Apoptosis Inhibitor Showed a Significant Prognostic Marker of Relapsed Oral Cavity Cancer after the Curative Resection Surgery

Wirote Lausoontornsiri MD*, Somjin Chindavijak MD**,
Ekapob Sangariyavanich MD**, Anantnuch Sakapiboonnan MD***

** Clinical Research Center, National Cancer Institute of Thailand, Bangkok, Thailand*
*** Department of Otolaryngology, National Cancer Institute of Thailand, Bangkok, Thailand*
**** Department of Pathology, National Cancer Institute of Thailand, Bangkok, Thailand*

Background: *Recurrence of oral cavity cancer after curative resection remains a major problem. Pathologic markers, which include positive margins, extracapsular nodal extension, lymphovascular invasion, and perineural invasion, predict likelihood of recurrence. However, there currently are no biomarkers that can be used to follow patients following the curative resection. Survivin, the anti-apoptotic protein is up-regulated in many types of cancer and is associated with poor prognosis and recurrence of cancer. We explored whether this biomarker predicted disease recurrence after curative resection of oral cancers.*

Material and Method: *Retrospective study of 47 patients with oral cancers who underwent curative surgery. Cases were assigned into two groups for analysis, with or without loco-regional recurrence/distant metastases. The study protocol was approved by the ethics committee of the National Cancer Institute. Biopsy sections both at tumor and margin were studied for expression of survivin and the tumor marker, CD44v6 by immunohistochemistry (IHC) technique.*

Results: *By using a scoring system, the surgical margin of the recurrent group showed a higher survivin score than non-recurrent group ($p = 0.003$). Interestingly, the primary tumor of the recurrent group showed a markedly higher survivin score than the non-recurrent group ($p<0.001$). By contrast, the CD44v6 scores of the primary and the margins showed no significant difference between either group.*

Conclusion: *The present study suggests that monitoring the survivin expression at the surgical margin may serve as a biomarker to evaluate the adequacy of the surgical margin and may serve to provide information to prepare a better preoperative plan for oral cancer surgery in order to improve the curative outcome.*

Keywords: *Oral cancer, Head and neck cancer, Survivin protein, Biological markers, Apoptotic regulatory protein*

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Oral cancer is a common cancer among the Thai population with more than 6,000 cases present annually. Treatment modalities include surgery, radiotherapy, and chemotherapy. The most important factor in management of oral cancer is the complete surgical removal of the tumor. Complete excision of the tumor is determined by histopathological assessment at surgical margin, however, local recurrences are still observed in histological free margin cases. The molecular marker with a capable of indicating the prognosis might contribute to the decision for adjuvant treatment. Recently, much attention has been focused on the evolution of molecular pathologic prognostic factors, for example, correlation between Survivin and CD44v6 expression and clinical outcome of laryngeal cancer has been reported[1].

Survivin is a member of the apoptosis inhibitor protein gene family. It is implicated in regulation of cell division, anti-apoptosis, and checkpoint mechanisms of genomic integrity. Survivin is up-regulated in many types of cancer and is associated with poor prognosis and recurrence[2,3].

CD44v6 was recently reported on its expression of cancer stem cell in head and neck cancer, which has tumorigenic potential when transplanted and self-renewing population. These cells are responsible for the development of metastasis by migrating and attaching to a new location. This marker is associated with advanced stages of tumor growth, increase

Correspondence to:
Lausoontornsiri W, 268/1 Rama 6 Road, Bangkok 10400, Thailand.
Phone: 0-2354-7025 ext. 2310
E-mail: wlausoon@yahoo.com

metastatic potential, and decrease overall survival in many types of cancer[4-6].

The aim of the present study was to determine the possibility of Survivin and CD44v6 expression to be prognostic factors for post-surgical oral cancer patients, which would influence the decision for adjuvant treatment.

