Received: 29 May 2023 | Revised: 11 December 2023 | Accepted: 18 December 2023

DOI: 10.1002/hed.27621

ORIGINAL ARTICLE

WILEY

Thirteen-gene DNA methylation analysis of oral brushing samples: A potential surveillance tool for periodic monitoring of treated patients with oral cancer

Davide B. Gissi PhD, DDS[1] | **Roberto Rossi DDS**[1] | **Jacopo Lenzi PhD**[2] | **Achille Tarsitano MD**[3,4] | **Andrea Gabusi PhD, DDS**[1] | **Tiziana Balbi MD**[5] | **Lucio Montebugnoli MD, DDS**[1] | **Claudio Marchetti MD, DDS**[3,4] | **Maria Pia Foschini MD**[6] | **Luca Morandi PhD, BSc**[7,8]

[1]Department of Biomedical and Neuromotor Sciences, Section of Oral Sciences, University of Bologna, Bologna, Italy

[2]Section of Hygiene, Department of Biomedical and Neuromotor Sciences, Public Health and Medical Statistics, University of Bologna, Bologna, Italy

[3]IRCCS Azienda Ospedaliero-Universitaria di Bologna, Maxillofacial Surgery Unit, Bologna, Italy

[4]Department of Biomedical and Neuromotor Sciences, Section of Maxillo-Facial Surgery at Policlinico S. Orsola-Malpighi, University of Bologna, Bologna, Italy

[5]IRCCS azienda ospedaliero universitaria di Bologna, Unit of Anatomic Pathology S. Orsola Hospital, Bologna, Italy

[6]Department of Biomedical and Neuromotor Sciences, Section of Anatomic Pathology at Bellaria Hospital, University of Bologna, Bologna, Italy

[7]Functional and Molecular Neuroimaging Unit, Bellaria Hospital, Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

[8]IRCCS Istituto delle Scienze Neurologiche di Bologna, Bologna, Italy

Correspondence
Davide B. Gissi, Department of Biomedical and Neuromotor Sciences, Section of Oral Sciences, University of Bologna, Via San Vitale 59, 40159 Bologna, Italy.
Email: davide.gissi@unibo.it

Funding information
University of Bologna

Section Editor: James William Rocco

Abstract

Background: We evaluated the prognostic role of 13-gene DNA methylation analysis by oral brushing repeatedly performed during the follow-up of patients surgically treated for oral cancer.

Methods: This is a nested case–control study including 61 patients for a total of 64 outcomes (2/61 patients experienced multiple relapses). Samples were collected at baseline (4–10 months after OSCC resection) and repeatedly every 4–10 months until relapse or death. DNA methylation scores were classified as persistently positive, persistently negative, or mixed.

Results: Twenty cases who had persistently positive scores and 30 cases with mixed scores had, respectively, an almost 42-fold ($p < 0.001$) and 32-fold ($p = 0.006$) higher likelihood of relapse, compared to 14 patients with persistently negative scores. The last score before reoccurrence was positive in 18/19 secondary events.

Conclusions: The 13-gene DNA methylation analysis may be considered for the surveillance of patients treated for oral carcinoma.

KEYWORDS

DNA-methylation analysis, epigenetic instability, follow-up surveillance, oral brushing, oral cancer

1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) has one of the highest mortality rates among all cancers[1] due to the high rate of loco-regional recurrence. A further cancer (including local recurrences [LRs], lymph-node metastases [LNMs] and second primary tumors [SPTs]) develops after multimodal therapy in 20%–50% of OSCC patients and accounts for most cancer-related deaths.[2]

OSCC recurrence remains a difficult condition to treat. Many authors considered surgical salvage as the primary option for recurrent OSCC,[3,4] because the efficacy of chemo/radiotherapy is still limited for loco-regional control and must be balanced with high toxicity.[5] So, a timely diagnosis of loco-regional recurrence is essential to achieve curative surgical excision and a good prognosis.[6] Standard methods for the evaluation of loco-regional control include clinical evaluation, incisional biopsy with histological examination, and imaging. However, repeated incisional biopsies are invasive and may not be appropriate for the follow-up of treated OSCC patients, and imaging is expensive and has insufficient specificity and positive predictive value.[7] Although incisional biopsy of visible lesions is an option, it does not provide useful information if performed for clinically unaltered mucosa.

The repeated analysis of OSCC biomarkers at adequate follow-up intervals using a minimally invasive sampling procedure may be an attractive strategy to evaluate loco-regional recurrence. Gene silencing by promoter methylation is an early and frequent event in oral carcinogenesis, and may occur even more frequently than structural inactivation of genes by mutations and deletions.[8] Some studies have found that the central pathogenesis of cancer involves a disrupted and unstable epigenome, which is usually caused by mutations and often preceded by epigenetic changes in normal tissues as a result of age and injury.[9] Normal mucosa with normal DNA methylation levels at the surgical site may exhibit aberrant methylation before OSCC recurrence because epigenetic alterations occur early during carcinogenesis.[10]

In a recent study, we developed a method for the early detection of OSCC based on the DNA methylation of 13 genes obtained from noninvasive oral brushing samples. The 13 genes showed an aberrant methylation pattern in patients with OSCC or high-grade dysplasia.[11–15] Based on the DNA methylation level of the most informative CpGs identified previously, a patented algorithm was developed to identify OSCC; an algorithm score >1.0615547 indicated epigenetic changes related to OSCC.[11] The diagnostic value of the 13-gene DNA methylation analysis in oral brushing samples was assessed in a multicenter Italian clinical trial, providing a sensitivity of 93.6% (CI 87.8–99.5), a specificity of 84.9% (CI 76.2–93.6), a PPV of 86.6% (CI 78.7–94.4), a NPV of 92.8% (CI 86.2–99.4), and accuracy of 89.4%.[16] The clinical relevance of 13-gene DNA methylation analysis was also evaluated for prognostic purposes. Specifically, the present study was based on a previous investigation in 2019 that identified an altered methylation profile in 16 (32.7%) of 49 brushing specimens obtained from clinically healthy mucosa that had replaced the surgical sites after tumor resection. In addition, patients with a positive score had a high risk of relapse.[17] However, the previous study collected a single sample from the patients at 6 months after surgical resection of OSCC. So far, the prognostic role of the analysis of epigenetic alterations performed longitudinally multiple times was described in a single case report: epigenetic alterations in 13-gene DNA methylation analysis were both found in a non-dysplastic oral leukoplakia that after 2 years transformed in OSCC and in the regenerative oral mucosa at 6 months after the resection of the primary OSCC before the development of a secondary OSCC. By contrast, oral brushing samples from the regenerative oral mucosa at 6 months after surgical resection for the secondary oral cancer did not show epigenetic alterations and the patient didn't develop further neoplastic manifestations.[18]

In the present study, the 13-gene DNA methylation analysis on oral brushing samples was collected at different times during the oncologic follow-up of patients surgically treated for OSCC.

