

Tachykinin Receptor 3 Distribution in Human Oral Squamous Cell Carcinoma

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Abstract. *Background:* Tachykinin 3 (TAC3) and its preferred tachykinin receptor 3 (TACR3) that are prominently detected in the central nervous system, play significant roles in physiological development and specifically in the human reproductive system. The roles of TAC3/TACR3 in oral squamous cell carcinoma are unknown. *Materials and Methods:* We examined the expression pattern of TAC3/TACR3 in clinically-resected oral squamous cell carcinoma samples using immunohistochemistry and immunofluorescence analysis. *Results:* We found that even though the expression level of TACR3 was negative in the normal epithelium, it was highly elevated in tumor cells. A more intense signal was observed in the invasive front of tumor cells that had migrated into the mandible bone matrix. TAC3 was not detected in tumor cells, but was expressed in PGP-9.5-positive sensory nerves in the mandible. *Conclusion:* Our results suggest that peripheral sensory nerve-derived TAC3 may affect gingival oral squamous cell carcinoma cells through TACR3 in the bone matrix.

Tachykinins are a family of neuropeptides distributed in the mammalian central and peripheral nervous system (1, 2). Tachykinins are characterized by a common C-terminal structure (Phe-Xaa-Gly-Leu-Met-NH₂) and include TAC1-3 (3). Their actions are mediated by three different receptors, TACR1-3 belonging to the superfamily of G protein-coupled receptors (4).

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Previously, the expression of TACR3 was considered to be restricted to the central nervous system, including the cortex, nuclei of the amygdala, hippocampus and midbrain (5, 6). TAC3 and TACR3 modulate the GnRH release at the hypothalamic-pituitary axis (7, 8) and their participation in the human reproduction system is clear from the fact that mutations of TAC3 and TACR3 are associated with human normosmic hypogonadotropic hypogonadism, a disease characterized by the failure of sexual maturation, impaired gametogenesis and infertility (9, 10). TAC3 is indispensable to physiological development and human reproductive system (11). The reproductive hormone signaling cascade have found extensive applications in treating a wide range of hormone-dependent diseases such as prostate (12) and lung cancer (13).

Sex hormone receptors have also been involved in head-and-neck squamous cell carcinoma (14). Despite this fact, the role of reproductive factors in oral squamous cell carcinoma remains unclear (15) and the role of TAC3/TACR3 in cancer has not yet been reported.

In this study, we analyzed the expression pattern of TACR3 in early and advanced oral squamous cell carcinoma, and how TACR3 signaling was involved in tumor invasion in bone matrix.

Materials and Methods

Patients. The eight patients included in the study were diagnosed and treated for lower gingival, tongue, mouth floor or buccal squamous cell carcinoma individually at Okayama University Hospital (Okayama, Japan) in the years 2000-2013, with clinicopathological confirmation of the diagnosis. The surgically resected mandibles were collected as part of routine care by the authors. No patient had received chemotherapy or radiation therapy before surgery. The retrospective study was approved by the Ethical Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (Protocol No: 1949). Written consent was not acquired, but an announcement of the study was posted prominently in adjacent clinics. The authors had access to patients' records prior to data anonymization.

Organ	Diagnosis	TACR3	Organ	Diagnosis	TACR3
Cerebral cortex	Normal		Maxilla	SCC	
Esophagus	Normal				
Kidney	Normal		Mandible	SCC	
Liver	Normal				
Lung	Normal		Mouth floor	SCC	
Ovary	Normal				
Pancreas	Normal		Pharynx	SCC	
Parathyroid	Normal				
Pituitary gland	Normal		Parotid gland	SCC	
Placenta	Normal				
Prostate	Normal		Head skin	SCC	
Spinal cord	Normal				

Figure 1. TACR3 expression in human normal organ tissue and head-and-neck disease tissue array. SCC: Squamous cell carcinoma.

Histochemical and immunohistochemical analysis of surgically resected samples. After resection, formalin-fixed mandible segments were embedded in paraffin and sectioned at 4 μm intervals. Also used in the tissue microarray were 35 types of normal human tissue, 90 cases/ 96 cores (MNO961, US Biomax, Rockville, MD, USA) as control samples, and a head-and-neck disease tissue samples, 48 cases/96 cores (HNT961, US Biomax)

