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Original article

DNA methylation analysis from oral brushing reveals a field cancerization effect in proliferative verrucous leukoplakia

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Summary

Objectives. The aim of the present study was to analyze the methylation status in patients who presented with an Oral Squamous Cell Carcinoma (OSCC) concomitantly with multifocal Proliferative Verrucous Leukoplakia (PVL)(PVL-OSCC).

Methods. Nine patients with OSCC and concomitant PVL lesions were selected. Two brushing samples were collected simultaneously from OSCC and PVL lesions in contralateral mucosa from each patient. 15 genes (272 CpGs) were used to compare methylation profiles of PVL-OSCC and paired OSCC. CpGs with a methylation level superimposable between PVL-OSCC and contralateral OSCC were selected for a comparative analysis between PVL-OSCC, 8 PVL patients with no history of OSCC (PVL) and 23 healthy donors. Samples were also tested using an algorithm that was recently validated for epigenetic alterations in OSCC.

Results. 220/272 CpGs islands (80%) showed a superimposable methylation level in OSCC and in PVL-OSCC. 10 genes (88 CpGs) and in particular *PARP15* and *ITGA4* (100% of the studied CpGs) were able to stratify PVL-OSCC from PVL and healthy donors. 3/4 (75%) PVL-OSCC patients with a "positive" algorithm score developed second neoplastic events compared to only 1/5 (20%) patients with a "negative" score.

Conclusions. The present study provides evidence that PVL shares an aberrant methylation profile with contralateral OSCC. In agreement with the theory of field cancerization, our data point towards the potential role of epigenetics in patients at risk of developing multiple neoplastic events.

Key words: oral squamous cell carcinoma, proliferative verrucous leukoplakia, methylation, oral field cancerization, oral brushing

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Introduction

Proliferative Verrucous Leukoplakia (PVL) is an aggressive clinical variant of Oral Leukoplakia (OLK) first described in 1985 by Hansen and colleagues [1].

The latest WHO blue book on Head and Neck tumors [2] defined PVL as a distinct form of multifocal oral leukoplakia characterized by a progressive clinical course, changing clinical and histopathological features and the

highest rate of cancer development compared to other OPMDs [3,4].

A recent systematic review reported a malignant transformation rate of 49.5% [5] (95% CI = 26.7–72.4%), while many reports describe PVL as being characterized by a marked tendency to evolve into multiple carcinomas [6].

Such behavior is deeply connected with the field cancerization effect described by Slaughter et al. [7] according to which a large portion of mucosa extending beyond the clinical and histological boundaries of the tumor is affected by genetic abnormalities. Hence, areas distant from the primary tumor may give rise to multiple additional neoplastic events.

Therefore, PVL is commonly accepted as one of the most important entities included among oral potentially malignant disorders (OPMDs) [3].

At present, the clinical management of patients affected by PVL relies on strict clinical observation to intercept malignant evolution at the earliest stage. Indeed, surgical management is poorly effective due to high rates of recurrence as well as not being protective against tumor development [8].

The introduction of a molecular biomarker ancillary to clinical examination that can recognize PVL patients at higher risk of malignant transformation during follow-up would be highly beneficial. Nevertheless, only few studies have investigated the molecular landscape of PVL.

Investigations of DNA ploidy in PVL have found abnormalities in DNA content in the majority of the studied cases [9].

Other studies found that the inactivation of *CDKN2A* (*P16INK4a*/*P14ARF*) which encodes the tumor suppressor proteins p16 (p16INK4a) and p14ARF is more frequently involved in PVLs than in OLKs [10].

Immunohistochemical studies exploring the expression of Ki-67 in PVL and normal epithelium have found a modest increase in Ki-67 in PVL and a moderate increase in PVLs undergoing malignant transformation [11,12]. Conversely, studies investigating the immunohistochemical expression of p53 in PVL have found conflicting results [11,13,14]. Therefore, none of the studied biomarkers can accurately predict malignant transformation [15].

DNA methylation plays an important role in various biological processes, such as gene expression, cell differentiation, development, and inflammation. Moreover, promoter methylation seems to be an early event in oral carcinogenesis and has been reported to occur even more frequently than mutations and deletions [16].

The identification of epigenetic alterations involved in the malignant degeneration of PVLs would be of great importance for the management of these OPMDs. However, few studies have investigated the epigenetic landscape of PVL [17,18].

The aim of the present study was to explore the methylation status of 9 patients with concomitant OSCC and PVLs and to compare the methylation profiles of both lesions in the same patient.

Materials and methods

All clinical investigations were conducted in accordance with the principles of the Declaration of Helsinki and the study was approved by the local ethics committee (520/2018/Sper/AOUBo 12 December 2018). All information pertaining to human material used in this study was managed using anonymous numerical codes.

We selected 9 consecutive patients with concomitant OSCC and PVLs referred to and/or followed up at the Department of Biomedical and Neuromotor Sciences, Section of Oral Sciences, University of Bologna.

To be included in the case series, patients had to display: a) a diagnosis of PVL based on clinical and histopathological examination. b) a diagnosis of squamous cell carcinoma formulated simultaneously or after the diagnosis of PVL.

The clinical diagnostic criteria adopted for PVL were those proposed by Cerero-Lapiedra et al. in 2010 [19] and in particular the following major criteria: a) the presence of a leukoplakia lesion with more than two different oral sites, b) the presence of a verrucous area, and c) lesions that have spread or engrossed during the development of the disease.

In all cases, malignant transformation was histologically confirmed using preoperative diagnostic biopsy.

In case of PVL diagnosis prior to OSCC development, all patients performed one or more biopsies during the oncological follow-up. In presence of simultaneous presence of OSCC and PVL lesions two incisional biopsies were always performed, one from the suspected malignant lesion and the other in the most representative area of the multifocal PVL lesions. Diagnostic biopsies and surgical specimens were subjected to histological analysis at the Sections of Anatomic Pathology of the University of Bologna at Bellaria and Sant'Orsola Hospitals. Biopsies of all cases were reviewed and the histological diagnosis of PVL was confirmed according to recently reported criteria [20]. Cases not fulfilling the aforementioned criteria were excluded.

Surgical resection of OSCC was performed in accordance with standard practices at the Maxillofacial Surgery Unit, Sant'Orsola Hospital.

Postoperatively, patients underwent routine follow-up, including clinical, instrumental, and radiological examinations in accordance with the International National

Comprehensive Cancer Network guidelines [21]. Demographic and clinical variables such as age, sex, smoke, site of malignant transformation, TNM, Grading and development of secondary tumors were recorded.
As reference samples, we also evaluated 8 PVL patients that were not transformed into OSCC (PVL Group) and 23 healthy donors (Healthy Group).

