

Clinicopathological significance of carbonic anhydrase 9, glucose transporter-1, Ki-67 and p53 expression in oral squamous cell carcinoma

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Abstract. Carbonic anhydrase 9 (CA9) is a glycoprotein present on the surface of cell membranes. It is expressed in 90% of renal cancer cells, but not in normal kidney tissue. Immunotherapy targeting CA9 is underway, and our group has also conducted a clinical trial using CA9 as a cancer vaccine and confirmed the induction of cytotoxic T lymphocytes, with efficacy in some cases. Expression of CA9 antigen in oral cancer has not been reported in Japan, but our results indicate that immunotherapy targeting CA9 might be possible. We immunohistochemically observed the expression of antigens such as CA9, Ki-67, glucose transporter-1 (GLUT-1) and p53 in 107 subjects with oral squamous cell carcinoma, and examined their correlation with clinicopathological parameters. Immunostaining analysis showed expression of CA9 in 98% of oral cancer subjects, and the survival rate was significantly lower in subjects with CA9 antigen expression in 50% or more cells ($P < 0.05$). Subjects with poorly differentiated, T4 and lymph node metastasis, or Stage IV cancer with high CA9 expression ($\geq 50\%$) had a worse outcome than those with low CA9 expression. Although GLUT-1 expression was observed in 98% of subjects, similarly to CA9 expression, no significant correlation between its expression and the survival rate was seen. However, subjects with lymph node metastasis had significantly higher GLUT-1 expression, demonstrating that GLUT-1 could be an indicator of lymph node metastasis. Ki-67 was expressed in 92% of subjects, but no correlation with outcome was observed. Expression of p53 was noted in 78% of subjects, and it was found that many oral cancers have p53 genetic abnormalities, but no correlation between

p53 and outcome was observed. It was confirmed that CA9 antigen is expressed in most oral cancer subjects, suggesting the possibility of immunotherapy targeting CA9 antigen in oral cancer.

Introduction

Among annual deaths of Japanese in 2009, >340,000 deaths were from malignant neoplasms (cancers), of which 6500 deaths (2%) were from oral malignant neoplasms. Many oral malignant neoplasms (oral cancer) are squamous cell carcinoma, and most of them develop in the tongue, with slightly higher frequency in men than in women (1). Approximately 50% of patients have cervical lymph node metastasis at the time of diagnosis, and the outcome of these patients is poor (2-4). In addition, the 5-year disease-free survival rate of patients with oral cancer decreases from 75% in Stage I to 22% in Stage IV (5), indicating that it is important to find markers for early detection of cancers and to develop more effective treatments.

Carbonic anhydrase 9 (CA9) is a glycoprotein present on the surface of cell membranes. It is absent in normal renal tissue, but is expressed in 90% of renal cell carcinomas (6). This is a cancer-associated antigen recognized by antibodies, produced by cervical cancer or renal cell carcinoma cells, and is expressed in normal tissues of gastrointestinal tract membranes, bile ducts (7) and skin (unpublished data). Recently, many research groups have reported its expression not only in renal cell carcinoma and cervical cancer, but also in bladder cancer, non-small cell lung cancer, breast cancer, head and neck cancer, esophageal cancer, stomach cancer, large intestine cancer, bile duct cancer, sarcoma and chronic myeloid leukemia (8-18). Yoshikawa *et al* prepared mouse-derived renal cell carcinoma cells expressing human CA9 antigen. They induced cytotoxic T lymphocytes (CTLs) using Balb/c mice with the same anchor motif as human HLA-A24, and identified an amino acid sequence of CA9 antigen-derived peptide that is recognized by CTL. Based on the results, they also conducted a clinical study using CA9 antigen-derived peptide as a cancer vaccine, and confirmed the safety of the peptide treatment and the induction potential of CTL (19).

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Currently, the principal treatments for oral cancer are surgery, chemotherapy and radiation therapy. However, it is anticipated that immunotherapy will bring about good results. If the expression of CA9 antigen can be confirmed in oral cancer patients, especially in those with a poor prognosis, immunotherapy targeting CA9 will be possible; however, there have been no studies conducted in Japan on the expression of CA9 antigen in oral cancer in Japanese patients.

