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ORIGINAL ARTICLE

Differences in oral habit and lymphocyte subpopulation affect malignant transformation of patients with oral precancer

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Background/purpose: In Taiwan, the combination of betel quid chewing, alcohol consumption, and smoking habits increases oral cancer risk by 123-fold compared to persons without these habits. Lymphocyte populations in patients may potentially affect the malignant transformation of oral precancer.
Methods: A total of 28 patients with oral precancer from our previous cohort were enrolled in this study, and their personal information and oral habits were documented. Their lymphocyte populations (CD4+, CD8+, CD19+, and CD56+) and activation markers (CD25 and CD69) were determined by flow cytometry from 1999 to 2004. After follow up till December 2014, data of

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patients with/without malignant transformation were recorded, and the relation between oral habits and percentage of initial lymphocyte markers was evaluated using the Student *t* test and Fisher's exact test.

Results: Ten precancer patients developed oral squamous cell carcinoma with a mean period of malignant transformation of 6.8 ± 2.1 years. Patients with malignant transformation had a mean age of 48.4 ± 5.0 years ($n = 10$), relatively more than that of patients without malignant transformation (41.6 ± 6.3 years, $n = 18$) ($p < 0.05$). An increase was noted in the population of peripheral blood mononuclear cells expressing CD4+CD69+, CD19+CD69+, and CD56+CD69+ ($p < 0.05$) in precancer patients with malignant transformation. Alcohol consumption showed an association with the malignant transformation of patients with precancer ($p = 0.030$), whereas betel quid and smoking showed little effect.

Conclusion: These results suggest that age, alcohol consumption, and early activation of T cells, B cells, and natural killer cells are crucial in the malignant transformation of oral precancer. Analysis of patient's lymphocyte populations may help predict the malignant transformation of oral precancer.

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Introduction

In recent decades, oral cancer has become an important health problem, and it is one of the top four causes of cancer death of male in Taiwan.[1] Many studies have revealed a strong correlation between oral cancer incidence and some oral habits. In Taiwan, people with alcohol drinking, betel quid (BQ) chewing, and smoking habits have a higher risk of oral precancers, such as oral leukoplakia, oral erythroplakia, oral submucous fibrosis (OSF), and even oral squamous cell carcinoma (OSCC).[2,3] Various factors such as ethnic groups, stages of diagnosis, gender, diagnostic age, anatomic site, morphologic type, BQ chewing, and treatment methods have been shown to affect the prognosis of OSCC.[4,5] Consumption of alcohol, BQ chewing, and cigarette smoking are highly associated with the initiation, promotion, and progression of oral cancer.[6–8] However, whether these oral habits may affect the malignant transformation of oral precancer is an intriguing issue awaiting further investigation. Clinically, various therapeutic modalities such as radical surgical excision, chemotherapy, and radiotherapy, separately or in combination, have been used for the treatment of OSCC. However, the overall survival rate for oral cancer patients was 61%,[9] and the survival rates were 75%, 65.6%, 49%, and 30%, respectively, for patients with stage I, II, III, and IV oral cancer.[10] Understanding the factors responsible for the malignant transformation of oral precancer is, therefore, important for early diagnosis and treatment of OSCC, to improve the prognosis.

Chemical carcinogens in alcohol, BQ, and cigarette have been shown to affect the normal structures of protein, lipid, and DNA of oral mucosal cells, leading to gene mutation, chromosomal aberrations, and even clinical cancer.[6,7] Most of the damaged DNA can be repaired, and the transformed cells can be destroyed by the immune system. However, some transformed cells may escape the host immune surveillance, resulting in cancer development. Accordingly, recent advances in tumor immunology reveal that an imbalance between effector cells and regulatory cells in different kinds of tumor microenvironments may affect oral cancer initiation, promotion, progression, and treatment outcomes.[11,12] Immunological changes occur in different stages of oral carcinogenesis. Presence of cytogenetic damage in lymphocytes isolated from precancerous patients and impairment of cellular immune responses in cancer patients have been reported.[12,13] Alterations of lymphocyte population and functional defects in lymphocyte were noted in patients with different stages of oral cancer.[14–16] Analysis of lymphocyte phenotypes in peripheral blood mononuclear cells (PBMCs) of patients with oral precancer and cancer, therefore, can be used potentially to predict disease progression and treatment outcomes. However, limited information is known about the changes in phenotypes of PBMCs in oral precancer patients and their relation to malignant transformation.

The purposes of this study were to investigate whether the populations of CD4+ T-helper cells, CD8+ T cells, CD19+ B cells, and CD56+ natural killer (NK) cells, and their activation markers (CD25+ and CD69+) in the initial diagnostic stage of oral precancer may have an effect on future malignant transformation. Furthermore, we clarified the relationship between age or oral habits and the malignant transformation of oral precancers.

