RESEARCH ARTICLE

Genetic Abnormalities in Oral Leukoplakia and Oral Cancer Progression

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Abstract

Background: The cancer progression of oral leukoplakia is an important watchpoint in the follow-up observation of the patients. However, potential malignancies of oral leukoplakia cannot be estimated by histopathologic assessment alone. We evaluated genetic abnormalities at the level of copy number variation (CNV) to investigate the risk for developing cancer in oral leukoplakias. Materials and Methods: The current study used 27 oral leukoplakias with histological evidence of dysplasia. The first group (progressing dysplasia) consisted of 7 oral lesions from patients with later progression to cancer at the same site. The other group (non-progressing dysplasia) consisted of 20 lesions from patients with no occurrence of oral cancer and longitudinal follow up (>7 years). We extracted DNA from Formalin-Fixed Paraffin-Embedded (FFPE) samples and examined chromosomal loci and frequencies of CNVs using Taqman copy number assays. Results: CNV frequently occurred at 3p, 9p, and 13q loci in progressing dysplasia. Our results also indicate that CNV at multiple loci–in contrast to single locus occurrences–is characteristic of progressing dysplasia. Conclusions: This study suggests that genetic abnormalities of the true precancer demonstrate the progression risk which cannot be delineated by current histopathologic diagnosis.

Keywords: Copy number variation - oral leukoplakia - biomarker - cancer progression

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Introduction

Oral leukoplakia is by far the most common oral premalignant lesion, representing 85% of such lesions. (Neville, 2002) Previous studies suggest that the malignant transformation potential of oral leukoplakia is 3~17%. (Silverman et al., 1984; Schepman et al., 1998). The criteria for determining the malignancy potential of oral leukoplakias are based on the presence and degree of epithelial dysplasia. However, more reliable indicators are needed because oral leukoplakias are mostly early lesions (mild and moderate epithelial dysplasia), and potential malignancies cannot be estimated by histopathologic assessment alone.

Various studies have been performed to find biomarkers of transforming oral premalignant lesions. One of them found genetic changes related to abnormal cells. The established methods of identifying these changes are array comparative genomic hybridization (aCGH), single nucleotide pleomorphism (SNP) array, and copy number assay. These can detect chromosomal anomalies in the specimen of the lesion. (Kallioniemi et al., 1992; Wang et al., 2007).

Copy number variations (CNVs) represent the copy number changes of DNA fragments, ranging from 1 kilobase or more, resulting from duplication or deletion. (Feuk et al., 2006; Oh and Nishimura, 2008). CNVs can contribute significantly to genomic polymorphism. Therefore, CNVs at specific sites are thought to be disease causative or susceptive. (Feuk et al., 2006).

One of the best ways to ascertain the genetic changes related to oral cancer progression is to compare genetic sequences of progressing and nonprogressing oral lesions. The malignant transformations arise from alterations of crucial regulatory genes. Therefore, progressing lesions may differ from nonprogressing ones even when they are histopathologically similar.

We have examined genetic abnormalities in oral leukoplakia using copy number assay. Moreover, we have verified differences between progressing and nonprogressing oral leukoplakias in terms of these abnormalities.

Materials and Methods

Sample collection

This study used Formalin-Fixed Paraffin-Embedded (FFPE) from the Department of Oral Pathology, Yonsei University Dental College. All lesions were diagnosed as mild or moderate epithelial dysplasia. Lesions with severe epithelial dysplasia were excluded from this study because they were thought to be undergoing neoplastic

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transformation.

Two sample groups were used. The first group, termed nonprogressing lesion, consisted of oral leukoplakias from patients without progression to head and neck cancer. These samples included 20 patients with early epithelial dysplasia.

The second group, termed progressing lesion, consisted of early epithelial dysplasia from 7 patients with progression to oral cancer. We confirmed that the preceding epithelial dysplasia and the following oral cancer occurred at identical sites using pathologic reports and medical records. In addition, we only included samples in which the onset of malignant lesions was more than 6 months since the onset of primary oral leukoplakias. All progressing lesions were confirmed by histopathologic diagnosis.

The gender and age distribution of nonprogressing and progressing sample sets are shown in Table 1.

Paraffin block microdissection and DNA extraction

Ten 20μm-thick sections were collected from 27 paraffin blocks using the microtome. We deparaffinized the 20 μm sections using xylene washes for DNA extraction, then washed them using ethanol. Next, we added proteinase to each tube and placed them in a 56°C heat block. The samples were then incubated for 1 hour in 90°C to reverse formalin crosslinking.

