Expression of transmembrane protein aquaporin-3 in oral epithelial dysplasia and oral squamous cell carcinoma

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Objectives. The objective of this study was to evaluate aquaporin-3 (AQP3) expression in patient samples of oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC), thereby assessing the potential of AQP3 as a molecular marker for tumor progression.

Study Design. An in vitro comparative study was done to determine the AQP3 expression on 20 surgical biopsy specimens each of OED and OSCC using immunohistochemistry. Twenty specimens of normal oral mucosa were kept as controls. The results were statistically analyzed using one-way analysis of variance and post hoc analysis.

Results. The expression of AQP3 was analyzed and further semiquantified using H-scores. The mean H-score showed a statistically significant difference between OSCC, OED, and normal oral mucosa ($P < .05$). There was a significant increase in the expression of AQP3 in OSCC and OED compared to normal oral mucosa. The highest expression was observed in OSCC ($P < .01$).

Conclusion. The observations of the study indicate that staining intensity of AQP3 increased from dysplastic noninvasive lesion to invasive OSCC, suggesting a possible role of AQP3 as a biomarker for tumor progression. (Oral Surg Oral Med Oral Pathol Oral Radiol 2021;131:202−208)

Oral squamous cell carcinoma (OSCC) is an aggressive epithelial malignancy representing a greater part of all malignant neoplasms of the oral cavity. In recent years, over 300,000 new cases of oral and oropharyngeal cancer have been reported worldwide, with an alarming increase in the mortality rate.[1] According to the latest reports of the International Agency for Research on Cancer, the projected incidence of cancer in India will increase from 1 million in 2012 to more than 1.7 million in 2035.[2,3]

A majority of OSCC cases are preceded by a variety of lesions and conditions, collectively referred to as oral potentially malignant disorders (OPMDs), which display an increased risk for malignant transformation. Among OPMDs, oral leukoplakia (OL) is the most commonly encountered entity in clinical practice, with a prevalence ranging from 0.4% to 2.6% and a rate of malignant transformation between 3.0% and 17.5%.[4-6] The World Health Organization (WHO) defines leukoplakia as a clinical term to describe "white plaques of questionable risk, once other specific conditions and other OPMDs have been ruled out."[7] OL can clinically mimic lesions that are reactive in nature or may represent oral epithelial dysplasia (OED), which is premalignant in nature.[8] It is important to recognize that progression of OED to OSCC is not a singular event but a gradual process of genetic and histologic changes that lead to malignant transformation.[9] Diagnosis and management in the early stages of the multistep carcinogenesis offers opportunities for better prognosis. Studies have shown that aquaporins (AQPs), a novel transmembrane protein, are closely associated with carcinogenesis and are expressed in more than 20 different human cancers.[10-12]

Aquaporins are a large family of water channel proteins (monomer size ~30 kDa), discovered by Peter Agre et al. and his colleagues in 1992, that facilitate transepithelial water movement across the cell membrane.[13,14] In humans, 13 isoforms (AQP0 to AQP12) have been identified, of which AQP3, AQP7, AQP9, and AQP10 are aquaglyceroporins that transport glycerol along with water.[15] Among the 13 AQPs, AQP3 has been detected to be expressed in many epithelial cells of various organs such as kidney, skin, lung, and gastrointestinal tract.[16,17] According to the literature, overexpression of AQP3 has been observed in several

Statement of Clinical Relevance

Lack of any potential marker to recognize the molecular alterations in oral epithelial dysplasia (OED) crucial to the progression to malignancy contributes to the alarming increase in the mortality rate of oral squamous cell carcinoma (OSCC). The present study was performed to assess the potential of aquaporin-3 as a biomarker in tumor progression of OED to OSCC.

