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The expression of NMDA receptor 1 is associated with clinicopathological parameters and prognosis in the oral squamous cell carcinoma

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BACKGROUND: Glutamate activates the *N*-methyl-D-aspartate (NMDA) receptors and this receptor is involved in the proliferation and migration of various tumour cells *in vitro*. However, the relationship between NMDA receptor expression and clinical parameters in cancer patients is unclear. Therefore, NMDA receptor 1 (NMDAR1) expression along with its clinical significance was examined in patients with oral squamous cell carcinoma (OSCC).

METHODS: Eighty-one tumour specimens from OSCC patients were used to determine the NMDAR1 expression level by immunohistochemical staining. The control was obtained from a matched normal adjacent mucosa. The cases were considered to be positive if reactivity was displayed in >25% of the cells.

RESULTS: The NMDAR1 reactivity was positive in 50 of 81 cases, while it was negative in the control. NMDAR1 expression was significantly associated with a lymph node metastasis ($P = 0.008$), the tumour size ($P < 0.001$), and the cancer stage ($P = 0.034$). The patients whose tumours expressed NMDAR1 had a significantly poorer survival than the patients who were NMDAR1-negative (log-rank = 6.45, d.f. = 1, $P = 0.011$).

CONCLUSIONS: The NMDAR1 overexpression was significantly associated with the prognosis-related factors. Therefore, it might be one of the prognostic markers of OSCC.

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Keywords: cancer stage; glutamate receptor; *N*-methyl-D-aspartate receptor 1; overall survival

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Introduction

Squamous cell carcinoma (SCC) is the most common cancer of the head and neck and the sixth most frequent cancer in the worldwide (1). Predicting the prognosis and clinical outcomes of patients is very important for the individualized management of oral SCC (OSCC). Until now, several molecular markers have been evaluated for their relationship with the clinicopathological factors in OSCC (2, 3). Among these, epidermal growth factor receptor (EGFR), transforming growth factor (TGF)-α, cyclin D1, and p53 are promising candidates (4). Therefore, more molecular markers need to be found in order to accurately predict the prognosis.

Glutamate is an essential amino acid and a neurotransmitter in the central nervous system (CNS). It activates various glutamate receptors including *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainite, and metabotropic receptors (5, 6). Glutamate plays a major role in excitotoxicity as well as learning and memory of the CNS (7). Although the expression of glutamate receptor has long been known to be restricted to the CNS, a recent study reported that non-neuronal tissues throughout the body have glutamate receptors (8). In addition, glutamate functions as a trophic factor in various cancer cells (9). This suggests that glutamate and its receptor are involved in tumour cell proliferation and migration. However, it has not been demonstrated that glutamate and the glutamate receptors are involved in the carcinogenesis of oral squamous cells.

The aim of this study was to investigate the expression profile of the NMDA receptor 1 (NMDAR1) as well as the relationship between its expression level and the various clinicopathological factors in OSCC.

Patients and methods

Patients

Eighty-one OSCC patients were examined in this study (mean age: 59.22 ± 12.96 years). The patients were

Table 1 Clinical characteristic of 81 patients with oral squamous cell carcinoma

Variable	*N (%)*
Gender	
Male	55 (67.9)
Female	26 (32.1)
Smoking	
No	33 (40.7)
Yes	48 (59.3)
Alcohol	
No	20 (24.7)
Yes	61 (75.3)
Site	
Tongue	29 (35.8)
Floor of mouth	6 (7.4)
Buccal mucosa	6 (7.4)
Gingiva	23 (28.4)
Retromolar trigone	6 (7.4)
Palate	3 (3.7)
Lip	6 (7.4)
Maxillary sinus	2 (2.5)

hospitalized and treated with surgery or surgery plus postoperative radiation therapy at Hallym University and the Oral Oncology Clinic of National Cancer Center in Korea. The control samples were taken from the normal mucosa in the resection margin. The clinicopathological data, including age, gender, alcohol intake, smoking, histological grade, cancer staging, and survival were collected from the clinical records. The average follow-up was 34.5 months, ranging from 6 to 60 months. The tumour was clinically classified according to the cancer staging system defined by the UICC (10). The grade of tumour differentiation was determined according to the criteria of the WHO histological typing of oral and oropharyngeal tumours (11). The clinicopathological profiles of the 81 patients are summarized in Table 1.