Material and Method

This was a retrospective study of 47 patients with oral cancers who underwent surgical resection of their primary tumor at the Department of Otolaryngology, National Cancer Institute Thailand between 1997 and 2007. The study protocol was approved by the ethics committee of the National Cancer Institute. The patient's medical records were fully reviewed and all the records showed a complete surgical resection and existing of pathological sections for further study. Patients who received neoadjuvant chemotherapy or radiotherapy were excluded from the present study. Patient age, gender, staging at diagnosis, surgical resection method, histopathology of the resected tumor, and follow-up data were harvested from medical records. From this data, 26 patients were found with a clinical course of locoregional or distant metastasis, and 21 patients were found without locoregional or distant metastasis for at least two years. From the archive, formalin-fixed, paraffin-embedded tissue of every patient was examined by immunohistochemistry for detection of survivin and CD44v6.

Immunohistochemistry

Immunohistochemical staining (IHC) was performed on 3 μm–thick sections of archived biopsy tissue from all 47 subjects. The sections were prepared from formalin-fixed paraffin embedded by tissue microarray (TMA) tissue blocks and were dried in a 60°C oven overnight. The sections were placed in a Bond Max Automated Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Wetzlar, Germany) according to the manufacturer's protocol (Protocol created by Lorena Maestre-Monoclonal Antibodies Unit, Centro Nacional de Investigaciones Oncologicas). Briefly, tissues were deparaffinized and pre-treated with Epitope Retrieval Solution (ethylenediaminetetraacetic acid (EDTA)-buffer pH9) at 100°C for 20 minutes. After washing steps, peroxidase blocking was carried out for 10 minutes using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH). Tissues were again washed and then incubated with the following primary antibodies: Survivin (1:800) and CD-44 (1:400) for 30 minutes. Subsequently, tissues were incubated with polymer for 10 minutes and developed with Diaminobenzidine tetrahydrochloride (DAB)-Chromogen for 10 minutes. Evaluation by the pathologist, a semiquantitative scoring system was used according to the percentage of the staining pattern under a microscope: score 0 = <5%, score 1 = 6-25%, score 2 = 26-50%, and score 3 = >50% (as Fig. 1-4). The pathologist was blinded from the clinical presentation of the subjects in order to reduce the bias for the pathological interpretation.

Statistical analysis

Association between variables were analyzed by the Chi-square test and when were cells that contained less than 5 or more than 20%, the Fisher's

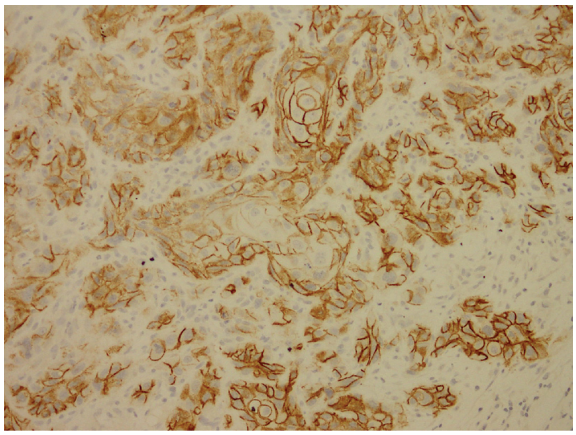

Fig. 1 Immunohistochemical staining for CD44v6 at tumor (x200).

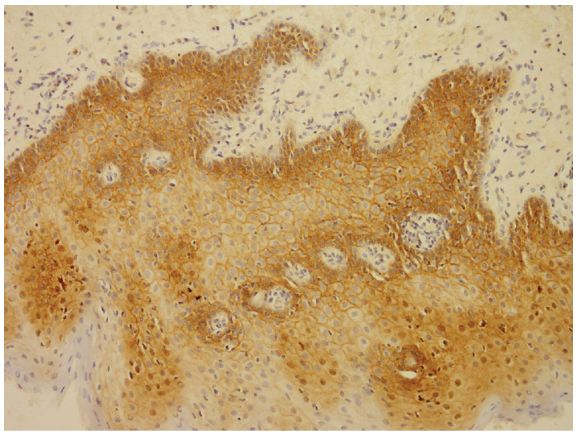

Fig. 2 Immunohistochemical staining for CD44v6 at tumor margin (x200).

exact test was performed. The association of the margin between the groups was performed using the Mann-Whitney U test. P level of less than 0.05 was considered statistically significant.