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cancers, including oral cancer, where it contributes to metastasis and proliferation.[17-22] The expression is related to tumor grade, with increased expression in tumor areas than in nontumor areas, as shown in a study of human primary SCC such as esophageal and lingual cancers.[23]

Multistep progression of squamous cells from normal to dysplastic epithelium and eventual malignancy does, however, undoubtedly offer clinicians a therapeutic window of opportunity to intervene during carcinogenesis. The overall objectives of this research project were to evaluate and compare the immunohistochemical (IHC) expression of AQP3 in OSCC, OED, and normal oral mucosa, hence assessing its potential as a molecular marker for tumor progression.

MATERIALS AND METHODS

Immunohistochemical detection of AQP3 antibody in human OSCC, OED, and normal oral mucosa biopsies

The guidelines were in accordance with standards of the Institutional Ethical Committee (IEC), PMS College of Dental Science and Research, Thiruvananthapuram, India (IEC No. PMS/IEC/2017/02). An in vitro comparative study was conducted on 60 formalin-fixed paraffin-embedded archived blocks of diagnosed cases of 20 OSCC and 20 cases of OED (10 mild epithelial dysplasias, 10 moderate to severe epithelial dysplasias). Twenty normal oral mucosa (control) were obtained from the Department of Oral Pathology and Microbiology, PMS College of Dental Science and Research. The OED samples were classified based on WHO criteria.[24] Mild dysplasia represents architectural disturbance present only in the lower third of the epithelium with cytologic atypia, whereas moderate to severe dysplasias exhibit architectural disturbance extending from middle third to greater than two thirds of the epithelium, with cytologic atypia.[24] Tissues from the posterior buccal mucosa excised during elective surgical removal of impacted third molars after relieving inflammation using medications were taken as normal oral mucosa samples for study controls.

Primary antibody for immunohistochemistry. IHC analysis was designed for the detection of AQP3 antibody using polyclonal rabbit anti-AQP3 (human) immunoglobulin G (Uniprot-Q92482, prediluted, Geno Technology Inc. USA). The antibody was specific to endogenous levels of total AQP3 with an amino acid spectrum ranging from 1 to 292. AQP3 has a subcellular location in the basolateral cell membrane. The antibody shows similarity to aquaporins containing 2 tandem repeats each containing 3 membrane-spanning domains and a pore-forming loop with the signature motif Asn-Pro-Ala (NPA) and belongs to the MIP/aquaporin (TC 1.A.8) family (Catalogue No. ITI05222). Human skin tissue with known positivity for AQP3 was used as positive control tissue. For negative control, the primary antibody was replaced with serum from the routine IHC protocol.

Immunohistochemistry protocol. Standard horseradish peroxidase immunohistochemistry was performed to detect the expression of AQP3 in OSCC, OL, and normal oral mucosa biopsy specimens. Briefly, 4-μm-thick tissue sections were mounted onto the APES (3-aminopropyltriethoxysilane)-coated slides, deparaffinized with xylene, and rehydrated through diminishing concentrations of ethanol in water. Antigen retreival was achieved by incubating the slides in sodium citrate buffer (95˚C) for 20 min in a pressure cooker, followed by 20 min of cooling at room temperature. Endogeneous peroxidases were quenched for 10 min with an incubation in 3% hydrogen peroxide (ImmunoTag). Slides were incubated overnight with primary antibody at room temperature in a humidified chamber, followed by 30-min incubation with biotinylated goat anti-rabbit IgG (ImmunoTag). The expression was visualized using a diaminobenzine tetrahydrochloride solutions detection system (VKAN Life Care, Chennai, India). The duration of diaminobenzine tetrahydrochloride incubation was determined through pilot experiments and was made constant for 35 min for all slides. Between each step, slides were washed with immuno wash buffer (VKAN Life Care). The sections were counterstained with hematoxylin, dehydrated with ethanol, and coverslipped using a xylene-based mounting medium.