The aim was to evaluate the association between the altered methylation level and secondary OSCC, exploring the predictive value of the 13-gene DNA methylation analysis for secondary oral carcinoma in treated OSCC patients.

2 | MATERIALS AND METHODS

The study included 61 consecutive patients who were diagnosed with OSCC at the Department of Biomedical and Neuromotor Sciences, Section of Oral Sciences,

University of Bologna, and underwent intent-to-cure surgical resection at the Maxillofacial Surgery Unit, Sant'Orsola Hospital, between 2014 and 2019. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the local Ethics Committee (study number 14092, protocol number 899/CE). Each participant gave informed consent. Surgical resection of OSCC was performed in accordance with standard practices.[19] Preoperative biopsy and surgical specimens were subjected to histological analysis at the Sections of Anatomic Pathology of the University of Bologna and Sant'Orsola Hospital.

This study included OSCC patients who underwent complete surgical resection and had no margin involvement. All patients included had no clinical or radiographic evidence of relapse 4 months post initial treatment. Postoperatively, patients underwent routine follow-up, including clinical, instrumental, and radiological examinations in accordance with the International National Comprehensive Cancer Network guidelines.[20] A head and neck multidisciplinary team, including ear-nose-throat specialists (ENT) and maxillofacial surgeons, radiation and medical oncologists, radiologists, serves as an outcome review panel. During the period of follow-up, patients received clinical and endoscopic evaluations. Both head and neck computed tomography and magnetic resonance imaging were performed every 6 months during the first 3 years for patients with advanced stage of disease and high risk of relapse, according to our Institutional guidelines. For patients having early stage of disease, only clinical assessment was performed; imaging was reserved for patients having local symptoms or suspect of local relapse. This study included some patients included in previous studies.[17,18]

2.1 | Oral brushing sample collection

Before any cancer treatment, oral brushing sample collection and DNA methylation analysis were performed to evaluate the presence of an altered methylation pattern in the tumor mass as previously described.[11]

During the follow-up oral brushing samples were collected from a wide regenerative area after surgical resection of the index tumor, exceeding margins of surgical resection regardless the type of surgery adopted (with or without reconstructive tissue transfer for surgical repair after resection). In presence of free-flap reconstruction of the surgical defect gentle brushing was performed on a wide area including both reconstructive tissue used for surgical repair and adjacent oral mucosa. Repeated sample collection in each patient was performed according to a previously described protocol.[17,21,22] Baseline brushing samples were obtained after 4–10 months of surgical resection of primary OSCC or after radiation therapy in case of multimodal therapy, and every 4–10 months thereafter, unless relapse or censoring occurred (Figure 1). Mean time between samples was 7.3 ± 1.5 months, with a median of 7.4 months and an interquartile range equal to 6.2–8.5. Brushing specimens were collected during follow-up visit at the Department of Biomedical and Neuromotor Sciences of the University of Bologna, and the Section of Oral Sciences and the Maxillofacial Surgery Unit of Sant'Orsola Hospital, between 2014 and 2019.

Preoperative clinical information (age, sex, smoking status, and tumor location), pathological information, and the staging results for surgical specimens (primary tumor type, regional lymph node involvement, tumor grade, depth of invasion [DOI], perineural invasion, resection margins, and tumor stage) were recorded in accordance with the 8th American Joint Committee on Cancer criteria.[23] Variables related to index OSCC treatment (i.e., postoperative radiotherapy and free-flap reconstruction of the surgical defect) were also evaluated.

Disease-free survival (DFS), defined as the interval between primary OSCC resection and occurrence of new loco-regional neoplastic manifestations, as tumor progression (which comprised LRs, LNMs or distant metastases) or as SPT and death were evaluated at the final follow-up visit in December 2019. LR and SPT were distinguished applying the criteria of Hong et al.,[24] that represent a modification of the definition given by Warren and Gates.[25] LR was defined as a second neoplastic lesion having the same histological features, appearing within 2 cm and occurring less than 3 years after the index tumor. SPT was defined as a second neoplastic lesion located at a distance greater than 2 cm from the index tumor or a second lesion occurring more than 3 years after the index tumor; any histopathologic differences between second and primary neoplastic lesions or the presence of Epithelial Precursor Lesions (EPL) associated with the second tumor supported the hypothesis of an SPT.

2.2 | Thirteen-gene DNA methylation analysis

A 13-gene DNA methylation analysis was performed as described previously.[11] Briefly, DNA from exfoliated cells was purified using the Quick DNA MagBead Plus kit (cat. no. D4081; Zymo Research, Irvine, CA, USA) and were treated with sodium bisulfite using EZ-96 DNA Methylation MagPrep (cat. no. D5041; Zymo Research)