CNV Analysis

We carried out CNV analysis using genomic DNA (gDNA) from twenty-seven FFPE tissue samples. The assay was confined to the eight genomic regions listed in Table 2. The gDNA from each FFPE sample, Taqman copy number assay (Life Technologies Corporation, Carlsbad, CA, USA), and Taqman copy number reference assay (Life Technologies Corporation, Carlsbad, CA, USA) were mixed together in a single well and duplex real-time polymerase chain reaction (RT-PCR) was performed. We loaded the reaction plates using the 7900 HT PCR system (Life Technologies Corporation, Carlsbad, CA, USA).

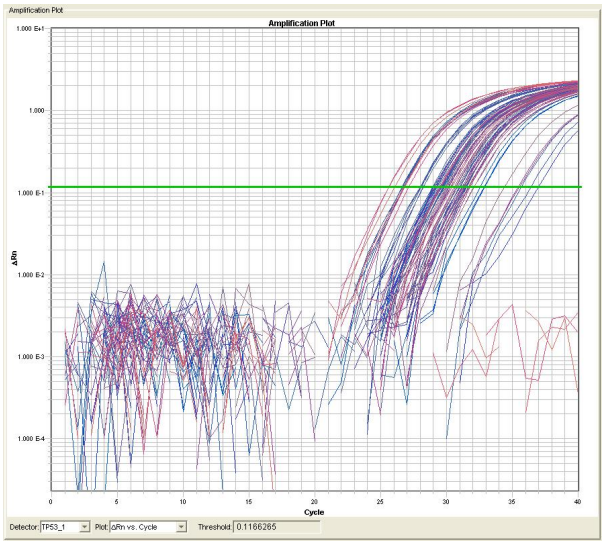

Figure 1. Cycle Threshold (Ct). The x axis represents the number of cycles and the y axis represents an arbitrary fluorescence unit

The copy number assay amplifies the target gene and the reference assay amplifies a reference sequence that is known to exist in two copies in a diploid genome. This study used the ribonuclease P RNA component H1 (H1RNA) gene (RPPH1) on chromosome 14, cytoband 14q11.2 as reference sequence.

When each Taqman probe binds to its target gene, the dye (FAM or VIC) generates fluorescence. Otherwise, the proximity of the quencher dye to the fluorescent dye blocks the fluorescent signal.

The copy number of the target sequence in each test sample is determined by relative quantitation (RQ) using the comparative cycle threshold (Ct) method, which measures the PCR cycles needed to obtain the intersection between threshold line and amplification curve. The greater the Ct value, the smaller the amount of DNA. (Figure 1).

This study measures the Ct difference (ΔCt) between

Table 1. Characteristics of Patients

	Non-progressing epithelial dysplasia	Progressing epithelial dysplasia
Sex (male/female)	15/5	3/4
Mean age	52	62

Table 2. Taqman Copy Number Assay

Cytoband	Gene symbol	Gene name	Assay location	Gene function	Assay ID
9p21.3c	CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	chr9:21976994 chr9:21987690	CDK inhibitor	Hs04367145_cn Hs03717798_cn
11q13.3d	CCND1	cyclin D1	chr11:69466166 chr11:69469088	Regulates CDK kinase	Hs02666923_cn Hs01978234_cn
17p13.1c	TP53	tumor protein p53	chr17:7572767 chr17:7578588	Regulates cell division	Hs02821479_cn Hs06423639_cn
3p21.31a	RASSF1	Ras association (RalGDS/AF-6) domain family member 1	chr3:50367666 chr3:50370782	Multiple functions of tumor suppression	Hs02560777_cn Hs03490926_cn
3p14.2c	FHIT	fragile histidine triad gene	chr3:60249964 chr3:60192696	Diadenosine triphosphate hydrolase	Hs03499888_cn Hs03469498_cn
8p21.3c	LPL	lipoprotein lipase	chr8:19802042 chr8:19823926	Instructions for making an enzyme	Hs03689798_cn Hs00778029_cn
18q21.2a	SMAD4	SMAD family member 4	chr18:48568378 chr18:48605262	TGF-β TF	Hs06436145_cn Hs02599864_cn
13q14.2b	RB1	retinoblastoma 1	chr13:48884045 chr13:48938126	Transcriptional repression; control of E2Fs	Hs07038675_cn Hs06369868_cn

target and reference sequences, then compares the ΔCt values of test samples to a calibrator sample known to have two copies of the target sequence. The Human Genomic DNA (Promega Corporation, Madison, WI, USA) was used as a reference sample having normal genetic information.