Oral brushing sample collection

The brushing specimens were collected according to a previously described protocol [22,23] using the kit SG-Oral Collection Kit (Studium Genetics Cat No. SG001-5, Italy). In brief, after collection each cytobrush sample was stored in a 2-ml tube containing a preservation solution at room temperature until DNA purification.
In the 9 patients with OSCC and concomitant PVL two oral brushing samples were collected before surgical resection of OSCC for DNA methylation analysis; one sample (OSCC Group) was collected from the histologically proven area of squamous cell carcinoma (Figs. 1a, 2a), and the other (PVL-OSCC Group) from white/verrucous PVL oral lesions in the contralateral mucosa (Figs. 1b, 2b).
In the PVL not evolved, oral brushing cell collection was performed in a wide more representative area of lesions, whereas in healthy volunteers the surface of different sites of the oral mucosa (cheeks, marginal, and dorsal tongue) was gently brushed with rotation and translational movements.

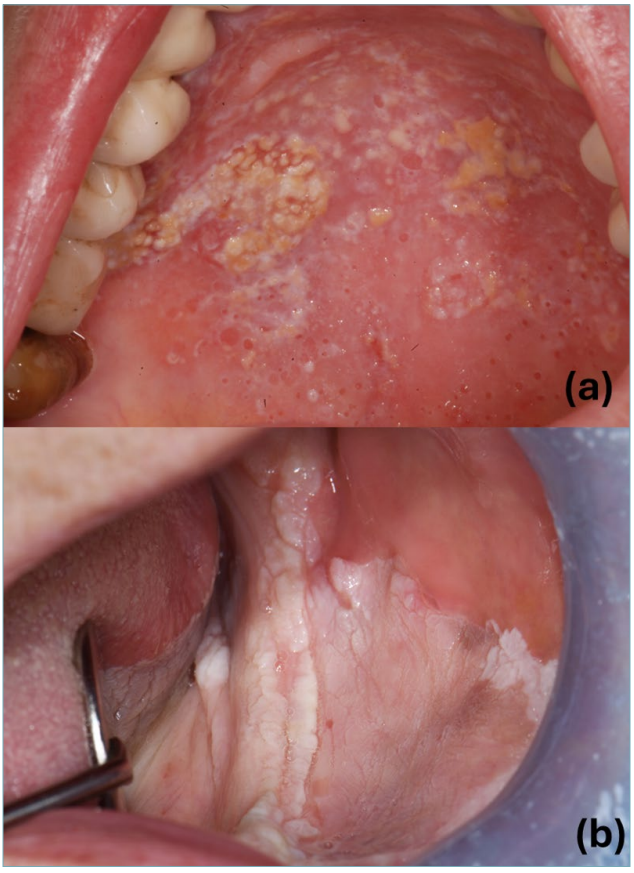

Figure 1. Clinical picture of the oral squamous cell carcinoma lesion from patient #2. Diagnosis confirmed by incisional biopsy (a). Clinical picture from the same patient showing the contralateral area affected by white verrucous lesions related to PVL. Both lesions were sampled for methylation analysis (b).

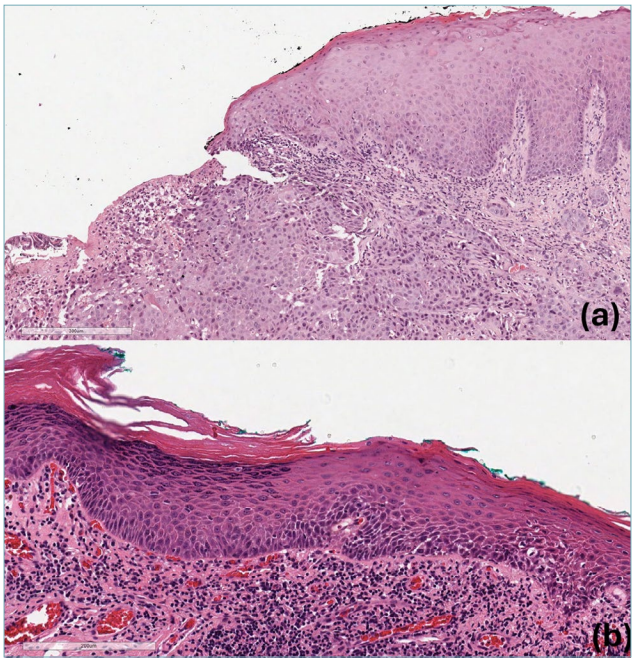

Figure 2. Squamous cell carcinoma: At the margin of the mucosal section, a solid-growing neoplasm is present, consisting of large clusters of cells with pink cytoplasm with no squamous pearls. The neoplastic cells show prominent intercellular bridges, and round, frequently hyperchromatic nuclei and mitotic figures (a). Proliferative verrucous leukoplakia: section of oral mucosa, lined by squamous epithelium with thickened hyperkeratotic growth accompanied by a lichenoid inflammatory response. The epithelium is characterized by orthokeratosis, with no cytological atypia and sharp transitions and skip zone of corrugated orthokeratosis (b).

DNA methylation analysis

Quantitative methylation analysis included next-generation sequencing of the following 15 genes: *KIF1A, ZAP70, GP1BB, LRRTM, PARP15, FLI1, NTM, EPHX, TERT, LINC0059, ITGA4, miR193, miR296, PAX1, miR137.* 13/15 genes (*KIF1A ZAP70 GP1BB LRRTM PARP15 FLI1 NTM EPHX TERT LINC0059 ITGA4 miR193 miR296)* are part of a panel of genes previously described to be aberrantly regulated in OSCC

[22]. The inclusion of *PAX1* was based on the results of Guerrero-Preston et.al. who found a high frequency of genomic and epigenomic alterations in the PAX gene family in head and neck cancers [24]. *Mir137* was also added to the panel as in a recent study by Dang et al., the miR-137 promoter was found to be methylated in 58.3% in patients with OSCC [25].

DNA from exfoliated cells was purified using the Quick-DNA ™ MagBead Plus Kit (ZymoResearch Cat. No. D4082). DNA ranging from 100–500 ng was treated with sodium bisulphite using the EZ DNA Methylation-Lightning Kit™ (D5031; Zymo Research) according to the manufacturer's instructions. The target enrichment was prepared amplifying the regions of interest [22] by multiplex PCR with Phusion U DNA polymerase (ThermoFisher, cod. F555L). Indexing was performed using the Nextera™ Index Kit (Illumina, San Diego, CA, USA, FC-121-1012) and libraries were loaded onto MiSEQ (Illumina, San Diego, CA, USA, cod. 15027617). Each NGS experiment was designed to allocate at least 1000 reads/amplicon, with the aim to reach a depth of coverage of 1000×.