Immunohistochemistry scoring system. The number (%) of cells expressing AQP3 in OSCC, OL, and normal oral mucosa was counted under light microscopy (high-power field) using a grid eyepiece in 5 consecutive fields per specimen.The evaluation was independently reviewed and confirmed by 2 oral pathologists. Plasma membrane staining (brown reaction product) was regarded as a positive staining result for AQP3. The intensity of immunohistochemical staining with antibody AQP3 was evaluated by scoring criteria as negative (−), weak (+), moderate (++), and strong (+++) with the staining distributed as focal/patchy for weak-moderate epression and more diffuse with increasing staining intensity. The scoring results were further evaluated by a semiquantitative approach (H-score, or “histo” score), which was calculated by adding the percentage of positive cells multiplied by the weighted intensity of staining.[25-27] H-score = 0 (% of nonstained cells) + 1 (% of weakly stained cells) + 2 (% of moderately stained cells) + 3(% of strongly stained cells), where the percentage of positive cells (0%-100%) was multiplied by intensity (weak = 1; moderate = 2; and strong = 3) to obtain a maximum score of 300.[28]

Statistical analysis

With a nondirectional risk of 0.05 and assuming a standard deviation of 0.83, a sample size of 20 specimens per group was selected. The mean H-score for each group was calculated and was used to compare and correlate within and between the groups. SPSS version 20.0 (IBM Corp., Armonk, NY) was used to perform all statistical analyses. One-way analysis of variance (ANOVA) was used to assess the difference in staining intensity among the groups. The comparison between 2 groups was done using *post hoc* tests. $P < .05$ for one-way ANOVA and $P < .01$ for post hoc analysis were considered to indicate a statistically significant difference. An additional comparison was made between mild and moderate to severe OED using unpaired *t* tests and $P < .05$ was considered a statistically significant difference.

RESULTS

The biopsy specimens of OSCC and OED were taken from anatomic sites including buccal mucosa, tongue, and alveolar mucosa within the oral cavity (Table I). A summary of patient characteristics including age- and sex-matched cohorts is provided in Table II.

Features of AQP3 staining

From the analysis it was found that AQP3 staining was restricted to the epithelium in all patients. In normal oral mucosa, scattered, weak, patchy staining was observed in the plasma membrane of basal and suprabasal cell layers of epithelium (Figures 1A, 1B). No staining reaction was detected within the stratum corneum of any study subject. In mild oral epithelial dysplasia, the expression was more focal, patchy, and weak in the plasma membrane of basal and suprabasal epithelial cells (Figures 1C, 1D). In moderate to severe dysplasia, the expression was diffuse and moderate to strong in the plasma membrane of basal, suprabasal, intermediate, and superficial epithelial cells (Figures 1E, 1F). The IHC expression of AQP3 in OSCC was diffuse and strong in the plasma membrane of tumor cells and a faint expression was also noted in the cytoplasm mainly in the cells of tumor islands (Figures 1G, 1H).

Quantitative analysis of AQP3 expression

The average (SD) H-score of AQP3 expression in groups according to OSCC, OED, and normal oral mucosa was 234.25 ± 28.66, 203.13 ± 21.29, and 170.57 ± 22.57, respectively (Figure 2). A comparison was also made between mild and moderate to severe epithelial dysplasia to analyze the difference in expression of AQP3 between these 2 groups using unpaired *t* tests. A significant difference was obtained ($P < .05$) and values increased with severe grades of dysplasia (Table III). The highest H-scores were seen with OSCC and numerically increased with increasing grades of dysplasia and decreased in normal oral mucosa. One-way ANOVA was applied to statistically analyze these findings between the 3 groups and the results were statistically significant ($P < .05$; Table IV). The *post hoc* analysis indicated a statistical significant difference between OSCC and OED ($P < .01$), OED and normal oral mucosa ($P < .01$), and OSCC and normal oral mucosa ($P < .01$; Table V). The observations in our study indicate a gradual increase in IHC expression of AQP3 from normal oral mucosa to OED to OSCC.