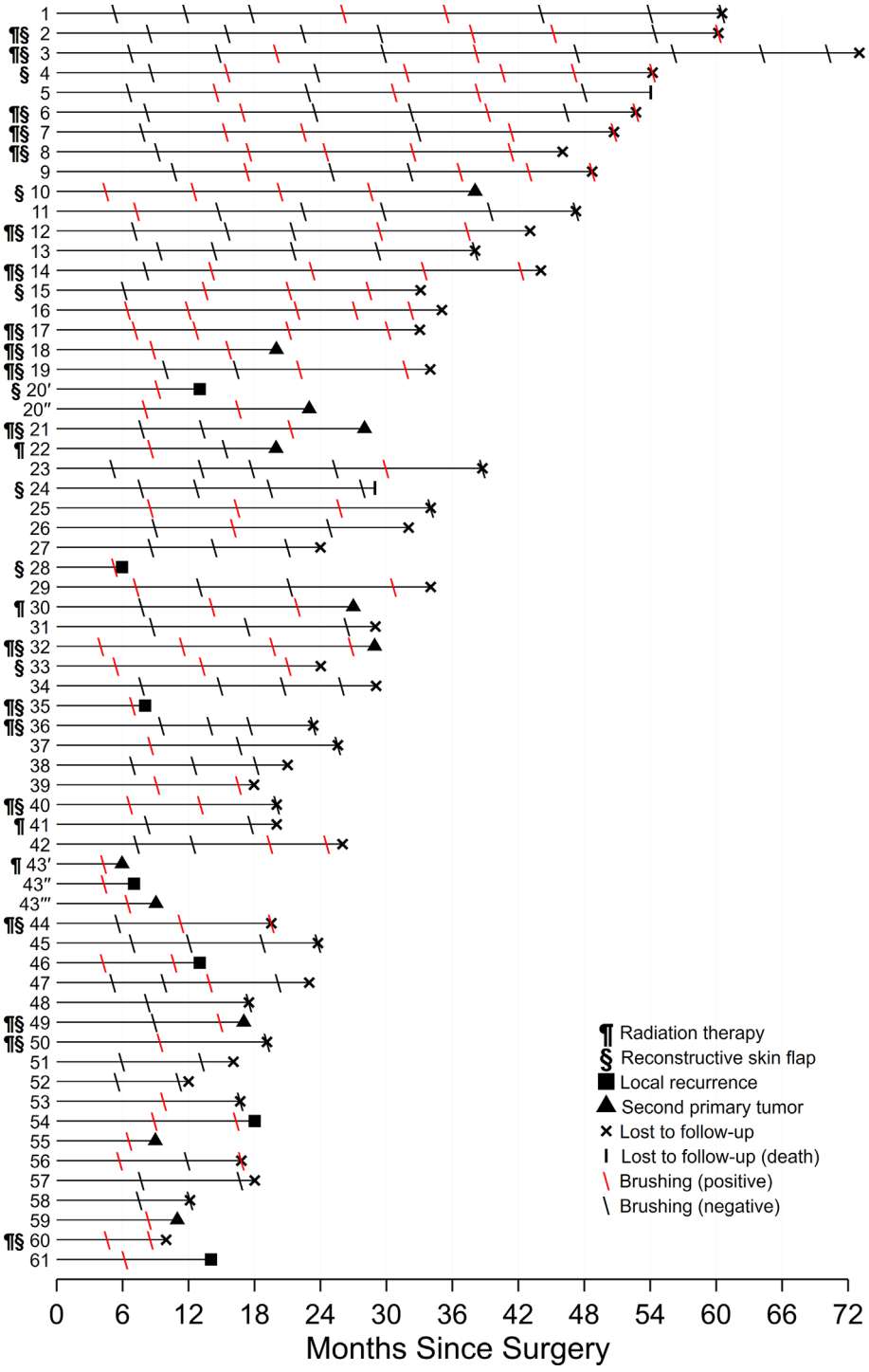

FIGURE 1 Time-to-relapse chart depicting the individual follow-up periods observed in the study. Patients No. 20 and No. 43 developed two and three tumors during the study period, respectively, and for this reason were included multiple times in the study design. [Color figure can be viewed at wileyonlinelibrary.com]

according to the manufacturer's instructions. The recommended minimum quantity of DNA is 100 ng (2 ng/μL in 50 ul of elution volume), corresponding to about 16 600 cells, as one single cell contains 6 pg. Quantitative DNA methylation analysis of the following genes was performed using next-generation sequencing: *ZAP70*, *ITGA4*, *KIF1A*, *PARP15*, *EPHX3*, *NTM*, *LRRTM1*, *FLI1*, *MIR193*, *LINC00599*, *MIR296*, *TERT*, and *GP1BB*. Libraries were prepared using Nextera™ Index Kit (Illumina, San Diego, CA, USA) and a locus-specific bisulfite amplicon approach.[11] The libraries were loaded onto the MiSeq instrument (15 027 617; Illumina), with ≥1000 reads/region allocated to obtain a coverage depth ≥1000×. FASTQ output files were subjected to quality control (>Q30) and evaluated using an oral risk score (https://galaxy.studiumgenetics.com).[26] In our previous study,[11] the best CpGs identified by receiver operating characteristic curve analysis were used to generate an algorithm

based on a multiclass linear discriminant analysis. This approach identified a threshold value for OSCC of 1.0615547; this value had the optimal sensitivity and specificity (area under the curve = 0.981). Values exceeding the threshold were considered positive.

2.3 | Statistical analysis

Quantitative variables are presented as means ± standard deviations, whereas categorical variables are presented as frequencies and percentages. DFS was estimated by the Kaplan–Meier method using the date of surgery as the time of origin.

The association between exposure (oral risk score) and outcome (relapse) was assessed using a nested case–control design, which can be seen as a case–control in a cohort study.[27] Cases were patients who experienced relapse during follow-up and, for each, four time-matched controls were randomly selected from among those in the cohort who had not relapsed by the time of disease occurrence in the case. An example of this technique, which is called "risk-set sampling" or "incidence density sampling," is provided in Figure S1. This approach was selected to ensure a similar time window for the measurement of methylation scores between cases and controls.[28] Cases and controls were classified into three mutually exclusive groups: persistently negative (scores persistently <1.0615547), persistently positive (scores persistently >1.0615547), and mixed (variable scores). Matched controls were excluded from the analysis if no samples were available between the dates of surgery and matching. The associations between score groups and relapse were estimated with a logistic regression model using Firth's method, which is similar to the penalization of the log-likelihood by the Jeffreys prior (which reduces the bias of maximum likelihood estimates and represents an ideal solution to the problem of separation or quasi-separation).[29] Unconditional Firth-type regression analysis was performed by controlling for the matching factor used in risk-set sampling (i.e., time of case occurrence) and including the matched follow-up period as a covariate in the model.[30] In a secondary analysis, the adjustment for confounders was enhanced by adding propensity scores based on baseline patient characteristics as additional covariates, including an age of >70 years, smoking, and hard palate tumor location. These variables were considered potential confounders because of their significant association with the outcome ($p = 0.10$) in simple (crude) regression analysis. The results are expressed as odds ratios (ORs). The regression analysis was replicated on LRs and SPTs separately, treating competing outcomes as censoring events. *P*-values were computed by the penalized profile likelihood method.[29] Lastly, the regression analysis was replicated to confirm the impact of each methylation beta value included in the last available score before the matching date.