In this study, we immunohistochemically examined 107 patients with oral squamous cell carcinoma, confirmed the expression of antigens including CA9, and discussed the correlation with clinicopathological parameters.

Materials and methods

Patients. We conducted a study in 107 patients who attended the Department of Dentistry and Oral Surgery, Aichi Medical University Hospital between April 1992 and March 2009 and were diagnosed with squamous cell carcinoma according to UICC classification (20), underwent surgical removal of the tumor, and completed post-surgery treatment. Our protocol of this study was reviewed and approved by the institutional review board of Aichi Medical University School of Medicine. The subjects were followed up for a maximum of 60 months after primary tumor removal, and age and gender, tumor site, degree of histopathological differentiation, T classification, N classification and disease stage were used for clinicopathological analysis.

Immunohistochemical staining. Based on the normal method of histopathological examination, samples were fixed with formalin solution, embedded in paraffin, and 4- μ m paraffin sections were prepared. After deparaffination, antigen was activated with microwave, endogenous peroxidase was inactivated, attachment of non-specific protein was blocked, and rabbit anti-CA9 antibody (1:500, ab15086, Abcam, Cambridge, UK), rabbit monoclonal anti-Ki-67 antibody (1:100, clone: SP6, Abcam), mouse monoclonal anti-GLUT-1 antibody (1:200, clone: SPM498, Thermo, Rockford, IL, USA), and mouse monoclonal anti-human p53 antibody (1:50, clone: DO-7, Dako, Glostrup, Denmark) were reacted as primary antibodies overnight at 4°C. As secondary antibodies, horse anti-rabbit Ig (ImmPRESS™ Reagent, Vector Laboratories, Inc., CA, USA) was reacted with CA9 and Ki-67, and horse anti-mouse Ig (ImmPRESS Reagent, Vector Laboratories, Inc.) was reacted with GLUT-1 and p53 for 1 h at room temperature. Subsequently, they were developed with 3,3'-diaminobenzadine (DAB) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and counterstained with hematoxylin, dehydrated and encapsulated for microscopic examination. As a negative control, normal rabbit serum or normal mouse serum was used instead of primary antibodies. Observation was made with an Axioplan2 and Axiophot2 Universal Microscope (Carl Zeiss Co., Ltd., Jena, Germany), and digital images were obtained with an Axio Vision 4.5 (Carl Zeiss Co., Ltd.). In order to calculate the antigen prevalence, Image J software was used. Cancers with a positive rate of cells of 10% or more were considered positive, while those with a positive rate of <10% were considered negative.

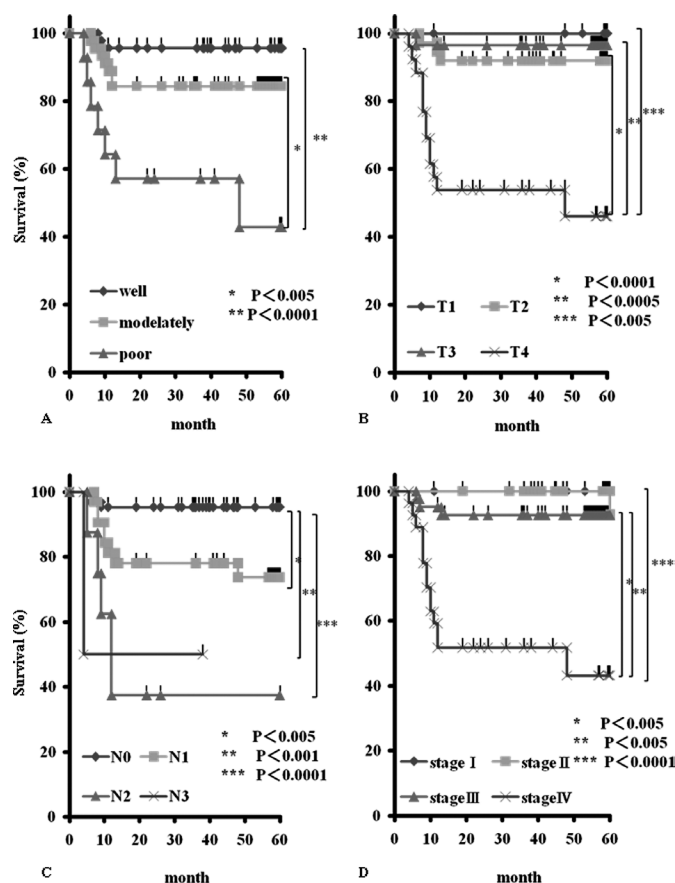


Figure 1. Clinicopathological survival analysis. The results indicate that subjects with poorly differentiated, T4, lymph node metastatic or Stage IV cancer had a poor outcome. (A) Tumor differentiation, (B) Stage, (C) T classification, (D) N classification.