Results

Patient characteristics

There were 26 cases in the recurrent group and 21 cases in the non-recurrent group that were analyzed. The mean age of the patients at the time of diagnosis in the recurrent group was 54 (range 30-86) and 52 (range 23-75) in the non-recurrent group; the male population was more prevalent than female in both groups. Staging of both groups were different in T and N stage. The most common primary site was tongue 69.2% in the recurrent group and 90.5% in the non-recurrent group. The distant of surgical margin was significantly larger in the non-recurrent group compared to the recurrent (the recurrent group = 0.48 cm and the non-recurrent group = 0.68 cm ($p = 0.029$) (Table 1)).

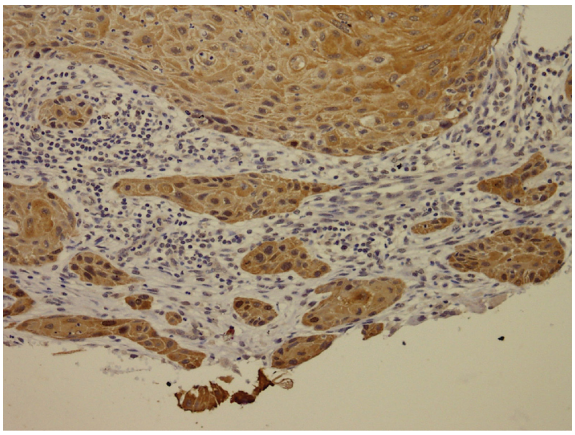

Fig. 3 Immunohistochemical staining for survivin at tumor (x200).

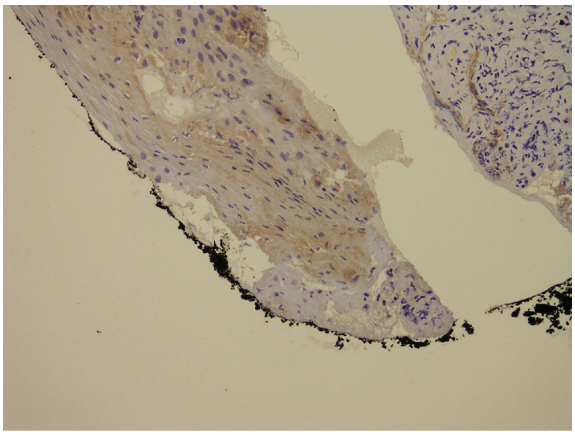

Fig. 4 Immunohistochemical staining for survivin at tumor margin (x200).

The median follow-up of recurrence group was 394.5 days (range 104-1,399 days) and in the non-recurrent group was 1,577 days (range 713-2,571 days). The median disease free survival of the recurrent group was 259.5 days while the non-recurrent group was not reached this point due to no relapse of the cancer. Among the recurrent cases, four cases had the cancer recurred at multiple sites, eight cases with recurrence at the neck, seven cases with recurrence at the primary, two cases of distant metastasis and four cases that developed skin metastasis (Table 2).

Outcome of survivin and CD44v6 immunohistochemical expression

There was no significant difference in survivin expression of the primary tumor between groups (Table 3).

However, in Table 4, the re-analysis of the survivin score at primary by grouping of score 0 and 1 compare with the group of score 2 and 3. The result was significant statistically difference between the recurrent and non-recurrent group. The recurrent group found more survivin score 2 and 3 (96.2%) compare to the non-recurrent group that found the group of survivin score 0 and 1 (76.2%) (Table 4). Survivin was positive at the margin in 57.7% (15/26) of the recurrent cases and 23.8% (5/21) in the non-recurrent group which was statistically significant $p = 0.020$ (Table 3).

The primary was positive for CD44v6 in both groups, 96.2% and 100%. The margin was positive for CD44v6 in 65.4% of the recurrent cases and 71.4% of the non-recurrent cases. Neither the expression at the primary or the margin were statistically significant between groups (Table 3).