Data were analyzed using Stata (version 17.0; StataCorp., College Station, TX, USA) and R (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria) software.[31] The significance level was set at $p < 0.05$ (two-sided).

3 | RESULTS

This study included 61 patients (35 [57%] females and 26 [43%] males) with a median age at the index OSCC presentation of 66.8 ± 13.0 (range: 36–91) years. In total, 22 patients were classified as T1N0M0, 12 as T2N0M0, 4 as T3N0M0, and 11 as T4N0M0; among patients with lymph node involvement, 2 had T2N2M0, 2 had T2N3M0, 1 had T3N3M0, 3 had T4N1M0, 1 had T4N2M0, and 3 had T4N3M0. Twenty-three (38%) of sixty-one patients received radiation therapy as adjuvant postoperative treatment. All baseline demographic, clinical, and histological characteristics of the patients are summarized in Table 1.

3.1 | Baseline prognostic variables

A time-to-relapse chart that illustrates all the individual follow-up periods included in the study is presented in Figure 1. During a median follow-up of 28.9 (interquartile range: 18–36.5) months, 19 secondary tumors were diagnosed. Two of the sixty-one patients developed multiple tumors (three in case No. 20 and two in case No. 43). As a result, there were 64 observations in total. Secondary tumors were classified according to the criteria established by Hong et al.[24]: seven patients had LRs (5/7 LRs limited to oral cavity and 2/7 LRs of the oral cavity also presented lymph node involvement) and 12 had SPT developed in oral cavity. In addition, seven patients died due to disease progression and two died due to unrelated causes.

Figure 2 presents the Kaplan–Meier DFS curve. The log-rank test showed that age >70 years was associated with decreased DFS ($p = 0.001$). The relapse rate was 2.05 per 100 person-months among patients aged >70 years and 0.39 per 100 person-months among those aged ≤70 years. A hard palate tumor location had a significant negative association with DFS ($p = 0.002$); the incidence rate was 3.78 per 100 person-months in this location and 0.86 per 100 person-months in the remaining tumor locations.

TABLE 1 Baseline characteristics at index OSCC manifestation ($n = 61$).

Characteristic	*n* (%)
Female	35 (57%)
Age >70 years	27 (44%)
Smoker	13 (21%)
OSCC location	
Gum	23 (38%)
Tongue	16 (26%)
Labial gingival mucosa	11 (18%)
Hard palate	8 (13%)
Soft palate	2 (3%)
Floor of mouth	1 (2%)
Grading	
1	27 (44%)
2	25 (41%)
3	9 (15%)
T3/T4 size and extent	23 (38%)
Lymph node involvement	12 (20%)
Radiation therapy	23 (38%)
Reconstructive skin flap	26 (43%)
Perineural invasion	9 (15%)
Vascular invasion	4 (7%)
Depth of invasion ≥4 mm	27 (44%)
Surgical margin	
Clear	57 (93%)
Close	3 (5%)
Dysplasia at surgical margin	1 (2%)

Abbreviation: OSCC, oral squamous cell carcinoma.

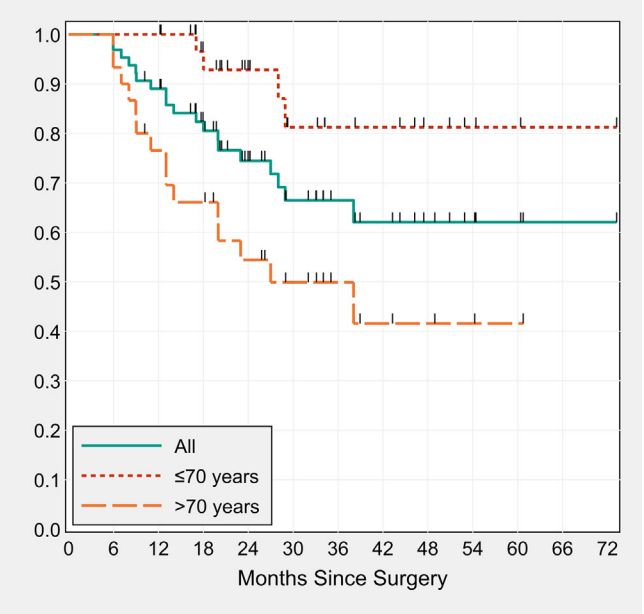

FIGURE 2 Kaplan–Meier survival estimates of time to relapse after surgical resection for oral squamous cell carcinoma (OSCC), overall and by age group. The spikes indicate censoring times. [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | Thirteen-gene DNA methylation analysis

A preoperative positive score has been detected in 61 OSCC patients of the population study (mean value 3.77 ± 1.37).

As shown in Figure 1, 221 oral brushing specimens were analyzed during follow-up. The DNA amount ranged between 100 and 500 ng. None of clinical variables (included radiation therapy) resulted significantly related with a significant lower DNA amount. A single oral brushing sample was collected in 9 patients (14%), all of whom experienced disease relapse, while two specimens were collected in 16 patients (25%), three in 11 patients (17%), four in 13 patients (20%), five in 5 patients (8%), six in 4 patients (6%), seven in 3 patients (5%), eight in 2 patients (2%), and nine in 1 patient (1%). Cases were classified into persistently negative ($n = 14$, 22%), persistently positive ($n = 20$, 31%), and mixed ($n = 30$, 47%) score groups. Among the 30 patients with mixed profiles, 21 tested negatives after the first oral brushing sample collection performed at 4–10 months after OSCC surgical resection, while 9 tested positive; in these two groups, 3/21 (14%) and 1/9 (11%) SPTs were observed, respectively.

3/61 OSCC cases showed close margins of surgical resection and finally one case showed histological dysplasia on the margin of resection. 2/3 close margins of the surgical resection margins showed a persistent positive score in oral brushing cell collection 6 months after OSCC resection. The only case with mild dysplasia on the margin of resection showed a negative score for oral brushing cell collection 6 months after OSCC resection. No significant relationship was found between the persistent positive score and the presence of a close resection margin.

A preoperative mean-score of 3.14 ± 0.3 has been calculated in the group of patients with persistently positive score during follow-up period, 2.8 ± 0.2 in the group of patients with mixed score and a mean score of 2.3 ± 0.4 has been detected in the group of patients with persistently negative score during follow-up period. A mean postoperative score of 2.75 ± 0.27 has been calculated in the group with persistently positive scores during the follow-up period, 0.96 ± 0.2 in the group with a mixed score during the follow-up period, and a mean

TABLE 2 Distribution of brushing score results in cases and matched controls obtained via risk-set sampling; values are count (percentage) or mean ± standard deviation [range].

