

# Clinical significance of metabotropic glutamate receptor 5 expression in oral squamous cell carcinoma

SO-YEON PARK<sup>2\*</sup>, SEOUNG-AE LEE<sup>1\*</sup>, IN-HEE HAN<sup>1</sup>, BYONG-CHUL YOO<sup>1</sup>,  
SEUNG-HOON LEE<sup>1</sup>, JO-YONG PARK<sup>1</sup>, IN-HO CHA<sup>3</sup>, JIN KIM<sup>4</sup> and SUNG-WEON CHOI<sup>1</sup>

<sup>1</sup>Research Institute and Hospital, National Cancer Center, Goyang, Gyeonggi; <sup>2</sup>Department of Pathology, College of Medicine, Seoul National University; Departments of <sup>3</sup>Oral and Maxillofacial Surgery, <sup>4</sup>Oral Pathology, College of Dentistry, Yonsei University, Seoul, Korea

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**Abstract.** The multifunctional G-protein-coupled metabotropic glutamate receptor (mGluR) family comprises eight subtypes, some of which participate in tumorigenesis. The purpose of this study was to evaluate mGluR5 expression in oral squamous cell carcinoma (SCC) tissues and oral cancer cell lines. We also investigated the prognostic significance of mGluR5 and its functional importance in the migration, invasion, and adhesion of oral cancer cells. We evaluated the expression of mGluR5 in samples from 131 oral SCC patients and in several oral cancer cell lines by immunohistochemistry and RT-PCR. We observed varying levels of mGluR5 in human oral SCC tissues and cancer cell lines. There was a significant association between strong mGluR5 immunoreactivity and overall survival ( $P=0.0109$ ). The functional significance of the expression of mGluR5 in oral cancer cells was then investigated in HSC3 oral tongue cancer cells. An mGluR5 agonist, DHPG increased tumor cell migration, invasion, and adhesion in HSC3 cells ( $P<0.05$ ). This was reversed by the mGluR5 antagonist MPEP. Our results strongly suggest that mGluR5 is a new prognostic marker and contributes to tumor cell migration and invasion in oral cancer.

## Introduction

Squamous cell carcinoma (SCC) is the most common cancer of the head and neck and is the sixth most frequent cancer worldwide (1). Oral SCC, frequently observed at various

subsites in head and neck cancer, is characterized by a high degree of local invasion, cervical lymph node metastasis, and local recurrence after initial treatment, possibly due to micro-invasion and/or micrometastasis (2). Clinical staging and cervical lymph node metastasis have been used as prognostic indicators in the management of oral cancer, but they have limited predictive value. Novel prognostic markers for assessing the biological aggressiveness of tumors are needed and should facilitate the individual tailoring of treatment.

There is increasing evidence that tumor growth, invasion, and metastasis are regulated by neurotransmitters that bind to serpine receptors, such as dopamine, somatostatin, substance P, calcitonin gene-related peptide, neuropeptide Y, and glutamate (3). Glutamate was originally identified as an excitatory neurotransmitter in the mammalian central nervous system, where it activates both ionotropic and metabotropic glutamate receptors. Ionotropic receptors include ligand-gated ion channels within the plasma membrane, whereas metabotropic glutamate receptors (mGluRs) are coupled to G-proteins and include eight subtypes (4). Abnormal glutamate signaling plays a critical role in the development and progression of diverse neurological disorders, such as Alzheimer's and Parkinson's diseases (5,6).

Although glutamate and its receptors are predominantly found within the central nervous system, they also exist in non-neuronal cells outside the brain (7,8). Glutamate signaling has been implicated in the growth and migration of various non-neuronal cancers (9-11). Rzeski and colleagues demonstrated that ionotropic glutamate receptor antagonists limit cell proliferation in various non-neuronal cancers, including colon, breast, lung, and thyroid (12). In addition, in two recent reports on melanoma tumorigenesis, Chen *et al* showed that the metabotropic glutamate receptor 1 (mGluR1) is ectopically expressed in a mouse model of TG3 melanoma (13,14). Other studies have shown that mGluR1 and mGluR5 are up-regulated in metastatic melanoma and lung cancer, respectively (15). These reports imply that metabotropic glutamate receptors are important in tumor progression, but there is currently no direct evidence for a role of mGluR1 and mGluR5 in oral cancer, even though glutamate receptors have been found in peripheral tissues and in different types of cancer.

