รายงานวิจัยฉบับสมบูรณ์

โครงการ การแสดงออกของ p53 ในรอยโรคก่อนมะเร็งในช่องปากที่ให้ผลบวกต่อโทลูอิดีนบลูและการแสดงออกของ Ki67 ในรอยโรคก่อนมะเร็งในช่องปากที่ให้ผลบวกต่อน้ำส้มสายชู

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คณะผู้วิจัย สังกัด

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

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Abstract

Objectives: The aims of this study were to investigate the sensitivity and specificity of toluidine blue and/or vinegar in oral cancer screening and to investigate the association between clinical screening using toluidine blue and vinegar and the expression of the tumor marker p53 and proliferation marker Ki67, respectively. Materials and methods: The study consisted of 87 participants suspected of having oral squamous cell carcinoma. Toluidine blue and/or vinegar were applied to the lesions, followed by incisional biopsies. The specimens were then microscopically examined for pathological diagnosis and underwent immunohistochemical investigation for p53 or Ki67. Results: The results revealed that the sensitivity and specificity of oral cancer screening using toluidine blue were 93% and 46%, respectively; whereas the sensitivity and specificity using vinegar were 85% and 81%, respectively. A statistically significant correlation between vinegar positive lesions and the expression of Ki67 ($p=0.019$) was observed. Although there was a difference in the expression of p53 between specimens that were positive and negative to toluidine blue, the correlation did not reach a significant level. Conclusions: Based on the results from this study, vinegar has a lower sensitivity than toluidine blue but a higher specificity in oral cancer screening. The results of the clinical screening using vinegar correlated with the expression of Ki67 at the cellular level. Clinical relevance: This study supports the use of toluidine blue and 5% acetic acid in oral cancer screening. Ki67antibody reaction can also be used for oral cancer treatment planning and prognosis determination.

Keywords Oral squamous cell carcinoma; oral cancer; toluidine blue; acetic acid

Introduction

Oral cancer is a global health problem [1]. The most common cancer occurring in the oral cavity is oral squamous cell carcinoma (OSCC) [2]. In Thailand, the current 5-year survival rate of 20-30% is quite low [3]. Early diagnosis is very important and can lead to improved survival rates. One diagnostic technique that is widely used internationally is the application of toluidine blue to suspected lesions [4, 5]. The proposed mechanism of toluidine blue in early cancer detection is that toluidine blue is taken up by dysplastic cells, which have an increased density of nuclear material [6]. Another potential screening method is the application of 3-5% acetic acid, which is used for cervical cancer screening [7]. The proposed mechanism of acetic acid in early cancer detection is that acetic acid causes dehydration and the surface coagulation of cellular proteins, thus reducing the transparency of the epithelium [8]. A clinical screening technique would be more trustworthy if it correlates with cellular markers used for cancer detection. Several investigators suggest that the protein p53, the product of a tumor suppressor gene, is one of the most promising candidates for oral cancer detection [9, 10, 11]. Mutation of p53 changes the property of p53 protein resulting in its accumulation in the nucleus [12]. A study has reported that toluidine blue positive cells have an allelic loss at chromosome 17p, which is the p53 locus [13]. Another marker to consider is Ki67, which functions to control cellular proliferation and is found only in proliferating cells [14]. In normal epithelium, Ki67 is found in the basal cell layer, but in malignant transforming tissue, Ki67 can be seen in every layer of the epithelium [15]. The proposed mechanism of an increase in amount of genetic material and protein in cancer tissues rendering them positive to vinegar application [8] can be confirmed by investigating the levels of Ki67 protein.

The purposes of this study were to investigate the use of toluidine blue and vinegar in the detection of oral squamous cell carcinoma and to determine the association between clinical screening using toluidine blue and vinegar and the expression of the tumor marker, p53 and the proliferation marker, Ki67, respectively.

Materials and Methods

Study Population

Eighty-seven patients with a clinical diagnosis of precancerous lesions or oral squamous cell carcinoma were included in our study. They were recruited from the Department of Oral Medicine, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand and the Department of Ear, Nose, and Throat, Rajvithi Hospital, Bangkok, Thailand. The study was conducted after both verbal and written informed consent using protocols approved by the Committees on Investigations Involving Human Subjects of both institutions.

Clinical application of toluidine blue and 5% acetic acid

After each patient agreed to participate in the research project, an investigator noted clinical findings, photographed the lesions, and chose the areas to be investigated. Toluidine blue application was conducted on 33 patients. Five percent acetic acid application was conducted on a second group of 30 patients. Both toluidine blue and 5% acetic acid were applied to a third group of 24 patients.

For the application of toluidine blue, a cotton bud soaked in 1% acetic acid was used to clean the lesion prior to the application of toluidine blue with a different cotton bud for 30 seconds. Subsequently, a third cotton bud soaked with 1% acetic acid was used to remove any excess toluidine blue on the lesion. The patient then rinsed out their mouth. A positive finding was designated as a lesion whose color changed to blue, while a negative finding was a lesion with no change. The lesion was then photographed and an incisional biopsy was performed at the blue-stained area.

