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Original article

The level and clinical significance of 5-hydroxymethylcytosine in oral squamous cell carcinoma: An immunohistochemical study in 95 patients

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ABSTRACT

Accumulating evidence has revealed that aberrant abundance of 5-hydroxymethylcytosine (5hmC) is critically involved in tumorigenesis. The aim of the present study was to investigate the level of 5hmC in primary oral squamous cell carcinoma (OSCC) and determine its clinical significance as well as prognostic value in predicting patients' outcomes. The expression levels of 5hmC in 95 human OSCC samples and 24 normal oral mucosa were evaluated by immunohistochemical staining. Moreover, the associations between the expression status of 5hmC and several clinicopathological parameters as well as patients' survival were further statistically assessed. Our immunohistochemical results revealed that 5hmC was significantly downregulated in a significant fraction of OSCC as compared their normal counterparts. However, elevated 5hmC level was found to be significantly associated with pathological grade and cervical node metastasis with *P*-values of 0.0239 and 0.0041, respectively. Results from Kaplan-Meier cumulative survival analyses indicated that high expression of 5hmC in OSCC was significantly associated with decreased overall survival, disease-free and disease-specific survival as compared to those with low 5hmC (Log-rank, $P = 0.0210$, 0.0313, 0.0415, respectively). Furthermore, the univariate and multivariate survival analyses further identified the expression status of 5hmC as an independent prognostic factor affecting patients' survival. Taken together, our results reveal a significant decrease of 5hmC level in a large subset of OSCC. However, high level of 5hmC associates with tumor aggressive features and unfavorable prognosis in a fraction of OSCC patients.

1. Introduction

Oral squamous cell carcinoma (OSCC) represents one of the common solid cancers worldwide with well-known etiologic factors and relatively high mortality. Despite considerable advances in the treatment of OSCC in the last decades, the prognosis for this malignancy remains far from satisfied, which becomes a serious concern and challenge for clinician [4]. The multi-disciplinary treatment for OSCC is largely depends on radical cancer ablation in combined with chemotherapy and radiotherapy. Local recurrence and cervical lymph node metastasis are commonly identified as the prominent factors affecting patients' prognosis [27]. The past decades have witnessed the continuous efforts to dissect the genetic and epigenetic abnormalities driving OSCC initiation and progression. It has become increasingly clear that OSCC is initiated and facilitated primarily by aberrant genetic and epigenetic alterations such as chromosomal abnormality, DNA and histone modifications [9,32]. However, until now, few cancer biomarkers have been unequivocally established for diagnostic and prognostic management of OSCC, which may hinder the improvement of patients' treatment outcome [15]. Thus, there is an urgent need to improve our understanding of the molecular underpinnings that drive OSCC development, and to identify novel biomarkers and therapeutic targets for OSCC that optimize diagnosis and treatment strategies.

Epigenetic aberrations have been well established and serve as oncogenic drivers, diagnostic biomarkers as well as therapeutic targets in human cancer [6]. In particular, DNA methylation of cytosine (5mC) in CpG dinucleotide is one of the most extensively investigated epigenetic modifications and usually associated with

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transcriptional silencing in cancer cells [11]. Recent breakthrough findings have revealed that conversion of cytosine into methylcytosine is catalyzed and maintained by DNA methyltransferases, while the reversal process is mediated by the Ten-Eleven-Translocation (TET) protein family consisting TET1, 2 and 3 [16]. These TET proteins mediate the conversion of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine and 5-carboxylcytosine in a stepwise manner [28]. Notably, relatively high levels of 5hmC have been detected in neuronal and stem cells, but highly variable depending on cell types [22]. Accumulating evidence has shown that 5hmC is not merely functioning as an intermediate of DNA demethylation, but also acts as a stable epigenetic marker with incompletely characterized functions [1]. Interestingly, global reduction of 5hmC was observed in most human cancers including melanoma, glioma, kidney and esophageal cancer, which was proposed to be resulted from inactivating mutations of Tet or inhibition of TET activity via isocitrate dehydrogenase 1 and 2 (IDH1/2) mutations [3,19,23,29]. Moreover, 5hmC loss is significantly associated with cancer aggressiveness and unfavorable prognosis, thus suggesting the potential roles of 5hmC as an novel biomarker for cancer diagnosis and prognostic prediction [8,26].

To the best of our knowledge, until now, there is only one report in which both levels of 5hmC and TET2 were found to be significantly decreased in OSCC as compared to healthy oral epithelium by immunohistochemistry [12]. However, relatively few amount of clinical samples in their study impeded further analyses of the clinicopathological significance of 5hmC loss in OSCC. Here, to bridge the knowledge gap, we attempted to analyze the expression level of 5hmC in a retrospective patient cohort with pathologist-verified and clinically annotated OSCC and investigate the relationship between 5hmC level and clinicopathological features as well as patients' prognosis.

