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DNA methylation analysis by bisulfite next-generation sequencing for early detection of oral squamous cell carcinoma and high-grade squamous intraepithelial lesion from oral brushing

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ABSTRACT

Purpose: Oral squamous cell carcinoma (OSCC) is commonly preceded by oral potentially malignant lesions (OPML). The aim of the present study was to assess, by bisulfite next-generation sequencing (NGS), the methylation status of a list of candidate genes obtained from oral brushings to early detect OPML and OSCC.

Material and methods: Oral brushing specimens from 11 OSCC, 11 high-grade squamous intraepithelial lesions (HG-SIL), 9 low-grade SIL (LG-SIL), 9 oral lichen planus (OLP), and 8 healthy donors were included in this study. We investigated, by means of bisulfite NGS, the promoter of GP1BB, ZAP70, KIF1A, p16 [CDKN2A], CDH1, miR137, and miR375. Statistical significance between lesions and a pool of healthy donors were evaluated with the Mann–Whitney *U* test.

Results: ZAP70 was found to be hypermethylated in 100% of OSCC and HG-SIL and in 28.6% of LG-SIL. GP1BB hypomethylation was detected in 90.9% OSCC and HG-SIL and in 37.5% of LG-SIL. MiR137 was hypermethylated in 100% of OLP, 44.4% of OSCC, 40% HG-SIL, and 25% LG-SIL. KIF1A hypermethylation was found to be associated with TP53 mutations ($p < 0.0001$).

Conclusion: In the present preliminary cohort of patients, DNA methylation analysis of GP1BB and ZAP70 seems to be a promising noninvasive tool for early detection of OSCC and HG SIL from oral brushing specimens.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent neoplastic disease of the oral cavity. OSCC mortality rates have remained unchanged, as patients are frequently diagnosed in an advanced stage, which is associated with worse prognosis and higher radio- and chemotherapy morbidity. Moreover, the patient quality of life in regard to the oral cavity is disproportionately compromised, as surgical therapy can be mutilating and often has significant effects on swallowing, speech, and physical appearance. OSCC is commonly preceded by oral potentially malignant lesions (OPML). OPML can present with a great variety of clinical patterns that are sometimes difficult to interpret. In addition, patients affected by OSCC can develop a second primary OSCC, with a frequency ranging between 17% and 30% (Acciarri et al., 1993; Braakhuis et al., 2002); therefore it is important to closely follow up the mucosal alteration in order to detect early squamous neoplastic lesions. All of these lesions are diagnosed usually on the basis of an incisional biopsy. Nevertheless, the incisional biopsy requires a minimally invasive surgical approach that can create discomfort and may be refused by the patient. Therefore the

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development of noninvasive methods for early OPML detection is an attractive strategy to reduce the burden of OSCC. Various authors have proposed to analyze the methylation status of a panel of genes, by using saliva and/or brushing specimens (Demokan et al., 2010; Langevin et al., 2010; Pattani et al., 2010; Nagata et al., 2012; Schussel et al., 2013). Quantitative methylation-specific polymerase chain reaction (qMSP PCR) preceded by bisulfite treatment has been proposed as a method to evaluate biomarkers useful in early OSCC detection and clinical management (Kagan et al., 2007). Various genes have been previously studied for promoter methylation status in OSCC tissues. It has also been shown that histologically normal tissue adjacent to tumors and OPML can have an aberrant methylation pattern in candidate genes, suggesting that such epigenetic modifications are early events in oral carcinogenesis. Clinically, they have been associated with tumor aggressiveness, invasiveness, and with the malignant transformation of high-grade squamous intraepithelial lesion (HG SIL) (Shaw, 2006). The ability to quantify methylation provides the potential for determination of a clinically meaningful threshold value of DNA methylation to improve sensitivity and specificity in detection of tumor-specific signals. Usually the promoter region of genes spans more than 1000 base pairs and contains approximately 100 potential methylation sites. To characterize methylation patterns at base pair resolution, bisulfite conversion of DNA followed by next-generation sequencing is considered to be the gold standard approach. Bisulfite treatment converts unmethylated cytosines to uracil, such that U is read as T after PCR amplification and sequencing. This conversion does not affect methylated cytosines, which remain C in the sequence.

The aim of the present study was to develop a noninvasive method for early detection of OPML by epigenetic modifications analysis in the oral mucosa. For this purpose, a preliminary series of OSCC, HG-SIL, low-grade SIL (LG SIL), and oral lichen planus (OLP) were evaluated by investigating the promoter DNA methylation pattern in a panel of 7 genes (*ZAP70*, *GP1BB*, *KIF1A*, *p16/CDKN2A*, *miRNA137*, *miRNA375*, *CDH1*) using a bisulfite next generation sequencing approach. *TP53* mutation analysis was also performed for exons 4–9.