For acetic acid application, a piece of gauze soaked with 5% acetic acid (vinegar) was applied to a clean and dry lesion for 1 minute. After the removal of the gauze, the lesion was photographed, and the investigator recorded the characteristics of any changes. A positive finding was defined as a lesion whose color changed to opaque white. A negative finding was a lesion that did not change or changed to transparent white as found in leukoedema. An incisional biopsy was performed at the area that had turned opaque white.

For the 24 patients to which both substances were applied, application of 5% acetic acid was conducted first, followed by the application of toluidine blue. A positive finding was designated as a lesion whose color changed to both opaque white and blue, while a negative finding was a lesion not demonstrating these combined changes. An incisional biopsy was performed at the area that turned opaque white and had stained blue. If the areas that had stained blue

and turned opaque white were not coincident, incisional biopsies were performed at both areas. If the lesion did not stain or change color, an incisional biopsy was performed at the central area of the lesion.

Immunohistochemical study

The biopsy tissue was routinely processed for histology, and sections were prepared and then stained with hematoxylin and eosin for histopathological diagnosis. For tissues obtained from patients receiving toluidine blue application, the consecutive tissue section was used for the immunohistochemical study of p53 as described by Kurokawa et al. (2005) [16]. For tissues obtained from patients receiving vinegar application, the consecutive tissue section was used for the immunohistochemical study of Ki67. A monoclonal antibody, anti-Ki67 (MIB 1, diluted 1:100; Dako, Denmark) was used as the primary antibody. The Envision plus kit (Dako, Denmark) was used according to the manufacturer's instructions for the application of secondary antibody and 0.03% diaminobenzidine (DAB) solution was used to visualize the reaction products. Both p53 and Ki67-positive cells were counted under a light microscope at 400x magnification. Three areas on each section; on the right, in the middle, and on the left of each tissue section were chosen for evaluation. The cells were quantified by two different investigators and averaged. The number of brown stained cells divided by the total number of epithelial cells was calculated as the percentage of positive cells. Positive controls were sections of squamous cell carcinoma with known Ki67 overexpression. The negative controls were sections processed through the same procedure but omitting the primary antibody. The tissues from the 24 patients who had both substances applied on the lesions were not included in the immunohistochemical analysis.

Statistical analyses

Descriptive analyses were used for the computation of sensitivity and specificity of the application of toluidine blue and/or vinegar for oral cancer detection. The correlation between the results of the vinegar application and the histopathological diagnoses was determined using Fisher's exact test. Differences in the percentage of p53/Ki67 positive cells between toluidine blue/vinegar positive specimens and toluidine blue/vinegar negative specimens were compared using the Mann-Whitney test. All statistical analyses were performed using SPSS 10.00 for Windows (SPSS Inc., Chicago, IL) with statistical significance considered at a p-value less than 0.05.

Results

As seen in Table 1, our study comprised 87 subjects (56% male/44% female) ranging in age from 25-86 years (average age 61.5 ± 12.38 years). Sample lesions were most commonly found on the lateral tongue (31.0%), buccal mucosa (20.7%), and floor of mouth (18.4%). Sixty-seven lesions received toluidine blue application, of which 58 were positively stained. Of these, 52 of were disease positive by histopathological diagnosis (Table 2). Of the 9 lesions negative to toluidine blue staining, 4 received a positive histopathological diagnosis. Thus, we found that the sensitivity of toluidine blue application for oral cancer detection was 92.86%, whereas the specificity was 45.45%. Five percent acetic acid was applied to 83 lesions, with 56 lesions showing positive results (Table 3). Upon histopathological diagnosis, 56 of these were seen to be disease positive. Of the 27 lesions negative to 5% acetic acid treatment, 9 of these were deemed disease positive. These results indicated that the sensitivity of 5% acetic acid application for oral cancer detection was 85.25%, while the specificity was 81.82%. We applied both toluidine blue and 5% acetic acid to 27 lesions, finding 23 lesions positive to both substances (Table 4). Twenty-two of these were positive for disease by histopathological diagnosis. Negative staining results were observed for 5 lesions, 1 of which was found to be disease positive. The sensitivity and specificity when using both reagents for oral cancer screening were thus 95.65% and 80%, respectively. Using Fisher's exact test to evaluate the relationship between the results of oral cancer screening using toluidine blue or 5% acetic acid and the results of the histopathological diagnoses revealed significant correlations ($p=0.000$ and $p=0.004$, respectively).

Figure 1 shows the results of the immunohistochemical staining for p53. Normal mucosa demonstrated little to no observable staining (Fig. 1a). Scattered cells with positive staining were noted in samples of dysplasia (Fig. 1b). Oral squamous cell carcinoma samples displayed many positively stained cells arranged in cord-like structures, suggesting a clonogenic origin (Fig. 1c). Although the average percentage of cells with positive p53 staining in all toluidine blue positive specimens at 4.93±1.32% was higher than that of the toluidine blue negative specimens at1.49±0.97%, the difference between these two groups was not significant ($p=0.198$) (Table 5). The results of the immunohistochemical staining for Ki67 are seen in Figure 2. Scant, if any, staining was noted in normal oral mucosa (Fig. 2a). Dysplastic samples, however, showed robust staining in the suprabasal epithelial layer (Fig. 2b). In contrast, in samples of oral squamous cell carcinoma widespread positive staining was seen throughout the epithelial cell layers (Fig. 2c). We found increased numbers of specimens positive for Ki67 staining as the histopathological diagnosis rose in severity from normal

mucosa (1/33%), to epithelial hyperplasia and chronic inflammation (4 /44.4%), through epithelial dysplasia and carcinoma in situ (9 /81.8%), and oral squamous cell carcinoma (24/88.9%) (Table 6). The percentage of positive stained cells based on severity was seen to follow the same trend (Fig. 3). The average percentage of cells stained positive for Ki67 in all vinegar positive specimens was 3.23±0.58% and that of the vinegar negative specimens was 1.45±0.45% , with this difference being significant (p=0.018) (Table 7).

