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The Association between Microsatellite Alteration and Survival of Oral Cavity Squamous Cell Carcinoma Patients from an Endemic Betel Quid Chewing Area

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Abstract

Background: The incidence of microsatellite alterations at the endemic betel quid chewing area and its association with the survival of patients with oral cavity squamous cell carcinoma (OCSCC) is not clear. Here we studied their possible relationship.

Methods: Subjects were 135 patients with histological confirmed OCSCC. From these patients we obtained their cancerous tissues, corresponding surgical margins, and peripheral blood samples. From these specimens, we analyzed the microsatellite alterations base on 10 oligonucleotide markers. Specifically, specimens were assessed by automatic fragment analysis following amplification by polymerase chain reactions.

Results: Of these specimens, 45 (33.3%) showed microsatellite instability (MSI) and 78 (57.8%) showed loss of heterozygosity (LOH) for at least one marker. Using Kaplan-Meier's analysis method, microsatellite alterations of patients did not associate with their disease-specific survival. However, the presence of MSI in surgical margins of the cancer increased the risk of local recurrence (odds ratio: 7.49; 95% confidence interval: 3.34 ~ 16.80; $P < 0.001$).

Conclusion: The prognosis of OCSCC patients was not associated with microsatellite alterations in region where betel quid chewing is prevalent. However, genomic examination of surgical margin can possibly find out OCSCC patients who are prone to develop local recurrence.

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Introduction

Microsatellite instability (MSI) and loss of heterozygosity (LOH) are the most common types of microsatellite alterations which have been reported to be associated with various type of cancer in the literature. Microsatellites are repeating segments of 1 to 6 base pairs in eukaryotic genomes [1]. Such repeated sequences are susceptible to inaccurate repetition during DNA duplication, and the failure to repair such errors leads to MSI [2]. For example, MSI is well-known to be related to the development and prognosis of colorectal cancer [3]. In addition, head and neck cancer patients with MSI are more likely to develop a second primary cancer [4]. Our previous study also showed that MSI in the dysplasia-free surgical margin of head and neck squamous cell carcinoma is associated with its local recurrence [2]. However, the relationship between survival of OCSCC patients and MSI remains controversial [5]. The loss of a functional allele at a heterozygous locus, or LOH, is correlated with allelic loss of a number of tumor suppressor genes [6]. A tumor suppressor gene adjacent to LOH can be deactivated, leading to uncontrolled cell growth [7]. A previous study found that LOH at D9S162 is associated with a poor recurrence-free survival in oral cancer patients [8]. Lee et al. [9] in a study on hypopharyngeal cancer patients also found that LOH is correlated with lymph node metastasis. However, most of abovementioned studies are in Western countries and little is known in Asian countries regarding MSI and LOH on the survival of OCSCC patients. Here we investigated microsatellite alterations in a betel quid-prevalent region and its association with the survival of OCSCC patients.

Table 1: Markers used for microsatellite alteration analysis.

Marker	Forward (5'→ 3')	Reverse (3' → 5')	Size (bp)	Repeat	Location
D9S1748	CACCTCAGAAGTCAGTGAGT	GTGCTTGAAATACACCTTTCC	130	Dinucleotide	9p21-9p21
D3S1079	GGGAGATAGGTAGTATCATCT	ATCTACCATTAAGGCAACCTG	136	Dinucleotide	3p25
THRB	GATCACAAGGATGCTAGAGT	TCAAAGGAGTCAGGCTGTAG	197	Dinucleotide	3p24.1-3p22
D3S1234	CCTGTGAGACAAAGCAAGAC	GACATTAGGCACAGGGCTAA	111	Dinucleotide	3p21.1-3p14.2
D3S1300	ACAAAGGAACGTCATGTGGTAGG	GCTGTTTATTCTTCGTGGAATGCC	155	Dinucleotide	3p21.1-3p14.2
IFNA.PCR2	TGCGCGTTAAGTTAATTGGTT	GTAAGGTGGAAACCCCCACT	138 -150	Dinucleotide	9p22-9p22
D2S206	TTAAAAATTAAGTAGGCTTTTGGTT	GTCCTCATGTGTTTATGCTGT	238	Dinucleotide	2q33-37
D21S236	CCCAAATAAAAAAGAGAACAG	CTAAAGAGGACTTCAGAGTAAGG	104	Dinucleotide	21q11.1
D21S1433	GCGGGCACTGTAGTCTCAG	CTATTTTCAGGCCAAGCCTT	240	Tetranucleotide	21p-q12
D21S11	ATATGTGAGTCAATTCCCCAAG	TGTATTAGTCAATGTTCTCCAG	223	Tetranucleotide	21q21