Table I. Anatomic site of samples

Groups	*Total number of samples*	*Anatomic site*		
		Buccal mucosa	*Tongue*	*Alveolar mucosa*
Oral squamous cell carcinoma	20	6	10	4
Oral epithelial dysplasia	20	15	2	3

Table II. Patient characteristics

Groups	*Total no. of samples*	*No. of males*	*No. of females*	*Age range (years)*
Oral squamous cell carcinoma	20	13	7	40-80
Oral epithelial dysplasia	20	15	5	40-70
Normal oral mucosa	20	12	8	25-40

DISCUSSION

There are several reports on the expression of AQP3 in various human cancers.[29-33] Increasing evidence suggests that AQP3 plays a pivotal role in cancer metastasis.[34] However, there are few reports regarding the expression of AQP3 in OSCC and grades of oral epithelial dysplasia. In the present study, using an immunohistochemical approach, we demonstrated the significantly higher levels of expression of AQP3 in OSCC and OED. The expression was analyzed using staining intensity, which was further semiquantified using H-scores. The mean H-scores of the 3 groups were statistically analyzed using one-way ANOVA and post hoc tests. The observations indicate that expression increased with increasing severity of dysplasia with highest in OSCC and weak among normal oral mucosa and indicate the potential of AQP3 as a biomarker for tumor progression.

Our results showed a marked increase in the expression of AQP3 in OSCC compared to normal oral mucosa, which is similar to the findings of Kusayama et al.[23] The authors reported significantly higher levels of expression of AQP3 protein in both human esophageal and lingual