Discussion

The average age of the patients in our study was 61.8±11.8 years old with a male to female ratio of 6 to 5. The average age of our patients is comparable with that of other studies [1, 17]. As betel nut chewing, an important risk factor for oral cancer, is still common in the Thai elderly female population [18], the proportion of female patients in our study was higher than that of other studies. The lesion location distribution is also consistent with those found in other studies [1, 17], with the exception of a higher finding of oral cancer of the buccal mucosa (23%) in our study.

When we compared the sensitivity and specificity of toluidine blue in the present study to the sensitivity and specificity of toluidine blue used for oral cancer screening from other studies [19-22], our results had a comparable sensitivity, but a much lower specificity. The reported low specificity here could stem from the low number of control lesions and the inclusion of all blue stained lesions. It has been reported that pale royal blue stained lesions are unrelated to any histological features [23]. When the sensitivity and specificity of the 5% acetic acid used in this study were compared with studies when it was used in cervical cancer detection, it was found that both values were higher [7, 8, 24-26]. When both substances were used on the same lesion, the sensitivity reached 96% and the specificity was as high as 80%. While using both substances will increase the reliability of oral cancer screening; it would be time consuming and not cost effective.

The significant relationship found between clinical screening using toluidine blue or 5% acetic acid and the histopathological diagnoses indicated that toluidine blue and 5% acetic acid were more likely to react with dysplastic or malignant tissues rather than with normal tissues. These findings support the use of toluidine blue and 5% acetic acid in oral cancer screening. As 5% acetic acid has a comparable sensitivity to toluidine blue but a higher specificity in oral cancer screening, we recommend further study and evaluation of 5% acetic acid (vinegar) for use in oral cancer screening in rural communities.

Our results revealed that the observable changes due to vinegar application in oral cancer screening and the results of histopathological staining were significantly correlated, with a significant difference in Ki67 positive cells between tissues that were positive to vinegar screening and those that were not. However, we found that the observable changes due to toluidine blue application in oral cancer screening and the results of the p53 staining were not significantly correlated. Although the number of p53 positive cells was higher in tissues that were positive to toluidine blue than those negative to toluidine blue, the difference did not reach a significant level. This could stem from the difficulty in separating lesions that are truly positive to toluidine blue (dark royal blue stain) and false positives (pale blue stain) as stated by a report that pale blue lesions have no histological significance [23]. .

The average percentage of cells positive to Ki67 antibody in specimens of oral squamous cell carcinoma, carcinoma in situ, and epithelial dysplasia was significantly higher than less affected specimens. This revealed that the immunohistopathological diagnoses of oral precancerous and cancerous lesions are correlated with the number of cells positive to Ki67 antibody. Thus, Ki67antibody reaction can also be used for oral cancer treatment planning and prognosis determination.

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Table 1: Characteristics of the patients

Number of Patients	87
Sex	Male 49 (56.3%) Female 38 (43.7%)
Male : Female	6.5 : 5
Age Range	25-86
Average Age	61.5 ± 12.38
Location of Lesions (Each patient may have more than one lesion)	Lateral tongue 27 (31.0%) Buccal mucosa 18 (20.7%) Floor of mouth 16 (18.4%) Lower lip 9 (10.3%) Soft palate 7 (8.0%) Hard palate 5 (5.7%) Alveolar ridge 5 (5.7%)

Table 2: Results from toluidine blue (TB) application and the histopathological diagnoses (67 specimens from 54 patients)

		Histopathological Diagnosis		
		Disease + [a]	Disease - [b]	Total
Toluidine Blue Application	+ result	52[c]	6	58
	- result	4	5[d]	9
	Total	56	11	67

[a]Disease positive are dysplasia, carcinoma in situ and squamous cell carcinoma.

[b]Disease negative are hyperplasia, inflammation and normal mucosa.

[c]Sensitivity = 52/56 = 92.86%; Positive predictive value = 52/58 = 89.66%

[d]Specificity = 5/11 = 45.45%; Negative predictive value = 5/9 = 55.56

Table 3: Results from 5% acetic acid application and the histopathological diagnoses (83 specimens from 67 patients)

		Histopathological Diagnosis		
		Disease +[a]	Disease –[b]	Total
Acetic acid application	+ result	52[c]	4	56
	- result	9	18[d]	27
	Total	61	22	83

[a]Disease positive are dysplasia, carcinoma in situ and squamous cell carcinoma.

[b]Disease negative are hyperplasia, inflammation and normal mucosa.