Statistical analysis. StatView (ver. 5.0, SAS Institute Inc., NC, USA) was used for statistical analysis. For Kaplan-Meier survival analysis, log-rank test of the clinical samples was used. Chi-squared test was used for between-group comparisons. $P < 0.05$ was considered statistically significant.

Results

Clinicopathological parameters and survival rates. Table I shows the characteristics of the subjects. In this study, 107 subjects (61 male and 46 female) with mean age of 63 (range: 20-93) years participated. The most common site of the primary tumor was the tongue in 52 subjects, followed by gingiva (43 subjects), floor of mouth (5 subjects), pharynx (3 subjects), lips (3 subjects) and buccal mucosa (1 subject).

According to the degree of histological differentiation, 48 subjects had carcinomas of well-differentiated type, 45 had moderately-differentiated type and 14 had poorly-differentiated type. According to T classification, 16 subjects had T1, 37 had T2, 28 had T3, and 26 had T4 carcinomas. According to N classification, 65 subjects had N0, 32 had N1, 8 had N3, and 2 had N3 carcinomas. No subject had distant metastasis. According to the disease stage classification, 14 subjects were Stage I, 25 Stage II, 41 Stage III and 27 Stage IV.

We examined the correlation between these clinicopathological parameters and the survival rate, and found that

Table I. Characteristics of patients.

Characteristics	Number of patients (%)
Gender	
Male	61 (57)
Female	46 (43)
Age	63 (20-93)
Anatomic site	
Tongue	52 (48)
Gingiva	43 (40)
Oral floor	5 (5)
Pharynx	3 (3)
Lip	3 (3)
Buccal	1 (1)
Degree of differentiation	
Well	48 (45)
Moderately	45 (42)
Poor	14 (13)
T classification ^a	
T1	16 (15)
T2	37 (35)
T3	28 (26)
T4	26 (24)
N classification ^b	
N0	65 (61)
N(1+2+3)	42 (39)
N1	32 (30)
N2	8 (7)
N3	2 (2)
Stage	
I	14 (13)
II	25 (23)
III	41 (39)
IV	27 (25)

^aT, tumor; ^bN, lymph node.

subjects with poorly-differentiated type carcinoma had a significantly worse outcome than those with well- ($P<0.0001$) or moderately- ($P<0.005$) differentiated type (Fig. 1A). Subjects with T4 carcinoma had a poorer outcome than those with T1 ($P<0.005$), T2 ($P<0.0001$) or T3 ($P<0.0005$) carcinomas (Fig. 1B). A significant difference was observed between subjects with N0 and those with N1 ($P<0.005$), N2 ($P<0.0001$) or N3 ($P<0.001$) (Fig. 1C). Outcome was poor in subjects with lymph node metastasis. Outcome was significantly different among disease stages; Stage IV subjects had a significantly poorer outcome than Stage I ($P<0.0001$), Stage II ($P<0.005$) or Stage III ($P<0.005$) subjects (Fig. 1D).

Expression of antigens in oral cancer. CA9 was stained positive in cancer cell membranes and a part of the cytoplasm, and

