OBJECTIVES: Accumulating evidence indicates that aberrant DNA methylation is closely related to oral carcinogenesis, and it has been shown that methylation changes might be used as prognostic biomarker in oral squamous cell carcinoma. Oral lichenoid disease (OLD) is the most common oral potentially malignant disorder in our region. The aim of this study was to perform the first wide DNA methylation study in OLD in order to investigate the relevance of DNA methylation changes in this premalignant disorder.

MATERIALS AND METHODS: Two different Illumina microarray platforms, namely the GoldenGate Cancer Panel I and the HumanMethylation27 DNA Analysis BeadChip, were utilized in the discovery phase to interrogate the methylation profile of 59 OLD cases and 9 healthy individuals. Top-ranked genes were further validated by pyrosequencing in a second sample set consisting of 160 OLD and 65 controls.

RESULTS: Our results show that the frequency of aberrant DNA methylation is rare in OLD, and this finding was further corroborated by pyrosequencing in the biological validation.

CONCLUSIONS: These findings reinforce the notion that molecular alterations associated with oral carcinogenesis do not seem to be common events in OLD, which in turn might explain the low rate of malignization of this disorder.

Keywords: oral lichenoid disease; genome-wide DNA methylation

Introduction

Epigenetic regulation is central to the biological function of all cells. Aberrant DNA methylation patterns, particularly at sites with the ability to affect gene function (such as gene promoter regions), are common in many human diseases. In cancer, epigenetic hallmarks include both decreases in global DNA methylation levels, commonly observed in DNA repetitive elements, and concomitant increases in gene-specific methylation. Loss of repetitive element methylation has been shown to activate the expression of proto-oncogenes, increase mutational events and enhance chromosomal instability, whereas gene-associated hypermethylation is often associated with phenotypic silencing of tumor suppressor genes (Feinberg, 2010).

Oral lichenoid disease (OLD) represents the most common oral potentially malignant disorder in our region (Basque Country). It is a chronic disorder with an immunological basis which affects between 0.5% and 2.2% of the population, being more frequently observed in women (Al-Hashimi et al., 2007; Scully and Carrozzo, 2008; Cortes-Ramirez et al., 2009).

Although this pathology is considered as an oral potentially malignant disorder, its real malignant potential is controversial (Cortes-Ramirez et al., 2009; van der Waal, 2009). The transformation rate usually does not exceed 1% (Cortes-Ramirez et al., 2009), and there are no reliable means of predicting this risk in an individual patient (Gandolfo et al., 2004; van der Meij et al., 2007).

The etiopathogenetic factors are unknown and so far, and despite the relatively high number of studies investigating the molecular characteristics of the OLD, the molecular portrait of this disorder has not been conclusively defined. Studies investigating the prevalence of cancer-related hallmarks such as p53 mutations, microsatellite instability or aberrant promoter hypermethylation are far contradictory. Most of the studies on p53 expression, associated with mutated p53, have found significantly higher expression rates in OLD than in normal mucosa (Georgakopoulou et al., 2012), although opposite results have also
been reported by Crosthwaite et al (Crosthwaite et al., 1996) who found no p53 expression in normal and oral lichen planus (OLP) tissue. Loss of heterozygosity (LOH) has been observed in OLP-associated dysplasia (Zhang et al., 2000; Kim et al., 2001), but not in non-dysplastic OLP lesions (Accurso et al., 2011). With respect to the changes in DNA aneuploidy, it has also been reported in some atrophic OLP lesions (Femiano and Scully, 2005; Hosni et al., 2010), although these results did not find the statistical significance in a study by Acha-Sagredo et al (Acha-Sagredo et al., 2011). When it comes to epigenetic alterations just two studies have been published, using small sample sizes and a candidate gene approach, showing inconsistent results (Ruesga et al., 2007; Dang et al., 2013). Therefore, our aim was to investigate the role that epigenetic alterations may be playing in this disorder using a genome-wide approach.

**Materials and methods**

**Samples**

A total of 177 oral rinse samples from patients diagnosed with OLD and 70 disease free controls were collected at the Oral Medicine Unit of the Dental Clinical Service of the University of the Basque Country (UPV/EHU) as previously reported (Lopez et al., 2003). Additionally, oral scrapes were also collected as described previously (Lopez et al., 2004).