The FASTQ output files underwent quality control processing (> Q30) and were processed in a Galaxy Project environment (https://galaxy.studiumgenetics.com/) [26]. The methylation ratio of each CpG was calculated in parallel using BWAmeth followed by MethylDackel.

In a previous study [27], the best CpGs identified by receiver operating characteristic curve analysis were used to generate an algorithm based on a multiclass linear discriminant analysis of the best CpGs identified by ROC analysis in a panel of 13 gene (Oral Carcinoma Risk Algorithm, SG-OCRA ™). 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 of 1.06 were considered as "positive" results.

Statistical analysis

Quantitative methylation analysis of CpGs islands of PVL-OSCC lesions and contralateral OSCC from the same patient was performed using paired t test if the standardized skewness and kurtosis values were within the data range for a normal distribution or, alternatively, the paired samples Wilcoxon signed-rank test.

CpGs that displayed a methylation level not significantly different between PVL-OSCC lesions and contralateral OSCC were selected. A comparative analysis of the methylation level of PVL-OSCC samples with respect to PVL samples and healthy subjects was performed using Kruskal Wallis test and Multiple Range Test for each CpG.

For each analysis a value of $p < 0.05$ was considered statistically significant.

PCA analysis and the methylation plot were created using ClustVis, a web tool for visualizing clustering multivariate data (http://biit.cs.ut.ee/clustvis/) [28]. Methylation Plotter tool (http://maplab.cat/methylation_plotter) [29] was used to generate the Supplementary Figure 1 showing the methylation level for each CpG.

Results

Study population and follow-up

The cohort included a group of 9 patients with concomitant OSCC and PVL. Clinical information from each of the PVL patients in PVL-OSCC group is shown in Table I: median age was 69±13 years [range: 55-92], 7/9 patients (77%) were females, and no patients (0%) were smokers.

Table I. Demographic and clinical characteristics of 9 patients with OSCC tumor mass associated with PVL lesions.

Patient	Age	Sex	Smoke	Alcohol	PVL Site Brushing	OSCC Brushing Site	OSCC Histology	TNM	Grading	OSCC Score	PVL-OSCC Score	Multiple OSCCs
1	75	F	No	No	Left Cheek	Tongue	Squamous	pT2N0M0	G1	2,767352*	0,916179	Yes
2	92	F	No	No	Lower Gingiva	Palate	Squamous	pT1N0M0	G1	3,434539*	0,954786	No
3	57	M	No	No	Left cheek	Right cheek	Squamous	pT2N0	G2	1,657414*	-1,161367	No
4	60	F	No	No	Right cheek	Tongue	Squamous	pTis	G1	2,460917*	0,143952	No
5	64	M	No	No	Left cheek	Palate	Squamous	pT1N0M0	G2	1,912301*	-0,859304	No
6	85	F	No	No	Left cheek	Right cheek	Verrucous	pT1N0Mx	G2	5,799223*	7,6*	Yes
7	55	F	No	No	Right cheek	Palate	Squamous	pTis	G1	3,220426*	13,367421*	Yes
8	65	F	NO	No	Left cheek	Right mandibular gingiva	Squamous	PTis	G1	5,1337852*	2,8964698*	Yes
9	86	F	NO	No	Left Cheek	Palate	Squamous	pT1N0M0	G1	4,94078884*	7,28468145*	No

PVL Proliferative Verrucous leukoplakia OSCC Oral squamous cell carcinoma associated with proliferative verrucous leukoplakia "*" values with asterisk refer to values exceeding the threshold of 1.06.

In 2/9 patients the diagnosis of OSCC and PVL were simultaneous, while in the remaining 7/9 the diagnosis of PVL preceded the development of an OSCC. Median time to malignant transformation was 59.4±62.8 months (range 0-163 months).
Four of 9 patients developed a second oral neoplastic event after the resection of primary tumor. In detail, median time from primary tumor to second events (calculated as the interval between the two diagnosis dates) was 31±25 months (range 8-54). Four of 4 second neoplastic events were histologically classified as oral squamous cell carcinoma. Three of 4 lesions, being located at more than 2 cm and / or having occurred more than 3 years after the index tumor, were classified as second tumor according to Hong et al. criteria, [30] whereas 1/4 was classified as local recurrence.
Nine patients with PVL associated with OSCC (PVL-OSCC Group and OSCC Group) were cumulatively followed for a median time of 98±57 months (range 11-150).
Brushing specimens from 8 PVL patients (mean age 65±10 years range 46-77) who never developed OSCC (PVL Group) in a median follow-up period of 82±81 months (range 11-209) and 23 healthy subjects (mean age 53±17 years range 28-90) were also included in DNA methylation analysis.

15 gene-DNA Methylation analysis

A total of 272 CpGs from the panel of 15 genes were analyzed.
Paired test analysis revealed that 220 (80%) out of 272 CpGs analyzed showed a superimposable mean methylation level between the PVL-OSCC Group and OSCC Group (p = ns). The percentage of studied CpGs with superimposable degree of methylation between PVL-OSCC and OSCC Group for each gene is shown in Table II.
These CpGs were selected to compare the methylation level of PVL-OSCC samples with healthy subjects and PVLs not transformed in oral cancer (PVL).
A statistical difference among groups was found in 122 CpGs from 10 genes (*KIF1A, ZAP70, LRRTM, PARP15, FLI1, NTM, EPHX, ITGA4, MIR 296, MIR 137*). Specifically, Kruskall-Wallis test with multiple range test revealed that in 88 CpGs the level of methylation in PVL-OSCC was significantly different from both PVL and Healthy subjects. More in detail, in *PARP15* and in *ITGA4* 100% of the studied CpGs could stratify PVL-

Table II. Comparative analysis of methylation level for different groups of specimens.