Materials and methods

By the approval of Ethics Committee, National Taiwan University Hospital, 28 consecutive male patients with oral precancerous lesion were recruited from the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital, from 1999 to 2004.[17] Patients with autoimmune diseases, those who were taking immunoactive drugs, and those with evident viral/bacterial infection within 1 month were excluded from this study. The analytic cohort consisted of 28 male patients with precancerous lesions, with a mean age of 44.0 ± 6.2 years. Basic information, and clinical and histopathological data of these patients were recorded during that period (Table 1). Expression of

Table 1 Demographics of the cohort of precancer patients with/without malignant transformation.

	Without transformation	With transformation	Total	*p*
Male	$n = 18$	$n = 10$	$n = 28$	
Age[a]	41.6 ± 6.3	48.4 ± 5.0	44.0 ± 6.2	**0.02***
Malignant transformation ($n = 10$)				
<3 y	1			
<5 y	5			
<10 y	8			
<15 y	10			
Initial precancer diagnosis	($n = 18$)	($n = 10$)	($n = 28$)	0.128[b]
Leukoplakia	9	8	17	
OSF	8	1	9	
Lichen planus	1	0	1	
Erythroplakia	0	1	1	
Histological finding	($n = 18$)	($n = 10$)	($n = 28$)	0.352[b]
Hyperplasia	1	1	2	
Hyperkeratosis	6	1	7	
Mild dysplasia	1	0	1	
Moderate or severe dysplasia	6	7	13	
Lichen Planus	1	0	1	
No report	3	1	4	
Malignant transformation	($n = 10$)			
Tongue Ca.	3			
Buccal Ca.	6			
Gingival Ca.	1			

*Statistically significant difference ($p < 0.05$).
Ca. = cancer; OSF = oral submucous fibrosis.
[a] Age of initial diagnosis (analyzed by Student *t* test).
[b] Chi-square test was used for statistical analysis.

lymphocyte markers in these patients were determined by flow cytometry, as published previously.[17] All patients were followed for their precancerous lesions with/without malignant transformation till December 31, 2014.

Isolation of peripheral blood mononuclear cells

In brief, peripheral blood samples (2 mL) were collected from patients with precancerous lesions prior to incisional or excisional biopsy. These blood samples were stored at 4°C, and the lymphocyte populations were examined within 24—48 hours.

Flow cytometric analysis of lymphocyte populations

Identification of lymphocyte phenotypes was performed directly on blood samples collected in anticoagulated EDTA tubes. Various specific monoclonal antibodies, including CD4-phycoerythrin(PE)/CD3-fluorescein-isothiocyanate (FITC), CD8-PE/CD3-FITC, CD19-PE, CD56-PE, CD4-PE/CD25-FITC, CD8-PE/CD25-FITC, CD19-PE/CD25-FITC, CD56-PE/CD25-FITC, CD4-PE/CD69-FITC, CD8-PE/CD69-FITC, CD19-PE/CD69-FITC, and CD56-PE/CD69-FITC, were used to label cell surface markers. Briefly, 50 μL of whole blood was taken from each patient, and then 10 μL of antibodies was added to these tubes. The tubes were vortexed and then incubated at room temperature for 30 minutes in the dark. Then, 750 μL of 1× FACS lysis solution was added to each tube, vortexed gently, and incubated in the dark for 30 minutes. After centrifugation, the cell pellets were resuspended in phosphate-buffered saline with 0.5% bovine serum albumin and analyzed by flow cytometry. The percentage of lymphocyte expressing various CD markers was estimated for analysis.[15,17]

Statistical analysis

Data were calculated using Microsoft Excel 2003 and presented as the mean ± standard error (SE). Differences in all categories were analyzed for statistical significance using the Student *t* test, chi-square test, and Fisher's exact test. A value of $p < 0.05$ was considered to have statistically significant difference between groups.

Results

The 28 precancer lesions were diagnosed as leukoplakia (17 cases), OSF (9 cases), lichen planus (1 case), and erythroplakia (1 cases). Histopathological observation revealed the presence of hyperplasia (2 cases), hyperkeratosis (7 cases), mild dysplasia (1 case), moderate to severe dysplasia (13 cases), and lichen planus (1 case). Four cases lacked a biopsy report (Table 1). Two OSF cases received no treatment, and three cases (1 lichen planus, 1 OSF, and 1 leukoplakia) received incisional biopsy only. Excisional biopsy alone was performed in seven cases, whereas in 14

Table 2 Differences in age and CD markers (mean ± SE) in precancer patients with/without malignant transformation.