Analysis of survivin and CD44v6 at margin

Analysis of the data at the margin by survivin score combined with CD44v6 score demonstrated that the 57.7% of the recurrent group was found to be survivin+/CD44v6+ and 47.6% of the non-recurrent group were found to be survivin-/CD44v6+. No cases of survivin+/CD44v6- phenotype were found in either group (Table 5).

Three cases in the recurrent group had a margin more than 1 cm, and the result of survivin was positive. In the non-recurrent group, five cases with

Table 1. The patient and tumor characteristics of the recurrent group and the non-recurrent group

	Recurrent 26 cases	Non-recurrent 21 cases	p-value
Age	30-86 (mean = 54)	23-75 (mean = 52)	
Sex, No. (%)			
Male	15 (57.7)	15 (71.4)	
Female	11 (42.3)	6 (28.6)	
T, No. (%)			
1	9 (34.6)	14 (66.7)	
2	8 (30.8)	7 (33.3	
3	2 (7.7)	0	
4	7 (26.9)	0	
N, No. (%)			
Negative	16 (61.5)	5 (23.8)	
Positive	10 (38.5)	16 (76.2)	
Stage, No. (%)			
I	6 (23.1)	10 (47.6)	
II	1 (3.8)	6 (28.6)	
III	5 (19.2)	3 (14.3)	
IV	14 (53.8)	1 (4.8)	
Site, No. (%)			
Tongue	18 (69.2)	19 (90.5)	
Alveolar ridge	6 (23.1)	1 (4.8)	
Floor of mouth	0	1 (4.8)	
Lip	2 (7.7)	0	
Margin, cm (mean)	0.1-1.5 (0.48)	0.3-1.5 (0.68)	0.02
Follow-up, days (median)	104-1,399 (394.5)	713-2,571 (1,577)	

Table 2. Site of recurrent in 26 recurrent cases

	Cases
Primary recurrent	7
Regional recurrent	8
Distant metastasis	2
Skin nodule	4
Multiple site metastasis	4

a margin of less than 5 mm had negative survivin (Table 6, 7).

Discussion

The incidence of oral cancer is the highest among head and neck cancer. The mainstay of treatment is surgery. The decision for adjuvant treatment depends on the pathological report after the operation. The margin after surgical resection is one of factors that influence the treatment decision, determined by the distance from the primary tumor. However, recurrence is still reported in the cases that have free margins and even in close margin cases some patients did not develop recurrence. This study demonstrated margins of 0.1 to 1.5 cm in the recurrence group and 0.3-1.5 cm in the non-recurrent group. In 2008, Zhao et al reported the prognostic significance of survivin and CD44v6 in laryngeal cancer and suggested that surgical margins which expressed survivin and CD44v6 could be a potential novel molecular marker of recurrence[1]. Therefore, in this report, we assessed the potential of survivin and CD44v6 expression at the surgical margin as prognostic factors for oral cancer.

Survivin is a member of the inhibitor of apoptosis protein family (Ambrosini et al 1997)[1,7] that regulates cell division and suppresses apoptosis[8]. Survivin is expressed in almost all tumor cells and is absent in normal adult differentiated cells[1]. In the present study, the survivin score at the margin was significantly different between the groups. There were 57.7% of recurrent cases positive for survivin whereas 76.2% of control group was negative ($p = 0.02$). Zhao et al reported that the incidence of recurrence in laryngeal cancer was higher in the survivin positive subgroup than in the negative margin

Table 3. The survivin score and CD44v6 score in recurrent and non-recurrent group

	Recurrent group	Non-recurrent group	p-value
Survivin score at primary			
Negative	0	1 (4.8%)	0.447
Positive	26 (100%)	20 (95.2%)	
Survivin at margin			
Negative	11 (42.3%)	16 (76.2%)	0.020
Positive	15 (57.7%)	5 (23.8%)	
CD44v6 score at tumor			
Negative	1 (3.8%)	0	1.000
Positive	25 (96.2%)	21 (100%)	
CD44v6 score at margin			
Negative	9 (34.6%)	6 (28.6%)	0.659
Positive	17 (65.4%)	15 (71.4%)	