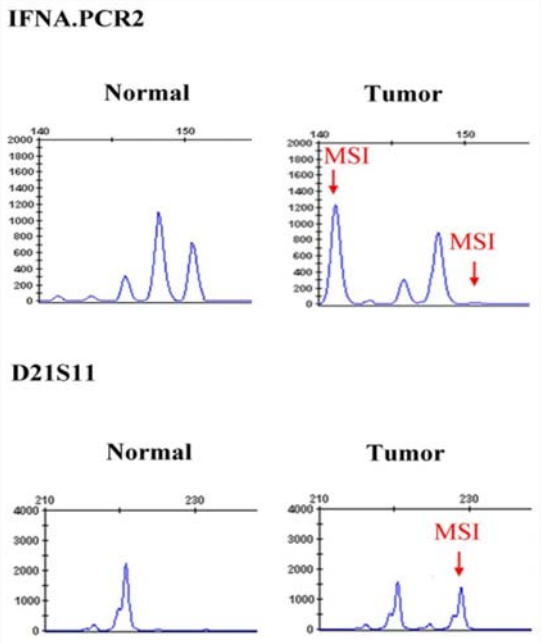

Figure 1: Representative sample of microsatellite instability (MSI) in selected microsatellite markers.

$$LOH = \frac{(\text{peak height of normal allele 2}) / (\text{peak height of normal allele 1})}{(\text{peak height of tumor allele 2}) / (\text{peak height of tumor allele 1})}$$

Figure 2: Scoring formula for loss of heterozygosity (LOH).

Materials and Methods

This study was reviewed and approved by the Institutional Review Board of the host Hospital. Potential participants were oral cavity cancer patients planned for surgical intervention during a four-year from April 2012 to April 2016. The detailed protocol was first explained to participants and written consent obtained prior to subject recruitment. We excluded those subjects who either had declined for surgery, non-squamous cell carcinoma, incomplete medical records, or refused to participate the study. Pathological stage was determined according to the guidelines of the American Joint Committee on Cancer (7th edition, 2009). Habits of participants on cigarette smoking and betel quid chewing were quantitatively recorded as follows. "One pack-year" represented smoking 20 cigarettes (1 pack) per day for 1 year and "one quid-year" represented chewing one betel quid per day for 1 year. Due to various kinds of alcoholic beverages were consumed by participants, we only divided participants into three groups: non-user, social user, and heavy user. Treatment plans for all participants were conducted in accordance with the consensus guidelines of the oral cavity cancer team of the host Hospital.

