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Detection of Genetic Alterations in Oral Squamous Cell Carcinoma Using Multiplex Ligation-Dependent Probe Amplification (MLPA)

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KEYWORDS

Biomarkers, DNA copy number, dysplasia, chromosome alteration

ABSTRACT

Deletions and amplifications of genes often occur during multistep progression from oral precancer, seen as oral epithelial dysplasia (OED) to cancerous stage. These genetic alterations could be used as markers to aid in detection of oral squamous cell carcinomas (OSCC). This study explored the use of multiplex ligation-dependent probe amplification (MLPA) technique in detecting OSCC and OED specific genetic alterations. MLPA was used to detect gains and losses of 106 genes in DNA extracted from frozen tissue samples of 10 OSCC and 10 non-cancer patients. Two biopsies of OED were analyzed to explore the alterations in oral potentially malignant disorders. There were significant differences ($p<0.001$) in the number of alterations in OSCC and dysplasia compared to non-cancer samples respectively. The most frequently altered genes in OSCC were PTP4A3, RECQL4, ATM, and KLK3 (60%). Five genes (MYC, SLA, TNFRSF1A, MESDC1, MIF) were altered in 50% of OSCC samples. These nine genes were specific to OSCC samples ($p<0.05$). Some genes, including MYB, MET, CASP2, SLA and PTEN occurred in 50% of OED samples. MLPA was able to detect genetic alterations, that are present only in the OSCC samples and showed potential to be used as an adjunctive tool in early diagnosis of OSCC.

INTRODUCTION

In the most recent global cancer statistics (2021), about 377,713 new cases of oral cancer were reported worldwide, and an estimated 177,757 individuals died from this disease [1]. Oral squamous cell carcinoma (OSCC), which arises from oral mucosal linings of the oral cavity, constitutes 90% of all oral cancers. About 50% of OSCC patients still succumb to this disease within 5 years of diagnosis despite the advancements in treatment modalities [1]. Therefore, there is a need to reduce the burden of OSCC-related mortality and morbidity, which could be achieved through early detection. One of the more promising methods for early detection of OSCC is through the screening of genetic markers such as copy number alterations of oncogenes and tumour suppressor genes. These alterations have the potential to act as screening markers [2-4].

Chromosomal alterations, such as amplification of oncogenes and deletion of tumor suppressor genes have been widely documented as one of the major drivers in cancers [5]. Techniques such as array comparative genomic hybridization (aCGH) and fluorescence in situ hybridization (FISH) have identified gains at chromosome 3q, 5p, 7p, 8q, and 11q, and losses at chromosome 3p, 7q, 8p, 11q, 13q, 18q, 19q, and 20q [6,7] in OSCC. Many genes have also been shown to be frequently altered in OSCC including PIK3CA (3q26.3), TRAILR1 (8p23), ATM (11q22-23), MYC (8q24.21), EGFR (7p12), TP63

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(3q26), Serpine1 (7q21.1), FGF4/FGF3 (11q13.3), DMD (Xp21.2), Caspase8 (2q33–34) and MTAP (9p21) [8,9]. However, the frequencies of most alterations detected are low due to genetic heterogeneity between tumors [10].

High-throughput technologies, such as aCGH and gene expression microarray were developed to screen the entire genome in a single rapid assay. These techniques are vital tools in the discovery of genes that are involved in carcinogenesis. A robust method was developed known as multiplex ligation-dependent probe amplification (MLPA). Compared to aCGH, this method is faster and can detect genetic alterations using smaller DNA amounts. This method is also able to measure numerical chromosomal alterations at around 40 target locations using only 20ng of DNA [11,12]. This feature allows analysis of a large number of oncogenes and tumour suppressor genes that have been found in chromosomal regions with known copy number changes in OSCC and even at the early stage of cancer known as oral potentially malignant disorder (OPMD). MLPA has been shown to be a promising tool for screening genetic markers, although only a limited number of studies have been done [13,14]. Because of its advantages and potential use as an adjunctive early diagnostic tool, MLPA has been employed in this study to identify genetic alterations in frozen tissue samples, which are specific to OSCC and OPMD.