Immunohistochemistry

Paraffin-embedded tissue sections (5 μm thickness) were made and dewaxed sections were immersed for 15 min in 10 mM sodium citrate buffer (pH 6.0) at 100°C for antigen retrieval. In order to block the endogenous peroxidase, the sections were placed in 0.5% hydrogen peroxide in methanol for 30 min and rinsed in distilled water and followed by rinsing in phosphate-buffered saline (PBS). The sections were then incubated with a 1:100 dilution of the antibody against the glutamate receptor subtype, NMDAR1 (Zymed, San Francisco, CA, USA) overnight at 4°C in a humidified chamber. After 12 h, the slides were rinsed in PBS and incubated with the second antibody (1:500; Dako, Copenhagen, Denmark) for 30 min, and again rinsed and incubated with the Streptavidin biotin peroxidase reagents (Strept-ABC complex; Dako) for 30 min. The sections were visualized using diaminobenzine hydrochloride (DAB). Finally, the sections were rinsed in distilled water, and counterstained with Mayer's haematoxylin. As negative controls, the sections were treated with PBS instead of the primary antibody. As a positive control, the human brain cortex was used for the detection of NMDAR1.

Evaluation of samples

The localization of NMDAR1 was visualized using an Olympus microscope (Olympus, Japan) under ×100 magnification. The criteria for evaluation were referenced from the published article (12). The staining intensity was scored as being weak, moderate or strong. Positive cells were defined as those with a moderate or strong intensity. The entire tissue section was examined. NMDAR1 expression in the cancer was defined as being negative if the positive cells comprised 0–25%, and positive when the proportion of positive cells comprised >25%. Two investigators, who were unaware of the clinical information, examined all the stains independently.

Statistical analysis

The difference in NMDAR1 expression between the control and the cancer samples was compared using a paired sample *t*-test. The clinicopathological factors were dichotomized for bivariate analysis. A Mann–Whitney *U*-test was used to determine the association between NMDAR1 expression and the clinicopathological factors. The statistical significance of the coefficients in the logistic regression models was tested using the Wald statistics and a *P*-value of <0.05 was used to determine which variables were to be included in the regression model. The odds ratios and confidence intervals were calculated from the regression coefficients. The cause-specific survival rates were evaluated using the Kaplan–Meier method and compared using the log-rank test. The factors found to be significant were chosen for a stepwise Cox's multivariate proportional hazard model in order to evaluate their prognostic values.

Results

Immunohistochemical analysis of NMDAR1

All the samples from normal oral mucosa showed weak immunoreactivity in the basal layer of the oral epithelium and were defined as negative (Fig. 1a). NMDAR1 immunoreactivity was found to be negative in 31 cases (Fig. 1b), and positive in 50 cases (Fig. 1c). NMDAR1 expression was distributed in the cytoplasm of most of the cancer cells. The difference between the cancer specimens and control was significant ($P < 0.001$).

The relationship between clinicopathological parameters and the expression level of NMDAR1

The clinicopathological parameters investigated in this study were age, gender, tumour site, smoking, alcohol drinking, tumour size, nodal metastasis, and histological grade. There was no statistically significance between NMDAR1 expression and age, gender, and the histological grade (Table 2). In addition, the tumour site, smoking or alcohol drinking was not significantly related to NMDAR1 expression level (data not shown). However, a lymph node metastasis, the tumour size, and the cancer stage were significantly related to NMDAR1 expression ($P < 0.05$, Table 2).