15 Genes Panel	Number of interrogated CpGs	% of CpGs with superimposable level of methylation between OSCC and paired PVL*	% of CpGs with a significantly altered methylation level in OSCC-PVL group respect to PVL group not evolved in OSCC and healthy donors**
KIF1A	27	27/27 100%	6/27 (22.2%)
ZAP70	20	15/20 75%	5/20 (25%)
GP1BB	18	2/18 11%	0/18 (0%)
LRRTM1	22	22/22 100%	15/27 (55.6%)
PARP15	19	19/19 100%	19/19 (100%)
FLI1	12	11/11 100%	1/11(9%)
NTM	15	15/15 100%	6/27 (22.2%)
EPHX3	29	29/29 100%	13/27 (48.2%)
TERT	6	6/6 100%	0/6 (0%)
LINC00599	20	14/20 70 %	0/14 (0%)
ITGA4	14	14/14 100%	14/14 (100%)
miR193	26	8/26 30%	1/26 (4%)
miR296	15	15/15 100%	0/15 (0%)
PAX1	13	7/13 54%	0/13 (0%)
miR137	16	16/16 100%	8/16 (50%)

*paired t test analysis orWilcoxon signed-rank test between OSCC and paired OSCC-PVL.

**Kruskall-Wallis analysis with multiple range test between PVL-OSCC, PVL and healthy donors groups. Analysis limited to genes that displayed a 100% superimposable level of methylation between OSCC and paired PVL.

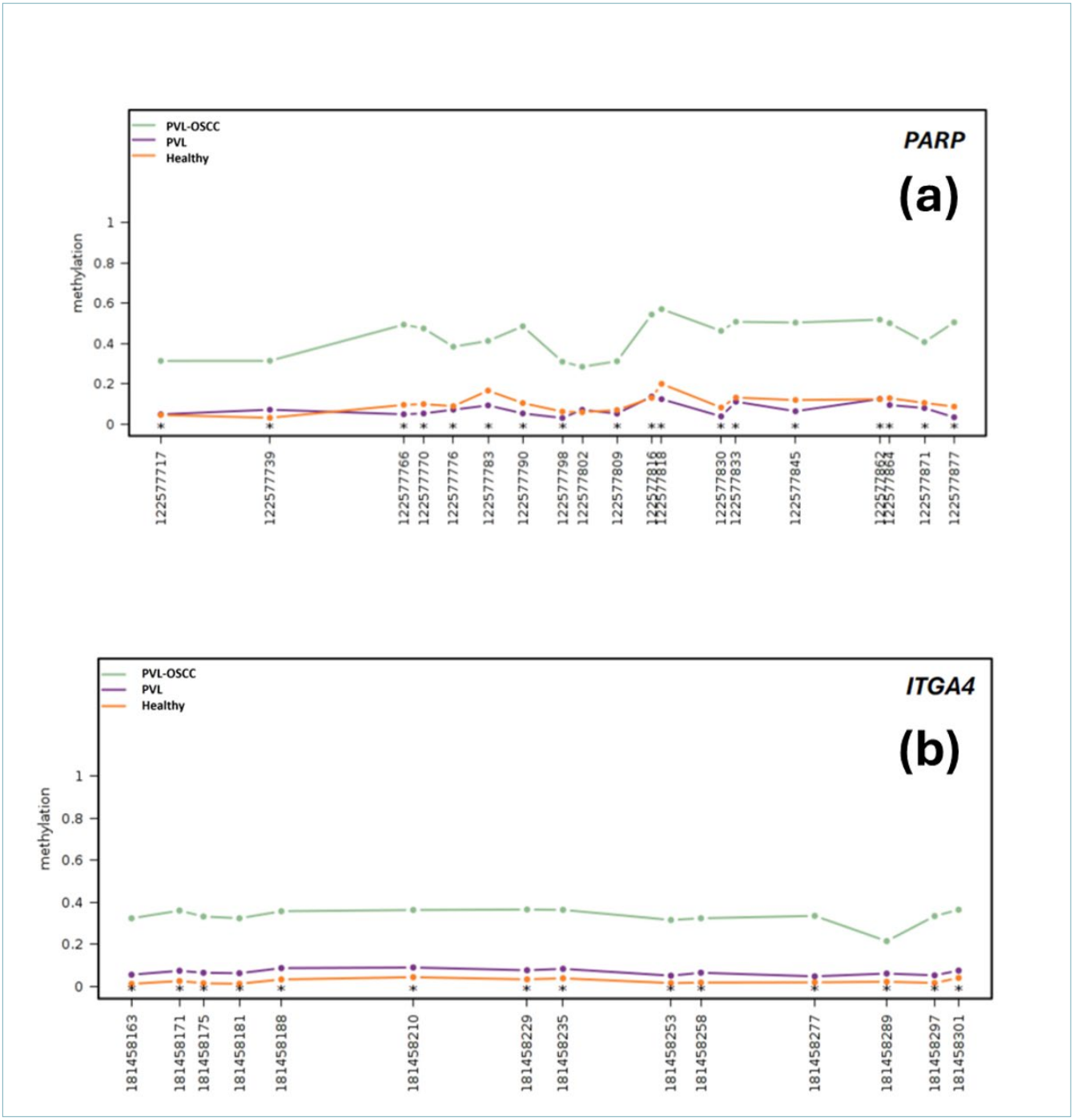

Figure 3. Methylation plots of *PARP15* (a*)* and *ITGA4* (b) genes. The statistical significance in each locus was highlighted by an asterisk and emerged in all the CpGs investigated. A significant higher quantitative methylation level of PVL-OSCC respect to PVL and healthy donors group can be noted.

OSCC from other groups *(*Fig. 3a-3b*)*. See Table II for details.

Supplementary Figure 1 points out in detail for the genes of interest the methylation level at each locus and the statistical significance highlighted with an asterisk. All methylation data for each gene are available in Supplementary File 1.