MATERIALS AND METHODS

24 frozen tissue samples were included in this study. The tissue samples consisted of 10 primary OSCC tumor and 10 non-cancer tissues, which were obtained from gingival mucosal flaps removed during wisdom tooth removal from non-cancer patients. The selection criteria for primary tumour including cases diagnosed histopathologically as OSCC and had not undergone any treatments before surgical removal of the tumour. Of the four tissue samples obtained from biopsies of OPMD, two samples were found to be hyperplasia while the other two samples were histologically graded as moderate dysplasia. Blood DNA from non-cancer patients were used as controls. All samples with respective socio-demographic and clinico-pathological data shown in Table 1 were obtained from the Malaysian Oral Cancer Database and Tumor Bank System (MOCDTBS) coordinated by Oral Cancer Research and Coordinating Centre, University of Malaya (OCRCC, UM). This study was approved by Medical Ethics Committee, Faculty of Dentistry, University of Malaya [MEC number: DFOP1109/0084(L)] and carried out in accordance with the Declaration of Helsinki. All patients have given their written consent.

Frozen tissue specimens were mounted in optimal cutting temperature (OCT) compound prior to sectioning. The sections were stained with hematoxylin and eosin (H&E) for histological assessment of tumors and gauging tumor cell percentage. DNA was extracted from tissues with tumour content of more than 70%. Genomic DNA from all tumour tissue were isolated using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Briefly, samples were lysed using proteinase K and the lysates were loaded onto the DNeasy Mini spin column. During centrifugation. DNA was selectively bound to the DNeasy membrane as contaminants passed through. Remaining contaminants and enzyme inhibitors were removed in two efficient wash steps and DNA was then eluted in buffer. DNA concentration and quality were measured using Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

The probe sets used were P005, P006, and P007 MLPA kit (MRC-Holland, Amsterdam, Netherlands) which allows the detection of 106 genes. These kits were selected as they were able to detect a variety of known oncogenes and tumor suppressor genes found in various locations across the genome. The experiments were performed based on supplier's protocol as described previously [10]. Briefly, purified DNA samples were denatured, followed by overnight incubation with MLPA probe oligos. Each probe was hybridised to adjacent target DNA sequences via ligation step. Ligated probes were amplified in a multiplex polymerase chain reaction (PCR) using a single universal primer pair. PCR products were loaded onto a capillary electrophoresis device and separated by length. Each fragment separation corresponded to a specific MLPA probe. In each experiment, one negative control and at least three reference controls were included. The amount of DNA used for each reaction was 50 ng.

Data analysis was done using Coffalyser.NET software (MRC-Holland, Amsterdam, Netherlands). The relative copy number ratio for each gene was calculated by dividing the normalised mean peak areas of each gene in a sample tissue with the normalised mean peak areas of the same gene in a control DNA from non-cancer peripheral blood samples. The mean value for genes with more than one probe was used for further analysis.

Copy number cut-off points for each gene were calculated using a 99.99% confidence interval based on non-cancer blood samples. The upper limit was defined as the cut-off point for gain and the lower limit as the cut-off point for loss. Chi-square test was used to test the difference between the number of genetic alterations in non-cancer and cancer or dysplastic tissue samples. Only two dysplastic samples were used for analysis. Fisher's exact test was used to determine genes, which can differentiate OSCC from non-cancer samples. The statistics were analyzed using the Excel spreadsheet and IBM SPSS version 20.

RESULTS

Overall, non-cancer tissue samples showed 3.0% (32/1060) alterations while the OSCC samples showed 20.7% (219/1060) alterations. There were significant differences ($p<0.001$) in the number of alterations between OSCC and non-cancer samples. There was an average of 22 (20.8%) alterations per OSCC sample. The highest number of alterations in one sample was sample 50 (47.2%), while the lowest was two (1.9%). The average number of alterations per non-cancer sample was three. The non-cancer frozen tissue sample with the highest number of alterations was 14 (13.2%).