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*Correspondence to:* Dr Sung-Weon Choi, Research Institute and Hospital, National Cancer Center, 809 Madu-1dong, Ilsandong-gu, Goyang-si, Gyeonggi-do 411-769, Korea  
E-mail: choiomfs@ncc.re.kr

\*Contributed equally

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Although oral SCC has a different origin than melanoma or lung cancer, we extended these studies to oral cancer by examining the expression of mGluR1 and mGluR5 in oral cancer tissues and cell lines. Since we did not detect mGluR1 in oral cancer tissues and cell lines, we focused on the role of mGluR5 in oral cancer cell migration, invasion, and adhesion and the relationship between its expression and prognosis.

## Materials and methods

**Cell culture.** Cal27 and KB human oral squamous cell carcinoma cell lines were obtained from American Type Cell Collection (Manassas, VA, USA). HSC-3, the human oral tongue squamous cell carcinoma cell line, was purchased from Japanese Cell Resource Bank. YD-17 and YD-10B oral squamous cell carcinoma cell lines were kindly donated by the Department of Oral Pathology, Dental College, Yonsei University (16). Cal27, KB and HSC-3 were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Brooklyn, MA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. YD-17 and YD-10B were cultured in a 1:3 mixture of DMEM and F12. All cultures were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

To evaluate the possible role of the mGluR5, we used mGluR5 agonist (DHPG, 3,5-dihydroxyphenylglycine) and antagonist (MPEP, 2-methyl-6-(2-phenyl-1-ethynyl)pyridine), which were purchased from Tocris (Bristol, UK).

**Tumor specimens.** Eight oral squamous cell carcinoma tissues were harvested at surgery and immediately snap frozen at -80°C until RNA extraction. Institutional Review Board-approved, written informed consent was obtained from all the eight patients donating specimens for RNA extractions, through the Oral Cancer Clinic, National Cancer Center. In total, 131 oral squamous cell carcinoma tissue samples were obtained from patients who underwent primary surgery between 1996 and 2000 at the Department of Oral and Maxillofacial Surgery, College of Dentistry, Yonsei University. All specimens were fixed in formalin and paraffin-embedded. Carcinomas were morphologically classified according to WHO specifications. Staging was based on the TNM classification of the American Joint Committee of Cancer (AJCC) criteria of 1997. In all cases, final staging was adjusted on the basis of postoperative pathological reports. The clinical outcomes of the oral cancer patients were followed until death or August 31, 2005 and median follow-up time was 76 months (range 3-118 months).

**Reverse transcriptase-polymerase chain reaction (RT-PCR).** Total RNA was isolated from cells and tumor tissues with a RNeasy mini kit (Qiagen Sciences, MD, USA). RNA samples were stored at -80°C until use. Reverse transcription was performed with 1  $\mu$ l total RNA using the SuperScript First-Strand Synthesis System (Invitrogen). To determine mGluR1 mRNA expression, 2  $\mu$ l cDNA was amplified with 1.25 units of Takara EX Taq™ (Takara Bio Inc., Japan) in a 25  $\mu$ l reaction containing 0.2 mmol/l dATP, dCTP, dGTP, and dTTP, and 2 mmol/l MgCl<sub>2</sub>. PCR was performed for 30 cycles (1 min at 95°C, 45 sec annealing at 58°C, 2 min at 72°C). The

following primers were employed for amplification of mGluR1: amplicon 453 bp; forward, 5'-ACC CGG TCC TCC TGC CCA ACA-3', and reverse, 5'-CGT CCA TTC CGC TCT CCC CAT AA-3'. Electrophoresis was performed by loading 10  $\mu$ l of each sample on a 1.5% agarose gel, and proteins visualized by ethidium bromide staining using the Bio-imaging System (Ultra-Violet Products, UVP, Cambridge, UK).

**Western blot analysis.** Cells were washed twice with PBS, and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) for the detection of mGluR5 protein. Proteins were separated by SDS polyacrylamide gel electrophoresis (7.5%), and transferred to nitrocellulose membrane (BioRad, Italy) for 1 h. Filters were blocked overnight in Tween-20 Tris-buffered saline (TTBS; 100 mM Tris-HCl, 0.9% NaCl, 1% Tween-20, pH 7.4) containing 4% non-fat dry milk. Blots were incubated for 2 h at room temperature with primary antibody against mGluR5 (1:1000, Upstate Biotechnology, Lake Placid, NY, USA), washed 3 times with TTBS buffer, and incubated for 1 h with peroxidase-coupled anti-rabbit secondary antibody (Upstate Biotechnology). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) according to the specifications of the manufacturer (Amersham Biosciences, Piscataway, NJ).