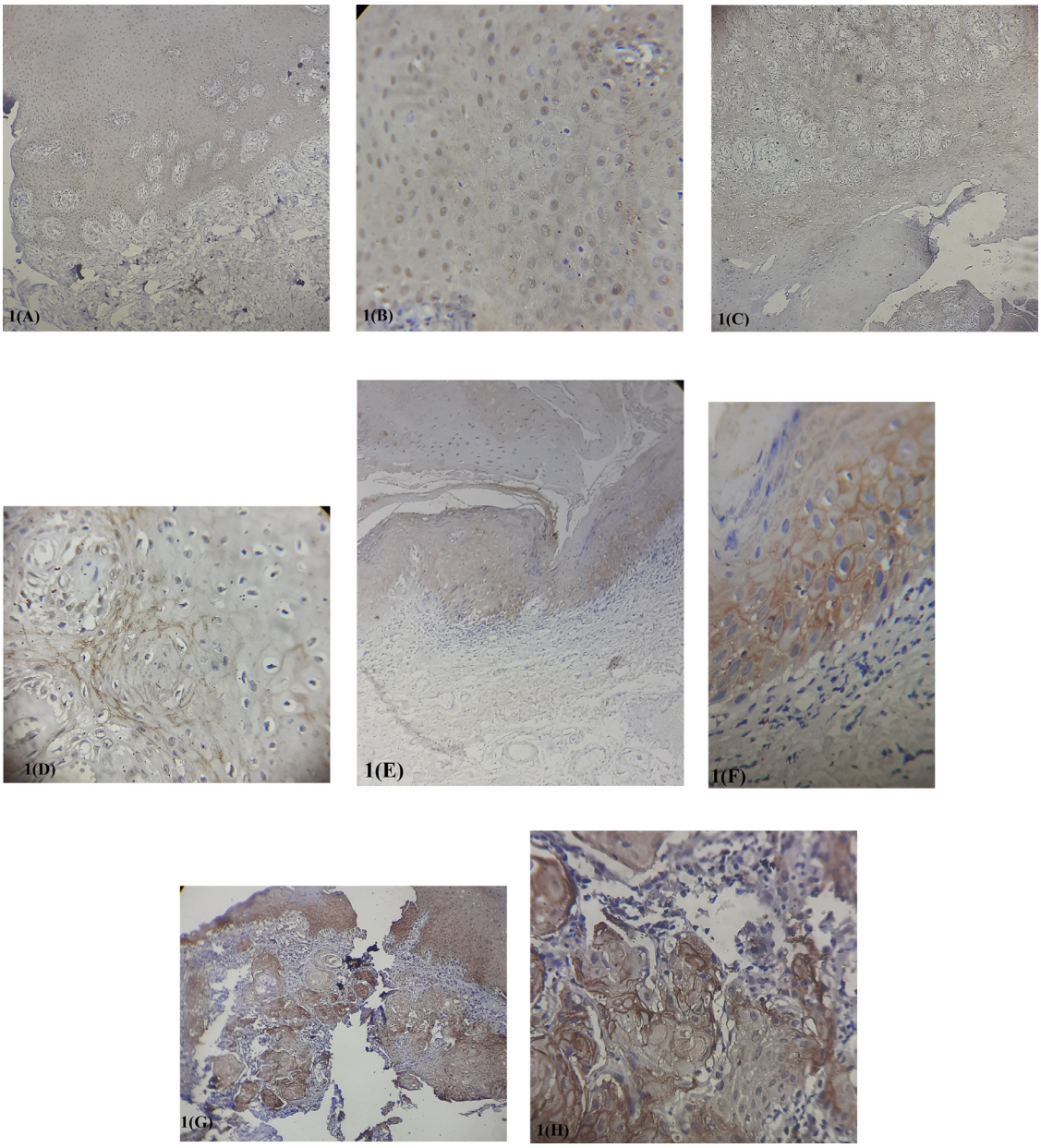

Fig. 1. Aquaporin-3 (AQP3) expression in human normal oral mucosa, oral epithelial dysplasia (OED), and oral squamous cell carcinoma (OSCC). Photomicrographs of immunohistochemistry (IHC) results using rabbit anti-AQP3 IgG. The chromogen is diaminobenzine tetrahydrochloride (brown) and the counterstain is hematoxylin (blue). **(A)** Representative image of normal oral mucosa (n = 20; AQP3 IHC staining original magnification × 10). **(B)** Scattered weak positive staining of plasma membrane was noted in the basilar and suprabasilar layers of normal oral mucosa (AQP3 IHC staining original magnification × 40). **(C)** Representative image of mild OED (n = 10; AQP3 IHC staining original magnification × 10). **(D)** Scattered focal, patchy, and weak positive staining of plasma membrane seen on the basilar and suprabasilar layers of mild OED (AQP3 IHC staining original magnification × 40). **(E)** Representative image of moderate to severe OED (n = 10; AQP3 IHC staining original magnification × 10). **(F)** Diffuse and moderate to strong positive staining of plasma membrane seen on the basal, suprabasal, intermediate, and superficial layers of moderate to severe OED (AQP3 IHC staining original magnification × 40). **(G)** Representative image of OSCC (n = 20; AQP3 IHC staining original magnification × 10). **(H)** Diffuse strong positive staining of plasma membrane and a faint cytoplasmic expression seen on the keratin pearls and tumor islands of OSCC (AQP3 IHC staining original magnification × 40).

cancer tissues. The expression of AQP3 in tumor tissues was much higher than that in nontumor areas in the same tissue samples. They surmised that AQP3 may be involved in the focal adhesion kinase-mitogen−activated protein kinase pathway, which regulates tumor progression and growth in OSCC.[23]