	Precancer without malignant transformation (n = 18), mean ± SE	Precancer with malignant transformation (n = 10), mean ± SE	p (t test)
Age[a]	41.6 ± 6.3	48.4 ± 5.0	**0.02***
CD4+[b]	38.0 ± 5.5	42.5 ± 4.6	**0.06**[#]
CD8+[b]	24.5 ± 5.0	23.5 ± 4.3	0.36
CD19+[b]	14.8 ± 3.6	15.1 ± 3.9	0.44
CD56+[b]	14.7 ± 8.1	15.9 ± 7.8	0.39
CD4+/CD8+[b]	1.7 ± 0.5	1.9 ± 0.5	0.20
CD4+ plus CD8+[b]	62.4 ± 6.8	66.0 ± 5.9	0.17
CD4 + CD25+[b]	6.5 ± 2.1[c]	7.0 ± 1.7	0.32
CD8 + CD25+[b]	2.2 ± 1.8[c]	1.1 ± 0.6	**0.11**
CD19 + CD25+[b]	0.6 ± 0.6[c]	0.6 ± 0.3	0.49
CD56 + CD25+[b]	0.3 ± 0.3[c]	0.5 ± 0.3	**0.09**[#]
CD4 + CD69+[b]	0.4 ± 0.2	0.7 ± 0.2	**<0.01***
CD8 + CD69+[b]	2.2 ± 1.5	1.9 ± 0.7	0.35
CD19 + CD69+[b]	0.1 ± 0.1	0.3 ± 0.2	**0.01***
CD56/CD69+[b]	0.5 ± 0.3	1.1 ± 0.7	**0.01***

Student t test was used for statistical analysis.
[#] A p value of <0.1 was considered borderline significant.
* A p value of <0.05 was statistically significant.
SE = standard error.
[a] Age of initial diagnosis.
[b] Percentage of cells expressing CD markers.
[c] Data collected (n = 15).

cases, excisional biopsy was performed after initial incisional biopsy and in three cases, a large surgical section was created, possibly due to histological findings of dysplasia.

Ten precancer patients developed OSCC in the follow-up period (Table 1). The mean period of malignant transformation was 6.8 ± 2.1 years from the date of initial diagnosis. The mean age of precancer patients without malignant transformation (n = 18) was 41.6 ± 6.3 years compared with 48.4 ± 5.0 years in precancer patients with malignant transformation (n = 10) ($p < 0.05$). Chi-square test showed no marked effect of clinical diagnosis (p = 0.128) and histopathological diagnosis (p = 0.352) on the malignant transformation of oral precancer ($p > 0.05$) (Table 1).

The PBMCs expressing CD4+CD69+, CD19+CD69+, and CD56+CD69+ populations showed a statistically significant difference between the precancer patients with and without malignant transformation (Table 2). Moreover, a tendency of changes in CD4+, CD8+CD25+, and CD56+CD25+ (but p value as 0.06, 0.11, and 0.09, respectively) of PBMC populations in precancer patients with and without malignant transformation was also noted.

We analyzed whether oral habits may affect the malignant transformation of oral precancer and found an association between alcohol consumption and the malignant transformation of patients with precancer (p = 0.030). However, BQ chewing and smoking habits showed little effects, because most of the precancer patients have BQ chewing and smoking habits (Table 3).

Discussion

In this cohort study, we followed 28 oral precancer patients for more than 10 years. The malignant transformation to OSCC was found in 35.7% of oral precancer patients, with an average of 6.8 years from initial diagnosis to OSCC. Our 9-

Table 3 Difference in oral habits (mean ± SE) in precancer patients with/without malignant transformation.

		Precancer without Malignant transformation (n = 18), mean ± SE	Precancer with Malignant transformation (n = 10), mean ± SE	p
Alcohol	(+)	11	10	**0.030***
	(−)	7	0	
Betel	(+)	15	10	0.532
	(−)	3	0	
Smoking	(+)	17	10	>0.99
	(−)	1	0	

Analyzed by Fisher exact test.
* A value of $p < 0.05$ was considered statistically significant.
SE = standard error.

year follow-up study showed a malignant transformation rate of 2.6% for oral lichen planus patients. Similarly, Warnakulasuriya et al[18] and Wang et al[19] also reported that the severity of epithelial dysplasia is a critical factor of malignant transformation of precancer. For this reason, excisional biopsy or wide surgical removal of diseased tissue was performed in cases with dysplasia.