**Immunohistochemistry.** Sections (4  $\mu$ m) were deparaffinized, rehydrated in graded alcohol, and processed using the avidin-biotin immunoperoxidase method. Briefly, antigen was retrieved by exposure in a microwave oven for 15 min in 10 mM citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked with a 3% H<sub>2</sub>O<sub>2</sub>-methanol solution, and slides incubated in 10% normal goat serum for 30 min to prevent non-specific staining. Sections were incubated with a primary polyclonal antibody against mGluR5 (1:100 dilution, Upstate Biotechnology) for 1 h at room temperature, followed by treatment with biotinylated secondary antibodies and avidin-biotin peroxidase complexes for 30 min. Diaminobenzidine was used as the chromogen and Mayer's hematoxylin as counterstain. As a negative control, non-immune serum was substituted for the primary antibody. Human brain cortex was employed as a positive control. The specificity of mGluR5 antibodies has been reported (17). Both membranous and/or cytoplasmic expression of mGluR5 were regarded as positive. Immunostaining was evaluated by two pathologists. Based on the percentage of immunostained cancer cells and staining intensity, the following grades were issued: 0 (negative), no or weak staining in <10% of tumor cells; 1+ (weakly positive), weak to moderate staining in >10% of tumor cells; 2+ (strongly positive), strong staining in >10% of tumor cells.

**Flow cytometry.** HSC3 cells was adjusted to a concentration of 2x10<sup>7</sup> cells/ml in FACS buffer (PBS containing 0.5% BSA and 0.05% sodium azide). The cells were incubated for 1 h at room temperature with primary anti-mGluR5 polyclonal antibody (Upstate Biotechnology) and washed with PBS and followed by incubation with secondary Alexa™ 488-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Negative