Detailed laboratory procedures were same as those in our previous report [2]. In brief, histologically confirmed OCSCC specimens and corresponding surgical margins were promptly stored in liquid nitrogen. Peripheral blood (10 ml) was drawn before operation and was placed in an EDTA-treated tube. The sample was then centrifuged and the plasma was transferred to a 1.5-ml microtube. The mononuclear cell layer was transferred into a clean 50 ml centrifuge tube, washed twice with a balanced salt solution, and re-centrifuged. Samples were stored at –30°C until use. Total DNA was extracted using the QIAamp DNA Mini kit (QIAGEN) according to its instructions. The final DNA was dissolved in double-distilled water and frozen at -30°C until further processing. Five binucleotide microsatellites (D9S1748, D3S1079, THRB, D3S1234, D3S1300) were selected based on literature review [10-12]. Three additional binucleotide microsatellites (IFNA.PCR2, D2S206, D21S236) and two tetranucleotide microsatellites (D21S1433, D21S11) were also selected according to our previous work (Table 1). Multiplex PCR reactions were performed with fluorescent-labeled forward primers and the amplified PCR products were analyzed through capillary array electrophoresis with the software Gene Scan (Applied Biosystems Inc., Foster City, USA). All PCR products were purified and sequenced with the ABI Big Dye Terminator (version 3.1) cycle sequencing ready reaction kit and the ABI PRISM 3100 sequencer (Applied Biosystems Inc., Foster City, CA). MSI was defined as the presence of novel sized fragments in DNA obtained from tumor subjects. These fragments were absent in the DNA of leukocytes taken from peripheral blood (Figure 1). In addition, to provide a tumor imbalance factor [11], the ratio of both microsatellite alleles (allele 2/allele 1) in the peripheral blood leukocyte DNA was divided by the corresponding ratio found in tumor DNA (Figure 2). Imbalance factor of values < 0.67 or > 1.5 were classified as LOH.

Statistical analyses

We used descriptive statistics to present the demographic data. Student's *t* test was used to compare continuous variables between

Table 2: Descriptive and bivariate analysis of oral cavity squamous cell carcinoma patients with or without microsatellite instability (MSI).

Variables	Total no. of patients (% in column)	No. of patients (%)		*P* value
		MSI (n=45)	Without MSI (n=90)	
Age at presentation (year)		50.9 ± 9.9	53.3 ± 11.6	0.225
Body mass index (Kg/M²)		24.6 ± 4.5	25.6 ± 4.5	0.223
F/U duration (month)		26.2 ± 14.6	25.2 ± 14.8	0.699
Gender (Female/Male)	10/125	2/43	8/82	0.495 [a]
Smoking				0.999
Yes	107(79.3%)	36(33.6%)	71(66.4%)	
No	28(20.7%)	9(32.1%)	19(67.9%)	
Alcohol				0.676
Heavy	40(29.6%)	15(37.5%)	25(62.5%)	
Social	56(41.5%)	19(33.9%)	37(66.1%)	
No	39(28.9%)	11(28.2%)	28(71.8%)	
Betel quid				0.836
Yes	99(73.3%)	34(34.3%)	65(65.7%)	
No	36(26.7%)	11(24.4%)	25(69.4%)	
Primary tumor sites				0.996
Lip	4(3.0%)	1(25.0%)	3(75.0%)	
Gum	11(8.1%)	3(27.3%)	8(72.7%)	
Floor of mouth	7(5.2%)	2(28.6%)	5(71.4%)	
Tongue	55(40.7%)	20(36.4%)	35(63.6%)	
Buccal	49(36.3%)	16(32.7%)	33(67.3%)	
Palate	6(4.4%)	2(33.3%)	4(66.7%)	
Retromolar trigone	3(2.2%)	1(33.3%)	2(66.7%)	
Histological features				0.307
Well differentiated	9(6.7%)	2(22.2%)	7(77.8%)	
Moderately differentiated	102(75.6%)	32(31.4%)	70(68.6%)	
Poorly or undifferentiated	24(17.8%)	11(45.8%)	13(54.2%)	
Perineural invasion				0.032
Yes	29(21.5%)	15(51.7%)	14(48.3%)	
No	106(78.5%)	30(28.3%)	76(71.7%)	
Angiolymphatic invasion				0.700
Yes	26(19.3%)	10(38.5%)	16(61.5%)	
No	109(80.7%)	35(32.1%)	74(67.9%)	
Extracapsular invasion				1.000
Yes	12(8.9%)	4(33.3%)	8(66.7%)	
No	123(91.1%)	41(33.3%)	82(66.7%)	
Pathological stage				0.078
Stage I-II	67(49.6%)	17(25.4%)	50(74.6%)	
Stage III-IV	68(50.4%)	28(41.2%)	40(58.8%)	
Postoperative radiotherapy				0.075
Yes	59(43.7%)	25(42.4%)	34(57.6%)	
No	76(56.3%)	20(26.3%)	56(73.7%)	
Local recurrence				0.006
Yes	33(24.4%)	18(54.5%)	15(45.5%)	
No	102(75.6%)	27(26.5%)	75(73.5%)	
Survival status				0.042
Alive	114(84.4%)	35(30.7%)	79(69.3%)	
Death	21(15.6%)	10(47.6%)	11(52.4%)	