[c]Sensitivity = 52/61 = 85.25%; Positive predictive value = 52/56 = 92.86%

[d]Specificity = 18/22 = 81.82%; Negative predictive value = 18/27 = 66.67%

Table 4: Results from both toluidine blue (TB) and 5% acetic acid application and the histopathological diagnoses (28 specimens from 24 patients)

		Histopathological Diagnosis		
		Disease + [a]	Disease - [b]	Total
TB and Acetic acid application	+ result	22[c]	1	23
	- result	1	4[d]	5
	Total	23	5	28

[a]Disease positive are dysplasia, carcinoma in situ and squamous cell carcinoma.

[b]Disease negative are hyperplasia, inflammation and normal mucosa.

[c]Sensitivity = 22/23 = 95.65%; Positive predictive value = 22/23 = 95.65%

[d]Specificity = 4/5 = 80.00%; Negative predictive value = 4/5 = 80.00%

Table 5: Percentage of cells positive to p53 antibody by the results of toluidine blue application

Percentage of cells positive to p53 ± standard errors	
Positive to toluidine blue application	Negative to toluidine blue application
4.93±1.32	1.49±0.97
p = 0.198[a]	

Note: [a]Analyzed by Mann-Whitney Test

Table 6: Number of specimen positive to Ki67 in each group of histopathological diagnosis

Histopathological diagnosis (number of specimens)	Number of specimens that are positive to Ki67 (%)
Oral squamous cell carcinoma (27)	24 (88.9%)
Epithelial dysplasia and carcinoma in situ (11)	9 (81.8%)
Epithelial hyperplasia and Chronic inflammation (9)	4 (44.4%)
Normal Mucosa (3)	1 (33.3%)

Table 7: Percentage of cells positive to Ki67 antibody by the results of vinegar application

Percentage of cells positive to Ki67 ± standard errors	
Positive to vinegar application	Negative to vinegar application
3.23±0.58	1.45±0.45
p = 0.019[a]	

Note: [a]Analyzed by Mann-Whitney Test

Fig. 1 Immunohistochemical staining for p53. A) normal oral mucosa B) epithelial dysplasia C) oral squamous cell carcinoma

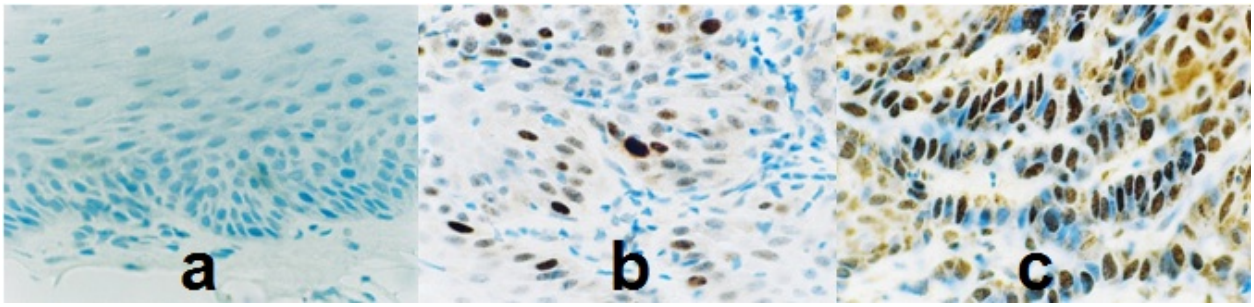


Fig. 2 Immunohistochemical staining for Ki67. A) normal oral mucosa B) epithelial dysplasia C) oral squamous cell carcinoma