After carrying out RT-PCR, we analyzed the results in terms of copy numbers using comparative Ct (ΔΔCt) relative quantitation analysis using CopyCaller software (Life Technologies Corporation, Carlsbad, CA, USA). To conceal the identities of the samples, groups were labeled alpha numerically for handling during DNA extraction and analysis for genetic anomalies.

Results

Clinical impression

Characteristics of patients: Patients' overall incidence age of oral leukoplakias was from 25 to 72 years old, the average age being 54.3 ± 12.6 years. The average age when the nonprogressing patients were first diagnosed with oral epithelial dysplasia is 51.7 ± 13.8 years old. The average age when the progressing patients were first diagnosed with oral epithelial dysplasia was 61.6 ± 1.3 years old.

In the case of nonprogressing samples, the gender ratio was fifteen men and five women. In the case of progressing samples, the gender ratio was three men and four women.

Follow-up observation period: The follow-up observation period for nonprogressing cases was 9.8 ± 2.4 years on average, and at least 7 years. In progressing cases, the average period to onset of oral cancer was 43.0 ± 36.7 months from the time of diagnosis of oral epithelial dysplasia, with a 13-month minimum period of onset.

Distribution by site of occurrence: Of the 27 total samples of oral epithelial dysplasia, 12 cases occurred in tongue, 8 in buccal mucosa, 6 in gingival, and 1 case in the palate. Of these, malignant progression occurred in 4 cases in tongue, 2 in gingiva, and 1 case in buccal mucosa. (Tables 3, 4)

The frequency of CNV

Of the 27 oral epithelial dysplasias subjected to RT-PCR, four samples (patient numbers 1, 2, 5, and 21) failed PCR amplification, apparently due to FFPE sample characteristics, insufficient DNA, and excessive fragmentation.

CNV was revealed in 12 cases (52.2%) of the entire 23 oral epithelial dysplasias on which CNV analysis was performed. The frequency of CNV was greater in progressing lesions. While all progressing lesions showed CNV at more than one site, only 42.9% of nonprogressing lesions showed any CNV. Moreover, three or more CNVs appeared mainly in progressing lesions (66.7%), though infrequently in nonprogressing lesions (5.9%). Genetic changes in progressing lesions appeared, on average, at 3.3 sites. However, those of nonprogressing lesions appeared, on average, at 2.0 sites. (Tables 5, 6)

Types of CNV

In all specimens, CNV appeared most frequently at 3p and 9p (32.6% and 17.4%, respectively). CNV of 3p was most common in nonprogressing lesions. CNVs of 3p, 9p, and 13q appeared frequently in progressing lesions. In nonprogressing lesions, deletions in 3p14 and 3p21 appeared in 29.4% and 23.5% of cases, respectively. In contrast, deletions in 3p14, 3p21, and 9p21 all appeared in 50% of progressing lesions.

In nonprogressing lesions, CNV of sites besides 3p were rare, CNV occurrences in 9p, 18q, and 17p totalling only 5.9%. The frequency of CNV occurrence in sites besides 3p was greater in progressing lesions, increasing

Table 3. Non-progressing Epithelial Dysplasia

Patient number	Age	Sex	Site	Follow-up (Year)
1	67	M	Buccal mucosa	15
2	60	F	Mn. Gingiva	14
3	52	M	Mn. Gingiva	10
4	53	F	Tongue	10
5	59	M	Tongue	15
6	25	M	Tongue	8
7	38	M	Buccal mucosa	11
8	51	M	Mx. Gingiva	11
9	57	M	Tongue	10
10	24	M	Palate	8
11	67	M	Tongue	9
12	45	F	Mx. Gingiva	9
13	57	M	Buccal mucosa	7
14	40	M	Tongue	7
15	53	M	Buccal mucosa	8
16	68	M	Buccal mucosa	9
17	62	F	Buccal mucosa	8
18	72	F	Tongue	9
19	50	M	Buccal mucosa	12
20	34	M	Tongue	8

Mx: maxilla, Mn: mandible, M: male, F: female

Table 4. Progressing Epithelial Dysplasia

Patient number	Age	Sex	Site	Interval (Month)
21	64	M	Buccal mucosa	45
22	61	M	Tongue	17
23	62	F	Mn. Gingiva	13
24	60	F	Tongue	42
25	62	F	Tongue	54
26	61	F	Tongue	117
27	61	M	Mn. Gingiva	13