[a] Fisher's exact test

Table 3: Descriptive and bivariate analysis of oral cavity squamous cell carcinoma patients with or without loss of heterozygosity (LOH).

Variables	Total no. of patients (% in column)	No. of patients (%)		*P* value
		LOH (n=78)	Without LOH (n=57)	
Age at presentation (year)		51.3 ± 11.2	54.2 ± 10.7	0.143
Body mass index (Kg/M^2)		25.3 ± 5.1	24.8 ± 3.0	0.436
F/U duration (month)		26.8 ± 14.7	23.8 ± 14.7	0.239
Gender (Female/Male)	10/125	3/75	7/50	0.095 [a]
Smoking				0.999
Yes	107(79.3%)	62(57.9%)	45(42.1%)	
No	28(20.7%)	16(57.1%)	12(42.9%)	
Alcohol				0.585
Heavy	40(29.6%)	25(62.5%)	15(37.5%)	
Social	56(41.5%)	33(58.9%)	23(41.1%)	
No	39(28.9%)	20(51.3%)	19(48.7%)	
Betel quid				0.783
Yes	99(73.3%)	56(56.68%)	43(43.4%)	
No	36(26.7%)	22(61.1%)	14(38.9%)	
Primary tumor sites				0.820
Lip	4(3.0%)	2(50.0%)	2(50.0%)	
Gum	11(8.1%)	6(54.5%)	5(45.5%)	
Floor of mouth	7(5.2%)	6(85.7%)	1(14.3%)	
Tongue	55(40.7%)	30(54.5%)	25(45.5%)	
Buccal	49(36.3%)	29(59.2%)	20(40.8%)	
Palate	6(4.4%)	3(50.0%)	3(50.0%)	
Retromolar trigone	3(2.2%)	2(66.7%)	1(33.3%)	
Histological features				0.051
Well differentiated	9(6.7%)	4(44.4%)	5(55.6%)	
Moderately differentiated	102(75.6%)	65(63.7%)	37(36.3%)	
Poorly or undifferentiated	24(17.8%)	9(37.5%)	15(62.5%)	
Perineural invasion				0.459
Yes	29(21.5%)	19(65.5%)	10(34.5%)	
No	106(78.5%)	59(55.7%)	47(44.3%)	
Angiolymphatic invasion				0.124
Yes	26(19.3%)	19(73.1%)	7(26.9%)	
No	109(80.7%)	59(54.1%)	50(45.9%)	
Extracapsular invasion				0.729
Yes	12(8.9%)	8(66.7%)	4(33.3%)	
No	123(91.1%)	70(56.9%)	53(43.1%)	
Pathological stage				0.441
Stage I-II	67(49.6%)	36(53.7%)	31(46.3%)	
Stage III-IV	68(50.4%)	42(61.8%)	26(38.2%)	
Postoperative radiotherapy				0.885
Yes	59(43.7%)	35(59.3%)	24(40.7%)	
No	76(56.3%)	43(56.6%)	33(43.4%)	
Local recurrence				0.072
Yes	33(24.4%)	24(72.7%)	9(27.3%)	
No	102(75.6%)	54(52.9%)	48(47.1%)	
Survival status				0.860
Alive	114(84.4%)	65(57.0%)	49(43.0%)	
Death	21(15.6%)	13(61.9%)	8(38.1%)	