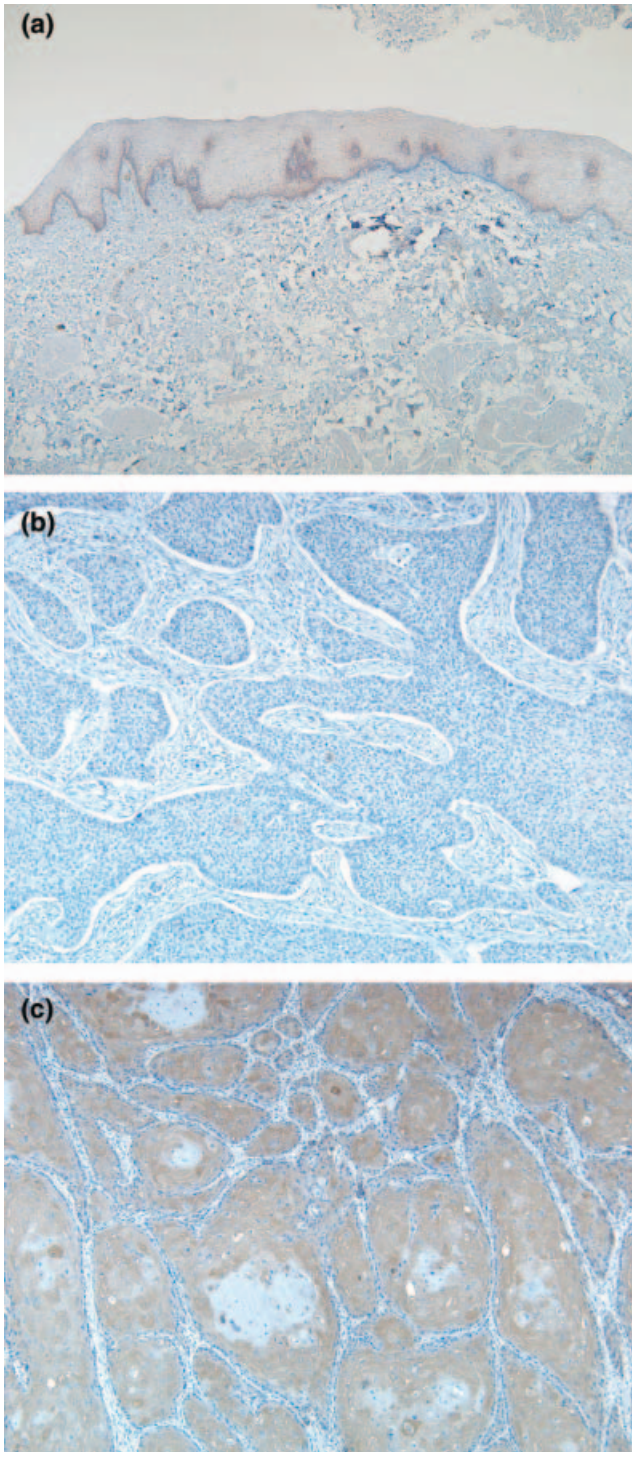

Figure 1 Immunohistochemical staining of the glutamate receptor subtype, *N*-methyl-D-aspartate receptor 1 (NMDAR1), in the normal oral mucosa and oral squamous cell carcinoma (OSCC). (a) NMDA1 expression in the normal oral mucosa is very weak and is observed only in the basal layer (original magnification ×40). (b) The weak NMDAR1 expression in OSCC (original magnification ×100). (c) The strong NMDAR1 expression in OSCC (original magnification ×100).

Table 3 shows the association between NMDAR1 expression and the clinicopathological factors of the 81 patients. Logistic regression analysis showed that the T2, T3, and T4 cases were 9.57 times more likely to be NMDAR1-positive in OSCC than those in the T1 group ($P < 0.0001$). The patients who had a regional lymph node metastasis were 3.53 times more likely to be NMDAR1-positive in OSCC than those who did not have a lymph node metastasis ($P = 0.0086$). The stage III/IV cancers (stage III/IV) were 2.74 times more likely to be NMDAR1-positive in OSCC than the stage I/II cancers ($P = 0.0347$).

Using Kaplan–Meier analysis, those patients whose tumours expressed NMDAR1 had a significantly poorer survival than those patients who were NMDAR1-negative (log-rank = 6.45, d.f. = 1, $P = 0.0111$; Fig. 2).

