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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

Luca Morandi ^{a, 1}, Davide Gissi ^{c, 1}, Achille Tarsitano ^{b, *}, Sofia Asioli ^a, Valentina Monti ^a, Giacomo Del Corso ^b, Claudio Marchetti ^b, Lucio Montebugnoli ^c, Maria Pia Foschini ^a

^a Department of Biomedical and Neuro-Muscular Sciences, Section of Anatomic Pathology "M.Malpighi" at Bellaria Hospital (Head: Prof. Maria Pia Foschini, MD), University of Bologna, Italy

^b Department of Biomedical and Neuro-Muscular Sciences, Section of Maxillofacial Surgery (Head: Prof. Claudio Marchetti, MD), University of Bologna, Italy ^c Department of Biomedical and Neuro-Muscular Sciences, Section of Oral Science (Head: Prof. Lucio Montebugnoli, DDS), University of Bologna, Bologna, Italy

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.

Keywords:

Oral squamous cell carcinoma
DNA methylation
Oral scraping
Next generation sequencing

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

* Corresponding author. Department of Biomedical and Neuromuscular Sciences, Section of Maxillofacial Surgery, University of Bologna, Italy, Via Massarenti, 9 40139 Bologna, Italy. Tel.: þ39 051 2143415; fax: þ39 051 2144160.

E-mail addresses: achille.tarsitano2@unibo.it, achilletarsitano@gmail.com (A. Tarsitano).

¹ Luca Morandi and Davide Gissi contributed equally to this work.

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 4e9.

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 purposes 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) 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

Table 1
Characteristics of patients and tumors in the study.

Type of lesion	Number of cases	Sex (n)	Mean age	Smoker (n)	Site (n)	Clinical appearance (n)
OSCC	11	(8) M, (3) F	65.23 ± 10.04	(4) Yes (7) No	(2) Cheek (4) Gingiva (4) Tongue (1) Floor of mouth	(4) Exophytic (5) Ulcerated (2) Verrucous
HG SIL	11	(7) M, (4) F	73.75 ± 11.41	(2) Yes (9) No	(4) Gingiva (6) Tongue (1) Floor of mouth	(5) Omogeneous (5) Nonomogeneous (1) Verrucous
LG SIL	9	(4) M, (5) F	66.77 ± 9.09	(2) Yes (7) No	(4) Cheek (3) Gingiva (2) tongue (9) Cheek	(7) Omogeneous (2) Verrucous
OLP	9	(4) M, (5) F	64.87 ± 11.54	(2) Yes (7) No	(2) Cheek	(8) Reticular lesion (1) Atrophic lesion
Controls	8	(4) M, (4) F	58.36 ± 13.51	(4) Yes (4) No	(2) Cheek (2) Gingiva (3) Tongue (1) Floor of mouth	

Table 2
CpG position along the seven genes studied with respect to the Transcriptional Start Site (TSS) using hg19 as reference.

Gene name	Chrom. number	RefSeq	Position	UCSC h19 Blat coordinates	Amplicon length	Position from TSS (5')	CpG evaluated
<i>ZAP70</i>	chr2	NM_001079	Exon 3	98340728e98340908	180 bp	þ10705	21
<i>GP1BB</i>	chr22	NM_000407	Exon 1	19710806e19710983	192 bp	þ315	18
<i>KIF1A</i>	chr2	NM_001244008	Exon 1	241759567e241759750	189 bp	-24	28
<i>MIR137</i>	chr1	NR_029679	Exon 3	98511621e98511836	216 bp	-49	10
<i>MIR375</i>	chr2	NR_029867	Exon 1	219866350e219866461	287 bp	-176	31
<i>CDH1</i>	chr16	NM_004360	Exon 1	68771008e68771219	221 bp	-120	15
<i>p16/CDKN2A</i>	chr9	NM_058195	Exon 1	21994173e21994427	255 bp	þ197	27
<i>p16/CDKN2A</i>	chr9	NM_058195	Intron 1	21974660e21974920	262 bp	þ19705	28

generate completely methylated DNA. Serial dilutions of this DNA spiked in untreated DNA were used to construct a calibration curve (Morandi et al., 2010).