Principal component analysis (Fig. 4) showed a partial

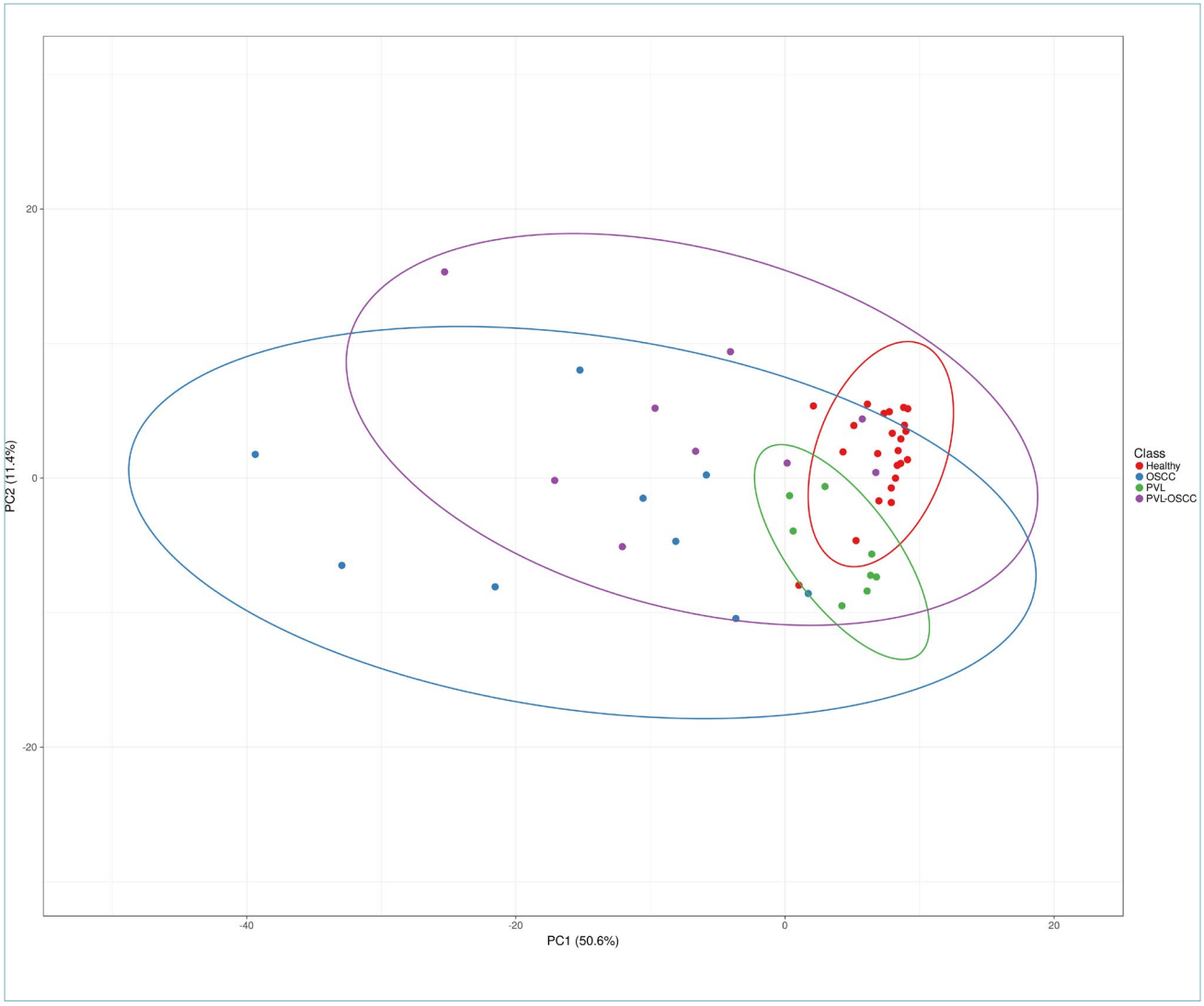

Figure 4. Principal component analysis (PCA); Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y axis show principal component 1 and principal component 2 that explain 50.6% and 11.4% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. N = 49 data points. The graph shows that OSCC elements (OSCC, blue) are sparse at the bottom of the plot, nearby PVL with OSCC (PVL-OSCC, violet), while healthy donors (Healthy, red) are clustered in a well-defined and restricted area on the center nearby PVL with no OSCC (PVL, green).

superimposable methylation profile of OSCC Group a PVL-OSCC Group. These data were also pointed out by the HeatMap in Figure 5, where some PVL-OSCC cases and relative OSCC clustered together.

Algorithm score

Concerning the score deriving from the algorithm recently validated for OSCC, 100% of the studied OSCCs had a score exceeding the threshold of 1.06 and were consequently recorded as "positive."

tralateral OSCCs in a selected series of nine patients. It emerged that 80% of the CpGs in the PVL-OSCC group had a methylation level superimposable to contralateral OSCC. In terms of genes, this study identified a panel of 10 genes that were epigenetically dysregulated in both OSCC (OSCC Group) and contralateral PVL-OSCC (*KIF1A, LRRTM, PARP15, FLI1, NTM, EPHX, TERT, ITGA4, MIR296, MIR193*) in 100% of the analysed CpGs.