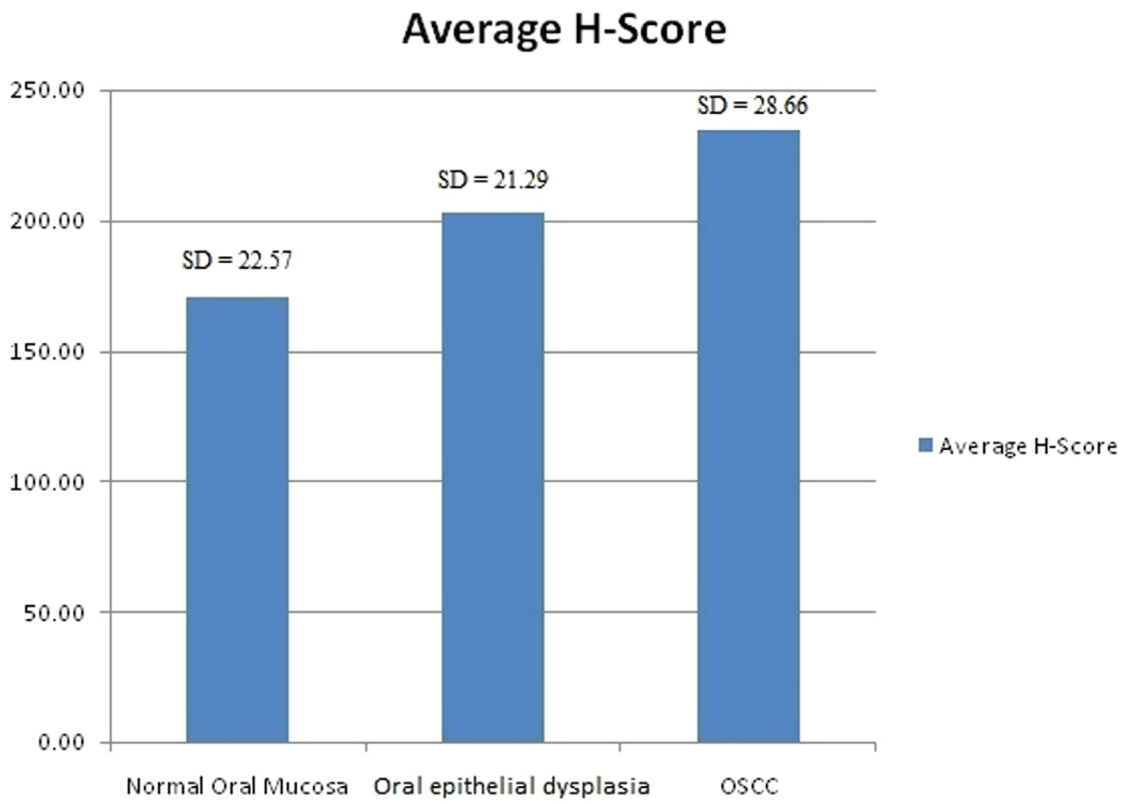

Fig. 2. Quantitative analysis of the average H-score values of aquaporin-3 (AQP3) positive staining in oral squamous cell carcinoma (OSCC; $n = 20$), oral epithelial dysplasia (OED; $n = 20$), and normal oral mucosa ($n = 20$). The average (SD) H-score of AQP3 expression among groups according to OSCC, OED and normal oral mucosa was 234.25 ± 28.66, 203.13 ± 21.29, and 170.57 ± 22.57, respectively. OSCC showed the strongest plasma membrane staining for AQP3, followed by OED, with weak expression in normal oral mucosa.

Table III. Comparison between 2 groups of oral epithelial dysplasia using unpaired *t* tests

Groups	*Variable*	*Total no. of specimens*	*Difference in mean*	*t*	*Standard error*	*P value*
Mild dysplasia	Aquaporin-3	10	24.933	3.0643	8.136	.0067
Moderate to severe dysplasia		10				

Table IV. Analysis using one-way analysis of variance

Groups	*Variable*	*Total no. of specimens*	*Mean H-score*	*Mean ± standard deviation*	*F ratio*	*P value*
Oral squamous cell carcinoma	Aquaporin-3	20	234.25	234.25 ± 28.66	32.40	.00001
Oral epithelial dysplasia		20	203.13	203.13 ± 21.29		
Normal oral mucosa		20	170.57	170.57 ± 22.57		

In our study, AQP3 expression was confined to the epithelium with membranous staining noted in the basal and suprabasal layers, consistent with reported IHC expression of AQP3 in stratified squamous epithelium.[35] No staining reaction was detected within the stratum corneum in all study patients. The normal oral mucosa showed a weak patchy expression, whereas a strong diffuse pattern was noted within OSCC samples. A slight cytoplasmic staining was noted within the keratin pearls of tumor islands in OSCC.

The present study further evaluated the AQP3 expression in mild and moderate to severe cases of OED, classified based on WHO 2017 criteria.[24] A statistically significant difference was obtained between the groups and expression increased with higher grades of dysplasia.