2.6. In silico prediction of CpG island and primer design

To identify putative CpG island on promoter region of *ZAP70*, *GP1BB*, *KIF1A*, *MIR137*, *MIR375*, *CDH1*, *p16/CDKN2A*, genomic sequence, as stored on Ensembl genome browser (<http://www.ensembl.org/index.html>) including 1000 bp upstream the ATG site, were used as query sequence. MethPrimer (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>) (Li and Dahiya, 2002) designing was applied to identify CpGs and the best primers of choice (see Supplementary information, Table 4). Table 2 indicates the position of evaluated CpGs along the 7 genes with respect to the Transcriptional Start Site (TSS). For GSJunior/454 Image processing and amplicon pipeline analysis was performed using default settings of the GS RunBrowser software version 2.7 (Roche Applied Science). SFF files were used as a source to generate FASTA and FASTQ files by SFFFile, SFFinfo, and Perl. In the first-step DNA sequence, reads were recognized trimming multiplex identifiers (MIDs; short barcode sequences used to label samples/patients when multiplexing), tail A and B, and gene-specific sequence primers.

2.7. TP53 mutation analysis

TP53 mutation analysis was performed by GS Junior-454 platform as previously described with some modifications (Morandi et al., 2015), using primers reported in Table 4 (see Supplementary information) covering exons 4 to 9. Cycling conditions for the first template-specific PCR were as follows: initial incubation at 98 °C for 3 min followed by 30 cycles at 98 °C for 10 s, 61 °C for 20 s and 72 °C for 20 s. A final extension step at 72 °C for

5 min was added at the end of the last cycle. The same conditions were used for a second round of PCR for barcoding, except for annealing temperature at 54 °C using Phusion Hot Start II High fidelity DNA polymerase (Thermo Scientific, Pittsburg, PA, USA) for both protocols. Processed and quality-filtered reads were analyzed with the GS Amplicon Variant Analyzer (AVA) software version 2.7 (454 Life Sciences).

2.8. Statistical analysis

We calculated the total percentage of methylated CpGs in each sample for each gene selected by QUMA software (<http://quma.cdb.riken.jp/>) (Kumaki et al., 2008). We compared the methylation pattern of each lesional sample with respect to the pool of normal healthy donors using the Mann-Whitney *U* test *p* value. A *p* value of <0.05 was considered statistically significant. The pool of healthy donors were compared with DNA from Human genomic DNA: female (catalog no. G1521, Promega, Madison, WI, USA). Methylation pattern results for each gene were dichotomized (i.e., no methylation versus hyper- or hypomethylation). The Fisher exact test was used to evaluate the presence of any significant between group difference in the methylation pattern. The Fisher exact test was also used to disclose any significant relationship between promoter hypermethylation of *KIF1A* and *TP53* mutations in each group. The flow chart of the entire assay is provided in Fig. 1.

3. Results

3.1. Gene methylation status

Table 3 summarizes the methylation status in OSCC, HG SIL, LG SIL, and OLP, comparing them with 8 healthy donors for each of the seven genes tested in this study. None of the healthy donors showed an altered methylation pattern. *ZAP70* was hypermethylated in 11 of