The sections were deparaffinized and subjected to antigen activation with 1.25% hydrogen peroxide solution (Wako, Osaka, Japan) containing methanol (Wako) for 30 min. Slides were incubated in Tris-EDTA (Sigma-Aldrich, St. Louis, MO, USA) for 2 min using a pressure cooker. After reaching room temperature, they were blocked with the peroxidase-blocking reagent included in the EnVision FLEX Mini Kit High pH (Dako, Carpinteria, CA, USA) for 5 min. Anti-TACR3 (#bs-0166R, rabbit IgG, BIOSS, Woburn, MA, USA), anti-TAC3 (#NB300-201SS, rabbit IgG, Novus Biologicals, Littleton, CO, USA), anti-cytokeratin-13 (CK-13, #ab16112, mouse IgG, Abcam, Cambridge, UK), and anti-cytokeratin-17 (CK-17, #M7046, rabbit IgG, Dako) antibodies were

used for the immunohistochemical analysis. The specimens were incubated with antibody overnight at 4°C, followed by washes with PBS. The slides were then treated with HRP (Dako) for 20 min at room temperature. The immunoreaction was visualized using DAB+ Chromogen (Dako), and counterstaining was performed with Hematoxylin QS (Vector, Burlingame, CA, USA).

Immunofluorescence. Control and disease specimens were deparaffinized and incubated in Tris-EDTA (Sigma-Aldrich) for 2 min using a pressure cooker. Sections were blocked with Block Ace (DS Pharma, Osaka, Japan) for 30 min, then immunolabeled with primary antibody anti-TAC3 (Novus Biologicals) and anti-Protein Gene Product 9.5 (PGP-9.5) (#ab8189, mouse IgG, Abcam) overnight at 4°C. The target protein was visualized by incubation with anti-rabbit IgG Fab2 Alexa Fluor 488 (#4412S, Cell Signaling, Danvers, MA USA) and Anti-mouse IgG Fab2 Alexa Fluor 647 (#4410S, Cell Signaling) for 30 min. Coverslips were mounted with ProLong Gold Antifade with DAPI (#8961S, Cell Signaling) and sealed with nail polish.

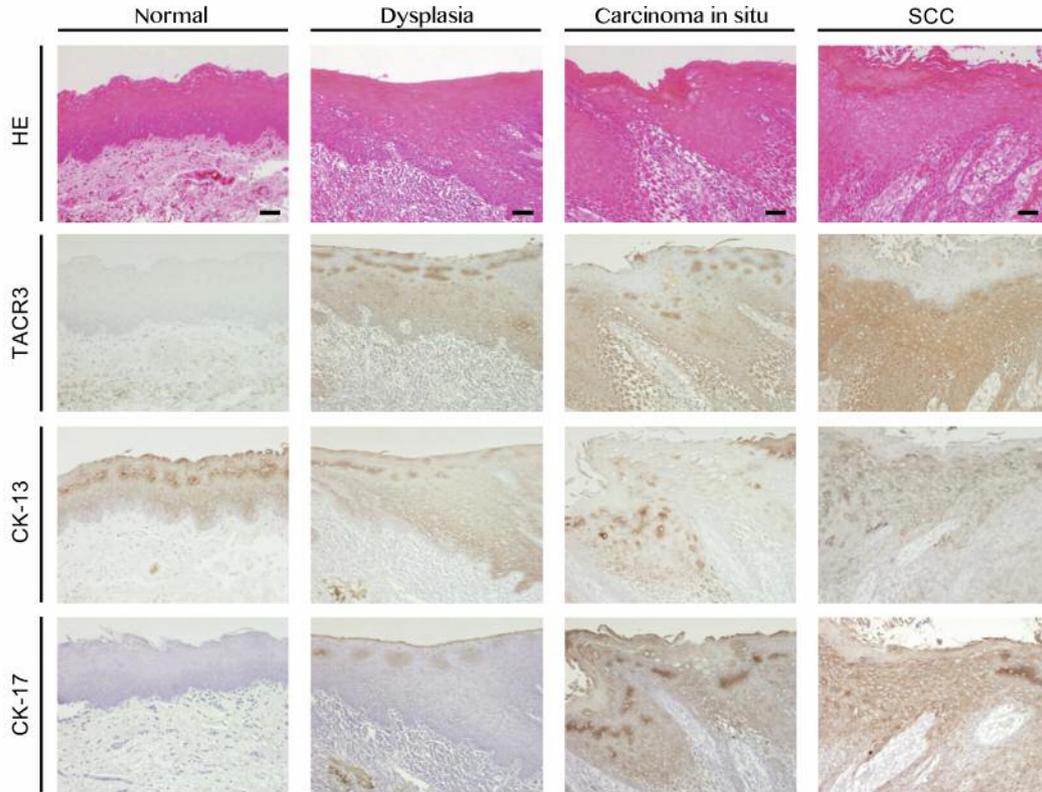


Figure 2. Expression of TACR3 in normal, dysplasia, carcinoma in situ and carcinoma region in the gingival epithelium. Hematoxylin and eosin (H&E) staining, immunostaining for TACR3, CK-13 and CK-17. Bar, 50 μ m.