The novelty of the present study is the comparison

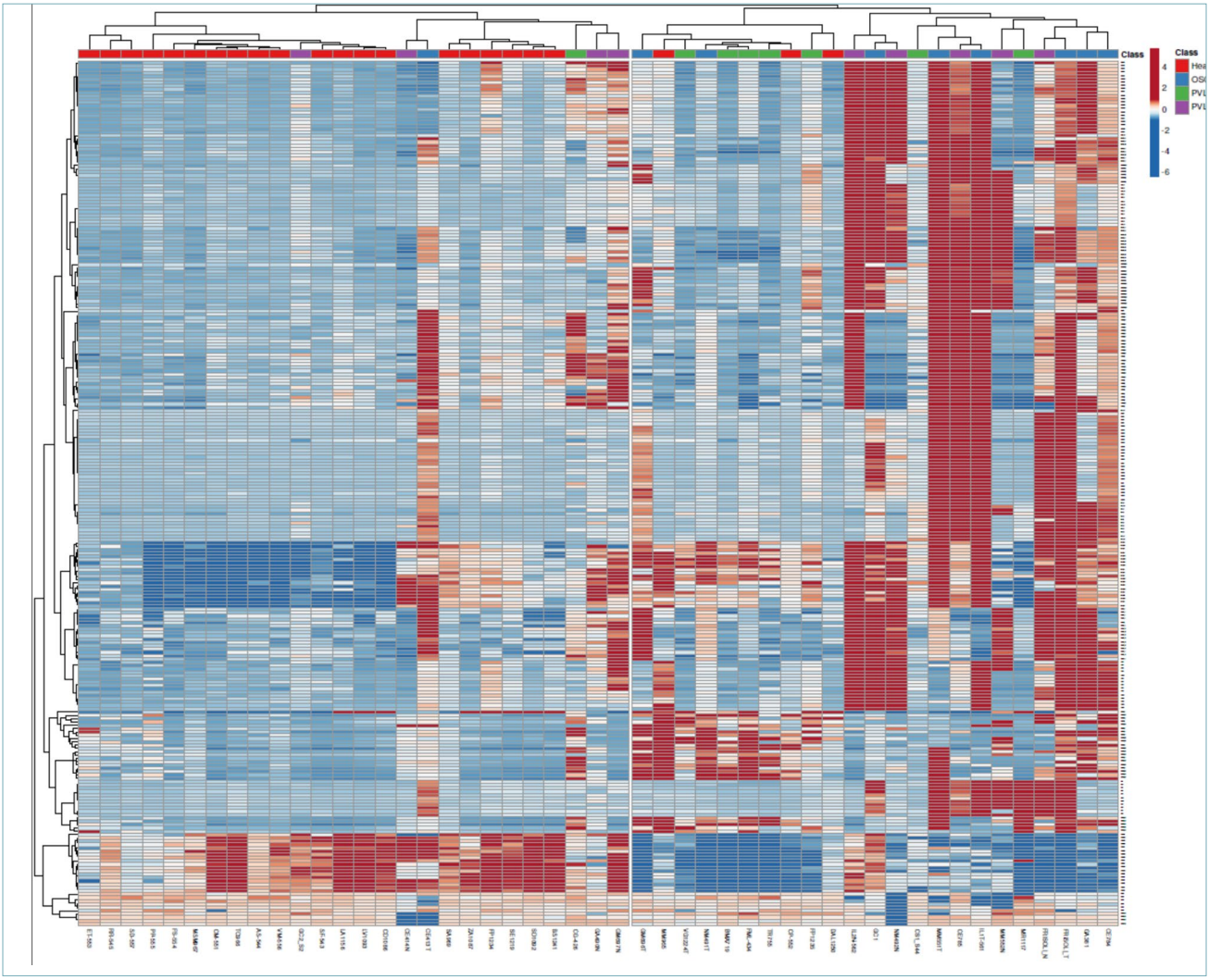

Figure 5. Cluster analysis: in the HeatMap, rows are centered; unit variance scaling is applied to rows. Imputation is used for missing value estimation. Columns are clustered using correlation distance and complete linkage. There are 261 rows, 49 columns. Two main clusters are present: the one on the left includes 20 healthy cases (Healthy, red), 4 PVL with carcinoma (PVL-OSCC, violet), one OSCC (OSCC, blue) and one PVL with no OSCC (PVL, green); the other on the right includes 8 OSCC (blue), 5 PVL with OSCC (violet), 7 PVL with no OSCC (PVL, green) and 3 healthy cases (red).

When the algorithm was tested in the group of 9 PVL-OSCC specimens, it emerged that 4/9 (44%) patients had a positive score. Three out of four (3/4, 75%) patients with a "positive" score also developed second neoplastic events (2 diagnosed as second tumors and 1 local recurrence) after the first malignant transformation (respectively after 36, 8 and 54 months respect to oral brushing cell collection. By contrast, only 1 out of 5 (20%) patients in the group of PVL lesions with a "negative" algorithm score developed a secondary OSCCs (15 months after primary OSCC treatment resection).
In the PVL group of 8 patients who did not develop OSCC during the follow-up (mean duration 82 months±81 range 11-209), 5/8 showed a score exceeding the value of 1.06 (see Supplementary files).
None of healthy individuals included in DNA methylation analysis showed a positive score (see Supplementary files).

Discussion

The aim of this study was to compare the methylation status of PVL lesions with the corresponding con-

between the methylation status of PVL lesions with related OSCCs in the contralateral mucosa. Indeed, matched samples obtained from the same individual simultaneously facilitate the identification of DNA methylation changes during oral cancer progression and reduce the impact of potential confounding factors. Specifically, the similarities in the dysregulated methylation profiles between OMPDs lesions and the corresponding OSCC support the role of epigenetics as an early event in oral carcinogenesis.

In addition, these findings strengthen the idea that the theory of field cancerization proposed by Slaughter et al. can be applied to patients with PVL [7]. This hypothesis is in agreement with Bagan et al. who clinically demonstrated that second primary tumors and field cancerization are frequent in PVL with decreasing time intervals between the appearance of one tumor and the next over time [17]. Epigenetic distinct dysregulation in oral cancers arising from PVL lesions compared to OSCCs not preceded by premalignant lesions was also highlighted in a recent study by Herreros-Pomares [31]. These data suggest that DNA methylation could be a regulatory mechanism.

Evidence of epigenetic alterations in the development of field cancerization has been also described in other cancers where the development of multiple carcinomas after the primary tumor frequently occurs [32]. In particular, increased incidence of aberrant methylation in the non-cancerous liver tissues of cases with hepatocellular carcinomas has been described by Kondo et al.[33] Similar results have been reported by Eads et al. in Barrett's esophagus and associated adenocarcinoma where APC and CDKN2A were found to be hypermethylated in Barrett's metaplasia and dysplasia [34]. Epigenetic field cancerization has also been suggested by Guo et al. for lung cancer. Histologically negative bronchial margins of resected non-small cell lung cancer have been found to exhibit frequent hypermethylation changes in multiple genes [35].

Present findings, with the limit of small number of specimens analyzed, also point out that PVLs associated with cancer show a different methylation level with respect to PVLs not associated with cancer and healthy subjects. Specifically, a panel of 8 genes (*KIF1A, LRRTM, PARP, FLI, NTM, EPHX, ITGA4, MIR 137*) stratified PVLs associated with cancer from other groups. Of note, *ITGA4* and *PARP15* showed 100% of the studied CpGs able to stratify PVL-Ca from other groups.

Integrin α4β1 *(ITGA4)* is a major receptor for FN1 on human eosinophils (Anwar et al. 1994) and involves in tumor cell proliferation, migration and survival. *ITGA4* is involved in gastric and colorectal cancer [36,37]. Previous reports indicate *ITGA4* as a promising marker for prognosis of oral squamous cell carcinoma [38,39].

PARP is a sensor of DNA damage and the recognition of DNA breaks by the PARP-1 enzyme has been identified as one of the earliest events that occur with DNA damage [40]. Moreover, the use of PARP inhibitors is of particular relevance for radiation oncology as it seems to interfere with DNA repair thus playing a role in radiosensitization of head and neck squamous cell carcinoma [41].

Concerning PVLs, the present data confirm the results of a previous study that identified epigenetic changes in three PVLs with a methylation pattern closer to OSCCs than to normal donors [22]. These findings also agree with Herreros-Pomares et al., who studied the methylation profile of 10 patients with PVL.

They found 4647 differentially methylated regions with a prominent state of hypermethylation in PVL with respect to 5 healthy individuals [17]. Their analyses also showed that PVL and healthy donors had clearly differentiated methylation patterns. Interestingly, Herreros-Pomares also demonstrated that PVLs and homogeneous leukoplakias present differential methylation patterns that could be linked to their differential clinical behavior [18].

To the best of our knowledge, this is the first study highlighting that PVLs associated with OSCC differ in terms of DNA methylation from those who do not undergo malignant transformation. This finding reinforces the hypothesis that epigenetics may play a pivotal role in oral carcinogenesis of PVL.