Brushing score	Relapse cases ($n = 19$)	Matched controls ($n = 66$)[a]
Results over follow-up period		
Negative	0 (0%)	28 (42%)
Positive	15 (79%)	23 (35%)
Mixed (both neg. and pos.)	4 (21%)	15 (23%)
Last result before matching date		
Negative	1 (5%)	32 (48%)
Positive	18 (95%)	34 (52%)

[a]Sample size is 66 instead of $19 \times 4 = 76$ because $76 - 66 = 10$ controls had no available cytological samples between the date of surgery and the matching date, and were thus discarded.

postoperative score of -0.35 ± 0.29 was detected in the group of patients with persistently negative scores during the follow-up period. No correlations exist between preoperative and postoperative score during follow-up period.

Table 2 presents the scores for the cases and matched controls. Controls were matched to cases at a ratio of 4:1 based on the follow-up duration, to ensure a similar time window for the measurement of methylation scores between the two groups. Among the 19 relapsed cases, 15 (79%) had persistently positive results before recurrence, whereas 4 (21%) had mixed results and none had persistently negative results. In particular, among the 15 cases with persistently positive results, 9 (5 LRs and 4 SPTs) were diagnosed after a single oral brushing sample collection, 4 (2 LRs and 2 SPTs) after two collections, and 2 SPTs after four collections. Four patients with mixed results experienced a secondary neoplastic event (Figure 1). The first case (No. 21) developed SPT after two negative tests at 7 and 13 months, and had a positive test at 21 months after the diagnosis of the index cancer and 6 months before the recurrence. The second case (No. 22) developed SPT following a first positive test 8 months after OSCC treatment, and had a subsequent negative test 5 months before the SPT was detected. The third case (No. 30) developed SPT after a first negative test, and had two positive tests at 14 and 21 months after index tumor excision. The fourth case (No. 49) developed SPT following a first negative test 8 months after OSCC treatment and a positive test 14 months after diagnosis and excision of the index cancer.

The median time between the last oral brushing sample collection and secondary tumor appearance was 2.6 (range: 0.7–9.5) months (see Table 3 for detailed data on relapsed cases).

Table 4 presents the results of the regression analysis. Compared to persistently negative patients, persistently positive patients had an almost 42-fold higher relapse likelihood (OR = 42.15, $p < 0.001$), whereas patients with mixed results had a 32-fold higher likelihood (OR = 31.96, $p = 0.006$). No significant differences were observed between the persistently positive and mixed groups, even after adjustment for the baseline risk factors. Compared to persistently negative patients, persistently positive patients had a 58-fold higher LR likelihood (OR: 57.96) and 20-fold higher SPT likelihood (OR: 19.31) (see Tables S1 and S2). No significant differences were observed between mixed groups and negative groups. Nearly all single methylation beta values included in the last available score before the matching date were significantly associated with increased relapse likelihood: *ZAP70-16* (OR = 4.06, $p = 0.000$), *GP1BB-1* (OR = 2.41, $p = 0.010$), *MiR193-12* (OR = 2.02, $p = 0.010$), *NTM-14* (OR = 2.00, $p = 0.000$), *LRRTM1-3* (OR = 2.00, $p = 0.000$), *KIF1A-22* (OR = 1.92, $p = 0.000$), *PARP15-2* (OR = 1.60, $p = 0.050$), *EPHX3-1* (OR = 1.60, $p = 0.030$), and *LINC00599-1* (OR = 1.57, $p = 0.040$). These results indicate an increase in the odds of relapse of one standard deviation with an increase in the methylation beta values.

4 | DISCUSSION

In the present study, we evaluated whether 13-gene DNA methylation analysis from oral brushing can be repeated to determine the time-related risk of OSCC development and identify LRs and SPTs. To our knowledge, this is the first study to use a minimally invasive tool based on DNA methylation analysis performed at different times to determine the risk of relapse during follow-up of patients treated for primary OSCC.

The 13-DNA methylation analysis of repeat oral brushing specimens was used to categorize patients into persistently negative ($n = 14$; score <1.06457), persistently positive ($n = 20$; score >1.06457), and mixed ($n = 30$) groups.

The present results showed that patients with persistently positive (OR = 42) or mixed (OR = 32) scores had a significantly higher risk of OSCC relapse compared to those with persistently negative scores. Specifically, none of the 14 patients with persistently negative scores developed a secondary tumor. In comparison, 15 (7 LRs and 8 SPTs) of the 19 secondary carcinomas had persistently

TABLE 3 Distribution of the scoring results during oncological follow-up in patients who developed multiple oral squamous cell carcinoma during oncological follow-up.

Cases	Index tumor date	Time distribution of oral brushing sampling collection, score calculation and secondary neoplastic manifestations					
Case 10	December 2015	April 2016: POS (2.19)*	December 2016: POS (1.45)*	August 2017: POS (1.35)*	April 2018: POS (1.26)*	February 2019: SPT‡	
Case 18	August 2016	April 2017: POS (2.76)*	November 2017: POS (1.18)*	April 2018: SPT‡			
Case 20	August 2016	May 2017: POS (3.39)*	September 2017: LR†	May 2018: POS (1.91)*	January 2019: POS (3.12)*	August 2019: SPT‡	
Case 21	September 2016	April 2017: NEG (−1.37)	October 2017: NEG (−0.56)	June 2018: POS (2.35)*	January 2019: SPT‡		
Case 22	September 2016	May 2017: POS (3.35)*	December 2017: NEG (0.49)	May 2018: SPT ‡			
Case 28	November 2016	April 2017: POS (8.88) *	May 2017: LR†				
Case 30	December 2016	July 2017: NEG (0.68)	February 2018: POS (2.08)*	September 2018: POS (4.06)*	March 2019: SPT‡		
Case 32	March 2017	June 2017: POS (1.27)*	January 2018: POS (2.54)*	September 2018: POS (3.04)*	April 2019: POS (1.79)*	July 2019: SPT‡	
Case 35	May 2017	November 2017: POS (2.34)*	January 2018: LR†				
Case 43	November 2017	March 2018: POS (1.15)*	May 2018: LR†	September 2018: POS (1.67)*	December 2018: SPT‡	June 2019: POS (1.64)*	September 2019: SPT‡
Case 46	December 2017	April 2018: POS (3.99)*	October 2018: POS (3.86)*	January 2019: LR†			
Case 49	March 2018	November 2018: NEG (0.96)	May 2019: POS (2.66)*	August 2019: SPT‡			
Case 54	June 2018	February 2019: POS (2.22)*	October 2019: POS (2.8)*	December 2019: LR†			
Case 55	June 2018	December 2018: POS (3.59)*	March 2019: SPT‡				
Case 59	August 2018	April 2019: POS (1.15)*	July 2019: SPT‡				
Case 61	March 2019	September 2019: POS (1.86)*	May 2020: LR†				

Abbreviations: LR†, local recurrence; NEG, negative test; POS*, positive test; SPT‡, second primary tumor.