Table V. Comparison between 2 groups using post hoc analysis

Groups	*Variable*	*P value*
Oral squamous cell carcinoma and oral epithelial dysplasia	Aquaporin-3	.001
Oral epithelial dysplasia and normal oral mucosa	Aquaporin-3	.001
Oral squamous cell carcinoma and normal oral mucosa	Aquaporin-3	.001

A study performed by Udompatanakorn et al.[36] assessed the immunostaining patterns of 3 AQP3 antigen recognition sites including the amino acid (AA) 250-C terminus, AA180-228, and N terminus AA1-80 in oral squamous cell carcinoma and compared the adjacent areas of high-grade epithelial dysplasia and normal oral mucosa. Their results showed that strong membranous immunostaining was observed for AQP3 antigen recognition sites at the AA250-C terminus and AA180-228 in normal oral mucosa and staining intensity decreased with high-grade epithelial dysplasia and OSCC. Conversely, in the AQP3 antigen recognition site at N terminus AA1-80, negative or slightly positive staining was observed in normal oral mucosa and expression increased with high-grade epithelial dysplasia and invasive front of OSCC.[36] In our study, unlike Udompatanakorn et al.,[36] we assessed total AQP3 antibody with an amino acid spectrum ranging from 1 to 292. With this antibody we were able to obtain a significant difference in the expression of AQP3 in normal oral mucosa, OED, and OSCC. Our results showed increased expression with higher grades of dysplasia and OSCC samples, which was in accordance with the expression of the N terminus AA1-80 antigen recognition site in the study by Udompatanakorn et al.[36]

In addition to overexpression of AQP3 in OSCC, we found that the staining pattern and intensity of AQP3 changed dramatically between the borders of normal oral mucosa and the cancerous tissue. In our study there was a statistically significant difference in the expression of AQP3 in OSCC, OED, and normal oral mucosa ($P < .05$). OSCC displayed the highest expression compared to OED and normal oral mucosa ($P < .01$) and OED exhibited an increased expression of AQP3 compared with normal oral mucosa ($P < .01$), suggesting the role of AQP3 as a potential biomarker in tumor progression.

Another study on AQP3 expression in OSCC was done by Matsuo and Kawano,[37] who observed the immunohistochemical distribution and morphometric analysis of AQP3 in OSCC. Their results showed that the immunostaining pattern of AQP3 in OSCC tissue was irregular and weaker than that in normal epithelium, which is discrepant with our results, which showed the highest expression in OSCC compared to normal oral mucosa.

In this study, we assessed the endogenous levels of total AQP3 with an amino acid spectrum ranging from 1 to 292. Instead of an antigen recognition site−specific antibody, we preferred a more sensitive total AQP3 antibody, so that even minimal expressions from lower grades of dysplasia could be assessed. We were able to observe a significant difference in the expression of AQP3 in the study groups; that is, increased expression with higher grades of dysplasia. We suggest that anti-AQP3 antibody with specificity to total AQP3 could be a potential biomarker for tumor progression. Future research on specific antigen recognition sites of AQP3 antibody with larger samples and clinical follow-ups may provide additional insight into the role of AQP3 as a potential biomarker of oral carcinogenesis.

CONCLUSION

Identification and management of oral epithelial dysplasia, which presents a high risk of malignant transformation, holds great promise for successful secondary prevention of OSCC, potentially reducing oral cancer morbidity and mortality. Oral leukoplakia, the most common potentially malignant disorder exhibiting different grades of dysplasia, has been shown to have a higher malignant transformation rate. Hence, we assessed the expression of total AQP3 antibody in these pathologies, because there is currently limited research in this area. Instead of using an antigen recognition site−specific antibody, we preferred a more sensitive total AQP3 antibody, so that even minimal expressions from lower grades of dysplasia could be assessed. Analysis of H-scores indicated that immunohistochemical expression of AQP3 is increasing in OED, with more expression in moderate to severe dysplasia, and was highest in OSCC compared to normal oral mucosa. Based on our results, we infer an important and novel role of total AQP3 as a potential biomarker for predicting the malignant transformation of OED and tumor progression.

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