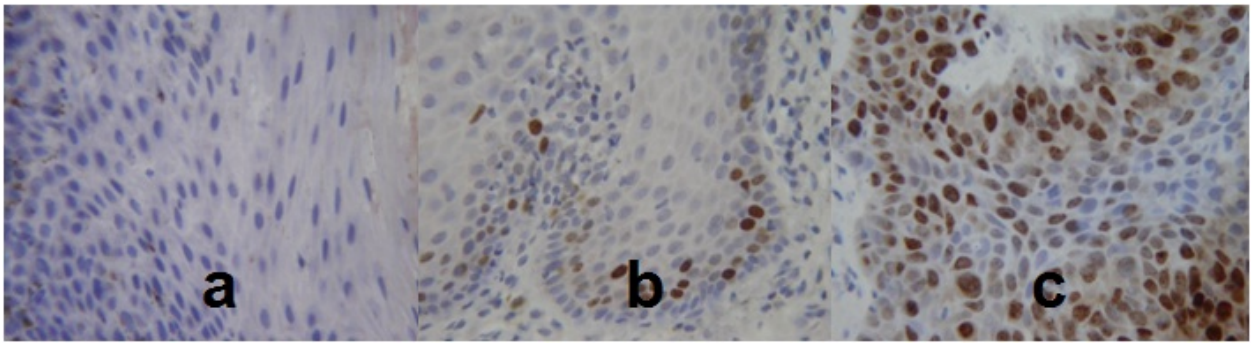
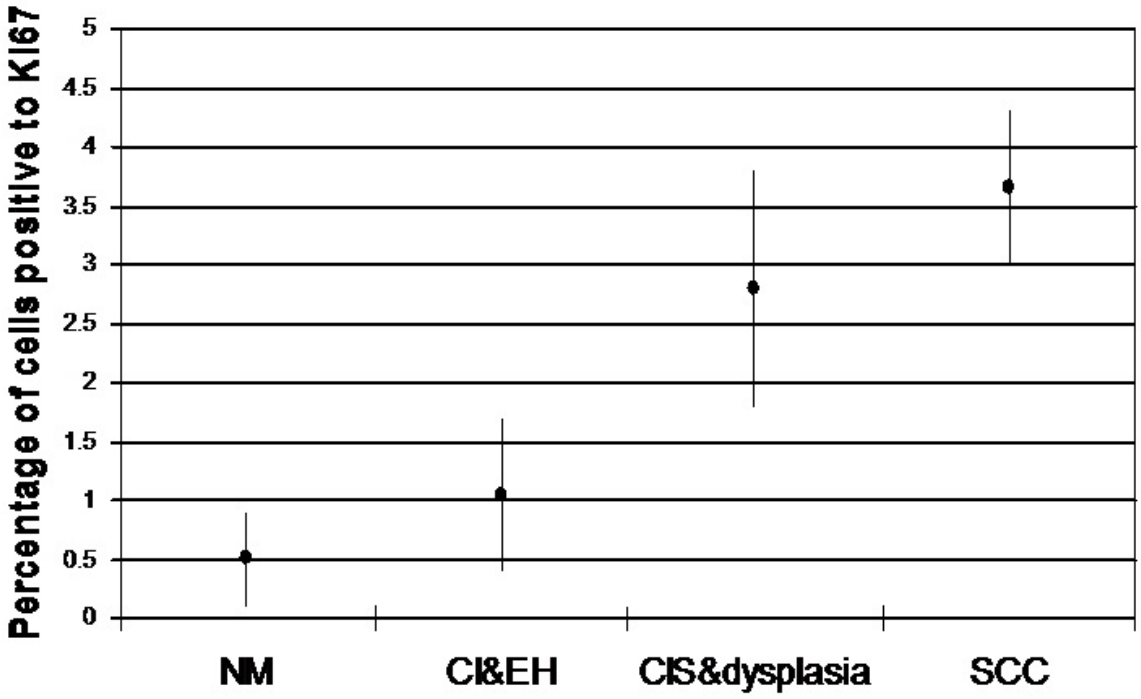


Fig. 3 Percentage of cells positive to Ki67 antibody ± standard deviations in each group of specimens (NM = normal mucosa; CI = chronic inflammation; EH = epithelial hyperplasia; CIS = carcinoma in situ; SCC = squamous cell carcinoma)



ภาคผนวก

The application of acetic acid in the detection of oral squamous cell carcinoma

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Background. Oral cancer is the fourth most common cancer in males and the seventh most common cancer in females in Thailand. The survival rates and quality of life of oral cancer patients will significantly be improved if they receive treatment for lesions that are less advanced or premalignant. Early diagnosis is therefore of paramount importance. A number of techniques have been developed to supplement clinical examination for oral malignancy. One interesting screening method is the application of 3% to 5% acetic acid, which has been used for cervical cancer screening.

Objectives. The primary objective of this study was to assess the sensitivity, specificity, and accuracy of using vinegar (5% acetic acid) for the examination of oral cancer. The secondary objective was to investigate the association between clinical examination using acetic acid and expression of the tumor marker, p53.

Methods. The study included 30 participants suspected of having oral squamous cell carcinoma. Five percent acetic acid was applied to the lesions, followed by incisional biopsy. The specimens were microscopically examined for pathological diagnosis and p53 immunohistochemical investigation.

Results. The sensitivity, specificity, and accuracy of using acetic acid for oral cancer examination were 83.33%, 84.21%, and 83.64%, respectively. There was a statistically significant association between clinical examination using acetic acid and expression of p53 protein ($P = .000$).

Conclusions. The results of this study suggest that 5% acetic acid has high sensitivity, specificity, and accuracy in detecting oral squamous cell carcinoma and might be used as an adjunct for oral cancer examination. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;106:371-6)**

Oral squamous cell carcinoma (OSCC) is the most common cancer in the oral cavity. It accounts for more than 90% of all oral cancer.[1] Each year, globally, there are 222,000 new cases of oral cancer diagnosed in men (5% of all cancer) and 90,000 new cases diagnosed in women (2% of all cancer).[2] The 5-year survival rate of OSCC is estimated to be about 50%.[3] In Thailand, the 5-year survival rate is lower than the average rate, at 20% to 30%.[4,5] Oral cancer is usually first diagnosed when symptoms are present. Thus, two thirds of patients already have advanced disease that leads to poor prognosis. The 5-year survival rate increased from 21% if the cancer was identified after distant metastasis to 46% when the cancer had only regional extension, to as high as 82% when it was found only locally.[6] For this reason, diagnostic aids for oral cancer have been developed. These include exfoliative cytology and flow cytometry.[7,8] A few substances have also been used for this purpose. One of the most widely used agents is toluidine blue. It is proven to be helpful in diagnosis of oral cancer because of its high sensitivity and uncomplicated protocol.[9,10] Interesting is the use of 3% to 5% acetic acid for the detection of cervical cancer in developing countries since it is inexpensive and very easy to use. Sankaranarayanan and colleagues[11] investigated the detection of cervical cancer in India using 4% acetic acid and reported the sensitivity and specificity of 88% and 78%, respectively. Since the anatomy of and the types of cancer found in the oral cavity and cervix are comparable, acetic acid seems to be an appropriate clinical marker for the detection of oral cancer as well. As for cellular markers, several investigators reported that mutation of the *p53* gene is one of the most commonly identified mutated genes in oral can-