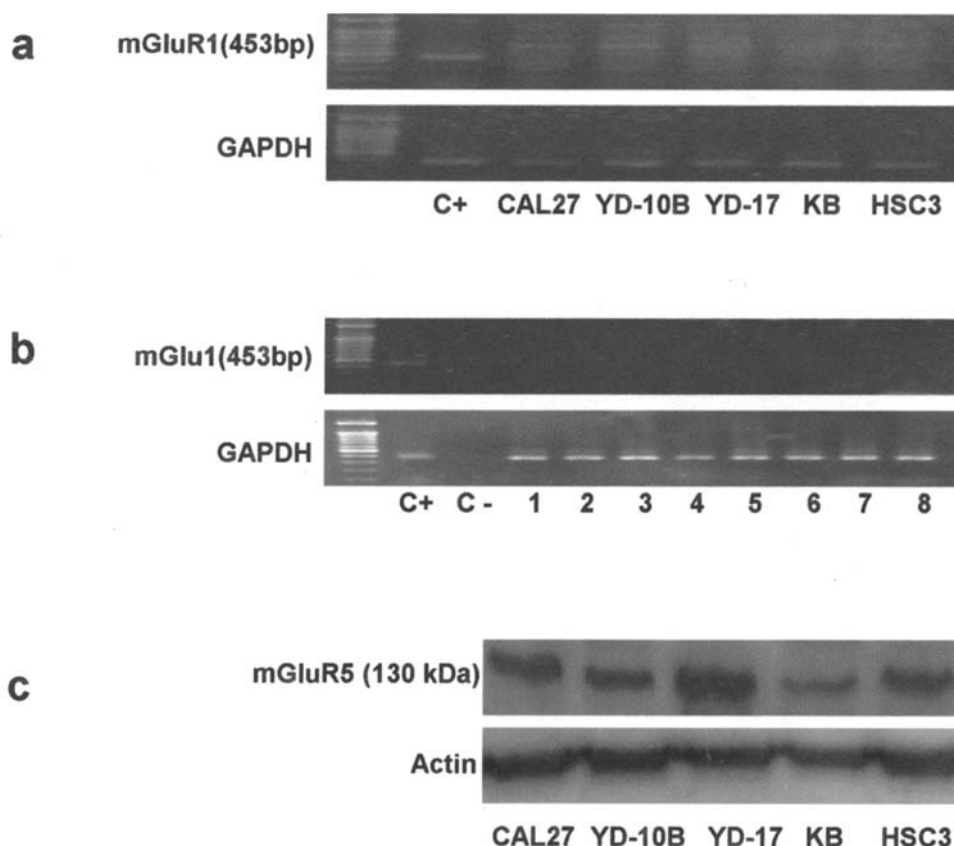


Figure 1. Expression of mGluR1 and mGluR5 in oral cancer cell lines and tissues. Transcripts for mGluR1 were not detected in oral cancer cells (a) and oral cancer tissues (b) by RT-PCR. C+, human cerebral cortex (positive control); C-, diethyl pyrocarbonate-treated water H<sub>2</sub>O (negative control); lanes 1-8, tumor tissue samples from eight patients. (c) Expression of mGluR5 protein in the cell lines was confirmed by Western blot analysis.

controls consisted of cells in the absence of primary antibody. Fluorescence was detected by flow cytometry on a FACSCalibur (Becton Dickinson, Heidelberg, Germany) and analysed using Cell Quest software.

**Immunofluorescence staining of mGluR5.** Cells were plated on glass cover slips for 24 h. After washing with cold PBS, samples were fixed with 3.8% paraformaldehyde, permeabilized with cold 0.2% Triton X-100 for 10 min at room temperature, and washed in PBS. Cells were incubated for 1 h at room temperature with primary anti-mGluR5 polyclonal antibody (Upstate Biotechnology), and washed twice with PBS, followed by incubation with secondary Alexa 488-labeled goat anti-rabbit IgG antibody (Molecular Probes) for 1 h at room temperature. Confocal microscopy was performed using LSM 50 (Zeiss, Oberkochen, Germany).

**Migration assay.** Migration of HSC3 cells were assayed using 6.5 mm Transwell chambers with 8  $\mu$ m pore filters (Transwell, 24-well cell culture, Coster, Cambridge, MA). Cells ( $1 \times 10^6$ ) were resuspended in DMEM containing serum-free 0.1% BSA, and added to the upper compartment of each Transwell. Next, 10, 50, 100  $\mu$ M DHPG with or without MPEP was added to the lower compartment of each Transwell, while 0.5 ml 0.1% BSA-containing media were added to the lower chamber. After 17 h of incubation at 37°C, cells on the upper surface of the filter were removed with a cotton swab, and filters were fixed and stained with Diff-Quick reagent (Dade Behring, Dugen,

Switzerland). The number of migrating cells in 10 fields per filter was counted microscopically at x100 magnification.

**Invasion assay.** Invasion of cancer cells was assayed using a Biocat Matrigel invasion chamber (Becton Dickinson, Bedford, MA), which consists of an 8  $\mu$ m pore size polyethylene terephthalate (PET) membrane that has been overlaid with Matrigel (basement membrane matrix). HSC-3 cells ( $1 \times 10^6$ ) were resuspended in DMEM containing serum-free 0.1% BSA, and added to the upper compartment of each Transwell. Next, 10, 50, 100  $\mu$ M DHPG with or without MPEP was added to the lower compartment of each Transwell, while 0.5 ml 0.1% BSA-containing media were added to the lower chamber. After 17 h of incubation at 37°C, cells on the upper surface of the filter were removed with a cotton swab, and filters were fixed and stained with Diff-Quick reagent (Dade Behring). The number of migrating cells in 10 fields per filter was counted microscopically at x100 magnification.

**Adhesion assay.** The adhesion of oral cancer cells to the elements of extracellular matrix (ECM) was evaluated. Oral cancer cells were grown to subconfluent state and then harvested by 0.25% trypsin/EDTA (Invitrogen Corp., Carlsbad, CA). Cells were preincubated with DHPG or with DHPG plus MPEP for 3 h. Preincubated cells were plated onto a 96-well microplate which was precoated with various ECM elements, that is, 0.01 mg/ml fibronectin, collagen IV (Becton-Dickinson Japan, Todyo, Japan). Then the cells were incubated

for 2 h at 37°C in 5% CO<sub>2</sub>/95% air to allow cell attachment. Cells were washed gently with PBS 3 times to remove detached cells. The number of adherent cells was measured by MTT assay. Experiments were repeated 3 times in triplicate wells.