The Cox forward stepwise regression multivariate survival analysis of the clinicopathological parameters, and NMDAR1 expression revealed that overall stage entered at the first step (Wald = 4.7718, RR = 5.0993, 95% CI: 1.1822–21.9941; $P = 0.0289$), and NMDAR1 expression entered at the second step (Wald = 3.9260, RR = 3.0535, 95% CI: 1.0122–9.2120; $P = 0.0475$). The other parameters did not enter the equation.

Discussion

This study is the first to demonstrate that OSCC overexpresses the glutamate receptor, NMDAR1, and the up-regulation of NMDAR1 is well-correlated with the TN stages in OSCC. This indicates a new role of glutamate and its receptor in tumour progression.

The NMDA receptor has two distinct subunits: the main subunit, NMDAR1, and modulatory subunit, NMDA2A-2D (13). NMDAR1 is essential and sufficient to form an active NMDA receptor channel, which is in contrast to the NMDAR2 subunits (14). This study investigated NMDAR1 expression. These receptors are glutamate-gated ion-channels that are characterized by a very high Ca^{2+} conductance (15).

In these results, the normal oral mucosa showed only mild immunoreactivity in the basal layer of the oral epithelium while it showed strong or moderate immunoreactivity in 50 of 81 OSCC cases. The difference in the immunoreactivity between the cancer and normal tissues was significant ($P < 0.001$). The meaning of the up-regulation of NMDAR1 in OSCC is unclear at this point with respect to the cancer characteristics such as cell proliferation, apoptosis, angiogenesis and metastasis.

Recent research has been focused on the trophic function of glutamate in developing mammals and cancer. It regulates the proliferation, migration, and survival of the neuronal progenitors (16, 17) and cancer cells (9, 15). Therefore, glutamate and its receptor might provide a new therapeutic target in cancer treatment.

Many prognostic markers have been evaluated with respect to the various clinicopatholigical parameters. EGFR, TGF-α, cyclin D1, and p53 have been reported to be good prognostic markers (18). The cases were positive to the vascular endothelial cell growth factor-C also had a poor prognosis in OSCC (12). When the expression of p53, Bcl-2, Cyclin D1, *c-myc*, p21ras,

Table 2 Association between NMDAR1 and clinicopathological factor

Factors	NMDAR1 Negative (%) (n = 31)	NMDAR1 Positive (%) (n = 50)	P-value[a]
Age (year)			NS
<65	14 (30.4)	32 (69.6)	
≥65	17 (48.6)	18 (51.4)	
Sex			NS
Male	22 (40.0)	33 (60.0)	
Female	9 (34.6)	17 (65.4)	
Tumour size			
T1 (<2 cm)	21 (70.0)	9 (30.0)	0.000
T2, T3 and T4 (≥2 cm)	10 (19.6)	41 (80.4)	
Lymph node metastasis			
Present	11 (25.0)	33 (75.0)	0.008
Absent	20 (54.1)	17 (45.9)	
Tumour differentiation			NS
Well	19 (33.9)	37 (66.1)	
Moderate/poorly	12 (48.0)	13 (52.0)	
Cancer stage			0.034
I/II (30)	16 (53.3)	14 (46.7)	
III/IV (51)	15 (29.4)	36 (70.6)	

[a]Mann–Whitney *U*-test; NS, not significant; NMDAR1, *N*-methyl-D-aspartate receptor 1.

Table 3 Logistic regression analysis in NMDAR1 expression

Factors	Odd ratio	95% confidence level	P-value
Age (<65/≥65)	0.4633	0.1859–1.1545	NS
Sex (male/ female)	1.2593	0.4766–3.3268	NS
Tumour size (T1/T2, T3 and T4)	9.5659	3.3714–27.1417	0.0000
Lymph node (present/absent)	3.5290	1.3785–9.0344	0.0086
Differentiation (well/moderate, poorly)	0.5563	0.2130–1.4529	NS
Cancer stage (I, II/III, IV)	2.7429	1.0750–6.9981	0.0347

NS, not significant; NMDAR1, *N*-methyl-D-aspartate receptor 1.

c-erb B2, cytokeratin-19, and the factor VIII-related antigen in SCC of the tongue are compared, *c-myc* expression was the second significant prognostic factor next to the cancer stages (19). However, there are few reports that show a clear correlation between expression of the marker and the cancer stages in OSCC. The T and N stage in gingival SCC are significant predictors of survival (20). Therefore, the cancer stage is an important factor for predicting the prognosis.