This is also in agreement with recent studies that showed that OSCCs preceded by PVL seem to exhibit different methylation profiles[31].

Finally, 3/4 (75%) patients who received a "positive" score in the PVL-OSCC sample at the algorithm developed second neoplastic events, compared to 1/5 (20%) patients who received a "negative" score in the PVL-OSCC specimen. Our group recently developed a patented algorithm (SG-OCRA) based on the DNA methylation analysis of CpG islands of 13 genes that were also included in the analysis in the present study. The algorithm discriminates lesions in "positive" or "negative" according to a pre-calculated threshold value. A multicenter clinical study collected 220 oral brushing samples (110 from OSCC patients and 110 from healthy volunteers) and, on the basis of this algorithm score, detected OSCCs with a sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of 93.6%, 84.9%, 86.6%, 92.8%, and 89.4%, respectively [27].

It may be speculated that a positive score of the algorithm may linked to the development of second neoplastic events in patients with OSCC proceeded by PVL lesions. However, since a positive score was also found in 5/8 patients from the group of PVLs that did not develop an OSCC during the follow-up it is prema-

ture to draw any conclusion regarding the predicting value of the score in terms of prediction of malignant transformation of PVL. Nevertheless, in the future it might be interesting to investigate whether a positive score in PVL, as a consequence of the field cancerization effect, can predict an aggressive local behavior associated with additional neoplastic events.

Conclusions

In conclusion, the results of the present study provide further evidence that PVL lesions share an aberrant methylation profile with OSCC even if spatially distant. Common epigenetic alterations between OSCC and related OPMDs strengthen the idea that methylation abnormalities reflect a field cancerization effect. Furthermore, results of the present study highlight the potential role of DNA methylation analysis in predicting the risk of developing multiple neoplastic events.

Conflicts of interest statement

Luca Morandi, Davide Gissi, and Achille Tarsitano are the founders and have a minority share in Studium Genetics S.r.l., a spin-off company of the University of Bologna. They are also inventors of the patent related to the algorithm based on 13 gene methylation in OSCC cited in this work. The remaining authors declare no competing interest related to the present study.

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Authors' contributions

GA was responsible for analysis and interpretation of data, drafting of the manuscript, final approval of the version to be published, GDB was responsible for conception and design, analysis and interpretation of data final approval of the version to be published, QG was responsible for analysis and interpretation of data, SA was responsible for analysis and interpretation of data, RR was responsible for analysis and interpretation of data, LE was responsible for analysis and interpretation of data TA was responsible for conception and design, analysis and interpretation of data, final approval of the version to be published, ML was responsible for conception and design, final approval of the version to be published FMP was responsible for conception and design final approval of the version to be published, ML was responsible for conception and design, analysis and interpretation of data, final approval of the version to be published

Ethical consideration

The study was approved by the local ethics committee (520/2018/Sper/AOUBo 12 December 2018).
The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki.
Written informed consent was obtained from each participant/patient for study participation and data publication

References

1 Hansen LS, Olson JA, Silverman S. Proliferative verrucous leukoplakia. A long-term study of thirty patients. Oral Surg Oral Med Oral Pathol. 1985;60(3):285-298. https://doi.org/10.1016/0030-4220(85)90313-5

2 WHO Classification of Tumours Editorial Board. Head and Neck Tumours [Internet]. Lyon (France): International Agency for Research on Cancer; 2023 (WHO Classification of Tumours Series, 5th Ed.; Vol. 9). Available from: Https://Tumourclassification.Iarc.Who.Int/Chapters/52.

3 Warnakulasuriya S, Kujan O, Aguirre-Urizar JM, et al. Oral potentially malignant disorders: A consensus report from an international seminar on nomenclature and classification, convened by the WHO Collaborating Centre for Oral Cancer. Oral Dis. 2021;27(8):1862-1880. https://doi.org/10.1111/odi.13704

4 Muller S, Tilakaratne WM. Update from the 5th Edition of the World Health Organization Classification of Head and Neck Tumors: Tumours of the Oral Cavity and Mobile Tongue. Head Neck Pathol. 2022;16(1):54-62. https://doi.org/10.1007/s12105-021-01402-9

5 Iocca O, Sollecito TP, Alawi F, et al. Potentially malignant disorders of the oral cavity and oral dysplasia: A systematic review and meta-analysis of malignant transformation rate by subtype. Head Neck. 2020;42(3):539-555. https://doi.org/10.1002/hed.26006

6 Bagán JV, Murillo J, Poveda R, Gavaldá C, Jiménez Y, Scully C. Proliferative verrucous leukoplakia: unusual locations of oral squamous cell carcinomas, and field cancerization as shown by the appearance of multiple OSCCs. Oral Oncol. 2004;40(4):440-443. https://doi.org/10.1016/j.oraloncology.2003.10.008

7 Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer. 1953;6(5):963-968. https://doi.org/10.1002/1097-0142(195309)6:5 < 963::aid-cncr2820060515 > 3.0.co;2-q

8 Kerr AR, Lodi G. Management of oral potentially malignant disorders. Oral Dis. 2021;27(8):2008-2025. https://doi.org/10.1111/odi.13980

9 Gouvêa AF, Santos Silva AR, Speight PM, et al. High incidence of DNA ploidy abnormalities and increased Mcm2 expression may predict malignant change in oral proliferative verrucous leukoplakia. Histopathology. 2013;62(4):551-562. https://doi.org/10.1111/his.12036

10 Kresty LA, Mallery SR, Knobloch TJ, et al. Frequent alterations of p16INK4a and p14ARF in oral proliferative verrucous leukoplakia. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol. 2008;17(11):3179-3187. https://doi.org/10.1158/1055-9965.EPI-08-0574

11 Gouvêa AF, Vargas PA, Coletta RD, Jorge J, Lopes MA. Clinicopathological features and immunohistochemical expression of p53, Ki-67, Mcm-2 and Mcm-5 in proliferative verrucous leukoplakia. J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol. 2010;39(6):447-452. https://doi.org/10.1111/j.1600-0714.2010.00889.x

[12] Thennavan A, Byatnal AA, Solomon MC, Radhakrishnan RA. The role of Ki-67, p16, CD34, Bcl-2, cyclooxygenase-2 in the pathogenesis of proliferative verrucous leukoplakia. Indian J Cancer. 2015;52(4):498-502. https://doi.org/10.4103/0019-509X.178424