Since the 5-year survival rate of OSCC is 61%,[9] prediction and early diagnosis of the malignant transformation of oral precancer to OSCC is a critical health issue in Taiwan. In this study, age was found to be a contributing factor for malignant transformation. A recent study also reported that after surgical excision of oral cancer, elder patients showed a poor prognosis compared to younger patients.[20] This is possibly due to alterations of lymphocyte and immune response during aging, accumulation of genotoxic damage, or aberrant DNA repair and DNA methylation in elder patients, which are important for the pathogenesis of oral cancer.[6,12,21,22]

Previous studies have found that BQ chewing, smoking, and alcohol consumption have correlation with the incidence of OSF, oral leukoplakia, and other verrucous lesions. BQ chewing and smoking show a synergistic effect on these lesions.[23] *In vitro* cell culture models and *in vivo* animal models also reveal the tumor-promoting and inflammatory effects of BQ components.[7,8,24] However, using statistical models, oral habits including BQ chewing and cigarette smoking were suggested to be the factors responsible for the malignant changes of OSCC.[25] Using the three-state Markov model, BQ chewing habit increases the risk of oral leukoplakia, but not that of malignant transformation. Smoking plays a role in leukoplakia, but only a minor role in malignant transformation, whereas alcohol is crucial for malignancy but not for leukoplakia.[26] In our longitudinal study, we followed oral precancer patients for 10 years and found that alcohol consumption can be an important factor of malignant transformation to OSCC. Since all precancer patients have BQ chewing and smoking habits, no conclusion can be made regarding the effects of both habits on malignant transformation. The tumor-promoting effect of alcohol can be due to its local or systemic effects, such as disturbance of systemic metabolism of nutrient, and alteration of redox metabolism and signaling pathways.[27] Alcohol may also enhance the permeability of oral mucosa, act as a solvent of tobacco carcinogen, induce basal cell proliferation, and generate free radicals and acetaldehyde that have the abilities to induce DNA damage, malnutrition, and immune suppression, to promote carcinogenesis.[28]

A number of studies have suggested that immune response plays critical roles in the pathogenesis of oral precancer and oral cancer. Evaluation of lymphocyte phenotypes in peripheral blood can help in knowing the systemic immune status of patients.[12,29] It is generally known that CD4+ T-helper cells, CD8+ T cells, and CD56+ NK cells may inhibit and kill tumor cells to impede tumor growth,[30] whereas B cells are important to control immune response and as an inflammatory regulator.[31] In this study, no marked difference in CD4+, CD8+, CD4+ plus CD8+, CD19+, and CD56+ cell populations and in CD4+/CD8 ratio was noted in precancer patients with and without malignant transformation. This suggests that these populations of T cells, T-helper cells, NK cells, and B cells are not involved in the malignant transformation of oral precancer. While cancer cells have been shown to induce the apoptosis of T cells and NK cells,[32] this event can be a late cancer stage response.

In addition, CD8+CD25+ ($p = 0.11$), CD4+CD25+, CD19+CD25+, and CD56+CD25+ ($p = 0.09$) populations showed no statistically significant difference in precancer patients with and without malignant transformation. Not all T cells are antitumor effector immune cells. CD4+CD25+Foxp3+, termed regulatory T cells, may support tumor growth and progression, and inhibit immune response against cancer.[30] An increase in CD25+ regulatory T cells in the blood has been found in pancreatic and breast cancer patients relative to normal donors.[30] The results of this study suggest that CD25+ lymphocyte population may be not directly involved in the malignant transformation of oral precancer. However, more studies with an increased sample size are necessary to further confirm the results.

Interestingly, we found that CD4+CD69+, CD19+CD69+, and CD56+CD69+ populations, but not CD8+CD69+ population, showed a significant difference in precancer patients with and without malignant transformation. An elevation of CD4+CD69+Foxp3− regulatory T cells in hapatocellular carcinoma patients was reported, and these cells may suppress T-cell response via membrane-bound transforming growth factor-β and may be involved in tumor progression.[33] CD69 is an early activation marker of lymphocytes.[34] Increases in early activation of CD4+CD69+ T cells, CD19+CD69+ B cells, and CD56+CD69+ NK cells in precancer patients with malignant transformation suggest that CD69+ expression in T, B, and NK cells might be important for the malignant transformation of oral precancer. These alterations in lymphocyte phenotypes may be due to the toxic effects of BQ, tobacco, alcohol, or other toxicants.

In conclusion, the etiology of oral cancer is multifactorial, including genetic, environmental, social, and behavioral factors. Age, alcohol drinking, and cellular immune response, in terms of their effects on the malignant transformation of oral precancer to OSCC, showed some differences between precancer patients with or without malignant transformation. Expression of CD4+CD69+, CD19+CD69+, and CD56+CD69+ lymphocyte populations plays important roles in this event, highlighting the importance of cellular immunity in the pathogenesis of OSCC. In addition, to stop usage of BQ, tobacco, and alcohol, an analysis of lymphocyte markers of peripheral blood in precancer patients may be useful for the prediction and early diagnosis of malignant changes to OSCC.

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