Table 4. Re-analysis of survivin at primary by group: group 1 = score 0, 1 and group 2 = score 2, 3

	Recurrent group	Non-recurrent group	p-value
Group 1 (score 0, 1)	1 (3.8%)	16 (76.2%)	<0.001
Group 2 (score 2, 3)	25 (96.2%)	5 (23.8%)	

Table 5. analysis of survivin and CD44v6 at margin

	Recurrent group	Non-recurrent group	p-value
Survivin+/CD44v6+	15 (57.7%)	5 (23.8%)	0.005
Survivin-/CD44v6-	9 (34.6%)	6 (28.6%)	
Survivin-/CD44v6+	2 (7.7%)	10 (47.6%)	

Table 6. Number of cases that survivin positive at margin in recurrent group

	Number of cases
<0.5 cm	6
0.5-1.0 cm	6
>1 cm	3

Table 7. Number of cases that survivin negative at margin in non-recurrent group

	Number of cases
<0.5 cm	5
0.5-1.0 cm	7
>1 cm	4

group[1] and was a significant independent predictor related to disease free survival after curative surgery of laryngeal cancer[1]. The analysis of the margin with the survivin marker found five cases with closed margin (which was less than 0.5 cm) that were survivin negative but none in the non-recurrent group. In the recurrent group there were three cases (margin were 1.2, 1.3, 1.5 cm) that were positive for survivin. The distant of the tumor from the mucosa may not be the ideal prognostic factor and these three cases were treated with postoperative radiation only for adjuvant therapy. Whereas the non-recurrent group with closed margin but survivin negative received postoperative radiation in two out of five cases.

CD44 positive cell was proposed as a cancer stem cell marker for head and neck cancer expressed in a subpopulation of cancer cells containing four characteristics: (1) tumorigenic potential when transplanted into immunodeficient mice (2) can be separated from the other cancer cells by distinctive cell surface markers (3) tumors resulting from the cancer stem cell contain the mixed tumorigenic and non-tumorigenic cells of the original tumor, and (4) the cancer stem cell population can be serially transplanted through multiple generations, indicating that it is a self-renewing population[9-14]. The cancer stem cell theory suggests that the presence of a single cancer stem cell under the right conditions can lead to tumor

repopulation, whereas a large mass of non-cancer stem cell may not be dangerous[9]. Gunthert et al reported that over expression of exon 11(v6) of the CD44 gene may cause tumor metastasis in rat pancreatic tumor cells4 and others reported the correlation of CD44 with faster progression of cancer, with metastasis and reduced survival in several cancers[1]. Therefore, in the present study, the authors assess CD44 expression at the margin and hypothesized that the margin of the recurrent group would have higher CD44 expression than the control cases. The result in this study showed that CD44 in both groups was not statistically different which is in contrast to the report from Zhao that found the difference of CD44 expression at the margin of positive and negative margins to be significantly different in laryngeal cancer[1].

The analysis of both Survivin and CD44v6 demonstrated that 57.7% of recurrence cases expressed the survivin+/CD44v6+ phenotype while only 28.6% of the non-recurrent group expressed a survivin-/CD44v6- phenotype. This may suggest that the status of survivin with CD44v6 may not be the ideal prognostic factor for detection of recurrence. The role of CD44v6 is not able to determine from our data.

In this pilot study, our data has determined that survivin, an apoptotic marker, is the good prognostic marker for recurrent oral cavity cancer after the surgical resection. The expression of survivin in the primary tumor and at the surgical margin are both likely to indicate the poor prognostic outcome for the oral cancer patient. This is the warrant for prospective randomize clinical trial of survivin in the future.

Conclusion

The surgical margin, which was conventionally determined by the distance from the tumor to the normal mucosa, might have to be reconsidered due to the finding that recurrence cases with free margins or even the close margins have high incidence of recurrence. Survivin is a promising marker at the margin that can predict recurrence and this can be used to determine which patients should receive adjuvant treatment after complete resection of oral cancer. The use of survivin as a predictive marker may lead clinicians to understand the molecular feature of oral cancer and to find a new paradigm treatment to improve overall survival in these patients.