Table 1 Sociodemographic and clinicopathological parameters of 10 non-cancer, 2 OED and 10 OSCC samples

Status	Gender	Ethnic	Tobacco smoking	Alcohol drinking	Betel quid chewing	pT	pN	pM	Tumour stage	Histological grade	Site
Normal	F	M	Never	Never	Never	N/A	N/A	N/A	N/A	N/A	Gingiva
Normal	M	M	Never	Never	Never	N/A	N/A	N/A	N/A	N/A	Gingiva
Normal	M	C	Never	Never	Never	N/A	N/A	N/A	N/A	N/A	Gingiva
Normal	M	M	Current	Never	Never	N/A	N/A	N/A	N/A	N/A	Gingiva
Normal	F	C	Never	Never	Never	N/A	N/A	N/A	N/A	N/A	Gingiva
Normal	M	C	Never	Never	Never	N/A	N/A	N/A	N/A	N/A	Gingiva
Normal	F	M	Never	Never	Never	N/A	N/A	N/A	N/A	N/A	Gingiva
Normal	M	M	Never	Never	Never	N/A	N/A	N/A	N/A	N/A	Gingiva
Normal	F	C	Never	Never	Never	N/A	N/A	N/A	N/A	N/A	Gingiva
Normal	M	C	Never	Never	Never	N/A	N/A	N/A	N/A	N/A	Gingiva
Hyperplasia	M	O	Never	Current	Stopped	N/A	N/A	N/A	N/A	N/A	Buccal Mucosa
Hyperplasia	F	I	Never	Never	Stopped	N/A	N/A	N/A	N/A	N/A	Buccal Mucosa
Dysplasia	F	I	Never	Never	Current	N/A	N/A	N/A	N/A	Moderate dysplasia	Buccal Mucosa
Dysplasia	F	O	Current	Never	Current	N/A	N/A	N/A	N/A	Moderate dysplasia	Buccal Mucosa
Cancer	M	C	Never	Stopped	Never	T4	N0	M0	Stage IV	Moderately differentiated OSCC	Tongue
Cancer	F	I	Never	Stopped	Current	U/A	U/A	U/A	U/A	U/A	Buccal Mucosa
Cancer	F	I	Never	Never	Stopped	T4	N0	M0	Stage IV	Well differentiated OSCC	Gingiva
Cancer	M	C	Stopped	Never	Never	T1	N0	Mx	Stage I	Well differentiated OSCC	Tongue
Cancer	F	I	Never	Never	Current	T4	N1	M0	Stage IV	Moderately differentiated OSCC	Tongue
Cancer	F	M	Never	Never	Current	T3	N0	M0	Stage III	Well differentiated OSCC	Buccal Mucosa
Cancer	M	I	Stopped	Stopped	Stopped	T1	N0	M0	Stage I	Moderately differentiated OSCC	Tongue
Cancer	M	C	Never	Current	Never	T4	N2B	M0	Stage IV	Moderately differentiated OSCC	Buccal Mucosa
Cancer	M	I	Never	Never	Current	T4	N0	M0	Stage IV	Well differentiated OSCC	Buccal Mucosa
Cancer	F	I	Never	Never	Current	T4	N1	M0	Stage IV	Moderately differentiated OSCC	Buccal Mucosa

Gender – M=Male; F=Female
Ethnic – M=Malay; C=Chinese; I=Indian; O=Others
N/A – not applicable
U/A – data unavailable