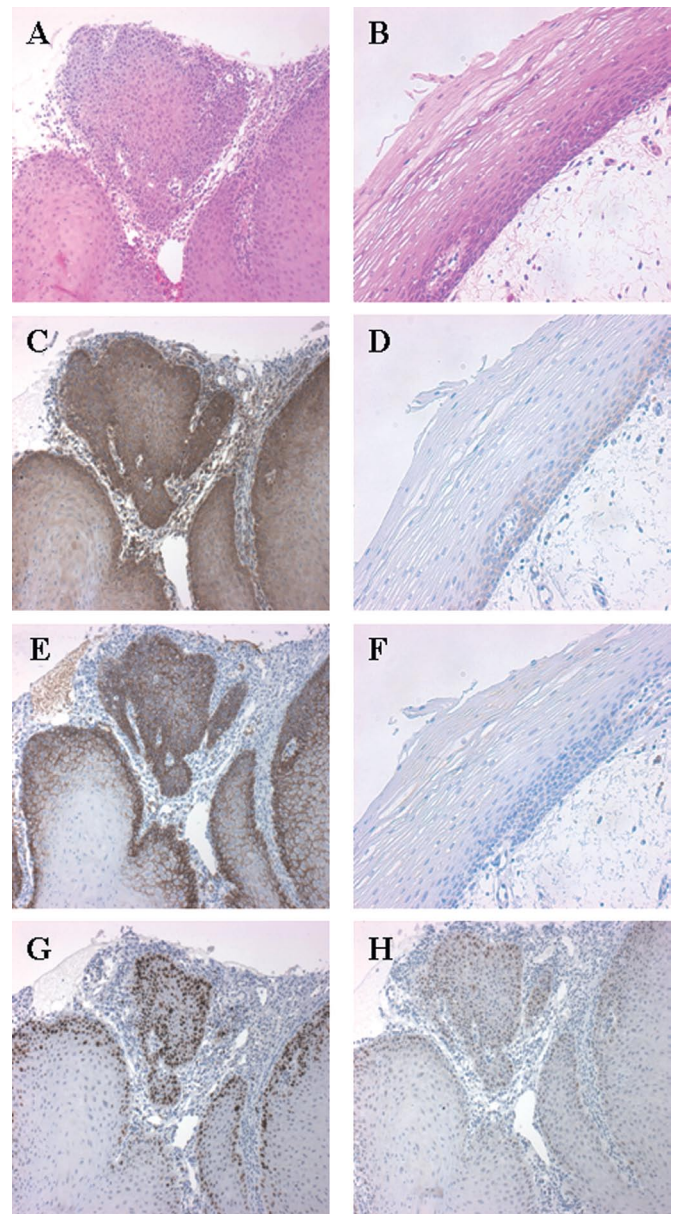


Figure 2. Immunohistochemical staining of CA9, GLUT-1, Ki-67 and p53 in oral squamous cell carcinoma and normal epithelial tissue (magnification, x20). (A) Hematoxylin-eosin staining of oral squamous cell carcinoma. (B) Hematoxylin-eosin staining of normal epithelial tissue. (C) CA9 expression; brown colored cytoplasmic and membranous staining. (D) In normal epithelial tissue, CA9 was stained slightly positive in the basal part of the mucosal epithelium. (E) GLUT-1 expression; brown colored membranous staining. (F) In normal epithelial tissue, GLUT-1 was not stained. (G) Ki-67 expression; brown colored nuclear staining. (H) p53 expression; brown colored nuclear staining.

GLUT-1 was stained positive in cancer cell membranes. In normal epithelial tissue, CA9 was stained slightly positive in the basal part of the mucosal epithelium. In most subjects, both Ki-67 and p53 were stained strongly positive in cancer cell nuclei (Fig. 2). Antigen prevalence and mean positive rate were 98 and 39%, respectively for CA9 antigen (Table II), 98 and 37% for GLUT-1 antigen, 92 and 34% for Ki-67 antigen, and 78 and 34% for p53 antigen.

Correlation between antigen expression rate and outcome. We examined the correlation between CA9 expression and

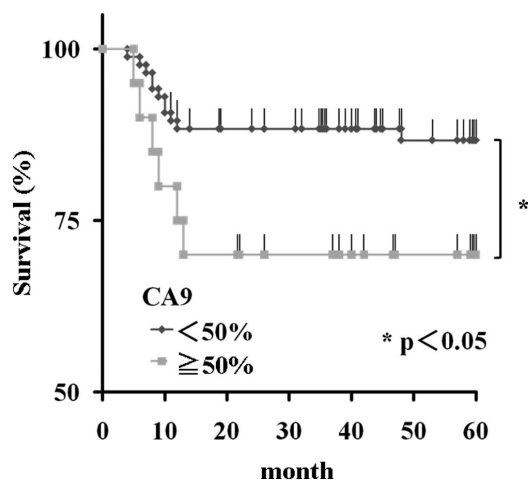


Figure 3. Correlation between CA9 and outcome. High CA9 expression (percentage of positively stained tumor cells: $\geq 50\%$) was significantly related to poorer survival as illustrated by Kaplan-Meier curves.