[a] Fisher's exact test

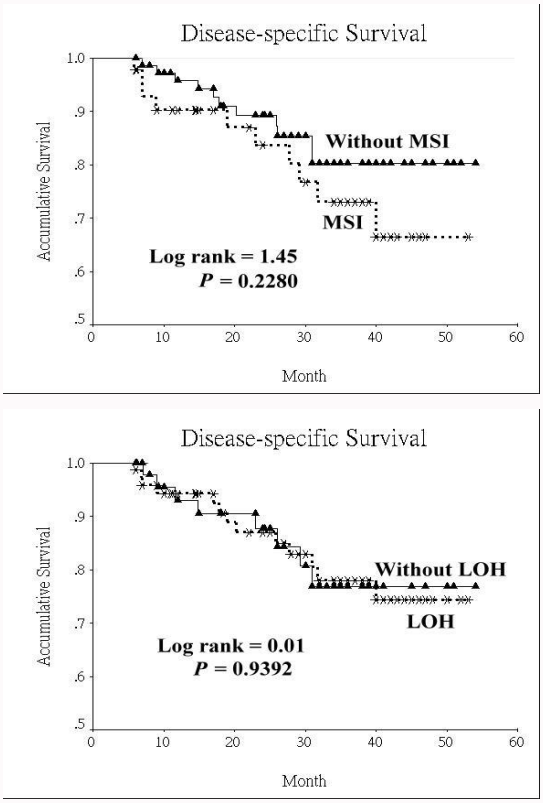

Figure 3: Disease-specific survival curves of oral cavity squamous cell carcinoma patients based on the status of microsatellite alteration. (MSI: microsatellite instability; LOH: loss of heterozygosity).

subgroups. Nominal or ordinal variables were analyzed using the Chi-square test or Fisher's exact test. The Kaplan-Meier method was used to calculate disease-specific survival. Differences among subgroups were assessed by the log-rank test. A backward stepwise logistic regression model was used to find independent factors correlated with local recurrence. All analyses were conducted in SPSS for Windows, version 12.1 (SPSS, Chicago, IL) and a $p< 0.05$ was considered statistically significant.

Results

There were totally 171 oral cavity cancer patients planned to receive surgical excision during the study period. Among these participants, 5 (2.9%) declined to participate in the study; 6 (3.5%) refused surgery and accepted organ preservation treatment instead, and 3 (1.8%) did not have squamous cell carcinoma in their final pathological reports. In addition to above-mentioned participants, we also excluded 13 (7.6%) who had inadequate surgical margins (< 5 mm) and 9 (5.3%) with dysplasia in at least one of the mucosa margins. Complete data were obtained from 135 participants. The average age of participants at presentation was 52.5+11.1 years and they were mostly men (n=125, 92.6%). The commonest primary site was the tongue (n=55, 40.7%), followed by buccal mucosa (n=49, 36.3%) and gum (n=11, 8.1%). In terms of personal habits of these patients, 107 (79.3%) were smokers, 106 (78.5%) consumed alcohol socially or heavily, and 99 (73.3%) habitually chewed betel quid. Regarding pathological stages of disease, 43 (31.8%) were in stage I, 24 (17.7%) in stage II, 16 (11.9%) in stage III, and 52 (38.5%) in stage IV. Only 33 (24.4%) of patients developed local recurrence during the follow-up period. Among these recurrent cases, 28 (20.7%) expired due to extensive local recurrence or complications during salvage treatments; 4 (3.0%) developed cervical lymph node recurrence. None of these showed distant metastasis. The average follow-up period was 25.5 (+14.7) months. A total of 45 participants (33.3%) had MSI in their cancerous specimens for one marker or more. Most MSI was localized to D21S236 (n=16, 35.6%), followed by IFNA.PCR2 (n=10, 22.2%), and THRB (n=9, 20.0%). More than half of these participants (n=78, 57.8%) had LOH in their tumor specimens for least one marker. The most frequently-detected positive marker for LOH was IFNA.PCR2 (n=40, 51.3%), followed by D9S1748 (n=27, 34.6%), and D3S1300 (n=21, 26.9%). Based on the detected microsatellite alterations, participants were divided into two groups. (Table 2 and 3)

Table 4: Factors associated with local recurrence based on logistic regression model.