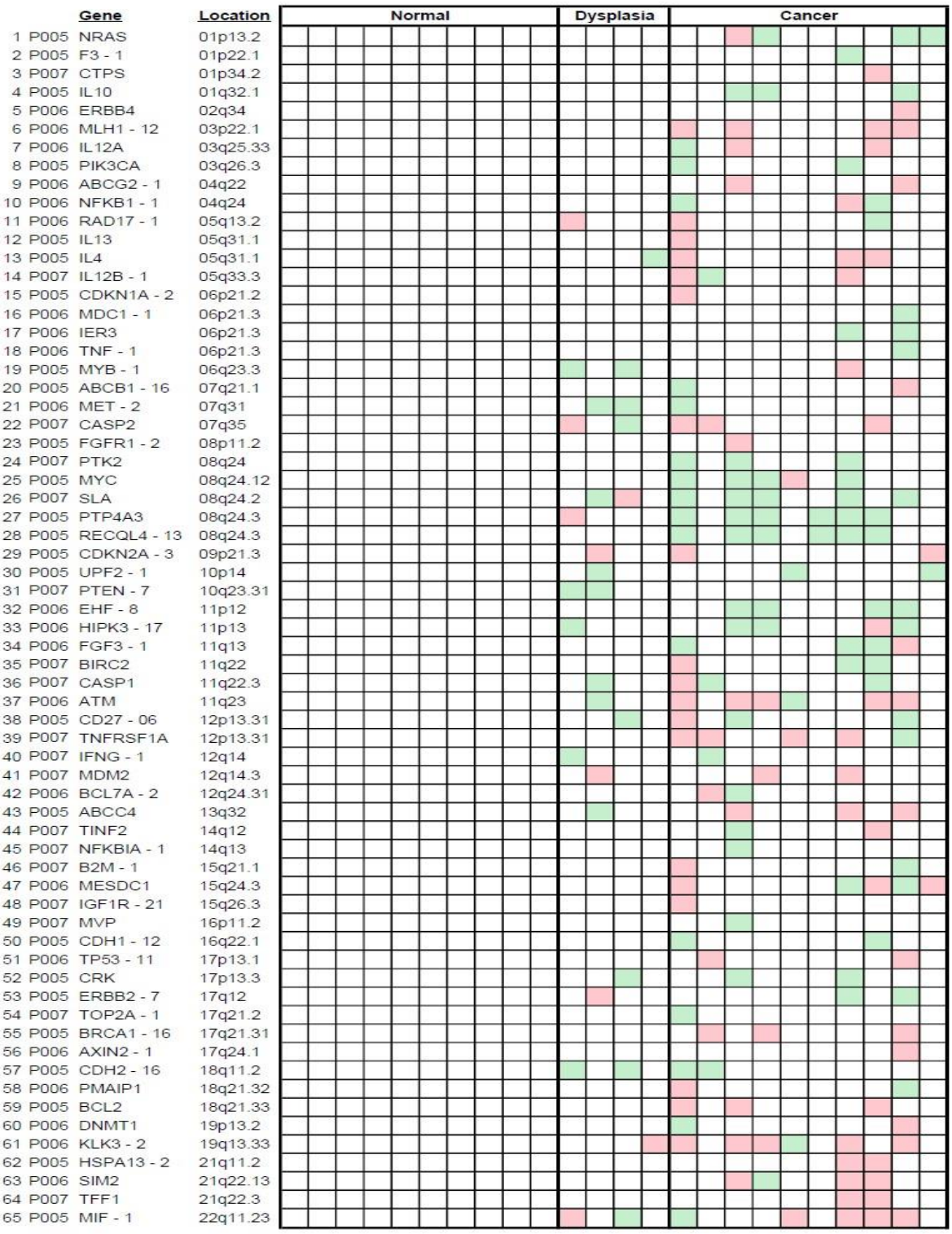

Figure 1 Heatmap of 65 genes which are altered only in cancer patients. Green colour represents gain while red represents loss. White colour represents no alterations.

Probes that show alterations in OSCC samples were selected and shown in Figure 1. Genes PTP4A3, RECQL4, ATM, and KLK3 showed alterations in 60% (6/10) of OSCC samples and were significantly different compared to the non-cancer samples (p=0.011). Five other genes namely MYC, SLA, TNFRSF1A, MESDC1 and MIF showed alterations in 50% (5/10) of OSCC samples and were also significantly different compared to the non-cancer samples (p=0.033). Genes that showed the highest frequency of gains (60%, 6/10), were PTP4A3 and RECQL4 while genes with the highest frequency of losses (50%, 5/10) were ATM and KLK3.

From OED samples, seven genes (MYB, MET, CASP2, SLA, PTEN, CDH2 and MIF) showed amplifications in two tissue samples. These dysplastic samples had 8.3% (35/424) alterations. The average number of alterations for each patient was nine. The highest number of alterations among dysplastic samples was 13 (12.9%), while the lowest was 2 (1.9%). We found that there was a significant difference ($p<0.001$) in the number of alterations between non-cancer versus dysplastic and dysplastic versus OSCC samples.