TABLE 4 Odds ratio estimates (*p*-values) for OSCC relapse obtained with unconditional Firth-type logistic regression; the full set of pairwise comparisons between the three exposure groups is presented.

	Reference: Low		Reference: High		Reference: Mixed	
Brushing score	**Unadjusted**	**Adjusted[a]**	**Unadjusted**	**Adjusted[a]**	**Unadjusted**	**Adjusted[a]**
Negative	1.00	1.00	0.02[b]	0.04[b]	0.03[b]	0.05[b]
	(·)	(·)	(<0.001)	(<0.001)	(0.006)	(0.020)
Positive	42.15[b]	28.12[b]	1.00	1.00	1.32	1.42
	(<0.001)	(<0.001)	(·)	(·)	(0.712)	(0.639)
Mixed (neg./pos.)	31.96[b]	19.75[b]	0.76	0.70	1.00	1.00
	(0.006)	(0.020)	(0.712)	(0.639)	(·)	(·)

[a]Controlled for age >70 years, smoking, and hard palate OSCC location via propensity-score covariate adjustment.
[b]Significant at the 5% level (*p*-value ≤0.05).

positive scores during follow-up: 9 further carcinomas (5 LRs and 4 SPTs) developed after the first oral brushing sample collection 4–10 months after OSCC surgical resection, 4 secondary OSCCs (2 LRs and 2 SPTs) developed after the second positive test, and 2 SPTs developed after four positive tests.

Interestingly, 30 surgically treated OSCC patients with mixed scores for the brushing samples collected every 6 months during follow-up were identified. As described previously, 21 (70%) of the 30 mixed results showed a negative score after the first oral brushing sample collection performed 4–10 months after OSCC surgical resection.

The four patients with mixed results who developed a SPT had characteristic epigenetic alterations: three had a first negative result 6 months after OSCC treatment, with subsequent samples showing a positive result. In the presence of a single oral brushing sample, patients may be erroneously considered to be at low risk of a secondary tumor. One only of these four patients developed a secondary event 5 months after a single negative test (No. 22).

The changes of the scores (i.e., positive and negative) in the mixed group may have been due to an insufficient number of adult cancer stem cells or cancer cells to repopulate the area of surgical intervention, leading to aberrant methylation patterns. Alternatively, this phenomenon might be explained by tumor heterogeneity, which may lead to insufficient altered epialleles to cross the threshold value.

The present results confirmed the predictive value of the 13-gene methylation analysis: 18 (95%) of 19 locoregional relapses developed after a positive score on oral brushing sample collection. Further studies are needed to understand the implications of the negative score obtained before SPT developed in case No. 22. A strategy characterized by a strict brushing sample collection interval (i.e., every 3–4 months) may enhance the ability of 13-gene based methylation analysis to identify patients at risk of secondary tumors.

In total, 9 of the 13 genes (*ZAP70-16*, *GP1BB-1*, *MiR193-12*, *NTM-14*, *LRRTM1-3*, *KIF1A-22*, *PARP15-2*, *EPHX3-1*, and *LINC00599-1*) showed a significantly altered methylation level in samples collected before the development of a secondary tumor compared to the remaining samples.

The present results confirm the role of DNA methylation as a molecular biomarker of microscopic and histological cellular alterations after OSCC treatment. This is the first study to reveal time-dependent epigenetic instability in the clinically healthy mucosa of patients surgically treated for OSCC.

Two different mechanisms have been proposed to explain the high rate of second neoplastic manifestations in the oral cavity. Slaughter et al. introduced the concept of "field cancerization": on the basis of histological examinations the authors hypothesized the persistence of abnormal tissue after surgery that explains the high rate of second neoplastic manifestations.[32] A secondary oral cancer may be also related to the possibility of an incomplete surgical resection of primary tumor.[2]

Previous studies have demonstrated that the presence of epithelial precursor lesions and/or molecular alterations in the negative surgical margins of resection beyond OSCC are powerful risk factors for secondary neoplastic events. Altered expression of genetic markers such as p53,[33–36] hLy6D,[37] and epigenetic markers has been associated with the presence of minimal residual disease and local recurrence. In contrast, dysplasia at the surgical margin of resection,[38,39] loss-of-heterozygosity (LOH),[40] and altered expression of Ki-67 40,[41] MMP9, and PTHLH[42] were observed in tumor-adjacent normal tissue and were associated with preneoplastic altered fields. Specifically, two recent studies demonstrated a relationship between the presence of dysplasia at surgical margins of resection and appearance of the SPT[38,39] whereas Carvalho et al. demonstrated that altered expression of MMP9 and PTHLH in the analysis of negative surgical margins of resection was associated with SPT.[42]

Data of the present study suggested that oral brushing cell collection in wide regenerative area after OSCC resection resulted in a minimally invasive procedure able to identify epigenetic modifications related to small clusters of residual tumor cells or a field effect undetectable in the histological analysis of the margins or both responsible for the development of a LR or SPT. The oral brushing sampling procedure showed potential limitations: data from the present study did not reveal whether an altered methylation level was related to the presence of minimum residual disease or the presence of a preneoplastic field. In case of a field effect responsible for multiple tumors brushing cell collection in a wide regenerative area after oral cancer surgical resection is not able to identify exactly the extension preneoplastic field, further investigations with a second oral brushing sampling collection in the opposite clinically normal mucosa (cheek opposite) may help us to identify the extension of the preneoplastic field.