[13] Akrish S, Ben-Izhak O, Sabo E, Rachmiel A. Oral squamous cell carcinoma associated with proliferative verrucous leukoplakia compared with conventional squamous cell carcinoma--a clinical, histologic and immunohistochemical study. Oral Surg Oral Med Oral Pathol Oral Radiol. 2015;119(3):318-325. https://doi.org/10.1016/j.oooo.2014.10.023

[14] Fettig A, Pogrel MA, Silverman S, Bramanti TE, Da Costa M, Regezi JA. Proliferative verrucous leukoplakia of the gingiva. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2000;90(6):723-730. https://doi.org/10.1067/moe.2000.108950

[15] Villa A, Hanna GJ, Kacew A, Frustino J, Hammerman PS, Woo SB. Oral keratosis of unknown significance shares genomic overlap with oral dysplasia. Oral Dis. 2019;25(7):1707-1714. https://doi.org/10.1111/odi.13155

[16] Mascolo M, Siano M, Ilardi G, et al. Epigenetic disregulation in oral cancer. Int J Mol Sci. 2012;13(2):2331-2353. https://doi.org/10.3390/ijms13022331

[17] Herreros-Pomares A, Llorens C, Soriano B, et al. Differentially methylated genes in proliferative verrucous leukoplakia reveal potential malignant biomarkers for oral squamous cell carcinoma. Oral Oncol. 2021;116:105191. https://doi.org/10.1016/j.oraloncology.2021.105191

[18] Herreros-Pomares A, Hervás D, Bagán L, Proaño A, Bagan J. Proliferative verrucous and homogeneous Leukoplakias exhibit differential methylation patterns. Oral Dis. Published online June 9, 2024. https://doi.org/10.1111/odi.15028

[19] Cerero-Lapiedra R, Baladé-Martínez D, Moreno-López LA, Esparza-Gómez G, Bagán JV. Proliferative verrucous leukoplakia: a proposal for diagnostic criteria. Med Oral Patol Oral Cirugia Bucal. 2010;15(6):e839-845.

[20] Thompson LDR, Fitzpatrick SG, Müller S, et al. Proliferative Verrucous Leukoplakia: An Expert Consensus Guideline for Standardized Assessment and Reporting. Head Neck Pathol. 2021;15(2):572-587. https://doi.org/10.1007/s12105-020-01262-9

[21] Caudell JJ, Gillison ML, Maghami E, et al. NCCN Guidelines® Insights: Head and Neck Cancers, Version 1.2022. J Natl Compr Cancer Netw JNCCN. 2022;20(3):224-234. https://doi.org/10.6004/jnccn.2022.0016

[22] 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. https://doi.org/10.1186/s13148-017-0386-7

[23] 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 Cranio-Maxillo-fac Surg Off Publ Eur Assoc Cranio-Maxillo-fac Surg. 2015;43(8):1494-1500. https://doi.org/10.1016/j.jcms.2015.07.028

[24] 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. https://doi.org/10.4161/epi.29025

[25] Dang J, Bian YQ, Sun JY, et al. MicroRNA-137 promoter methylation in oral lichen planus and oral squamous cell carcinoma. J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol. 2013;42(4):315-321. https://doi.org/10.1111/jop.12012

[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. https://doi.org/10.1093/nar/gky379

[27] 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: Italian multicenter study. Head Neck. 2021;43(5):1563-1573. https://doi.org/10.1002/hed.26624

[28] Metsalu T, Vilo J. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Res. 2015;43(W1):W566-570. https://doi.org/10.1093/nar/gkv468

[29] Mallona I, Díez-Villanueva A, Peinado MA. Methylation plotter: a web tool for dynamic visualization of DNA methylation data. Source Code Biol Med. 2014;9:11. https://doi.org/10.1186/1751-0473-9-11

[30] 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. https://doi.org/10.1056/NEJM199009203231205

[31] Herreros-Pomares A, Hervás D, Bagan-Debon L, et al. Oral cancers preceded by proliferative verrucous leukoplakia exhibit distinctive molecular features. Oral Dis. Published online March 9, 2023. https://doi.org/10.1111/odi.14550

[32] Ushijima T. Epigenetic field for cancerization. J Biochem Mol Biol. 2007;40(2):142-150. https://doi.org/10.5483/bmbrep.2007.40.2.142

[33] Kondo Y, Kanai Y, Sakamoto M, Mizokami M, Ueda R, Hirohashi S. Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis--A comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. Hepatol Baltim Md. 2000;32(5):970-979. https://doi.org/10.1053/jhep.2000.19797

[34] Eads CA, Lord RV, Kurumboor SK, et al. Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res. 2000;60(18):5021-5026.

[35] Guo M, House MG, Hooker C, et al. Promoter hypermethylation of resected bronchial margins: a field defect of changes? Clin Cancer Res Off J Am Assoc Cancer Res. 2004;10(15):5131-5136. https://doi.org/10.1158/1078-0432.CCR-03-0763

[36] Lam K, Pan K, Linnekamp JF, Medema JP, Kandimalla R. DNA methylation based biomarkers in colorectal cancer: A systematic review. Biochim Biophys Acta. 2016;1866(1):106-120. https://doi.org/10.1016/j.bbcan.2016.07.001

[37] Qu Y, Dang S, Hou P. Gene methylation in gastric cancer. Clin Chim Acta Int J Clin Chem. 2013;424:53-65. https://doi.org/10.1016/j.cca.2013.05.002

[38] Gissi DB, Fabbri VP, Gabusi A, et al. Pre-Operative Evaluation of DNA Methylation Profile in Oral Squamous Cell Carcinoma Can Predict Tumor Aggressive Potential. Int J Mol Sci. 2020;21(18). https://doi.org/10.3390/ijms21186691

[39] Yen CY, Huang CY, Hou MF, et al. Evaluating the performance of fibronectin 1 (FN1), integrin $\alpha 4\beta 1$ (ITGA4), syndecan-2 (SDC2), and glycoprotein CD44 as the potential biomarkers of oral squamous cell carcinoma (OSCC). Biomark Biochem Indic Expo Response Susceptibility Chem. 2013;18(1):63-72. https://doi.org/10.3109/1354750X.2012.737025

[40] Kraus WL, Hottiger MO. PARP-1 and gene regulation: progress and puzzles. Mol Aspects Med. 2013;34(6):1109-1123. https://doi.org/10.1016/j.mam.2013.01.005

[41] Lee TW, Wong WW, Dickson BD, et al. Radiosensitization of head and neck squamous cell carcinoma lines by DNA-PK inhibitors is more effective than PARP-1 inhibition and is enhanced by SLFN11 and hypoxia. Int J Radiat Biol. 2019;95(12):1597-1612. https://doi.org/10.1080/09553002.2019.1664787