**Statistical analysis.** The association between immunohistochemical staining grade and clinicopathological factors was assessed with the  $\chi^2$  and ANOVA tests. To establish the overall survival rate related to mGluR5 expression, the Kaplan-Meier method was employed for survival analysis. Differences in survival rate were evaluated using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression model. Differences in cell migration, invasion and adhesion were assessed with the unpaired Student's t-test.  $P < 0.05$  was considered significant in all statistical analyses.

## Results and Discussion

To examine the role of mGluR1 and mGluR5 in oral cancer, we first examined their expression in oral cancer cells and SCC tissues by RT-PCR. We were not able to detect mGluR1 transcripts in oral cancer cells (Fig. 1a and b). Further examination of five human oral cancer cell lines by Western blotting showed that they express various amounts of mGluR5 protein (Fig. 1c).

Immunohistochemical staining of oral SCC tissues showed no or very weak expression of mGluR5 in the basal layer of normal oral squamous cell epithelium (Fig. 2a). In oral cancer tissues, mGluR5 immunoreactivity was localized specifically in carcinoma cells (Fig. 2c-e). Moreover, the dysplastic mucosa adjacent to the main cancer lesion displayed weak staining of membranes that was confined to dysplastic cells (Fig. 2b). The staining for mGluR5 was mostly concentrated in the cell membrane, but cytoplasmic staining was observed in some specimens. Of the 131 cases of oral cancer examined, 37 (28%) displayed negative mGluR5 expression, 52 (40%) were weakly positive (1+), and 42 (32%) exhibited strongly positive expression (2+) of mGluR5.

Chen *et al* reported that, in melanoma, ectopically expressed mGluR1 participates in tumorigenesis and that mGluR5 is not oncogenic (13,14). Herein we found that only mGluR5 is expressed in oral cancer cells and tumors. This suggests that different mGluRs participate in the progression of different types of cancer. Microarray analysis has demonstrated that mGluR5 is overexpressed in lung adenocarcinoma (15). The current study is the first to examine the expression of mGluR5 in human cancer tissues.

Interestingly, we found a correlation between strong positive expression of mGluR5 and T stage T3/T4 ( $P < 0.0001$ ) and AJCC stage III/IV ( $P < 0.0001$ ). In contrast, mGluR5 expression was not significantly associated with smoking, sex, age, tumor differentiation, or lymph node metastasis (Table I). A significant association between mGluR5 expression and advanced AJCC stages (III and IV) suggests that mGluR5 is involved in progression of the primary tumor, consistent with recent reports that glutamate and its receptors are involved in tumor growth *in vitro* (10). Univariate analysis revealed that increased expression of mGluR5 correlated significantly

Table I. Correlation between mGluR5 expression and clinicopathological factors.

	mGluR5			P
	Negative	1+	2+	
Age (years)				
<60	21	25	19	P=0.315
≥60	16	27	23	
Sex				
Male	25	41	33	P=0.270
Female	12	11	9	
Tumor differentiation				
Well	24	28	26	P=0.697
Moderate/poorly	13	24	16	
Tumor size				
T1/T2	26	35	12	P<0.0001
T3/T4	11	17	30	
Lymph node metastasis				
Absent	28	27	24	P=0.108
Present	9	25	18	
Stage				
I/II	22	21	6	P<0.0001
III/IV	15	31	36	

(log-rank,  $P = 0.0109$ ) with a decreased 5-year survival rate compared with cases showing negative expression of mGluR5 (Fig. 3). In addition, the multivariate Cox proportional hazards model showed that there was a significant association between poor survival and lymph node metastasis ( $P = 0.016$ ) and mGluR5 expression ( $P = 0.026$  and  $0.038$  for 1+ and 2+ levels of mGluR5 vs. mGluR5-negative samples) but that there was not a significant association between poor survival and age, sex, primary site, or T stage (Table II). Close follow-up studies and aggressive treatment may be necessary in patients expressing high levels of mGluR5, but the mechanism underlying the poor prognosis in these patients remains to be determined.