Assay Flow Chart

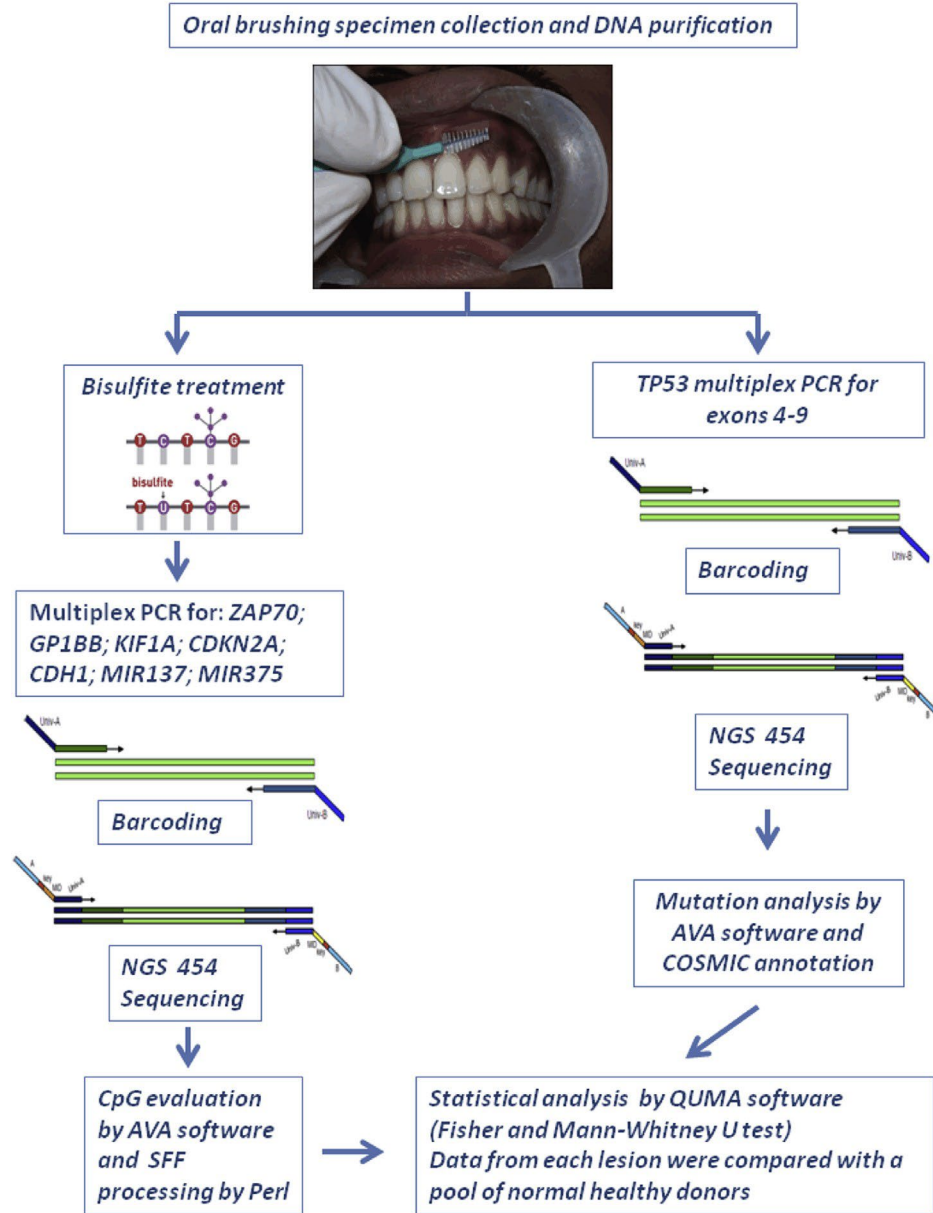


Fig. 1. Assay flow chart from oral brushing collection of specimens to statistical evaluation. After sample collection by a cytobrush, DNA was purified. A small fraction of this DNA serves as a target for TP53 PCR amplification (exons 4e9), and another fraction was bisulfite treated to convert unmethylated cytosines. Gene-specific PCR for GP1BB, ZAP70, KIF1A, p16[CDKN2A], CDH1, miR137, and miR375 was performed, and DNA from each patient was barcoded to be retrieved during sequence evaluation. To detect any possible aberrations, we evaluated the DNA methylation pattern by the QUMA software tool using the Mann-Whitney *U* test.

Table 3

DNA methylation analysis comparing OSCC, HG-SIL, LG-SIL, OLP vs a pool of normal healthy donors including 4 smokers and 4 non smokers.

Sample group	ZAP70 (hypermethylated)	GP1BB (hypomethylated)	KIF1A (hypermethylated)	MIR137 (hypermethylated)	MIR375 (hypermethylated)	CDH1 (hypermethylated)	P16(CDKN2A) (hypermethylated)
OSCC	11/11 (100%)	10/11 (90.9%)	5/11 (45.4%)	4/9 (44.4%)	0/7 (0%)	0/7 (0%)	1/11 (9%)
HG-SIL	11/11 (100%)	10/11 (90.9%)	3/10 (30%)	4/10 (40%)	0/10 (0%)	0/8 (0%)	0/11 (0%)
LG-SIL	2/7 (28.5%)	3/8 (37.5%)	4/8 (50%)	2/8 (25%)	0/9 (0%)	0/8 (0%)	0/9 (0%)
OLP	0/9 (0%)	0/9 (0%)	0/9 (0%)	9/9 (100%)	0/9 (0%)	0/9 (0%)	0/9 (0%)

We compared the methylation pattern of each lesional sample with respect to a pool of normal healthy donors (four smokers and four nonsmokers) using a Mann-Whitney *U* test *P* value and QUMA software. A *p* value < 0.05 was considered statistically significant. Methylation pattern results for each gene were dichotomized (i.e., no methylation versus hyper- or hypomethylation). The Fisher exact test was used to evaluate the presence of any significant between-group difference in the methylation pattern.