Table II. Cancer-related antigen positive rate of oral squamous cell carcinoma.

Markers	Positive (%)	Expression (max/min) (%)
CA9	105 (98)	39 (71/2)
GLUT-1	105 (98)	37 (73/0)
Ki-67	99 (92)	34 (90/4)
p53	84 (78)	34 (91/0)

Table III. Correlations of CA9 with cancer-related antigen.

Marker	CA9 <50% expression (n=87)	CA9 $\geq 50\%$ expression (n=20)	p (χ^2)
GLUT-1			
0 to <10%	2	0	0.638
≥ 10 to <30%	23	4	
$\geq 30\%$	62	16	
Ki-67			
0 to <10%	8	0	0.079
≥ 10 to <30%	32	4	
$\geq 30\%$	47	16	
p53			
0 to <10%	16	7	0.118
$\geq 10\%$	71	13	

outcome, and found that subjects with a 50% or higher positive rate had a poorer outcome compared to those with a positive rate of <50% ($P < 0.05$) (Fig. 3). We defined a positive rate of 50% or higher as 'high expression', and <50% as 'low expression', and further divided the subjects into two groups of well and moderate differentiated type and poorly differen-

Table IV. Correlations of Antigen expression with lymph node metastasis.

	Metastasis (+)	Metastasis (-)	p (χ^2)
CA9			
<50%	31	56	0.109
$\geq 50\%$	11	9	
GLUT-1			
<30%	6	23	0.016
$\geq 30\%$	36	42	
Ki-67			
<30%	13	31	0.085
$\geq 30\%$	29	34	
p53			
<10%	13	10	0.055
$\geq 10\%$	29	55	

tiated type, based on the degree of histological differentiation. In both groups, subjects with high CA9 expression tended to have a poor outcome. Similarly, we divided the subjects into T1-3 and T4 groups according to T classification, N0 and N1-3 groups according to N classification, and Stage I-III and Stage IV groups based on disease stage, and investigated the correlation with CA9 expression. In all comparisons, groups with high CA9 expression tended to have a poorer outcome, but the difference was not significant (data not shown).

The correlation between GLUT-1, Ki-67 and p53 antigen expression and outcome was also examined, but no correlation between antigen expression and outcome was observed. In addition, no correlation was observed with CA9 antigen expression (Table III). However, when we examined the correlation between the expression of antigens and lymph node metastasis, GLUT-1 had a significant correlation with lymph node metastasis in subjects with a positive rate of 30% or higher (Table IV).

Discussion

In this study, we observed immunohistochemically the CA9, Ki-67, GLUT-1 and p53 expression in 107 subjects diagnosed with and treated for oral squamous cell carcinoma, and examined the correlation between the antigen expression and clinicopathological parameters.

As with previous reports (21-23), our analysis on the correlation between clinicopathological parameters and outcome revealed that subjects with poorly-differentiated type (histological differentiation), T4 (T classification), N1-N2 (N classification) or Stage IV (disease stage at diagnosis) oral cancer had a poor outcome, and were confirmed to belong to a common patient group. However, 42 (39%) of 107 subjects had lymph node metastasis in this study, and had a 5-year survival rate of 65%. This indicates that our subjects had a better outcome compared to 30% in previous studies (24).

Expression of CA9 and GLUT-1 antigens, which was examined in this study, is regulated by hypoxia-inducible factor (HIF-1). Generally, HIF-1 α leads to ubiquitination and

degradation by the VHL tumour suppressor protein in normal oxygen conditions and accumulates without being destroyed in hypoxic conditions, by the action of prolyl hydroxylase domain containing protein (PHD) and factor-inhibiting HIF-1 (FIH-1) antigens which are expressed by acting as transcription factors (25,26).