We also performed a variety of functional studies to determine the phenotype of mGluR5-expressing oral cancer cells. We examined the effects of an agonist, DHPG and an antagonist, MPEP, of mGluR5 on the migration, invasion, and adhesion of HSC3 oral tongue cancer cells. Treatment of the cells for 24 h with 10, 50, or 100  $\mu$ M DHPG agonist did not affect their proliferation, and a 24-h treatment with 100  $\mu$ M DHPG had no effect on their viability or cell proliferation (data not shown). As shown in Fig. 4b and c, DHPG dose-dependently enhanced the migration and invasiveness of the HSC3 cells ( $P < 0.05$ ). This was inhibited by MPEP ( $P < 0.05$ ). In addition, DHPG dose-dependently stimulated the adhesion of the cells to collagen type IV and fibronectin (Fig. 4d and e). Cancer cells invade tissues by first migrating from their primary sites. Thus, the enhancement of cell migration,



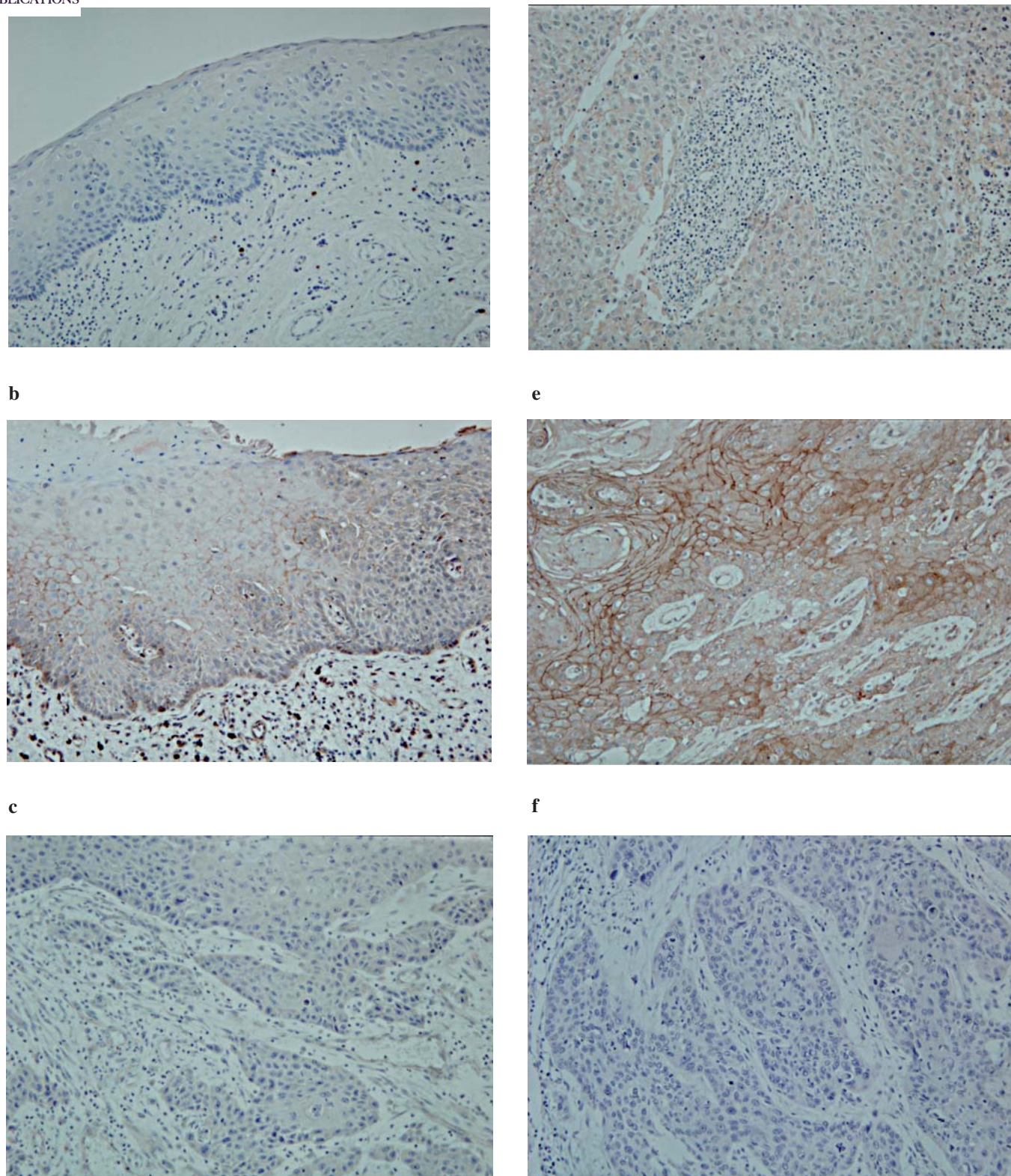


Figure 2. Immunohistochemical analysis of mGluR5 expression in human oral SCC tissues. (a) Normal oral epithelium showing very weak staining in the basal layer and discrete staining of melanocytes. (b) A dysplastic lesion adjacent to the cancer lesion. (c) Oral SCC displaying negative mGluR5 expression. (d) Oral SCC with weakly positive expression (1+). (e) Oral SCC showing strongly positive expression (2+) localized predominantly in the cell membrane, although cytoplasmic staining is observed in some cases. (f) Negative control. Original magnification, x200.

invasion, and adhesion of oral cancer cells with DHPG supports the idea that mGluR5 signaling is important in the invasion and metastasis of oral cancer.