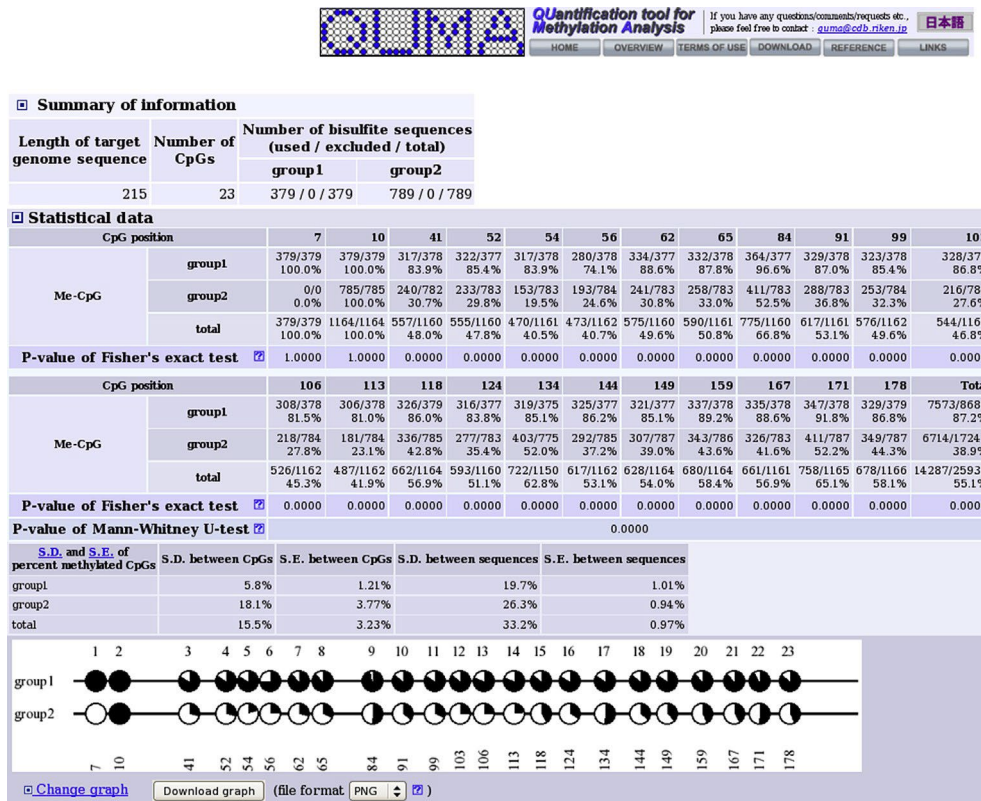


Fig. 2. Statistical analysis using QUMA for the ZAP70 gene, comparing case 22 (group 1) with respect to the pool of normal healthy donors (group 2). Each of the 23 CpG included in the amplicon was listed and details about CpG position, number of reads, methylated vs unmethylated cytosines ratio, and P value by Fisher test were visualized. Considering the 23 evaluated CpGs as a whole, a Mann-Whitney U test p value of <0.00001 was detected. A pie chart (bottom) relevant to each CpG pointed out graphically the high methylation level for case 22 (group 1) with respect to a pool of normal healthy donors (group 2). Black area of pie chart represents methylated CpGs; white area represents unmethylated CpGs.

11(100%) cases of OSCC and HG SIL, in 2 of 7 (28.6%) cases of LG SIL. Fig. 2 shows QUMA statistical analysis between case 22 and the pool of normal healthy donors for ZAP70. Fisher test results were found to be significant in 21 of 23 CpGs. Considering the total number of CpGs as a whole, for this case, with the Mann-Whitney U test a p value of <0.00001 was detected.

GP1BB was hypomethylated in 10 of 11 (90.9%) cases of OSCC and HG SIL, in 3 of 8 (37.5%) cases of LG SIL; these difference was statistically significant (p < .01) with respect to both OSCC and HG SIL respect to LG SIL for ZAP70 as well as for GP1BB. KIF1A was found to be hypermethylated in 5 of 11 (45.5%) cases of OSCC, in 3 of 10 (30%) cases of HG SIL, and in 4 of 8 (50%) cases of LG SIL. Mir137 was hypermethylated in 4 of 9 (44.4%) cases of OSCC, in 4 of 10 (40%) cases of HG SIL, in 2 of 8 (20%) cases of LG SIL, in 9 of 9 (100%) cases of OLP. No significant differences between groups were found for KIF1A and mir137. p16/CDKN2A was hypermethylated only in 1 (9%) case of OSCC. miR375 and CDH1 did not show any altered methylation pattern. OSCC and HG SIL groups displayed substantially the same methylation pattern. Concerning OLP samples, no altered methylation pattern was observed in regard to ZAP70, GP1BB, and KIF1A genes, whereas Mir137 was hypermethylated in all samples.

