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Immunohistochemical distribution and morphometric analysis of aquaporin-3 in oral squamous cell carcinoma

K. Matsuo[a], **K. Kawano**[b]
[a]Department of Health Promotion, Division of Oral Pathology, Kyushu Dental University, Kitakyushu, Japan; [b]Department of Dentistry and Oral-Maxillo-Facial Surgery, Faculty of Medicine, Oita University, Oita, Japan

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Abstract. This study aimed to evaluate the relationship of aquaporin-3 (AQP3) expression with clinico-pathological parameters and lymph node metastasis in patients with oral squamous cell carcinoma (SCC). The immunohistochemical distribution of AQP3 was investigated in normal squamous epithelium and SCC tissue of 48 cases of SCC of the tongue and floor of the mouth. The percentage of the total AQP3-positive SCC tissue area relative to the total tumour tissue area (T-AQP3) was calculated as a morphometric AQP3 parameter for each patient. Furthermore, the percentage of the AQP3-positive area relative to the SCC tissue area at the invasion front (F-AQP3) was calculated as another AQP3 parameter. The immunostaining pattern of AQP3 in SCC tissue was irregular and weaker than that in normal epithelium. Well-differentiated SCCs had higher T-AQP3 and F-AQP3 values than poorly differentiated SCCs. SCCs with an infiltrative invasion pattern had lower F-AQP3 than SCCs with expansive and intermediate patterns. SCCs with T-AQP3 <27% or F-AQP3 <17% showed an increased incidence of lymphatic metastasis, and multivariate analysis demonstrated that F-AQP3 was an independent prognostic factor of lymphatic metastasis. These results suggest that AQP3 is involved in keratinocyte differentiation and decreased AQP3 expression is associated with more aggressive tumour behaviour.

Key words: oral squamous cell carcinoma; aquaporin-3; immunohistochemical study; squamous epithelium; metastasis.

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Aquaporins (AQPs) are a family of small, hydrophobic integral membrane proteins (monomers of approximately 30 kDa) that function as osmotically driven transepithelial and transcellular water transporters.[1,2] Currently, 13 mammalian AQPs (AQP0–AQP12) have been characterized and divided into two groups.[3–5] Most members of the AQP family function primarily as water-selective transporters, whereas a subset known as 'aquaglyceroporins' (AQP3, 7, 9, and 10) transport glycerol, water, and possibly other small solutes.[6] Biological evidence suggests that AQPs play important roles under various physiological and pathological conditions, such as urinary concentration, exocrine glandular fluid secretion, fat metabolism, and brain oedema.[7–9] Furthermore, the recent discovery of the involvement of AQPs in cell migration, proliferation, and angiogenesis suggests that AQPs play key roles in tumour biology.[4,5]

AQP3 belongs to the aquaglyceroporin subtype of the AQP family. It is a relatively weak transporter of water but an efficient transporter of glycerol compared

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to other water-selective transporter AQPs, such as AQP1, 2, 4, and 5.[10] AQP3 was initially cloned from kidney collecting duct principal cells[11–13]; however, it is also expressed in various epithelial cells, including the urinary, digestive, and respiratory tracts, and the epidermis.[14–20] AQP3-expressing cells are localized on the basal cell side of epidermal, tracheal, and nasopharyngeal epithelia, and thus, it is considered to participate in water and glycerol entry into epithelial cells from the subepithelial side of connective tissue to protect against potential water loss from upper epithelial cells.[3,15] Because the skin is prone to water loss by evaporation, it is reasonable to hypothesize that AQP3-facilitated water permeability and glycerol transport are involved in hydration and elasticity of the squamous epithelium.[3,15]

Recent studies have demonstrated a relationship between AQP3 expression and squamous cell carcinomas (SCCs) of the skin, oesophagus, oral mucosa, and lungs.[3,4,21–24] Some reports suggest a potential role for AQP3 in the growth/tumorigenesis of SCC, although this hypothesis remains controversial. Because SCC is the most common type of oral cancer, efficient prevention and early diagnosis are important. There have been many immunohistochemical and molecular biological studies involving oral mucosal lesions, including pre-cancerous lesions, carcinoma *in situ*, and early and advanced stage SCCs, but there is little knowledge of the relationship between AQP3 and oral SCC.[23] Furthermore, there have been no detailed histopathological examinations of the relationship of AQP3 expression in oral SCC with cellular differentiation, invasive growth, and metastatic potential.

In the present study, we performed an immunohistochemical analysis of biopsy specimens obtained from 48 cases of SCC of the tongue and the floor of the mouth to determine the AQP3 distribution in oral SCC and evaluate any possible relationship between AQP3 and clinico-pathological parameters.