DISCUSSION

Carcinogenesis is known to be initiated by multistep accumulation of genetic alterations. These genetic alterations could potentially be used as biomarkers for early detection of OSCCs. Although a number of genes have been found to be frequently altered in OSCCs [8,15], no single genetic alteration has shown to be sufficient in accurately distinguishing OSCC patients from non-cancer patients. Furthermore, some studies have shown that the alterations in most genes were not widespread among tumors of a single cancer type due to tumor heterogeneity [10,16]. A genetic profile consisting of a list of frequently altered genes in a cancer type such as OSCC would be better suited in identifying OSCCs and potentially malignant lesions. From a list of genes consisting of oncogenes and tumor suppressor genes, which are found across the genome in various cancers [13], we identified those, which are repeatedly altered in OSCCs to explore the potential use of these genes in a genetic profile.

The 99.99% confidence interval of each probe was calculated and used as copy number cut-off points to increase the reliability of our results by reducing the effect of variation between probes [13,14]. Overall, we have shown that OSCC patients have a significantly higher number of alterations than non-cancer patients, which is consistent with other studies that used the same MLPA probe sets [13,17]. This difference was also shown in another study, which used a different MLPA probe panel [18]. However, the set of genes found altered in those studies were noticeably different compared to the genes found in our study. Among the possible reasons are the etiology or ethnicity differences of the patients in our study compared to others [13]. Our set of genes may highlight the genetic alterations that are specific to the etiology or ethnicity of patients in our study. The small sample size employed in this study could also explained the results were not consistent with previous studies. The availability of the characterized samples and the study feasibility within the resources and time, were among the limiting factor to increase sample size in this study.

Nine of the selected genes (MYC, SLA, PTP4A3, RECQL4, ATM, TNFRSF1A, MESDC1, KLK3 and MIF) were found to have a significantly higher number of alterations in OSCC compared to non-cancer samples. In tumorigenesis, gene amplification is an essential step in the activation of oncogenes. Notably, the most frequently amplified genes were MYC, PTP4A3, and RECQL4. The PTP4A3 gene (8q24.3) encodes an enzyme called human protein tyrosine phosphatase type IVA, member 3. The 8q24 region on which PTP4A3 is located has been shown to be a frequently gained region in OSCCs [6,19]. PTP3A4 copy number gains shown in our study is consistent with others found in OSCC and head and neck squamous cell carcinoma (HNSCC) patients [13,20]. In another study on HNSCCs, the gain of PTP4A3 was shown to predict tumor presence [21]. Overexpression of PTP4A3 has also been shown to be involved in the metastasis of cancers [22]. Also found in the 8q24 region is the RECQL4 gene (8q24.3) which encodes a DNA helicase. Other studies have shown similar gains of this gene in OSCCs and HNSCCs [13,20]. Both gains and losses of this gene was also reported in other cancers such as larynx and pharynx carcinomas, and colorectal cancers [20,23]. In our results, MYC, the well-known oncogene involved in cell cycle progression, apoptosis, and cellular transformation was altered in 50% of OSCC patients. In other studies, MYC was shown to be frequently amplified in OSCCs [8,15]. Of note, gains in chromosome 8q have been widely documented in dysplasia and invasive OSCC, which implies its significance in the malignant progression of OSCC. The focal gain of chromosome 8q24.3 is commonly attributed to the MYC gene and was identified as one of the frequently amplified gene in OSCC genome. Our study demonstrated that PTP4A3 and RECQL4 were also found amplified within this amplicon and is similar to the study by Ambatipudi et. al. in 2011 [24].