In line with our results, two studies reported hypermethylation of a single gene or panel of genes in saliva samples collected after the diagnosis and treatment of primary OSCC.[43,44] Finally, a recent study found that the minimally invasive procedure is feasible, thereby highlighting the value of oral brushing sample as a noninvasive surrogate for tissue biopsies for the epigenomic profiling of oral cancer.[45]

Another potential limit of this study, in addition to the small population, is the presence of a high number of

elderly patients (44%). OSCC is characterized by high prevalence in elderly age and it is well known that aging can affect global genome methylation.[46] However, an increase in the incidence of OSCC in patients younger than 45 has been recently reported and further investigation is needed to analyze the reliability of 13-gene DNA methylation analysis in selected younger than 45 years old OSCCs.

5 | CONCLUSIONS

The present study evaluated the application of a minimally invasive procedure based on 13-gene DNA methylation analysis of oral brushing samples collected at different times during the follow-up of patients surgically treated for oral cancer. A positive score on methylation analysis was associated with diagnostic accuracy of >90% for emerging secondary neoplastic events. Furthermore, epigenetic instability was observed in patients with OSCC. Further studies with long-term follow-up and a shorter surveillance interval (i.e., every 3 months) are necessary to verify our findings and determine the optimal brushing cell collection interval. Moreover, biological factors potentially related to OSCC influence the methylation status of patients surgically treated for this oral cancer.

ACKNOWLEDGMENTS

This study was supported by an academic grant (Proof of Concept—PoC 2019 assigned to Luca Morandi) from the University of Bologna. The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/FWS3Bn

CONFLICT OF INTEREST STATEMENT

As a possible conflict of interest, Luca Morandi, Davide B Gissi, and Achille Tarsitano submitted a patent (the applicant is the University of Bologna) in November 2016 to the National Institute of Industrial Property; however, we believe that this is a natural step of translational research (bench-to-bedside) and guarantee that the scientific results are true. The remaining authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Davide B. Gissi https://orcid.org/0000-0002-0195-7694

REFERENCES

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209-249. doi:10.3322/caac.21660
2. Braakhuis BJM, Tabor MP, Leemans CR, van der Waal I, Snow GB, Brakenhoff RH. Second primary tumors and field cancerization in oral and oropharyngeal cancer: molecular techniques provide new insights and definitions. *Head Neck*. 2002;24(2):198-206. doi:10.1002/hed.10042
3. Janot F, de Raucourt D, Benhamou E, et al. Randomized trial of postoperative reirradiation combined with chemotherapy after salvage surgery compared with salvage surgery alone in head and neck carcinoma. *J Clin Oncol*. 2008;26(34):5518-5523. doi:10.1200/JCO.2007.15.0102
4. Lee N, Chan K, Bekelman JE, et al. Salvage re-irradiation for recurrent head and neck cancer. *Int J Radiat Oncol Biol Phys*. 2007;68(3):731-740. doi:10.1016/j.ijrobp.2006.12.055
5. Ho AS, Kraus DH, Ganly I, Lee NY, Shah JP, Morris LGT. Decision making in the management of recurrent head and neck cancer. *Head Neck*. 2014;36(1):144-151. doi:10.1002/hed.23227
6. Menezes FDS, Fernandes GA, Antunes JLF, Villa LL, Toporcov TN. Global incidence trends in head and neck cancer for HPV-related and -unrelated subsites: a systematic review of population-based studies. *Oral Oncol*. 2021;115:105177. doi:10.1016/j.oraloncology.2020.105177
7. Cao C, Gan X, He Y, et al. Diagnostic efficacy of PET-CT, CT, and MRI in preoperative assessment of mandibular invasion caused by head and neck cancer: a systematic review and meta-analysis. *Oral Oncol*. 2021;116:105264. doi:10.1016/j.oraloncology.2021.105264
8. Herman JG. Hypermethylation of tumor suppressor genes in cancer. *Semin Cancer Biol*. 1999;9(5):359-367. doi:10.1006/scbi.1999.0138
9. Feinberg AP. The key role of epigenetics in human disease prevention and mitigation. Longo DL, ed. *N Engl J Med*. 2018;378(14):1323-1334. doi:10.1056/NEJMra1402513
10. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med*. 2003;349(21):2042-2054. doi:10.1056/NEJMra023075
11. Morandi L, Gissi D, Tarsitano A, et al. CpG location and methylation level are crucial factors for the early detection of oral squamous cell carcinoma in brushing samples using bisulfite sequencing of a 13-gene panel. *Clin Epigenetics*. 2017;9:85. doi:10.1186/s13148-017-0386-7
12. Guerrero-Preston R, Michailidi C, Marchionni L, et al. Key tumor suppressor genes inactivated by "greater promoter" methylation and somatic mutations in head and neck cancer. *Epigenetics*. 2014;9(7):1031-1046. doi:10.4161/epi.29025
13. Marsit CJ, Christensen BC, Houseman EA, et al. Epigenetic profiling reveals etiologically distinct patterns of DNA methylation in head and neck squamous cell carcinoma. *Carcinogenesis*. 2009;30(3):416-422. doi:10.1093/carcin/bgp006
14. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J. Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. *Cancer Res*. 2008;68(7):2094-2105. doi:10.1158/0008-5472.CAN-07-5194
15. Li YF, Hsiao YH, Lai YH, et al. DNA methylation profiles and biomarkers of oral squamous cell carcinoma. *Epigenetics*. 2015;10(3):229-236. doi:10.1080/15592294.2015.1006506