Genomic DNA was extracted using a Gentra Puregene Buccal Cell Kit (Qiagen, Valencia, CA, USA), and DNA was bisulfite-treated using the EZ DNA methylation Kit™ (Zymo Research, Irvine, CA, USA) following the manufacturer’s protocol.

Patients with a clinicopathologically diagnosed OLD were classified as OLP or oral lichenoid lesion (OLL) as previously described (van der Meij and van der Waal, 2003; Cortes-Ramirez et al., 2009). Smoking was defined as the use of 10 or more cigarettes per day and alcohol use as the intake of more than 10 units per week. The study was carried out according to the principles of the Declaration of Helsinki and approved by the Ethics Committee in Research of the Faculty of Medicine and Dentistry at the University of the Basque Country. Informed consent was obtained from all participating patients.

**Marker discovery**

**Methylation profiling.** The Illumina GoldenGate Methylation Cancer Panel I was used for the initial DNA methylation profiling on 51 OLD and six normal samples. Briefly, DNA was treated with sodium bisulfite using the EZ DNA Methylation Kit™ (Zymo Research) and hybridized to the GoldenGate Methylation Cancer Panel that analyses 1505 individual CpG loci within 807 genes. Extraction and normalization of intensity data were performed using the GenomeStudio software. Methylation levels were quantified by means of the \( \beta \) value, which represent the ratio of methylation and, as such, ranges between 0 (completely unmethylated) and 1 (completely methylated). This ratio was calculated as the ratio of preprocessed (background corrected and normalized) fluorescent signal from the methylated allele (M) and the sum of the signals from both methylated and unmethylated (U) alleles, i.e., \( \beta = M/(M + U + 100) \).

A more comprehensive DNA methylation profiling was conducted using the Illumina HumanMethylation27 DNA Analysis BeadChip assay, which interrogates 27 578 CpG sites from 14 495 protein coding gene promoters and 110 microRNA gene promoters at single-nucleotide resolution. 1 \( \mu g \) of genomic DNA was treated with sodium bisulfite, using the EZ DNA methylation Kit™ (Zymo Research). Bisulfite converted DNA from OLD (n = 17) and normal (n = 5) samples were hybridized to the HumanMethylation27 DNA Analysis BeadChip array. Extraction and normalization of intensity data as well as quantification of the methylation levels were performed following the same procedure described for the GoldenGate.

Overall, 11 samples (nine cases and two controls) were run on both Infinium and GoldenGate, whereas 46 samples (42 cases and four controls) were analyzed exclusively on GoldenGate, and 11 samples (eight cases and three controls) were analyzed exclusively on Infinium. All data were packaged and deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number [GEO: GSE54211].

**Computational analysis of the methylation levels.** Microarray data were analyzed to look for differences between OLD and normal samples. Preprocessing of the data was carried out using lumi package (Bioconductor). As a first global approach, principal component analysis (PCA) and hierarchical clustering were used to check whether the methylation level could separate cases from controls.

Methylation differences between cases and controls at site level were also assessed by implementing the Wilcoxon-Mann–Whitney test (R's stats package). The two-stage Benjamini and Hochberg method was used to correct the obtained \( P \)-values for multiple testing. Additionally, 100 bootstrap samples were randomly generated and used to re-compute the \( P \)-values, to test the robustness of the computed \( P \)-value. The median bootstrap \( P \)-values were computed and compared with the original ones. Sites where these two values were very different were regarded as non-robust (i.e., non-reliable).

Furthermore, the absolute value of the differences between the two groups was also computed as the average absolute difference (AAD). This value was estimated as the mean value of all the pairwise absolute differences between OLD and control samples. Sites were ranked according to the \( P \)-value and, as a second criterion, to the AAD. Sites with lowest \( P \)-values and highest AADs were considered as the top-ranked sites.

**Validation by pyrosequencing methylation analysis**

The selected markers were further validated in a validation set composed of 160 cases and 65 controls by pyrosequencing methylation analysis (PMA). Additionally, LINE-1, a global DNA methylation surrogate, was also tested following the protocol described in Daskalos et al (Daskalos et al., 2009). The primers for pyrosequencing analysis are provided in Table S1. Pyrosequencing analysis was conducted as previously described (Bediaga et al., 2010).
Furthermore, the levels of methylation of two genes between oral rinses and oral brushes were compared by pyrosequencing to test the validity of the oral rinses for methylation studies in this disorder.