Variables	No. of margins (N=392)	Odds Ratio	*P* value	95% Confidence Interval	
				Lower limit	Upper limit
Age					
<= 50 years	181	1.105	0.799	0.513	2.381
> 50 years *	211	1.000			
Gender					
Female	17	1.972	0.401	0.405	9.613
Male *	375	1.000			
Pathological stage					
Stage III, IV	204	3.865	0.003	1.584	9.427
Stage I, II *	188	1.000			
MSI					
Yes	46	7.494	<0.001	3.342	16.80
No *	346	1.000			
LOH					
Yes	89	1.702	0.194	0.763	3.797
No *	303	1.000			

* Reference group
Abbreviation: MSI: Microsatellite Instability; LOH: Loss of Heterozygosity

listed comparisons of variables between the two groups. Participants with MSI when compared with those without tended to have higher rates of both local recurrence and mortality. In addition, the proportion of perineural invasion was also higher in those with MSI. Significant differences were not found between the MSI and non-MSI groups in terms of age, gender, personal habits, primary tumor sites, histological characteristics, angiolymphatic invasion, extra capsular invasion, pathological stage, and postoperative radiotherapy (Table 2). Regarding LOH, all variables were not significantly different between those with LOH and those without (Table 3). In terms of quantitative data of personal habits, the average cigarette consumptions were similar between participants with MSI/LOH and those without (MSI vs. non-MSI: 32.5+23.2 vs. 29.5+15.6 pack-years, *P*=0.486; LOH vs. non-LOH: 27.9+16.2 vs. 34.2+20.8 pack-years, *P*=0.079). However, participants with microsatellite alterations showed betel quid more than those without (MSI vs. non-MSI: 511+433 vs. 331+275 quid-years, *P*=0.031; LOH vs. non-LOH: 415+366 vs. 363+321 quid-years, *P*=0.459). Although the 4-year disease-specific survival rate was lower in participants with MSI than those without, the difference was not significant. (80.3 % vs. 66.4 %, *P*=0.2280). On the other hand, the 4-year disease-specific survival rates were similar between participants with LOH and those without (76.8 % vs. 74.3 %, *P*=0.9392) (Figure 3).

There were totally 606 surgical margins were obtained from the defects after tumor extirpation. Among these specimens, 516 were from mucosa, and 90 from deep soft tissues. Surgical margins obtained from participants without microsatellite alteration were excluded. Consequently, 392 surgical margins (from 87 informative participants) were analyzed in the logistical regression model. Of these specimens, 46 (11.7%) had MSI, whereas 89 (22.7%) had LOH in at least one marker. For those patients who had local recurrence, 37 of their margins were found near the recurrent sites. Here, MSI was more likely to be found in such margins when compared with that of those without local recurrence (17 out of 37, 45.9% vs. 29 out of 355, 8.2%, *P*< 0.001). Also, the proportion of LOH in above-mentioned margins was also higher than those without local recurrence (14 out of 37, 37.8% vs. 75 out of 355, 21.1%, *P*=0.035). In the multivariate analysis, the presence of MSI in surgical margins increased risk of over 7 folds in developing local recurrence [Odds ratio (OR): 7.494; 95% confidence interval (CI): 3.342 ~ 16.80; *P*< 0.001]. Finally, late stage was another independent risk factor for local recurrence (OR: 3.865; 95% CI: 1.584 ~ 9.427; *P*=0.003). Detailed data are shown in (Table 4).