16. Gissi DB, Morandi L, Colella G, et al. Clinical validation of 13-gene DNA methylation analysis in oral brushing samples for detection of oral carcinoma: an Italian multicenter study. *Head Neck*. 2021;43:1563-1573. doi:10.1002/hed.26624
17. Gissi DB, Tarsitano A, Gabusi A, et al. 13-Gene DNA methylation analysis from oral brushing: a promising non invasive tool in the follow-up of oral cancer patients. *J Clin Med*. 2019;8(12):2107. doi:10.3390/jcm8122107
18. Rossi R, Gissi DB, Gabusi A, et al. A 13-gene DNA methylation analysis using oral brushing specimens as an indicator of oral cancer risk: a descriptive case report. *Diagnostics (Basel)*. 2022;12(2):284. doi:10.3390/diagnostics12020284
19. Kademani D, Bell RB, Bagheri S, et al. Prognostic factors in intraoral squamous cell carcinoma: the influence of histologic grade. *J Oral Maxillofac Surg*. 2005;63(11):1599-1605. doi:10.1016/j.joms.2005.07.011
20. Caudell JJ, Gillison ML, Maghami E, et al. NCCN Guidelines® insights: head and neck cancers, version 1.2022. *J Natl Compr Canc Netw*. 2022;20(3):224-234. doi:10.6004/jnccn.2022.0016
21. Gissi DB, Gabusi A, Tarsitano A, et al. Application of a non-invasive oral brushing procedure based on bisulfite sequencing of a 13-gene panel to study high-risk OSCC patients. *Cancer Biomark*. 2020;28(4):499-510. doi:10.3233/CBM-190422
22. Morandi L, Gissi D, Tarsitano A, et al. 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. *J Craniomaxillofac Surg*. 2015;43(8):1494-1500. doi:10.1016/j.jcms.2015.07.028
23. Amin MB, Edge S, Greene F, et al., eds. *AJCC Cancer Staging Manual*. 8th ed. Springer International Publishing; 2017. Accessed July 9, 2019. https://www.springer.com/gp/book/9783319406176
24. Hong WK, Lippman SM, Itri LM, et al. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med*. 1990;323(12):795-801. doi:10.1056/NEJM199009203231205
25. Warren S, Gates O. Multiple primary malignant tumors: a survey of the literature and statistical study. *Am J Cancer*. 1932;16:1358-1414.
26. Afgan E, Baker D, Batut B, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res*. 2018;46(W1):W537-W544. doi:10.1093/nar/gky379
27. Ernster VL. Nested case–control studies. *Prev Med*. 1994;23(5):587-590. doi:10.1006/pmed.1994.1093
28. Essebag V, Platt RW, Abrahamowicz M, Pilote L. Comparison of nested case–control and survival analysis methodologies for analysis of time-dependent exposure. *BMC Med Res Methodol*. 2005;5(1):5. doi:10.1186/1471-2288-5-5
29. Heinze G, Schemper M. A solution to the problem of separation in logistic regression. *Stat Med*. 2002;21(16):2409-2419. doi:10.1002/sim.1047
30. Pearce N. Analysis of matched case–control studies. *BMJ*. 2016;352:i969. doi:10.1136/bmj.i969
31. Heinze G, Ploner M, Jiricka L. logistf: Firth's Bias-Reduced Logistic Regression. R package version 1.24. 2020. https://CRAN.R-project.org/package=logistf
32. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer*. 1953;6(5):963-968. doi:10.1002/1097-0142(195309)6:53.0co;2-q
33. Brennan JA, Mao L, Hruban RH, et al. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. *N Engl J Med*. 1995;332(7):429-435. doi:10.1056/NEJM199502163320704
34. van Houten VMM, Tabor MP, van den Brekel MWM, et al. Mutated p53 as a molecular marker for the diagnosis of head and neck cancer. *J Pathol*. 2002;198(4):476-486. doi:10.1002/path.1242
35. Nees M, Homann N, Discher H, et al. Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: a possible molecular basis for the development of multiple tumors. *Cancer Res*. 1993;53(18):4189-4196.
36. Nakanishi Y, Noguchi M, Matsuno Y, et al. p53 expression in multicentric squamous cell carcinoma and surrounding squamous epithelium of the upper aerodigestive tract. Immunohistochemical analysis of 95 lesions. *Cancer*. 1995;75(7):1657-1662. doi:10.1002/1097-0142(19950401)75:73.0.co;2-g
37. Graveland AP, Bremmer JF, de Maaker M, et al. Molecular screening of oral precancer. *Oral Oncol*. 2013;49(12):1129-1135. doi:10.1016/j.oraloncology.2013.09.005
38. Slootweg PJ, Hordijk GJ, Schade Y, van Es RJJ, Koole R. Treatment failure and margin status in head and neck cancer. A critical view on the potential value of molecular pathology. *Oral Oncol*. 2002;38(5):500-503. doi:10.1016/s1368-8375(01)00092-6
39. Montebugnoli L, Gissi DB, Flamminio F, et al. Clinicopathologic parameters related to recurrence and locoregional metastasis in 180 oral squamous cell carcinomas. *Int J Surg Pathol*. 2014;22(1):55-62. doi:10.1177/1066896913511982
40. Tabor MP, Brakenhoff RH, van Houten VM, et al. Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. *Clin Cancer Res*. 2001;7(6):1523-1532.
41. Govindaraj PK, Kallarakkal TG, Mohd Zain R, Tilakaratne WM, Lew HL. Expression of Ki-67, Cornulin and ISG15 in non-involved mucosal surgical margins as predictive markers for relapse in oral squamous cell carcinoma (OSCC). *PLoS One*. 2021;16(12):e0261575. doi:10.1371/journal.pone.0261575
42. de Carvalho AC, Kowalski LP, Campos AHJFM, Soares FA, Carvalho AL, Vettore AL. Clinical significance of molecular alterations in histologically negative surgical margins of head and neck cancer patients. *Oral Oncol*. 2012;48(3):240-248. doi:10.1016/j.oraloncology.2011.10.018
43. Rettori MM, de Carvalho AC, Bomfim Longo AL, et al. Prognostic significance of TIMP3 hypermethylation in post-treatment salivary rinse from head and neck squamous cell carcinoma patients. *Carcinogenesis*. 2013;34(1):20-27. doi:10.1093/carcin/bgs311
44. Righini CA, de Fraipont F, Timsit JF, et al. Tumor-specific methylation in saliva: a promising biomarker for early detection of head and neck cancer recurrence. *Clin Cancer Res*. 2007;13(4):1179-1185. doi:10.1158/1078-0432.CCR-06-2027
45. Viet CT, Zhang X, Xu K, et al. Brush swab as a noninvasive surrogate for tissue biopsies in epigenomic profiling of oral cancer. *Biomark Res*. 2021;9(1):90. doi:10.1186/s40364-021-00349-x

46. Bollati V, Schwartz J, Wright R, et al. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mech Ageing Dev*. 2009;130(4):234-239. doi:10.1016/j.mad.2008.12.003

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Gissi DB, Rossi R, Lenzi J, et al. Thirteen-gene DNA methylation analysis of oral brushing samples: A potential surveillance tool for periodic monitoring of treated patients with oral cancer. *Head & Neck*. 2024;1-12. doi:10.1002/hed.27621