ATM, also known as ataxia telangiectasia mutated is located at 11q23 and plays an important role in maintaining genomic stability. In a study of HNSCC cell lines, the loss of the distal region of 11q, which encompasses ATM, has been found [25]. The ATM gene was found to be frequently lost in HNSCC patients [26]. A study by Freier et. al. in 2007 suggested that the loss of ATM may play a role in the pathogenesis of OSCCs [8]. KLK3 or prostate specific antigen (PSA) is located at 19q13.33. KLK3 is a serine protease and is an established tumor

marker for prostate cancer. KLK3, which is usually expressed in the prostate, is also found to be expressed in the salivary glands [27]. Our study found more frequent losses for this gene, which may result in lower or lack of expression of its mRNA or protein in OSCC patients. In a study of 30 individuals with low PSA concentrations, the deletion of KLK3 has been reported [28]. Gains of KLK3 has been found in gastric cancer and breast cancer cell lines [29,30]. The expression of KLK3 in OSCCs was found to be negative in a study using immunohistochemistry, cDNA microarray and qPCR [31].

A significant proportion of oral cancers are preceded by OPMD, which clinically may appear similar to OSCC [32]. Due to the limited number of characterized samples available during this study, we only tested two tissue samples of OED which were obtained from biopsies of OPMDs histologically graded as dysplastic to explore the feasibility of detecting genes in the early stages of carcinogenesis. These lesions would already have accumulated some genetic alterations [33]. Our results ($p<0.001$; number of alterations between non-cancer vs dysplastic) indicate that with the appropriate set of genes, it is possible to use MLPA to differentiate dysplastic from non-cancer tissues. A larger cohort would be better placed to test that possibility.

In this study, an attempt was made to compare the MLPA results of one OSCC patient to the previous aCGH result [19] of the same patient. The list of alterations and its associated genes from both MLPA and aCGH results were matched and we found that similar genes with high number of amplifications in OSCC identified by MLPA were also detected by aCGH. Some of the genes with high number of alterations including MYC, SLA, PTP4A3, RECQL4 and ATM has affirmed the reliability of the MLPA probe sets used. Again, a larger cohort of samples would be better to validate the MLPA probe sets by determining the correlation between the MLPA and aCGH copy number ratios.

Additionally, we have found non-cancer samples, which had higher number of alterations than OSCC samples. However, each alteration was found only once among all non-cancer samples and crucially, are not among the most altered genes in OSCC samples. Two such genes were CASP8 and FADD Like Apoptosis Regulator (CFLAR) and Interleukin 2 (IL-2). As an apoptosis regulator protein encoded by CFLAR, it may function as a crucial link between cell survival and cell death pathways in cells. Meanwhile, IL-2 involved in cytokine secretion that is important for the proliferation of T and B lymphocytes. It is possible that the alterations found in these non-cancer samples were not directly involved in OSCC carcinogenesis. The healthy subjects who enrolled in this study possibly had undergoing inflammatory responses at the point of sample collection, which could result in the genetic alterations.

Noninvasive screening is a method that has been frequently explored because the current gold standard requiring a tissue biopsy followed by histological examination, is not practical for routine screening [3,13,14,34]. Some noninvasive techniques involve the collection of DNA from saliva and exfoliated cells via brush biopsy. The testing of genetic alterations can be expanded by utilizing saliva or oral scrapings DNA. The amount of DNA obtained from saliva and exfoliated cells from brush biopsy is usually low. Hence, MLPA is a suitable method as part of the routine noninvasive screening due to its minimal DNA amount requirement and its relatively uncomplicated protocols. The MLPA technique was also fast and efficient as we managed to simultaneously detect large sets of genes using only 50 ng of DNA from a single patient. A few studies have shown the feasibility of noninvasive screening using the brush technique and saliva; however, further research is needed to further demonstrate its usefulness [13,14].

CONCLUSIONS

In conclusion, we found significantly more genetic alterations in OSCCs compared to non-cancer and OED samples. We were also able to identify nine frequently altered genes (MYC, SLA, PTP4A3, RECQL4, ATM, TNFRSF1A, MESDC1, KLK3 and MIF) that are specific to OSCCs and are possible candidates to be included in a genetic profile for OSCC detection. The MLPA technique has the potential to be used as a prospective application for community noninvasive genetic screening and diagnosis of OSCCs.

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DECLARATION OF INTEREST

None declared.

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