The mGluRs are members of the large family of seven-transmembrane domain G protein-coupled receptors, which can be divided into three subtypes according to their sequences and

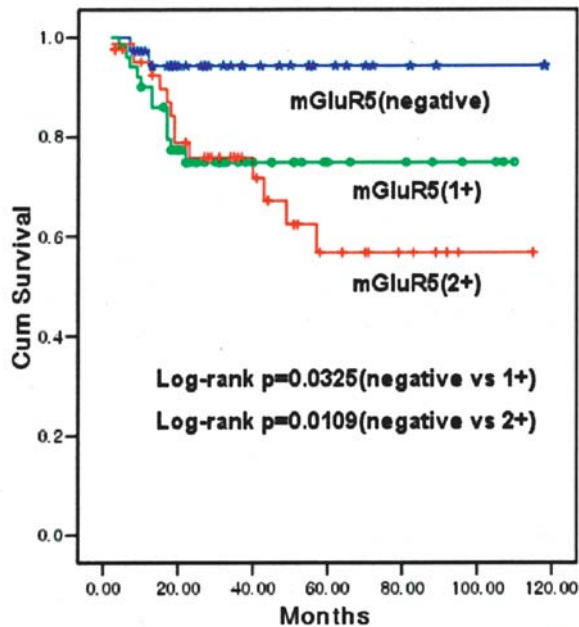


Figure 3. Kaplan-Meier survival curve of oral SCC patients in relation to mGluR5 immunoreactivity. Patients with oral SCC tissues strongly positive for mGluR5 expression have a decreased survival rate compared to those with tissues showing negative expression of mGluR5 ( $P=0.0109$ ).

intracellular effector systems (18). Both mGluR1 and mGluR5 are members of Group I mGluRs, proteins that are coupled to multiple intracellular signaling pathways, including phospholipase C, which stimulates the production of inositol 1,4,5-triphosphate and diacylglycerol, leading to the activation of protein kinase C (13). These downstream signaling pathways may contribute to the migration and invasive phenotype of mGluR5-expressing cells in oral cancer. Although there is limited evidence that mGluR5 participates in tumor progression, various G-protein coupled receptors are known to be ectopically expressed, overexpressed, or mutated in some tumors (19). These studies and our current results indicate that mGluR5 may be a novel therapeutic target in various cancers.

In conclusion, we found that mGluR5 is not only frequently overexpressed in oral SCC but also may be involved in tumor progression and that its expression may be linked to prognosis. Thus, mGluR5 may be a new prognostic marker and may play a role in the invasiveness of oral SCC.