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cers.[12-14] The wild-type *p53* gene is a tumor suppressor gene regulating a cell cycle checkpoint and the induction of apoptosis in response to DNA damage. When there is a genetic mistake, the protein p53 will stop the cell cycle in the G1 phase and so the mistake can be corrected through the DNA repair system.[15] Mutation of *p53* inactivates its growth suppressing activities. Elevated transcription of the mutant *p53* gene contributes to the overall high levels of the mutant protein in tumor cells and results in the accumulation of this protein in the nucleus that can be detected immunohistochemically.[16,17]

The main purpose of this study was to investigate the use of household vinegar (5% acetic acid) in the detection of OSCC. This study also investigated the relationship between clinical findings after acetic acid application and cellular expression of p53.

EXPERIMENTAL PROCEDURE

Study population

Thirty patients suspected of having OSCC were included in the study. They were recruited from the Department of Ear, Nose and Throat, Rajvithi Hospital, Bangkok, Thailand, and from the Department of Oral Medicine, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. The study was conducted with both written and verbal informed consent using protocols reviewed and approved by the Committees on Investigations Involving Human Subjects of both institutes. Information regarding patients' age, sex, and duration and locations of lesions as well as history of smoking, alcohol drinking, and betel nut chewing were recorded.

Five percent acetic acid

Thai household vinegar (Preserved Food Organization, Thai Q.P. Co., Ltd., Ratchaburi, Thailand), a rice-based clear fluid containing 5% acetic acid, was used for oral cancer detection.

Clinical application of 5% acetic acid

After each patient agreed to participate in the research protocol and signed the consent form, an investigator trained in the specialty of oral medicine recorded clinical findings, photographed the lesions, and selected the areas to be investigated. A piece of gauze soaked with 5% acetic acid was applied to a cleaned and dried lesion for 60 seconds. After the gauze was removed, the lesion was photographed again, and the investigator noted the characteristics of any changes. A positive finding was designated as a lesion that changed color to opaque white, while a negative finding was a lesion that showed no change or changed to transparent white as found in leukoedema. There were 39 lesions from varied locations in 30 patients (some patients had more than 1 lesion). At each site, if the lesion reacted differently to acetic acid from one area of the lesion to another, more than 1 specimen was excised for biopsy. Thus, a total of 55 specimens were taken from these lesions. The biopsy specimens were kept in 10% formalin.

Immunohistochemical study

The tissue was embedded in paraffin, cut into sections, and stained with hematoxylin and eosin for histopathological diagnosis. The consecutive tissue section was used for immunohistochemical study of p53 as adapted from the protocol of Kurokawa et al.[18] Monoclonal antibody, anti-p53 (DO-7, diluted 1:100; Dako, Glostrup, Denmark), which recognizes both wild-type and mutant forms of the protein, was used as the primary antibody. The Envision plus kit (Dako) was used for the application of secondary antibody, according to the manufacturer's instructions. The reaction products were visualized by adding 0.03% diaminobenzidine (DAB) solution. The tissue section was then washed in distilled water, counterstained with hematoxylin, dehydrated, and mounted. The p53-positive cells were quantified under light microscope at $\times 400$ magnification. The investigator chose 3 areas on each slide; on the right, in the middle, and on the left of each tissue section for evaluation. The percentage of positive cells was calculated as brown-stained cells to the total epithelial cells. The cells were counted by 2 different investigators. The results of the 2 counts were averaged. Sections of squamous cell carcinoma with known p53 overexpression were used as positive control. The negative control was done by omitting the primary antibody.

Statistical analyses

Descriptive analyses were used for the evaluation of sensitivity, specificity, and accuracy in the application of 5% acetic acid for oral cancer detection. Histopathological assessment was used as the gold standard. The association between the results of 5% acetic acid application and the histopathological diagnoses was analyzed by Fisher's exact test. Differences in percentage of p53-positive cells between acetic acid–positive specimens and acetic acid–negative specimens were compared using the Mann-Whitney test. SPSS 10.00 for Windows (SPSS Inc., Chicago, IL) was used for statistical analyses. A P value less than .05 was considered statistically significant.

RESULTS

Of 30 patients, 18 (60%) were male and 12 (40%) were female. The male-to-female ratio was 3:2. The age

Table I. The results from acetic acid application and the histopathological diagnoses

	Histopathological diagnoses		
Acetic acid application	*Disease +**	*Disease −†*	*Total*
+ result (%)	‡30 (90.9)	3 (9.1)	33
− result (%)	6 (27.3)	§16 (72.7)	22
Total	36	19	55

*Disease + are dysplasia, carcinoma in situ and squamous cell carcinoma.
†Disease − are hyperplasia, inflammation, and normal mucosa.
‡Sensitivity = 30/36 = 83.33%; positive predictive value = 30/33 = 90.91%.
§Specificity = 16/19 = 84.21%; negative predictive value = 16/22 = 72.73%.