Materials and methods

Patients

Forty-eight cases (32 men and 16 women; age range 40–83 years; average 62.8 years) of surgery for SCC of the tongue and floor of the mouth were examined. The clinical stage of the tumours was defined according to the TNM system (Union Internationale Contre le Cancer, 1997). This study was limited to T1 ($n = 21$) and T2 ($n = 27$) tumours. The site of the primary tumours was the tongue in 34 cases and the floor of the mouth in 14. Preoperative treatment was not observed in any case. Lymphatic metastasis was confirmed by histological examination of surgical specimens. Twenty-one of the 48 cases exhibited cervical node involvement, and 11 of them exhibited histologically positive nodes at initial treatment (primary lymphatic metastasis), while 10 exhibited secondary lymphatic metastasis during the follow-up period, without recurrence at the primary site. Twenty-seven cases exhibited no cervical node involvement during the follow-up period of at least 2 years.

Immunohistochemical staining for AQP3

Formalin-fixed, paraffin-embedded sections (6-μm) of biopsy specimens were deparaffinized in xylene and used for immunohistochemical analysis. Endogenous peroxidase was blocked using 0.03% H_2O_2 in methanol for 20 min. After incubation in 10% normal goat serum (Nichirei Biosciences Inc., Tokyo, Japan) for 10 min, sections were incubated with rabbit polyclonal antibody against AQP3 (V214, 1:100; Bioworld Technology, Inc., St. Louis Park, MN, USA) at room temperature for 1 h. Histofine Simple Stain MAX-PO (MULTI; Nichirei Biosciences Inc.) was used as the secondary antibody. AQP3-positive cells were detected using the ImmPACT DAB peroxidase substrate kit (Vector Laboratories, Inc., Burlingame, CA, USA). Nuclear counterstaining was performed using methyl green or haematoxylin stain.

Evaluation of AQP3 expression

The area containing AQP3-positive tumour cells (AQP3 AREA) and that containing the entire tumour tissue (SCC AREA) were measured on digital images of histological sections using the Adobe Photoshop CS5 Extended software (Adobe Systems Inc., San Jose, CA, USA). The percentage of the AQP3 AREA relative to the SCC AREA was calculated and defined as the total tumourous AQP3-positive area (T-AQP3) for each case. More than four high-power digital images (original magnification, 200×) of the tumour invasion front were analyzed in a similar manner. The percentage of the AQP3 AREA relative to the SCC AREA at the invasion front was calculated and defined as the frontal AQP3-positive area (F-AQP3) for each case. The percentage of AQP3-positive cells in the normal oral epithelium (N-AQP3) was assessed in a manner similar to that used for T-AQP3. The 'normal epithelium' selected for evaluation of N-AQP3 ($n = 25$) was present in specimens where oral SCC tissue was also observed. The intensity of AQP3-immunostaining was not taken into consideration when evaluating the T-, F- and N-AQP3 values.

Clinical and histological variables

Clinical and histological features examined with respect to T-AQP3 and F-AQP3 expression were age, sex, T classification, tumour thickness, degree of differentiation (World Health Organization, 2005), tumour invasion pattern, and lymphatic metastasis. Tumour thickness was measured vertically from an imaginary line that was reconstructed from the healthy oral mucosa to the deepest level of invasion using an ocular micrometre with slides of paraffin-embedded primary tumour sections. The degree of differentiation was categorized into well-, moderately, and poorly differentiated types; 34 cases of tongue SCC included 24 (70.6%) well-differentiated, 6 (17.6%) moderately differentiated, and 4 (11.8%) poorly differentiated, while 14 cases of SCC of the floor of the mouth included 4 (28.6%) well-differentiated, 6 (42.8%) moderately differentiated, and 4 (28.6%) poorly differentiated. The tumour invasion pattern (expansive, intermediate, or infiltrative) was assessed according to Jakobsson's criteria.[25]

Statistical analysis

The 48 cases were divided into two or three groups according to their age (<65 years and ≥65 years), sex (male and female), T classification (T1 and T2), tumour thickness (<4.5 mm and ≥4.5 mm), differentiation (well, moderate, and poor), invasion pattern (expansive, intermediate, and infiltrative), and lymphatic metastasis ('No' = no metastasis and 'Yes' = metastasis) (Table 1). The mean values of T- AQP3 and F-AQP3 were calculated and compared for each subdivided group using an unpaired *t*-test and one-way analysis of variance (ANOVA). If the difference among the three groups (i.e., groups subdivided according to differentiation or invasion pattern) was statistically significant, then post hoc multiple comparisons were performed using a Bonferroni correction with the level of significance set at $\alpha = 0.05/3$.

χ^2 tests were conducted to examine the association between lymphatic metastasis