Results

The expression of TACR3 in gingival squamous cell carcinoma. Most previous TACR3 studies identified the protein in the central nervous system. To evaluate the TACR3 expression in normal and diseased organs, paraffin tissue microarrays were performed. As shown in Figure 1, TACR3 was expressed not only in the cerebral cortex and pituitary gland, but also in the esophagus, kidney, liver, lung, ovary, pancreas, parathyroid, placenta, prostate and spinal cord. In the pathological condition, TACR3 was expressed in the oral squamous cell carcinoma in head-and-neck tumors (Figure 1).

To further investigate the expression of TACR3 in human gingival squamous cell carcinoma, we performed immunohistochemical staining using specific antibodies. TACR3 was not detected in the normal gingival epithelia (Figure 2). In sharp comparison with normal epithelia, TACR3 was detected in dysplasia and carcinoma *in situ* and was more expressed throughout the whole squamous cell carcinoma area (Figure 2). All cases with the invasive phenotype showed a strong intensity of TACR3 immunoreactivity. To confirm the stage of epithelial neoplasia, expressions of CK-13 and CK-

17 were examined. CK-13 was expressed in the normal epithelium and dysplasia, with a tendency of lower expression in malignant transformation. CK-17 was not expressed in the normal epithelium and dysplasia, but was found in carcinoma *in situ* and squamous cell carcinoma (Figure 2). These results suggested that TACR3 expression might be useful for detecting neoplastic lesions, specifically oral squamous cell carcinoma. This expression was also observed in all cases of tongue, mouth floor and buccal squamous cell carcinoma (n=8 individually, Figure 3).

TACR3 expression in osteolytic mandible squamous cell carcinoma. Figure 4 shows representative microscopic images of invasive bone destruction observed out of the 8 patients with oral squamous cell carcinoma in the mandible region. TACR3 was weakly expressed in tumor cells located at the surface of the epithelium. However, more intense TACR3 signals were observed in tumor cells that had invaded the bone matrix (Figure 4). On the other hand, TAC3 was not detected in tumor cells, *but strongly expressed* in PGP-9.5-positive the highly specific marker for neurons, mandibular sensory nerve (Figure 4).