3.2. TP53 mutation analysis

TP53 mutations were found in 5 of 11 (45.5%) cases of OSCC, 3 of 11 (27.2%) cases of HG SIL, and in 3 of 9 (33.3%) cases of LG SIL: no significant differences between groups were found. Table 5 (Supplementary information) summarizes TP53 mutation analysis in OSCC, HG SIL, and LG SIL. A significant association was found between KIF1A hypermethylation and TP53 mutations (C²[11.95];

p < 0.0001). Case 22 shows two concurrent mutations in cis: p.H233Q and p.L252P (see Supplementary information, Fig. 4 for details). This case revealed even the hypermethylation of KIF1A (see Supplementary information, Fig. 6).

4. Discussion

In the present study, we evaluated the utility of detection of aberrant promoter methylation using 7 genes (GP1BB, ZAP70, KIF1A, miR137, miR375, CDH1, and p16/CDKN2A) proposed previously (Sanchez-Cespedes et al., 2000; Rosas et al., 2001; Hasegawa et al., 2002; Lopez et al., 2003; Kato et al., 2006; Shaw, 2006; Sawhney et al., 2007; Viet et al., 2007; Takeshima et al., 2008; Cao et al., 2009; Marsit et al., 2009; Demokan et al., 2010; Kordi-Tamandani et al., 2010; Langevin et al., 2010; Pattani et al., 2010; Su et al., 2010; Nagata et al., 2012; Dang et al., 2013; Khor et al., 2013), to early detect HG SIL and OSCC and comparing these individuals with a normal population as a detection tool. None of the seven interrogated genes showed an aberrant methylation status in eight normal healthy donors recruited for this study as reference controls. Meanwhile we have found two genes showing high sensitivity and specificity for detection of OSCC and HG SIL: ZAP70 was hypermethylated in all brushing samples of both OSCC and HG SIL, and GP1BB was hypomethylated in the same lesions except in one case.

Interestingly, ZAP70 and GP1BB methylation status seems to be also a promising markers to discriminate HG SIL with respect to LG SIL. ZAP70 gene encodes the z-chain-associated protein kinase 70 kDa, which is a tyrosine kinase normally expressed by natural killer cells and T cells. Hypermethylation of ZAP70 gene predicted

an unfavorable disease course in terms of disease progression and overall survival in chronic lymphocytic leukemia (Amin et al., 2008; Claus et al., 2012, 2014).

In contrast, *GP1BB* revealed loss of methylation; this gene encodes heterodimeric transmembrane protein that constitutes the receptor for von Willebrand factor and mediates platelet adhesion in the arterial circulation. Mutations of *GP1BB* are associated with Bernard Soulier Syndrome, an extremely rare inherited bleeding disorder (Savoia et al., 2011). Further investigation will be helpful for determining whether the activation of this gene is related to the production of tumor-specific antigens, which can trigger immune rejection (De Smet and Loriot A., 2013).

In head and neck oncology, only Marsit et al. reported an altered methylation status of *ZAP70* and *GP1BB* (Marsit et al., 2009). They characterized DNA methylation profiles of primary human OSCC tumors by examining DNA methylation status of 1400 CpG sites in about 800 cancer-related genes in a population of primary HNSCC, starting from formalin-fixed paraffin-embedded (FFPE) samples.

An altered methylation status was also found for *KIF1A* and *miR137* genes in the present study cohort, although with lower levels of specificity, sensitivity, PPV, and NPV (see Supplementary information, Tables 5 and 6). *KIF1A* (Kinesin family member 1A) encodes a protein that is a microtubule-dependent molecular motor involved in important intracellular functions such as organelle transport and cell division (Okada et al., 1995). Demokan et al. found an hypermethylation of *KIF1A* in 98% of HNSCC tissues and in 38% of corresponding HNSCC salivary samples (Demokan et al., 2010). Accordingly, in our series, *KIF1A* was hypermethylated in 45.5% of OSCC, 33.3% of HG SIL, and 44.4% of LG SIL. Notably, we found a significant association between *KIF1A* hypermethylation and *TP53* mutations. To the best of our knowledge, this is the first report about this phenomenon, and further analysis is needed to investigate *KIF1A* and *TP53* interactions.