Table 5. CNV in DNA from Epithelial Dysplasia

	Nonprogressing[a]	Progressing[a]	p[b]
Number of samples	17	6	
Number of samples with CNV	6 (35.3)	6 (100.0)	0.014
More than three CNVs	1 (5.9)	4 (66.7)	0.008
3p14 CNV	5 (29.4)	3 (50.0)	0.621
3p21 CNV	4 (23.5)	3 (50.0)	0.318
9p21 CNV	1 (5.9)	3 (50.0)	0.04
18q21 CNV	1 (5.9)	2 (33.3)	0.155
17p13 CNV	1 (5.9)	2 (33.3)	0.155
13q14 CNV	0	3 (50.0)	0.011
11q13 CNV	0	2 (33.3)	0.059
8q21 CNV	0	2 (33.3)	0.059

[a]The values in parentheses are percentages; [b]Fisher's exact test

Table 6. Summary of CNV Findings for All Samples

Sample number	3p14.2	3p21.31	9p21.3	18q21.2	17p13.1	13q14.2	11q13.3	8p21.3
Non-progressing epithelial dysplasia								
3	+							
4								
6								
7		+			+			
8								
9								
10	+							
11								
12								
13								
14	+	+	+	+				
15	+	+						
16								
17	+	+						
18								
19								
20								
Progressing epithelial dysplasia								
22	+	+	+		+	+	+	
23	+							
24		-	-	-				-
25								-
26					+	+	+	
27	+	+	+	+		+		

+: copy number gain, -: copy number loss

about eightfold (50%) in the case of 9p and about sixfold (33.3%) in the case of 18q and 17p. Furthermore, only progressing lesions manifest CNVs of 13q, 11q, and 8p. (Tables 5, 6)

Discussion

The risk of cancer progression increases in oral leukoplakia including erythematous or erosive lesion, or having verrucoid hyperplastic pattern, or showing epithelial dysplasia. (Silverman et al., 1984). In determining the treatment of oral leukoplakia, one must consider the risk of malignant transformation, size of the lesion, usefulness of the excision, and degree of patient cooperation.

Since there is no clinical reference with which to separate the stages of oral leukoplakia nor standardized treatment, we cannot predict the effectiveness of surgical treatment. In addition, oral leukoplakia is difficult to treat since it occurs multiply, has variable features, and in many cases manifests no clear boundary. Moreover, it is difficult to treat based on histological evaluation alone, as it is impossible to accurately assess the prognosis of recurrence and malignant transformation.

In recent years, researchers have sought a biomarker to predict the prognosis of oral leukoplakia. As molecular events indicate a tumor's biological characteristics, it may be advantageous to base a prognosis on a molecular staging system rather than conventional clinicopathological features. (Partridge et al., 1999) Ideally, a genetic marker would yield information on protein, tissue, organization, and human body.

CNV is an important polymorphism that affects the expression of nearby genes or in the rearranged area. (Henrichsen et al., 2009) Normally, humans have two copies of each autosomal region. CNV might be caused by genomic rearrangement such as deletion, duplication, inversion, and translocation, producing a higher or lower level of transcription than in the case of a single replication. (Hastings et al., 2009). CNVs connect the gap between single-nucleotide change and microscopic chromosomal alteration in genetic changes. Since the finding of 21 trisomy in Down syndrome in 1959, many studies have drawn associations between CNV and genetic diseases such as cancer, immune disorders, and neurological disorders.

Methods for CNV discovery fall into two main categories: genome-wide detection, of which array CGH is representative, and target-specific detection, based on the RT-PCR used in this study. As described above, when a specific probe is bound to the target gene and amplified through the process of PCR, it will show a constant signal. The copy number can be estimated by comparing the signal detected in test samples to controls. This method has two advantages: the high resolution of the desired target gene and the fact that a probe, once produced, can be used repeatedly in approximately 230 samples.

Many studies of head and neck cancer have observed a genomic imbalance, commonly in 3p, 8p, 9p, 11q, 17p, and 13q. (Brennan et al., 1995; Choi et al., 2000; el-Naggar et al., 1993; Maestro et al., 1996; Martin et al., 2008; Partridge et al., 1999; Pateromichelakis et al., 2000; Singh et al., 2013; van der Riet et al., 1994; Ye et al., 2007). Among these, modification of 3p occurs quite frequently in oral cancer. This chromosomal region is known to contain at least three tumor suppressor genes. (Maestro et al., 1993; Wu et al., 1994).