Statistical analysis of the validation data
The methylation of the four gene promoters included in the biological validation was analyzed in two ways: as a continuous variable and as a binary variable by dichotomizing the data (methylation vs no methylation). The pooled control samples’ mean ± two times the standard deviation was used as a cutoff value to define the hyper/ hypomethylated status of the samples (Feng et al, 2007). The distribution of subjects’ epidemiological, clinical, and methylation characteristics was described separately for the training and validation datasets. One-sample Kolmogorov–Smirnov test was used to evaluate fitness to normal distribution of continuous parameters. Wilcoxon-Mann–Whitney test was used to test the methylation differences between cases and controls or different clinicopathological characteristics. Comparisons of categorical variables were made using Fisher’s exact test. Pearson’s correlation tests were used to assess the correlation between two continuous variables. All reported P-values are two-tailed and considered statistically significant if P < 0.05.

Results
Clinical data
The main clinical characteristics of the cases and controls included in this study are showed in Table 1. Controls and cases were epidemiologically matched for sex and age. OLD patients were mainly females (71.2%), with ages ranging from 21 to 90 years old [mean (±s.d.) age, 55.38 ± 12.44 years]. Regarding OLD subtypes, 130 were classified as OLP and 47 as oral lichenoid lesions (OLL). The average follow-up time of all patients was 4.8 years; transformation was observed in four cases (2.2%) of which two were classified as OLP and the other two as OLL. These cases were mainly females (3/4), non-smokers (3/4), with a mean age of 71.75 years and with no alcohol consuming history.

Aberrant promoter methylation in OLD
We assessed DNA methylation profiles in a discovery set of 59 OLD cases and nine controls. At the beginning of the current study, DNA methylation was screened using the Illumina GoldenGate Cancer Panel I (Figure S1b), but migrated to the more comprehensive Infinium platform (Figure S1a), as it became available.

The clustering-based visual approach shows that, globally, the DNA methylation information is not enough to distinguish between OLD and control samples. As shown in Figure 1, using the first two components returned by PCA is not enough to separate controls from OLD samples. Similarly, both heatmaps in Figure S1 clearly show that OLD cannot be separated from controls on their methylation level. These results suggest that there are not big differences in the methylation of OLD and control samples.

Regarding the analysis of the pairwise differences, after correcting the P-values for multiple testing, all of them were above 0.3 and 0.19 in the GoldenGate and Infinium data, respectively. Therefore, we can conclude that no significant differences can be observed in our data. Nonetheless, 4 of the most relevant top-ranked genes (those with lowest P-values and highest AAD between OLD and control groups) were selected for biological validation. With respect to the OLP vs OLL comparison, none of the CpG sites reached even the P nominal value.

Validation of differentially methylated genes in a large panel of OLD and controls
For the validation study, pyrosequencing assays for the four selected CpG loci were conducted in a cohort of 160 OLD samples and 65 controls (Table 2). We found that only MGC40178 was slightly hypomethylated in OLD samples when compared to controls (mean difference =

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>Screening set 1 (GoldenGate, n = 57)</th>
<th>Screening set 2 (Infinium, n = 22)</th>
<th>Validation set (Pyrosequencing, n = 225)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender% (n)</td>
<td>OLD: 74 (38)</td>
<td>Control: 50 (3)</td>
<td>OLD: 70.6 (113)</td>
</tr>
<tr>
<td></td>
<td>Male: 26 (13)</td>
<td></td>
<td>29.4 (47)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Mean (s.d.): 56.9 (6.5)</td>
<td>Mean (s.d.): 59.8 (6.6)</td>
<td>Mean (s.d.): 56.8 (13.3)</td>
</tr>
<tr>
<td>Tobacco% (n)</td>
<td>No: 70 (36)</td>
<td>83.3 (5)</td>
<td>No: 76.2 (122)</td>
</tr>
<tr>
<td></td>
<td>Yes: 30 (15)</td>
<td>16.7 (1)</td>
<td>Yes: 23.8 (38)</td>
</tr>
<tr>
<td>Alcohol% (n)</td>
<td>No: 72.5 (37)</td>
<td>83.3 (5)</td>
<td>No: 62.5 (100)</td>
</tr>
<tr>
<td></td>
<td>Yes: 27.5 (14)</td>
<td>16.7 (1)</td>
<td>Yes: 37.5 (60)</td>
</tr>
<tr>
<td>OLD% (n)</td>
<td>OLP: 72.5 (37)</td>
<td></td>
<td>OLP: 76.2 (122)</td>
</tr>
<tr>
<td></td>
<td>OLL: 27.5 (14)</td>
<td></td>
<td>OLL: 23.8 (38)</td>
</tr>
</tbody>
</table>