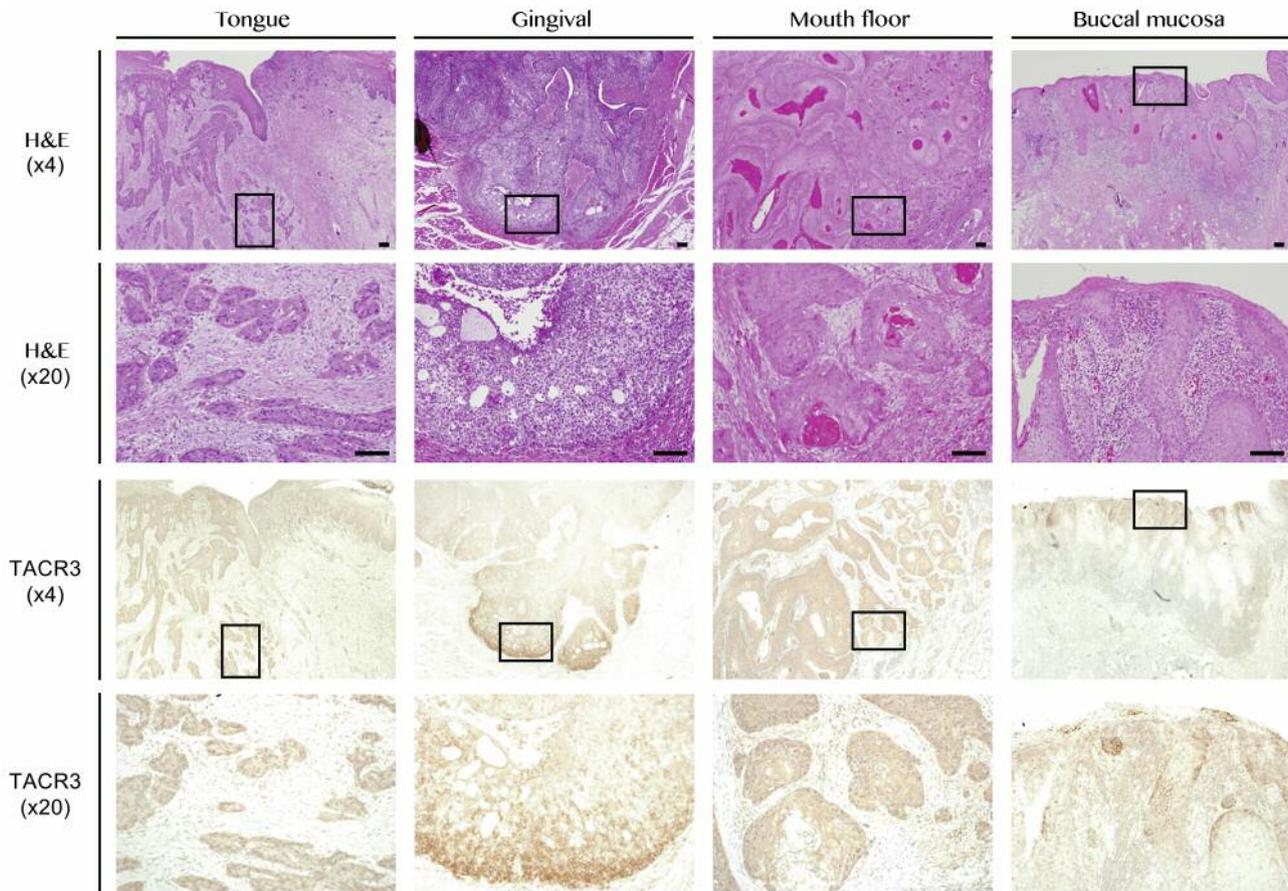


Figure 3. H&E staining and the immunohistochemistry results for TACR3 in tongue, gingiva, mouth floor and buccal oral squamous cell carcinoma. Bar, 50 μ m.

Discussion

Evidence is increasing that the regulation of the reproductive signaling pathways by the tachykinin peptide family and their receptors occurs not only at the CNS level, but also in the peripheral organs (16, 17). The exact pathological role of TACR3 in oral squamous cell carcinoma remains unclear. In this study, we analyzed the expression of TACR3 at the cellular level in oral squamous cell carcinoma. Our findings are as follows: (i) TACR3 expression was observed in oral squamous cell carcinoma, (ii) TACR3 was not expressed in CK-13-positive normal epithelium, (iii) TACR3 staining was especially intense in the invasive front of oral squamous cell carcinoma in the bone matrix compared to the tumor cells located at the surface of the epithelium.

CK-13 (type I cytokeratin) is an important component of mucosal-stratified squamous epithelium and CK-13-positive cells are present during normal differentiation and keratinization (18, 19). In the present study, the expression

of TACR3 was associated with CK-13-positive epithelial dysplasia, indicating that TACR3 may be involved in abnormal differentiation in dysplastic epithelia. CK-17 is also a type I cytokeratin that is expressed in oral squamous cell carcinoma and its expression is known to increase cell mobility and migration (19, 20). In this study, the CK-17 staining pattern was comparable to that of TACR3 in oral squamous cell carcinoma. This indicated that TACR3 may represent a highly specific marker for detecting neoplastic lesions, as it is associated with a marker for mobility and migration in oral squamous cell carcinoma.

TAC1 is a major excitatory neurotransmitter in the peripheral nervous system, while TAC3 is primarily involved in the CNS (21). In this study, histopathological examination demonstrated the importance of the expression of TAC3 in peripheral nerve in the mandible. TAC3 may play roles not only as neurotransmitters but also as local factors and are involved in almost all aspects of the regulation of physiological functions and pathophysiological processes. TAC3 signaling