Regarding the site of CA9 antigen expression, a study, in which an antibody recognizing CA9 antigen was created, showed its expression in normal tissues of uterine mucous membrane, gastrointestinal mucous membrane including stomach and intestines, and bile duct. Our study later confirmed its expression in the basal part of skin epithelium, and CA9 antigen expression in the basal part of the oral epithelium was observed, although it was weak. Regarding its expression in cancer tissues, expression was observed in cervical cancer and renal cell carcinoma, since monoclonal antibodies were made using a cervical cancer or renal cell carcinoma cell line as the immunogen. However, recently there have been studies on the expression of CA9 in many solid tumors including bladder cancer, non-small cell lung cancer, breast cancer, esophageal cancer, stomach cancer and sarcoma.

Since, in many studies, patients with CA9 expression had a poor outcome, CA9 has been believed to be a negative prognostic factor (8,10-16). On the contrary, among these solid tumors, there is one study indicating that patients with CA9 expression had a better outcome in renal cell carcinoma (9). Furthermore, CA9 expression was observed in acute myeloid leukemia (AML), a hematological malignancy, and it was reported that patients with CA9 expression had a better outcome than those without CA9 expression; thus, CA9 is considered a positive prognostic factor in AML (17,18). Nonetheless, no study exists on CA9 expression in oral cancers in Japan, and its correlation with outcome is still unknown. Nor is there any case report solely on the oral region in the scientific literature. Two studies noted different positive rates of ~27 and 92% for head and neck cancers (27,28). In our study, we observed CA9 expression in 98% of the subjects, and considered that CA9 has high prevalence in cancers in the oral region. We also examined the correlation between CA9 expression and survival rate, and found that subjects with a positive rate $\geq 50\%$ had a significantly lower survival rate compared to those with a positive rate $< 50\%$. Subjects with poorly differentiated, Stage IV, T4 or high N factor cancer had high CA9 expression ($\geq 50\%$) and a tendency for a poor outcome. These findings confirm that CA9 antigen is highly expressed in oral cancer in the Japanese, and immunotherapy targeting CA9 may be possible in the future. The possibility of using CA9 as a prognostic indicator is suggested, but we believe that its accuracy should be confirmed in studies in a larger number of subjects.

GLUT-1, which regulates glucose uptake by cells and promotes glycolysis (29), is not usually expressed in the epithelium or in benign tumors, but it is expressed in several tumors including head and neck cancers (30-33). There are many studies indicating that the outcome of patients with hypoxia with head and neck cancers is poor (34-36). In the present study, GLUT-1 expression was as high as 98%, but no significant correlation with the survival rate of the subjects was observed. A correlation between GLUT-1 and lymph node metastasis was reported in patients with tongue

squamous cell carcinoma (37), and our study also observed a significant correlation between GLUT-1 expression and lymph node metastasis in oral cancer, demonstrating that GLUT-1 could be an indicator of lymph-node metastasis.

Ki-67 is a nuclear protein expressed in G1, S and G2 phase (38,39), and it adjunctively controls cell proliferation. Therefore, the presence of Ki-67 is considered to be a 'proliferation index' of tumors (39). In oral cancers, patients with tumors with high positivity (92%) or lymph node metastasis had a poor outcome (40), and many studies indicated that Ki-67 could be an important prognostic factor (41-44). Similarly, 92% of subjects showed Ki-67 expression in our study. Furthermore, Ki-67 expression was seen in ~60% of tumor cells, indicating high cell proliferation of oral cancer. However, no significant correlation was observed with survival rate or CA9 expression.

p53 is a known tumor suppressor gene, and an abnormal p53 protein. Due to mutation of encoding genes, it escapes destruction by MDM-2 protein and accumulates so that it can be detected by immunostaining. p53 mutation frequency is believed to be as high as 50% in human cancers (45). One study (46) indicated low expression of p53 in oral cancer, but many other studies (5,47-49) observed expression of p53 in 50% or more tumors. In addition, there are many studies on oral cancer, especially on the correlation between p53 gene mutation, and outcome (50) and the development of oral cancer (51,52). Patients with a high expression rate are treatment-resistant and their outcome with treatment is considered to be poor (53). In this study, we tried to detect mutated p53 genes including those with genetic abnormality by immunostaining, and as a result, 78% of genes in the subjects stained positive, suggesting that many oral cancers have a p53 gene mutation. However, no significant correlation was observed between p53 expression and survival rate.

In conclusion, CA9 antigen was observed in most of the oral cancer patients. Its presence was related to patients' outcome, and it is useful as a target molecule for immunotherapy against oral cancer.

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