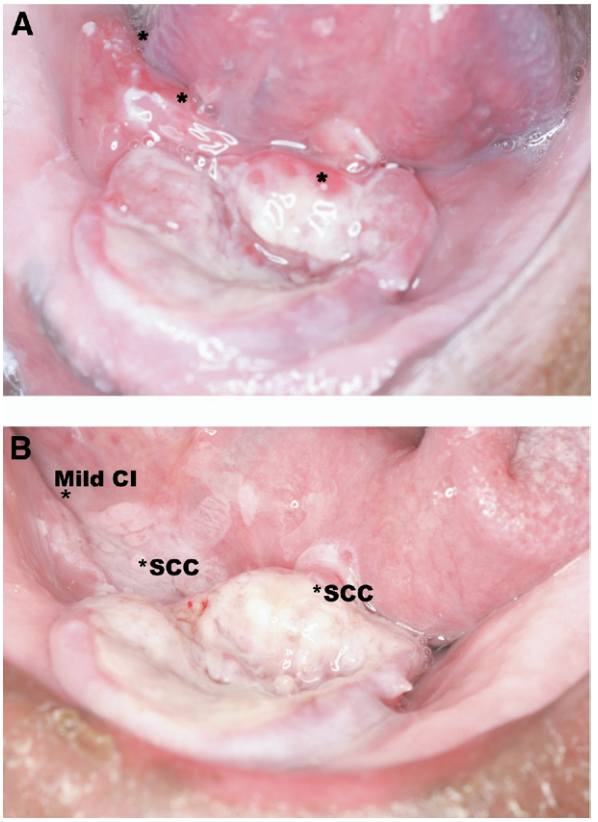

Fig. 1. **A,** Lesion on the floor of the mouth of a patient before acetic acid application (*asterisks*). **B,** The same lesion as in *A* after the application of acetic acid and the histopathological diagnoses. Mild CI, mild chronic inflammation; SCC, squamous cell carcinoma.

range was 39 to 77 with an average age of 60.50 ± 10.88 years. Duration of the lesions ranged from 1 to 24 months with the average duration of 6.13 ± 6.80 months. Locations of lesions were as follows: floor of the mouth, 8 (24.2%); lateral tongue, 8 (24.2%); buccal mucosa, 7 (21.2%); lower lip, 4 (12.1%); hard palate, 2 (6.1%); soft palate, 2 (6.1%); and alveolar ridge, 2 (6.1%).

Sixty-seven percent of our patients smoked, 50% consumed alcohol, and 23% chewed betel nut. When the patients were separated into 2 groups by sex, it was found that 100% and 77.8% of male patients smoked and consumed alcohol. On the other hand, only 16.7% and 8.3% of female patients smoked and consumed alcohol, while 66.7% chewed betel nut. No male patients had any history of betel nut chewing.

The results from 5% acetic acid application and histopathological diagnosis are shown in Table I. It was found that the sensitivity of 5% acetic acid application for oral precancer and cancer detection was 83.33%, whereas the specificity was 84.21%. In addition, the positive predictive value of the use of 5% acetic acid for oral cancer examination was 90.91% and the negative predictive value was 72.73%. The relationship between the results of oral cancer examination using 5% acetic acid (Fig. 1) and the results of histopathological diagnoses revealed significant association (P = .001).

The numbers of specimens positive to p53 in each group of histopathological diagnoses are as follows: oral squamous cell carcinoma, 21 (91.3%) of 23; epithelial dysplasia and carcinoma in situ, 12 (92.3%) of 13; epithelial hyperplasia, 2 (66.67%) of 3; chronic inflammation, 3 (50%) of 6; normal mucosa, 1 (16.67%) of 6; and others, 2 (50%) of 4. Average percentages of p53-positive cells from 3 areas of the tissue section under light microscope in each group of specimens are shown in Fig. 2. Of 55 specimens, 33 had clinically changed to an opaque white color upon application of 5% acetic acid. The average percentages of cells positive to p53 in all acetic acid–positive and –negative specimens were 33.49% ± 4.22% and 11.45% ± 5.18%, respectively. The difference in percentage of the p53-positive cells between these 2 groups was significantly different (P = .000).

DISCUSSION

The average age of our patients was 60.50 ± 10.88 with a male-to-female ratio of 3:2. These findings are consistent with age and gender of oral cancer patients reported by other studies[1,19,20]; however, the proportion of female patients in our study is slightly higher than that of other studies. This is probably because betel nut is still used by our elder female population. The distribution of lesions, from 24.2% at the lateral tongue and floor of mouth, to 6.1% at the hard palate, soft palate, and alveolar ridge is also comparable to the distribution