2. Material and methods

2.1. Ethics statement

All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by local Ethics Committee (study number 14092, protocol number 899/CE). All information regarding the human material used in this study was managed using anonymous numerical codes.

2.2. Sample population

We included all consecutive patients referred to the Department of Oral Sciences, University of Bologna, from January 2013 to July 2014. Lesions with an obvious etiology such as trauma and infective aphthous ulcerations were excluded. All patients presenting with oral lesions that required incisional biopsy to diagnostic purposed underwent also oral brushing of the same lesion. Oral brushing specimens were always picked before incisional biopsy for histological diagnosis and staging of each lesion.

Histological examination for the diagnosis of each lesion was performed on a blinded basis at the Department of Biomedical and Neuro Motor Sciences, Section of Anatomic Pathology M.Malpighi at Bellaria Hospital, University of Bologna, Italy. All of the cases were examined by two pathologists (M.P.F. and S.A.). A multi-head microscope discussion took place with regard to discordant cases, and a common diagnosis was obtained. Histological diagnoses were performed following World Health Organization (WHO) criteria (Thompson, 2006). The distinction between HG SIL and LG SIL was made according to the Ljubjana classification of 2014 (Gale et al., 2014). Finally, OLP histological diagnosis was characterized based on the presence of irregular acanthosis, degeneration of the basal cell layer of the epithelium, and an inflammatory infiltrate in the upper chorion composed almost exclusively of mature lymphocytes. The oral brushing sample series was composed of 48 patients: 11 diagnosed with OSCC, 11 with HG SIL, 9 with LG SIL, 9 with OLP. In addition, 8 samples were collected from healthy donors as normal controls (4 smokers and 4 nonsmokers). Table 1 provides information on patient age, sex distribution, and clinical appearance of each group of lesions.

2.3. Oral brushing method

A cytobrush was used to collect exfoliated cells from oral mucosa. In OSCC and OPML lesions all surface of lesions was gently brushed repeatedly five times. Brushing cell collection was always performed before incisional biopsy and without the use of any local anesthetic. After brushing, each cytobrush sample was placed in a 2-mL tube containing absolute ethanol for cell preservation.

2.4. DNA purification

DNA from oral cytobrush specimens was purified using The MasterPure™ Complete DNA extraction kit (Epicentre, Madison, WI, USA). Bisulfite treatment of genomic DNA was carried out with the EZ DNA Methylation-Lightning™ Kit (Zymo Research Europe, Freiberg, Germany) according to the manufacturer's protocol.

2.5. Library preparation

Locus-specific bisulfite amplicon libraries were generated with tagged primers (see Supplementary information, Table 4) using the High Fidelity FastStartTaq DNA polymerase (Roche Applied Science, Mannheim, Germany). Cycling conditions for the first template specific PCR were as follows: initial incubation at 95 °C for 3 min followed by 35 cycles at 95 °C for 20 s, 55 °C for 30 s and 72 °C for 30 s. A final extension step at 72 °C for 5 min was added at the end of the last cycle. Amplification products for each sample were diluted 1:100 in ultrapure water then used as template (2 μL) for the second round of PCR for barcoding. Sample-specific barcode sequences (MIDs = multiplex identifiers) and universal linker tags (454 adaptor sequences, A- or B-primer and key) were added in a second round of PCR where Universal Adaptors A and B are recognized as follows, including Adaptor A + key + MID + Universal Tail A or B (see Supplementary information, Table 4). Phusion Hot Start II High fidelity DNA polymerase (Thermo Scientific, Pittsburg, PA, USA) was used for this step to minimize PCR errors at 54 °C of annealing temperature. The amplicon products were purified using Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany), then quantified with the Fluorometer Quantus™ (Promega, Madison, WI, USA). The libraries were diluted, pooled, and clonally amplified in an emulsion PCR (emPCR). Sequencing was conducted on the Roche/454 GS junior system according to the manufacturer's protocol (Roche emPCR Amplification Method Manual–Lib-A and Roche Sequencing Method Manual, Roche, Branford, CT, USA). All data were generated using GS Junior Sequencer Instrument software version 2.7 (Roche Applied Science, Mannheim, Germany). Standardization for quantitative analysis was done by collecting DNA from human genomic DNA: female (catalog no. G1521, Promega, Madison, WI, USA) and subjecting to methylation in vitro with excess of *SssI* methyltransferase (New England Biolabs) to