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Potential conflicts of interest

None.

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การใช้ apoptosis inhibitor เป็นตัวพยากรณ์การเกิดกลับซ้ำของมะเร็งช่องปากในผู้ป่วยที่ได้รับการผ่าตัด

วิโรจน์ เหล่าสุนทรศิริ, สมจินต์ จินดาวิจักษณ์, เอกภพ แสงอริยวนิช, อนันท์นุช ศักดิ์อภิบุญนันท์

ภูมิหลัง: *การกลับมาเป็นซ้ำของมะเร็งช่องปากภายหลังการผ่าตัดยังเป็นปัญหาสำคัญ ตัวบ่งชี้ทางพยาธิวิทยาที่ใช้อยู่ ได้แก่ positive margin, extracapsular nodal extension, lymphovascular invasion และ perineural invasion สามารถทำนายโอกาสในการเกิดกลับซ้ำของโรคได้ แต่อย่างไรก็ตามปัจจุบันยังไม่มีตัวบ่งชี้ทางชีววิทยา ในการติดตามผู้ป่วยหลังการผ่าตัด มีการรายงานว่า survivin ซึ่งเป็นโปรตีนชนิด anti-apoptotic มีการทำงานเพิ่มขึ้นในมะเร็งหลายชนิด และมีความสัมพันธ์กับการพยากรณ์โรคที่ไม่ดีและการเกิดกลับซ้ำ ผู้นิพนธ์จึงได้ทำการศึกษาว่าโปรตีนดังกล่าวจะสามารถพยากรณ์การเกิดกลับซ้ำของมะเร็งช่องปากหลังการผ่าตัดได้หรือไม่*

วัสดุและวิธีการ: *ทำการศึกษาย้อนหลังผู้ป่วยมะเร็งช่องปากที่ได้รับการผ่าตัดในสถาบันมะเร็งแห่งชาติ 47 ราย โดยแบ่งเป็น 2 กลุ่ม คือกลุ่มที่มีโรคและกลุ่มที่ไม่มีการเกิดกลับซ้ำ และ/หรือ แพร่กระจาย โดยการทำการศึกษานี้ได้รับการรับรองจากคณะกรรมการวิจัยในคนของสถาบันมะเร็งแห่งชาติ ชิ้นเนื้อที่ผ่าตัดออกมาจะได้รับการตรวจที่บริเวณก้อนมะเร็ง และบริเวณขอบของชิ้นเนื้อจากการผ่าตัดเพื่อหา survivin และ CD44v6 ด้วยวิธีทาง immunohistochemistry*

ผลการศึกษา: *คะแนนของ survivin ที่ขอบของชิ้นเนื้อจากการผ่าตัดในกลุ่มที่มีการเกิดกลับซ้ำ และ/หรือ แพร่กระจายสูงกว่ากลุ่มที่ไม่มีการเกิดกลับซ้ำ และ/หรือ แพร่กระจายอย่างมีนัยสำคัญ ($p = 0.003$) นอกจากนั้นคะแนนของ survivin ที่ก้อนมะเร็งในกลุ่มที่มีโรคเกิดกลับซ้ำ และ/หรือ แพร่กระจายยังสูงกว่าอีกกลุ่มอย่างชัดเจน ($p<0.001$) ในทางตรงกันข้ามคะแนนของ CD44v6 ที่บริเวณก้อนมะเร็งและขอบของชิ้นเนื้อจากการผ่าตัดของทั้งสองกลุ่มไม่มีความแตกต่างกัน*

สรุป: *การตรวจหา survivin ที่ขอบของชิ้นเนื้อจากการผ่าตัดอาจใช้เป็น biomarker ในการประเมินความกว้างของขอบชิ้นเนื้อจากการผ่าตัดว่าเพียงพอหรือไม่ และยังเป็นข้อมูลในการวางแผนการรักษามะเร็งช่องปากก่อนการรักษาโดยการผ่าตัดเพื่อให้ผลการรักษาที่ดีขึ้น*