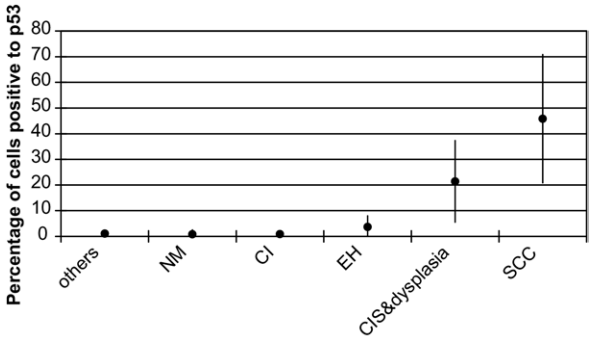

Fig. 2. Percentage of cells positive to p53 antibody ± standard deviations in each group of specimens. NM, normal mucosa; CI, chronic inflammation; EH, epithelial hyperplasia; CIS, carcinoma in situ; SCC, squamous cell carcinoma.

of oral cancer lesions obtained from other studies.[1,19,20] We, however, observed a higher occurrence of oral cancer on the buccal mucosa (21.2%) than that reported by others. This could stem from the use of betel nut as well.

It is widely accepted that smoking, alcohol consumption, and betel nut chewing are leading risk factors for the development of oral cancer. We found that 66.7% of our patients smoked, 50.0% drank alcohol, and 23.0% chewed betel nut. This confirms the danger of those risk factors. The association between sex and the risk factors indicates that sociological habits associated with gender determine the risk factors for oral cancer development.

This study aimed to examine the sensitivity, specificity, and accuracy of 5% acetic acid in the detection of OSCC, an area in which no researcher has explored the possibilities. When we compared the sensitivity and specificity of 5% acetic acid (83.33% and 84.21%, respectively) from this study to the reported sensitivity (77% to 100%) and specificity (44% to 93%) of toluidine blue from 9 studies,[21-29] we observed that, in general, acetic acid has lower sensitivity than toluidine blue but higher specificity. In addition, when the sensitivity and specificity of acetic acid used in this study were compared with its use in cervical cancer detection, we found that both values were, for the most part, higher than those reported in 8 studies of cervical cancer detection (49% to 90% and 49% to 92%, respectively).[11,30-36] This is likely due to the relative accessibility of oral cavity as compared to the cervix.

The significant relationship found between clinical examination using 5% acetic acid and histopathological diagnoses confirmed that acetic acid reacted better with tissues that had turned dysplastic or malignant than with normal tissues and warranted the use of acetic acid in oral cancer examination. Immunohistochemical study revealed that 21 (91.3%) of 23 OSCC specimens showed positive results using p53 antibody. Other studies reported a wide range of results, from 11% to 94%.[37,38] One normal mucosa specimen and 3 chronic inflammation specimens also showed positive results. The presence of p53 positively stained in normal tissues surrounding cancer lesions were also reported in other studies.[39,40]

From Fig. 2, the average percentages of cells positive to p53 by the results of histopathological diagnoses revealed the relationship between the severity of histopathological results and the number of cells positive to p53 antibody. The results support the use of p53 antibody reaction for treatment planning and prognosis.

We stated earlier that clinical changes due to acetic acid application in oral cancer examination and the results of histopathological diagnoses are significantly correlated. This finding is in line with the significant difference of p53-positive cells between tissues that are positive to acetic acid examination and those that are not.

Regarding patients' tolerance after the application of 5% acetic acid, we found that most patients had no complaint over the use of 5% acetic acid. Four patients had records of bleeding and 3 patients had burning sensation. These were patients with ulcerations of at least 1 cm in diameter.

This is a preliminary study investigating the use of acetic acid in oral cancer detection. Thus, any patients with lesions suspected of having OSCC were included in the study. The added values of acetic acid can be summarized as follows:

(1) A small aphthous-like ulcerated lesion that might not routinely be biopsied turned opaque white after the application of acetic acid and the pathologic result was moderate epithelial dysplasia.
(2) In 14 patients we also biopsied the sites that did not change to opaque white but were close to the lesions. We found that all those specimens were normal mucosa, chronic inflammation or epithelial hyperplasia. Thus, acetic acid helps demarcate dysplastic areas from nonmalignant tissue (Fig. 1). Interestingly, specimens from a lesion that we biopsied in 3 areas (lesion with opaque white color, clinically normal area that turned into a white line, and normal area with no change) were histopathologically identified as squamous cell carcinoma, lichen planus, and normal mucosa, respectively.
(3) There were 3 patients in whom we also biopsied normal areas that had turned opaque white but

were not part of the lesions of primary interest. We found that those areas were dysplastic.

This study, however, has few limitations. First, only a single investigator assessed patients with clinically known cancer or dysplastic lesions. Thus, the results of the acetic examination were influenced by the clinical examination. Second, only patients with lesions were included in this study; therefore, we recommend further study and evaluation of acetic acid used for oral cancer screening in rural communities because of its acceptable sensitivity, specificity, and accuracy. Five percent acetic acid (vinegar) can be conveniently obtained from any market, while toluidine blue has to be ordered from certain chemical companies. In addition, the price of toluidine blue is at least 1000 times higher than the price of vinegar (by weight). Thus, 5% acetic acid is suitable to be used for oral cancer examination, especially in developing countries.

In conclusion, acetic acid showed promising sensitivity, specificity, and accuracy for oral cancer examination. The results of clinical examination using 5% acetic acid also correlate with the expression of p53 in the cellular level. The expression of p53 is associated with the severity of the lesions.

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