the expression of TET2 enzyme corresponds to the expression of its product, 5hmC. In summary, it may be suggested that 5hmC and TET2 may have an important function in the epigenetic regulation of OSCC development.
References


Münzel M, Globisch D and Carell T (2011). 5-Hydroxymethylcytosine, the sixth base of the genome. *Angew Chem Int Ed* 50; 6460-6468.


Figures

Fig 1. 5hmC-positive cells in OSCC (A) and healthy mucosa (B). 5mC-positive cells in OSCC (C) and healthy mucosa (D). TET2-positive cells in OSCC (E) and healthy mucosa (F). Positive cells stain brown.
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<th>5hmC</th>
<th>TET2</th>
<th>5mC</th>
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<tr>
<td>OSCC</td>
<td>26 (0-100)</td>
<td>33 (0-302)</td>
<td>1091 (48-2622)</td>
</tr>
<tr>
<td>Healthy</td>
<td>1250 (427-2086)</td>
<td>773 (180-2099)</td>
<td>100 (0-1967)</td>
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Table 1. The table shows median values and ranges in positive stained cells/mm² for 5hmC, TET2 and 5mC in OSCC and healthy oral epithelium.
Fig 2. Amount of 5hmC, 5mC and TET2 positive cells/mm². The graph shows the distribution and median values. *p<0.05, ***p<0.001.