Studies of chromosomal abnormalities in oral precancerous lesions are ongoing. Mao et al. first studied chromosomal abnormalities in the oral leukoplakia, investigating the loss of 3p14 and 9p21 regions and finding that oral cancer was more likely to migrate when these sites were deficient (Mao et al., 1996). Partridge et al. studied allelic imbalances in the 3p21, 8p21-23, 9p21, 13q14.2, 17p13.1, and 18q21.1 regions. (Partridge et al., 1998). They established that the likelihood of progressive

squamous cell carcinoma is very large in lesions with an imbalance in two or more fields. Rosin et al. investigated sites of chromosomal deletion in patients with early oral premalignant lesions and confirmed that the risk of malignant transformation increased when chromosomal deletion appears not only at 3p and 9p, but also 4q, 8p, 11q, and 17p. (Rosin et al., 2000). Garnis et al. performed aCGH on oral premalignant lesions and squamous cell carcinomas, confirming that the genetic changes of mild epithelial dysplasia that has migrated to oral cancer are similar to those in severe lesions. (Garnis et al., 2009). This phenomenon contrasts with the histopathological similarity of progressing and nonprogressing epithelial dysplasia. In the study of Garnis et al. (2009) the frequent chromosomal deformation in progressing epithelial dysplasia involved 9p, 8q, 20p, and 20q.

We studied differences between CNVs in nonprogressing and progressing lesions using the Taqman copy number assay. CNV of 3p was most frequent in both progressing (50.0%) and nonprogressing (26.5%) lesions. Accordingly, we could confirm the results of previous studies that CNV of 3p reflects an important genetic imbalance related to epithelial changes. (Califano et al., 1996; Emilion et al., 1996; Mao et al., 1996; Partridge et al., 1998; Rosin et al., 2000). Also, similar to findings in a previous study, multiple CNVs of specific sites (3p, 9p, 13q, 18q, 17p, 11q, and 8p) in progressing lesions appeared more frequently than nonprogressing ones. Therefore, multiple genetic changes at those sites are considered indicators of progressing epithelial dysplasia. (Partridge et al., 1998).

In terms of the frequency of genetic variations, the results of the current study differ from those of previous studies. Hwang et al. found expression of p53 (17p13) in 29% of nonprogressing cases and 43% of progressing cases.(Jin Ha et al., 2002) However, this study found 5.9% and 33.3%, respectively. Moreover, Baek et al. studied the expression of cyclin D1 (11q13) and reported its presence in 20% of nonprogressing cases and 80% of progressing cases. (Ji Young et al., 2001) However, the current study found it in 0% and 33% of cases, respectively. According to the study by Rosin et al., allelic loss of 3p was found in 25% of nonprogressing lesions and 64% of progressing lesions, and in the case of 9p, 46% and 83%, respectively. (Rosin et al., 2000) In contrast, this study observed CNV of 3p in 26.5% of nonprogressing lesions and 50.0% of progressing lesions; CNV of 9p was observed in 5.9% and 50.0%, respectively. Moreover, this study differed from that of Garnis et al. in terms of the frequency of genetic changes at sites 3p and 9p. (Garnis et al., 2009). These differences may be attributed to small sample sizes, disparate methods for defining and classifying oral premalignant lesions, and different analytical approaches.

As confirmed in this study, genetic changes at 3p and 9p regions are involved in early cancer progression. (el-Naggar et al., 1995; Califano et al., 1996). In addition, depending on the histological stages from oral precancerous lesions to head and neck cancer, loss of heterozygosity and microsatellite instability increase. (Rosin et al., 2000; Ha et al., 2002; Garnis et al., 2009). However, we should keep in mind that progression of cancer depends on a tight web of interactions and that various genetic changes in lesions may appear simultaneously. We cannot make an absolute equation between specific genetic changes and the phases of lesion progression. The genetic abnormalities revealed in this study may occur elsewhere than in epithelial dysplasia and carcinoma in situ.

In conclusion, verifying chromosomal changes in several regions, including 3p and 9p in patients with oral premalignant lesions, may help in evaluating the risk of malignant transformation. If we combine progression risk factors based on the conventional classification system with those based on genetic imbalances, we may better understand the potential risk of oral leukoplakia progressing to malignancy and establish a strategy to improve the prognosis and clinical course.

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