2. Materials and methods

2.1. Patients and tissue specimens

A total of 95 patients diagnosed as primary OSCC from January 2008 to December 2013 and treated at our institution was included in the present study. Patient inclusion criteria were listed as follows: (i) primary OSCC without any prior history of chemotherapy or radiotherapy (ii) patients underwent radical tumor resection and neck dissection (elective or therapeutic neck dissection when indicated); patients without neck dissection were excluded here. (iii) detailed clinical, pathological, and follow-up data available. The archived tissue samples were retrieved and the haematoxylin and eosin (H&E) stained sections of each tumor were analyzed to confirm the previous histopathological diagnosis. In addition, 24 healthy oral mucosa samples were collected from non-tumor surgery such as debridement of oral trauma, surgical excision of mucous cyst, dental implantation and harvested from tongue (10), mouth floor (6), buccal (4) and gingiva (4), respectively. Furthermore, these samples were histologically verified as normal oral mucosa by senior pathologist during the same period. The enrolled OSCC samples were pathologically classified into well-, moderately- and poorly differentiated groups (Grade I–III) by histological examination in terms of the differentiation status of cancer cells. Together with clinical and histopathological data, each patient was further categorized by the well-established TNM classification and clinical staging systems. In brief, T was defined as the tumor size and these tumors were classified into T1-4 based on the largest dimension of tumor (2, 2–4,4-6 and more than 6 cm, each corresponding to T1-4). N was defined according to the presence (N +) or absence (N0) of nodal metastasis identified by postoperative routine pathological examination (H&E staining). Notably, when the presurgical examinations such as clinical palpation or CT/MRI scan indicate high possibility of cervical node metastasis, we usually perform neck dissection regardless of T status even T1 cases in our center. Written informed consent was obtained from all individual participants included in the study. This study and research protocol was reviewed and approved by the Ethical Board at Nanjing Medical University (2015-116). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2.2. Immunohistochemical staining and scoring

Immunohistochemical staining for 5hmC was performed on 4 μm-thick sections from formalin-fixed paraffin-embedded clinical samples. The staining procedure was carried out as our previous report [18]. Briefly, these OSCC samples were dewaxed in xylene and rehydrated through graded series of ethanol. Then, the tissue slides were blocked using 3% hydrogen peroxide as well as non-immune horse serum, and heated in a microwave in 10 mM citrate buffer (pH 6.0) for 15 min for antigen retrieval. These sections were further incubated with primary antibodies 5hmC (GTX629765, GeneTex, Irvine, USA; 1:200 dilution) at 4 °C overnight and horseradish peroxidase-conjugated secondary antibody for another 30 min. Positively stained cells were detected using 3,3′-Diaminobenzidine substrate. These sections were further counterstained using haematoxylin. Omission of primary antibodies served as a negative control in each staining run. A human glioma sample known to be 5hmC positive staining was also used as positive control.

5hmC immunoreactivity was scored independently by senior oral pathologists without knowledge of clinical data and recorded using a semi-quantitative and subjective system based on the staining intensity and distribution of cancer cells displaying positive nuclear reaction as we and others described before [5,7,18,20]. Intensity score was defined as: 0, negative; 1, weak; 2, moderate and 3, strong. When different staining intensity of 5hmc was simultaneously observed in a single sample, the score of the highest intensity was recorded. Moreover, the proportion score was defined as: 0, negative; 1, <10%; 2, 11–50%; 3, 51–80%; and 4, >80% positive cells. The total score ranged from 0 to 12. Subsequently, immunoreactivity of each sample was arbitrarily divided into three groups based on the final score: 0, negative; 1–4, low expression; and 5–12, high expression, similarly as previous studies [5,7,18,20].

2.3. Data acquisition and analyses from publically available database

The genetic variations of TET1, 2, 3 in the head neck cancer were analyses in online cBioPortal (http://www.cbioportal.org/) database by selecting TCGA-HNSCC cohort using default parameters. The mRNA expression of TET1-3 in OSCC samples are retrieved from Oncomine (https://www.oncomine.org/) database and further analyses.

2.4. Statistical analysis

All statistical analyses were performed using the Graphpad Prism 5 (La Jolla, CA, USA) and SPSS 18.0 (Armonk, NY, USA). The quantitative data was compared with unpaired Student-*t* test. The associations between different categorical variables (5hmC expression and various clinicopathological parameters) were evaluated by Pearson χ^2 test. The overall survival rates were estimated using Kaplan-Meier method and compared with Log-rank test. The Cox proportional hazards model was applied to assess the impact of