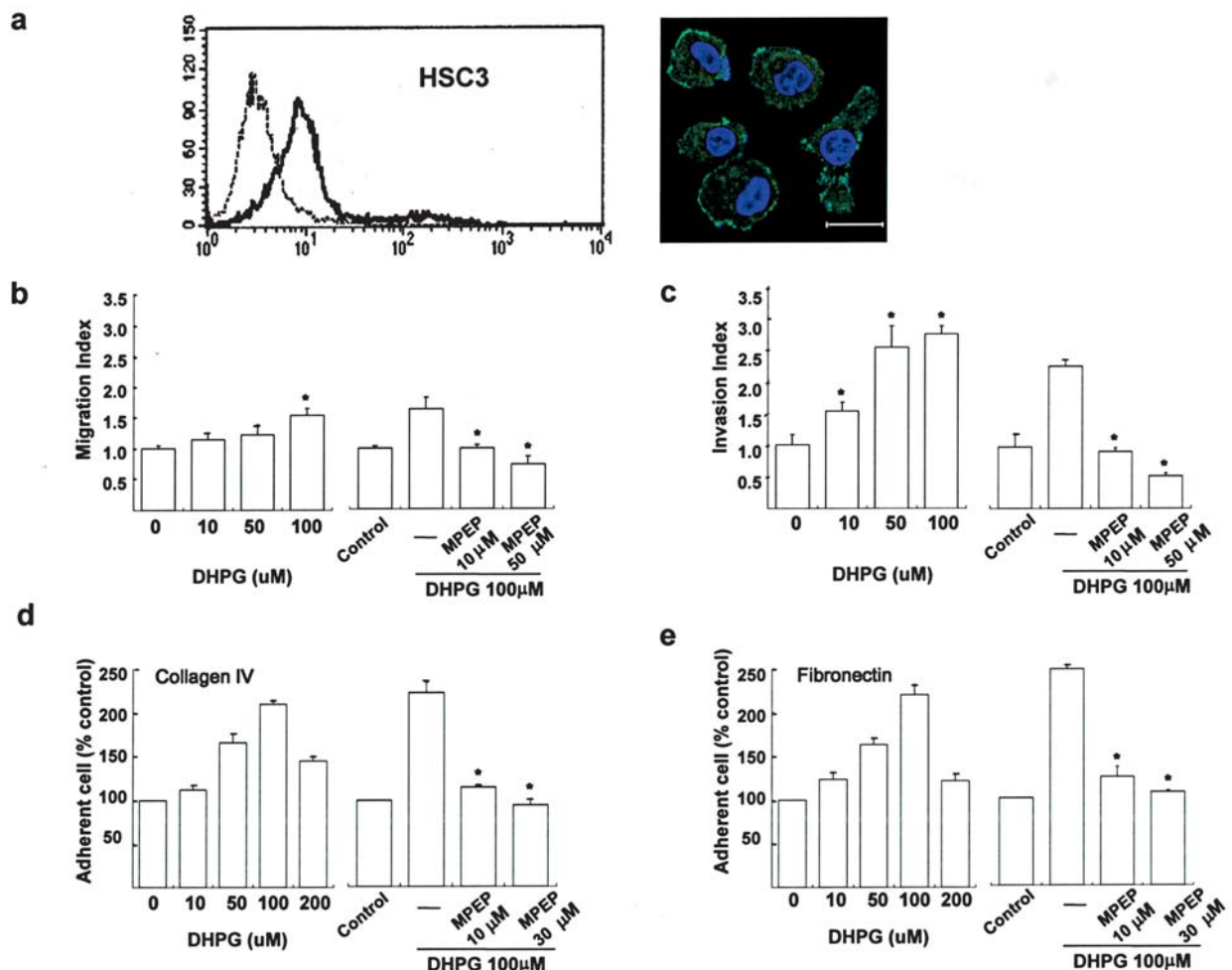


Figure 4. Effect of an mGluR5 agonist, DHPG, and antagonist, MPEP, on the migration, invasion, and adhesion of HSC3 oral tongue cancer cells. (a) Expression of mGluR5 in HSC3 cells as assessed by flow cytometry and confocal microscopy. Bar, 10  $\mu$ m. (b) Migration of HSC3 cells. DHPG stimulated cell migration. This effect of DHPG was inhibited by MPEP. (c) DHPG enhanced the invasiveness of HSC3 cells, and this effect of DHPG was suppressed by MPEP. (d and e) Adhesion of cells. DHPG enhanced the number of cells adhering to collagen IV (d) and fibronectin (e), and these effects of DHPG were inhibited by MPEP. In all panels, results represent means  $\pm$  standard deviation from three independent experiments. \* $P<0.05$  vs. control or 100  $\mu$ M DHPG.



Parameters	Case No.	Multivariate analysis		
		Univariate analysis (P)	Hazard ratio (95% CI)	P
mGluR5		0.0389		
Negative	37			
1+	52		5.225 (1.096-24.912)	0.038
2+	42		6.096 (1.236-30.061)	0.026
Age (years)		0.7172	1.499 (0.676-3.321)	0.319
<60	66			
≥60	65			
Sex		0.8797	2.077 (0.780-5.532)	0.144
Male	99			
Female	32			
Tumor size		0.0452	2.490 (0.977-6.346)	0.056
T1/T2	73			
T3/T4	58			
Tumor differentiation		0.5800		
Well	78			
Moderate/poorly	53			
Lymph node metastasis		0.0178	2.949 (1.224-7.108)	0.016
Absent	79			
Present	52			

CI, confidence interval.

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