Finally, in our series, *miR137* was hypermethylated in 44.4% of OSCC, 40% of HG SIL, and 20% of LG SIL. Remarkably it was the only gene with an altered methylation status in OLP patients with 100% of hypermethylated cases. This result is in accordance with Dang et al., who found an aberrant promoter methylation of *miR137* in OSCC and OLP patients (Dang et al., 2013), although recently Bediaga et al., comparing genome-wide methylation profiles between OLP and control samples, showed that the frequency of aberrant DNA methylation is rare in OLP (Bediaga et al., 2014).

MiR137 appears to play a role in cellular differentiation and cell cycle control, at least in part through negative regulation of Cdk6 expression (Kozaki et al., 2008; Silber et al., 2008). It is associated with a large CpG island and has been reported to undergo promoter methylation in OSCC (Kozaki et al., 2008), gastric cancer cell lines (Ando et al., 2009), and colon cancer (Bandres et al., 2009). Langevin et al. found increased *miR-137* DNA methylation level linked to poor OSCC survival rates, and it was detected specifically in patient oral rinse, suggesting that it might provide a readily detectable prognostic marker for OSCC (Langevin et al., 2010).

Late diagnosis of advanced-stage disease is the main cause of head and neck cancer morbidity and mortality (Mignogna et al., 2004). Although the oral cavity is an easy site for a physical examination, often a delay in seeking medical care leads to the advanced stage of the disease at the time of diagnosis (Siegel et al., 2013). Early diagnosis of OSCC and OPML at high risk may be of importance for clinical management, particularly in high-risk populations.

Cancer-related genetic alterations have been widely demonstrated in OSCC; however, recently epigenetic alterations, such DNA methylation alterations, have gained importance and have been shown to be promising markers related to oral cancer (Shaw, 2006;

Gasche and Goel, 2012; Mascolo et al., 2012; Jithesh et al., 2013; Towle et al., 2013). In our preliminary series, the methylation pattern of OSCC and HG SIL was found to be substantially identical. This reinforces the idea that epigenetic modifications such as DNA methylation are early events in oral cancerogenesis. Detection of aberrant DNA methylation status in exfoliated cell samples is proposed in the present study as a potential non-invasive method for early identification of patients at risk for developing OSCC and HG SIL.

The rapid development of NGS methods, which can generate millions of reads, each corresponding to the sequence of a single DNA molecule in one run without subcloning, has brought new opportunities for the wide use of the bisulfite sequencing method for genome-wide and single-gene promoter DNA methylation analysis.

This method, with respect to the other most-used methods, such as qMSP (which interrogates only a few CpG, usually 2e4), provides important advantages for DNA methylation analysis. First, it includes many CpG sites, such that complex methylation patterns of individual DNA molecules can be determined. Second, the longer reads can be easier and more accurately aligned to the reference sequence, especially in repetitive regions of the genome. Third, the long reads have a greater chance to cover more genotype information such as single nucleotide polymorphisms (SNPs) in the neighborhood of cytosines, making it possible to analyze the correlation between DNA methylation and genotype. Finally, this method is quantitative, and previously findings have demonstrated how important is DNA methylation level estimation for a correct identification of OSCC (Lim et al., 2014).

5. Conclusion

In summary, the present study showed that DNA methylation analysis by bisulfite NGS from oral brushing seems to be a promising tool for early detection of patients at risk for developing OSCC and HG SIL. The early diagnosis of OSCC/OPML with high risk of progression using a noninvasive, low-cost method could select patients who can benefit from more aggressive treatment protocols or intensive follow-up modalities. The main features of this approach were related to the noninvasiveness of specimen collection and a wide CpG island DNA methylation analysis of the promoter of various genes in parallel. However, in a preliminary cohort of patients, we found that aberrations in the methylation level of two genes, *GP1BB* and *ZAP70*, represented highly sensitive markers to discriminate OSCC and HG SIL with respect to normal healthy donors and OLP, whereas *miR137* seems to be a specific marker of OLP. However it should be stated that the group of patients enrolled in this study, and the number of healthy donors, are small in our study, so these data must be confirmed in a larger population of patients and normal controls, including smokers and nonsmokers, as this condition may influence the pattern of methylation level in monozygotic twins. Further work will be aimed at elucidating the functional roles of *GP1BB* and *ZAP70* in OSCC/HG SIL and *miR137* in OLP patients.

Conflict of interest

The authors have no conflicts of interest to declare.

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