OLD, oral lichenoid disease; OLP, oral lichen planus; OLL, oral lichenoid lesion.
In addition, we measured the methylation level of LINE-1, a surrogate marker for global DNA methylation, in both screening and validation sample sets. We found that OLD samples were statistically significantly hypomethylated (mean difference = 0.75%, \( P \)-value = 0.029), although the biological relevance of this difference is questionable.

We next evaluated the methylation status of those five markers by dichotomizing the data as described in Material and Methods. The results indicated that only MGC40178 showed a significant difference (\( P \)-value = 0.035) in the frequency of promoter hypomethylation between OLD patients and controls (7% vs 0%, respectively; Table 2).

### Table 2 Methylation level of validated markers in OLD and control samples

<table>
<thead>
<tr>
<th>Gene</th>
<th>OLD</th>
<th>Control</th>
<th>( P )-value</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADORA1</td>
<td>[76.86 ± 5.66] (7)</td>
<td>[78.16 ± 5.35] (3)</td>
<td>0.195</td>
<td>0.508</td>
</tr>
<tr>
<td>FBXO27</td>
<td>[53.99 ± 13.99] (0)</td>
<td>[51.25 ± 14.60] (0)</td>
<td>0.214</td>
<td>1.000</td>
</tr>
<tr>
<td>EML3</td>
<td>[56.11 ± 6.56] (1)</td>
<td>[55.00 ± 5.66] (0)</td>
<td>0.099</td>
<td>1.000</td>
</tr>
<tr>
<td>MGC40178</td>
<td>[45.98 ± 8.31] (7)</td>
<td>[51.92 ± 8.65] (0)</td>
<td>( 3 \times 10^{-5} )</td>
<td><strong>0.035</strong></td>
</tr>
<tr>
<td>LINE-1</td>
<td>[65.50 ± 2.51] (8)</td>
<td>[66.25 ± 1.91] (4)</td>
<td><strong>0.029</strong></td>
<td>0.532</td>
</tr>
</tbody>
</table>

OLD, oral lichenoid disease. Statistically significant \( P \)-values are shown in bold.

\*Positive rate using the sample mean methylation ± 2 × s.d. of the pooled normal samples as a cutoff point.

Correlation between methylation levels and clinical data
The analysis of DNA methylation data with regard to the clinical characteristics of the OLD samples revealed that methylation of tested markers was not associated with age, smoking or drinking habits, OLD clinical subtype, or clinical outcome (\( P > 0.05 \)).

Technical validation of the oral rinses for methylation studies
We quantified the promoter methylation levels of EML3 and MGC40178 in 17 paired salivary rinses and oral cytologies from patients with OLD and found strong correlations between the salivary rinses and oral cytologies for both MGC40178 (\( \rho = 0.89, P < 0.0001 \)) and EML3 (\( \rho = 0.91, P\)-value < 0.0001).

### Discussion

Oral cancer is characterized by global DNA hypomethylation and site-specific hypermethylation (Gasche and Goel, 2012), and multiple lines of evidence suggest that aberrant DNA methylation plays an early (Gasche and Goel, 2012) and central role in oral carcinogenesis (Jithesh et al., 2013). Unfortunately, few studies have examined DNA methylation profiles in oral premalignant lesions, and most of them are based on candidate gene approaches (Lopez et al., 2003; Hall et al., 2008; Takeshima et al., 2008).

This is, to the best of our knowledge, the first study that utilizes a genome-wide DNA methylation approach to dissect the differentially methylated genes in the OLD disease. We have used two different DNA methylation platforms, Illumina’s GoldenGate Cancer Panel I and HumanMethylation27 DNA Analysis BeadChip, to screen the methylation levels of 1505 and 27 578 CpG sites, respectively, in OLD. Further pyrosequencing analysis of some of the top-ranked genes in a cohort of 160 patients (121 OLP and 39 OLL) and 65 controls confirmed that
most of the methylation changes were either no significant or very small, thus indicating that aberrant methylation is not a common event in OLD disease.