Discussion

The relationship between MSI and the survival of colorectal cancer was well-documented [3]. Our OCSCC patients however demonstrated no relationship between microsatellite alterations and survival rates. The clinical implication and prevalence of MSI are different across tumors of different primary sites [5]. For example, MSI is associated with the survival of gastrointestinal cancer and endometrial cancer [13,14]. The prognostic value of MSI in OCSCC remains controversial. Lack of statistical power in the literature with a low prevalence of MSI in head and neck cancer may underly such controversy [5]. Murali et al. [8] reported that oral cancer patients with LOH at D9S162 have poor recurrence-free survival rates. Here, we found no difference in disease-specific survival between OCSCC patients with LOH and those without. Not only did we select a different set of microsatellite markers but we also used different techniques. For example, we adopted modern automatic fragment analysis after PCR amplification whereas their study resolved PCR amplified samples in poly-acrylamide gels and used silver staining for detection. Moreover, the cutoff value of tumor imbalance factor (or LOH ratio) in our study was < 0.67 or >1.5, while other studies used dissimilar cutoffs values (such as < 0.5 or >2) [5,15]. Such differences in methodology could account for the discrepancy in results. The incidence of MSI in head and neck cancer varies from 7.7 to 48 % and the occurrence of LOH ranges from 29.6 to 86.7% [4,5,7-12,16]. In our study, the prevalence of MSI was 33.3% and that of LOH was 57.8%. One possible explanation for the different frequency of MSI/LOH is the different microsatellite markers selected. Also, the studied populations are different across different studies and predisposing factors in different countries are also dissimilar. Interestingly, the average consumption of betel quid was higher in our patients with MSI than those without. A previous study reported that patients with genomic alterations in tumor DNA have higher consumption of betel quid (two-fold difference) than patients without. Therefore, apart from MMR system mutation, betel quid chewing is believed to cause genomic instability which can ultimately lead to carcinogenesis [17].

Despite the lack of association between MSI/LOH and disease-specific survival in our OCSCC patients, the presence of MSI in the tumor-free surgical margins was linked to a higher risk of developing local recurrence. Temam et al. [16] in their study on head and neck squamous cell carcinoma patients also reported a similar finding. MSI represents the molecular fingerprint of the deficient mismatch repair (MMR) system [3], which repairs errors that occurred in DNA replication [1]. Mutations of the MMR genes (such as MLH1, MSH2, MSH6, and PMS2) can lead to lynch syndrome, which is closely related to hereditary non-polyposis colorectal cancer [14]. Dysfunction of MMR system may lead to carcinogenesis as mutations accumulated in pivotal genes. Accumulation of genetic modifications, such as inactivation of the tumor suppressor genes may initiate oral carcinogenesis after several years [5]. Partridge et al. [18] reported that premalignant lesions with MSI likely lead to develop into head and neck cancer. That could well explain the 7-fold increment on the risk of local recurrence we found in our OCSCC patients with MSI present in their surgical margins. On the other hand, our study showed no connection between the presence of LOH in the surgical margin and local recurrence of the cancer. A previous study in head and neck cancer patients showed that the genetically altered margin (or LOH) is associated with a higher risk of local recurrence as well as second primary [19]. The discrepancy in results between the two studies might be related to dysplasia margins were included in their study whereas we excluded margins with any form of dysplasia.

There were some limitations in our study. First, the external validity of our findings is limited as our patients analyzed were from a single institute. Second, the statistical power was probably low due to the relatively small sample size. Third, we only collected MSI/LOH rather than tumor suppressor gene (such as P53) or gene methylation status in specimens. Furthermore, the follow-up period was likely not long enough to determine the survival benefit of MSI. Lastly, although the therapeutic guidelines are standardized in our institute, differences in treatment of patient could not be ruled out.

Conclusion

MSI and LOH in the microsatellite markers designated herein were not associated with disease-specific survival of OCSCC patients in Taiwan, which is a betel quid-prevalent country. MSI present in the dysplasia-free surgical margins increased the risk of local recurrence.

Genomic examinations of surgical margins could be helpful in screening out those patients in risk of developing local recurrence. Adjuvant treatments might therefore be provided for them to improve their prognosis.

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