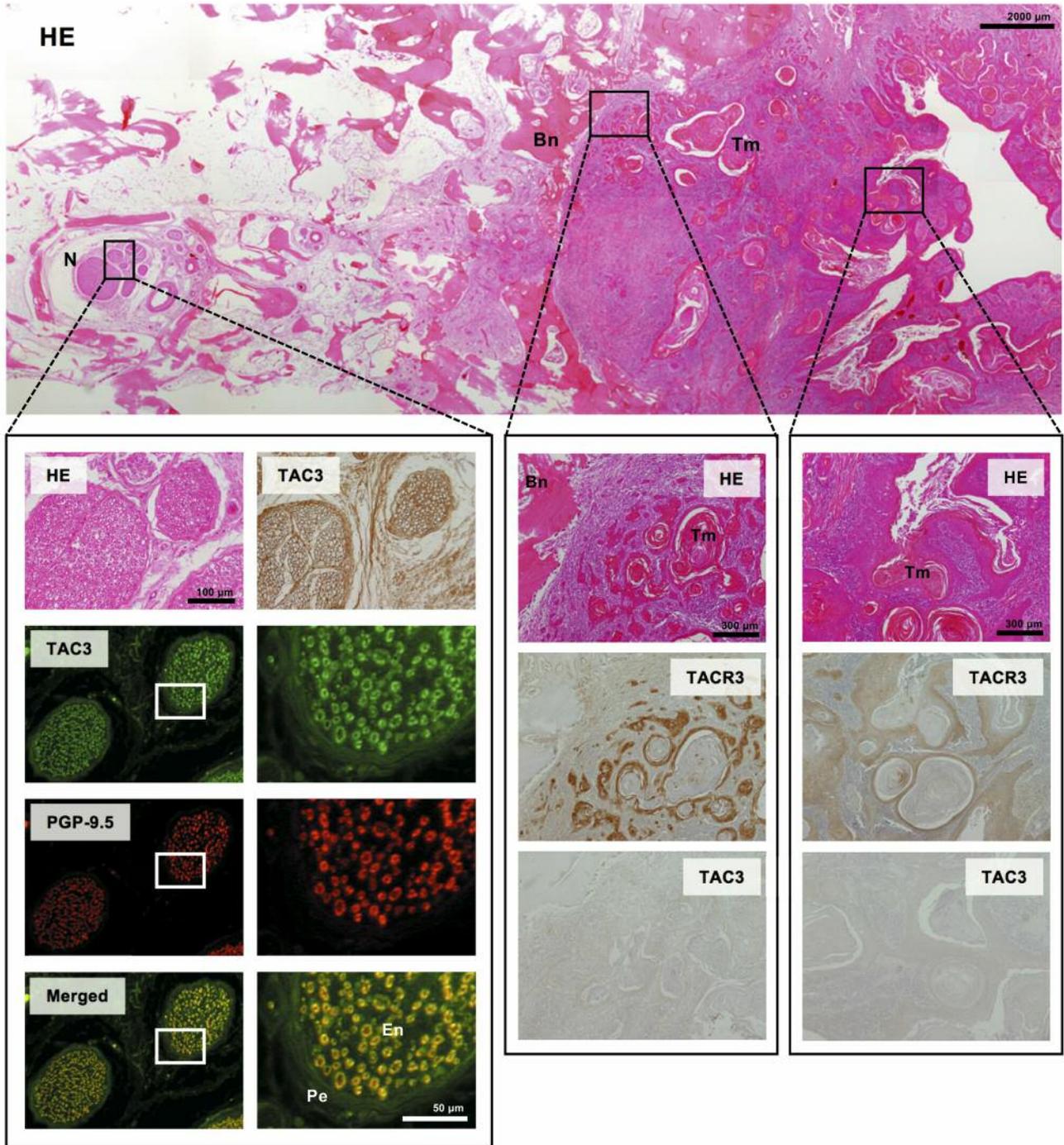


Figure 4. HE staining, immunofluorescence and immunohistochemistry results for TAC3, TACR3 and PGP-9.5 in resected mandible affected by lower gingival oral squamous cell carcinoma. N, inferior alveolar nerve; Bn, bone; Tm, tumor; En, endoneurium; Pe, perineurium; Bar, 150 µm.

system in the cancer bone microenvironment has been shown the possibility to play a crucial role in the regulation of tumor, but molecular biological studies are needed to elucidate novel function of TAC3 in tumor invasion in the bone matrix.

Expression of TACR3 seems to be regulated by estrogens in central and peripheral levels (22). Estrogen-related receptor alpha (ESRRA) has been shown to be up-regulated in several cancers such as breast, prostate, ovarian, colon and

oral squamous cell carcinoma (23-28). Further, ESRRA is known to promote the migration and invasion of oral squamous cell carcinoma (28). These observations suggest that TACR3 expression is upregulated by ESRRA signaling and that TACR3 has a role in tumorigenesis. Further studies analyzing estrogen levels from patient serum may establish an association between estrogen levels and TACR3 expression in human oral squamous cell carcinoma. Although the mechanism by which TACR3 expression is involved in oral squamous cell carcinoma is unknown, TACR3 protein showed a clear and significant overexpression compared with the adjacent normal epithelium, particularly in the invasive front of the tumor in the bone matrix. Bone resorption releases growth factors from the bone matrix, including estrogen that may further upregulate TACR3 in cells at the invasive front of tumors.

In summary, we have shown for the first time that TACR3 is highly expressed in the invasion front of oral squamous cell carcinoma in bone matrix. It is possible that TAC3 released by the peripheral sensory nerves may act in tumor cells and that TACR3 signaling may, in turn, contribute to tumor progression; however, further investigation is needed.

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