To date, there are just two previous studies (Ruesga et al, 2007; Dang et al, 2013), which have analyzed DNA methylation using a candidate gene approach and their results are contradictory. While Ruesga et al (Ruesga et al, 2007) found no p16INK4a hypermethylation in 34 oral scrapings from OLD patients, a recent work by Dang et al (Dang et al, 2013) observed that 25% (5/20) and 35% (7/20) of the OLD patients analyzed – using methyl-ation-specific PCR (MSP) – showed aberrant promoter methylation of p16INK4a and miR-137 genes, respectively. The contradictory results observed so far could be due to several factors, including small sample size, selection criteria, techniques utilized, and sample type among others. To avoid these possible pitfalls, in our study we have analyzed the methylation profile of a relatively high series of OLD cases (177 OLD cases overall between screening and validation phase) and used pyrosequencing as our validation technique to overcome the problems with false positives and gain quantitative methylation data. In addition, the diagnostic criteria used to identify OLD patients were based on both stringent clinical and histological criteria (van der Meij and van der Waal, 2003; Cortes-Ramirez et al, 2009), resulting in a cohort of patients with similar clinicopathological characteristics and transformation rate as those previously described (Gonzalez-Moles et al, 2008).

Regarding the sample type, it has been previously hypothesized that oral rinses collected without a cytological brush might not contain enough oral cells to meet cutoffs for biomarker findings. However, we found a strong correlation in promoter methylation levels of EML3 and MGC40178 genes between paired salivary rinses and oral cytolgies in accordance with previous reports (Sun et al, 2012). These results support the use of oral rinses without cytological brush for epigenetic studies. Furthermore, we consider that, when analyzing disorders that harbor multiple lesions in the oral mucosa such as OLD, the use of oral rinses might be a more appropriate collection method because cytological brushings are site specific.

Changes in DNA methylation during oral carcinogene-sis are not only circumscribed to gene-specific promoter regions; there is an increase recognition that global hypomethylation contributes to oral carcinogenesis (Poage et al, 2011). Unlike gene-specific promoter methylation, global DNA hypomethylation has never been reported before in any oral potentially malignant disorder. Here, we have observed that global hypomethylation is not a common epigenetic aberration in OLD, because only 12 of 160 OLD samples (8%) displayed hypomethylation of LINE-1.

Oral lichenoid disease is a frequent oral potentially malignant disorder whose etiopathogenesis and real malignant transformation capacity are still controversial (Gonzalez-Moles et al, 2008). While there is a consensus about the chronic inflammatory nature of the OLD condition, it remains unclear whether OLD has an intrinsic tendency to become malignant instead of just providing a fertile soil for carcinogens to work on, as it is an inflammatory lesion and has a higher cell turnover. According to our results, DNA methylation does not seem to be involved in this disorder. However, we cannot rule out that changes in DNA methylation patterns in OLD lesions might lead to transformation. Further studies comparing transforming and non-transforming OLD lesions and the corresponding tumors are needed to investigate the epigenetic mecha-nisms underlying malignant transformation in this pathology. Besides, additional research – in well-characterized OLD samples – is required to dissect the molecular portrait of this disease (profiling of mRNA, non-coding RNA, and epigenetic alterations). A better understanding of the molecular alterations taking place in this highly prevalent premalignant disorder could lead to an improved clinical management of these patients as well as to the discovery of new therapeutic targets.

In summary, the present study has compared for the first time the genome-wide methylation profiles between OLD and control samples and has demonstrated that the frequency of aberrant methylation changes is very rare in OLD. These results add to previous findings and reinforce the fact that molecular alterations associated with oral carcinogenesis do not seem to be common events in OLD, which in turn might explain the low rate of malignization of this disorder.

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Conflict of interest

None to declare.

Author contributions

NGB, JMAU, MMP and AAS contributed to the design of the study and identified the research questions. JMAU and MAEG were responsible for obtaining ethics approval and collecting the samples. BC, AAS and NGB planned the statistical analyses. XMM, NGB and ASS are responsible for the study implementation. JMAU, MMP and AAS are scientific reviewers for the project. All authors have read, revised and approved the final manuscript.

References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Unsupervised hierarchical clustering of oral lichenoid disease (OLD) and control (C) samples using the most variable 500 genes.

**Table S1** PCR and pyrosequencing primer sequences and PCR conditions.