For the statistical analysis, the tumour size was divided into two groups. T1 was one group and T2, T3 and T4 comprised the other. This grouping improved the statistical significance and T2 of the UICC classification (10) in the oral cavity cancer was T3 or T4 in the new classification proposed in the DÖSAK study (21). The T2, T3 and T4 cancers were 9.57 times more likely to be NMDAR1-positive in OSCC than the T1 cancers ($P < 0.0001$). The cancers that had a regional lymph node metastasis were 3.53 times more likely to be NMDAR1-positive in OSCC than the cancers that did not have a regional lymph node metastasis

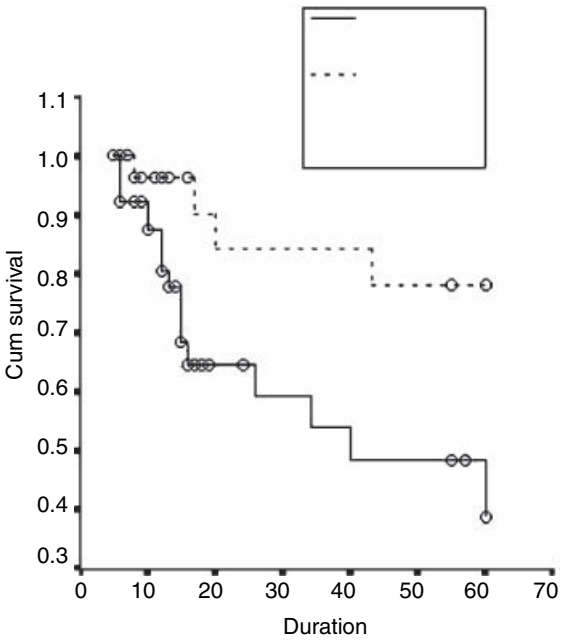

Figure 2 Kaplan and Meier survival curves of the patients showed that the tumours expressing *N*-methyl-D-aspartate receptor 1 (NMDAR1) had an unfavourable relapse-free survival than the tumours not expressing NMDAR1.

($P = 0.0086$). The stage III/IV cancers (stage III/IV) were 2.74 times more likely to be NMDAR1-positive in OSCC than the stage I/II cancers ($P = 0.0347$).

Using Kaplan–Meier analysis, the patients whose tumours expressed NMDAR1 had a significantly poorer survival than the patients who were NMDAR1-negative (Fig. 2). The Cox forward stepwise regression multivariate survival analysis showed that the cancer stage was the most significant prognostic factor followed by NMDAR1 expression. However, the other variables did not showed statistical significance. Although cancer stage was the most powerful prognostic factor, patients who had the same cancer stage might show a different clinical outcome. Therefore, it is important to identify new prognostic factors that can predict the biological aggressiveness of the cancer. NMDAR1 expression is a useful prognostic indicator and would supplement cancer staging for predicting the clinical features as well as the outcome.

These results suggest an interesting possibility that NMDAR1 overexpression is involved in cell proliferation. In a recent study, when a NMDAR blocker was administered, some OSCC cell lines (SCC-4, KB-9) showed the inhibition of cellular proliferation in a dose-dependent manner (date not shown). A further study will be needed to clarify the relationship between glutamate and its receptor and cell proliferation.

In conclusion, overexpression of the glutamate receptor, NMDAR1, significantly correlated with the tumour size, lymph node metastasis, and the cancer stage. Therefore, it is expected that NMDAR1 will be a useful marker for determining the prognosis of OSCC.

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