

# Assessing the Expression of Aquaporin 3 Antigen-Recognition Sites in Oral Squamous Cell Carcinoma

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**Abstract:** Aquaporin 3 (AQP3) serves as a water and glycerol transporter facilitating epithelial cell hydration. Recently, the involvement of AQP3 in cancers has been reported. However, the immunohistochemical expression of AQP3 in carcinomas remains controversial. We hypothesized that differences in aquaporin 3 antigen recognition (AQP3 AR) may influence their expressions. Thus, our study aimed to assess the immunostaining patterns of 3 AQP3 AR sites in oral squamous cell carcinoma (OSCC) and to compare the adjacent areas of high-grade epithelial dysplasia (HG-ED) and normal oral mucosa (NOM). The study group included formalin-fixed OSCC samples (n = 51) with adjacent regions of HG-ED (n = 12) and NOM (n = 51). The tissues were stained with anti-AQP3 antibodies (AR sites at amino acid (AA) 250-C terminus, AA180-228, and N terminus AA1-80) by immunohistochemistry. Our results showed that strong membranous immunostaining was observed for AQP3 AR sites at the AA250-C terminus and AA180-228 in all the samples for NOM and weak AQP3 immunostaining for both the AR sites in all the 12 samples for HG-ED. The invasive front of OSCC samples showed that AQP3 AR at the AA250-C terminus decreased in 42/51 samples (82.4%) and AA180-228 in 47/51 samples (92.2%). Conversely, in the AQP3 AR site at N terminus AA1-80, all samples of the NOM showed negative or slightly positive staining in the cytoplasm of the lower layers. AQP3 expression was increased in 12/12 cases (100%) and 46/51 cases (90.2%) in the HG-ED and invasive front of OSCC, respectively. AQP3 may be used as a biomarker for detecting malignant transformations. AQP3 AR site differences influence their immunohistochemical expression in OSCC.

**Key Words:** oral cavity, squamous cell carcinoma, epithelial dysplasia, aquaporin 3, immunohistochemistry

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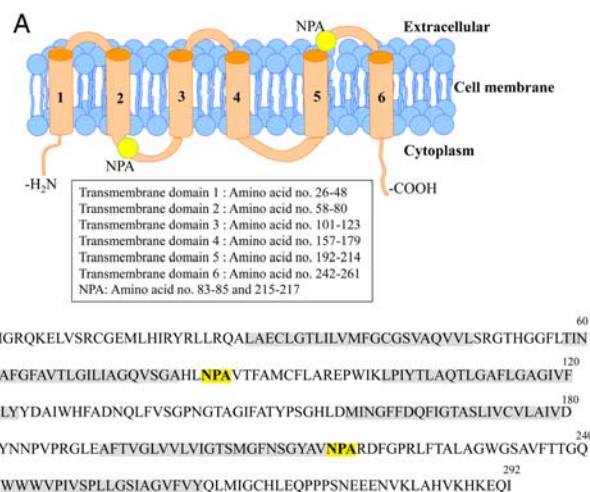
The authors declare no conflict of interest.

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Oral and oropharyngeal cancers together comprise the sixth most common form of cancer in the world.<sup>1</sup> More than 90% of oral cancers are oral squamous cell carcinomas (OSCCs).<sup>1</sup> OSCC is often preceded by oral potentially malignant disorders such as leukoplakia, which is defined as a white plaque of questionable risk, once other known diseases or disorders that carry no increased risk for cancer are ruled out.<sup>2</sup> The presence of epithelial dysplasia in oral potentially malignant disorders is an important prognostic indicator of malignant transformation.<sup>2</sup> At present, surgery is the preferred treatment for OSCC.<sup>3</sup> However, the 5-year survival rates (28% to 50%) remain unimproved despite progress in the treatment of OSCC over the past few decades.<sup>1,4,5</sup> Therefore, a number of biomarkers have been studied as potential prognostic factors and as therapeutic targets for treating OSCC.<sup>1</sup>

Aquaporins (AQPs) are water channel proteins that facilitate transepithelial water movement across the cell membrane. In humans, 13 isoforms (AQP0 to AQP12) have been identified. AQPs are categorized as aquaporins



**FIGURE 1.** Diagrammatic representation of the structure and amino acid sequence of aquaporin 3 (AQP3). A, Each AQP3 monomer has 6 transmembrane domains connected by 5 loops spanning the cell membrane. Conserved motifs, asparagine-proline-alanine (NPA), bend into the molecule and form the water channel. Both amino (-NH<sub>2</sub>) and carboxy (-COOH) termini are cytoplasmic. B, The polypeptide in the AQP3 structure is formed by a chain of 292 amino acids. The amino acids highlighted in gray are indicating the transmembrane domains of AQP3 protein.

**TABLE 1.** Clinicopathologic Features of 51 Oral Squamous Cell Carcinoma Samples

Characteristics	Cases (%)
Sex	
Male	32 (62.7)
Female	19 (37.3)
Age	
≥ 65	33 (64.7)
< 65	18 (35.3)
Location	
Tongue	38 (74.5)
Gingiva	5 (9.9)
Floor of the mouth	4 (7.8)
Buccal mucosa	4 (7.8)
Histologic grade	
Well	38 (74.5)
Moderate to poor	13 (25.5)
T status	
T1	28 (54.9)
T2+T3	23 (45.1)
Lymphatic metastasis	
Yes	22 (43.1)
No	29 (56.9)

(AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8), which exclusively transport water; aquaglyceroporins (AQP3, AQP7, AQP9, and AQP10), which can transport water, glycerol, and other small molecules; and super-aquaporins (AQP11 and AQP12), whose physiological roles remain unclear.<sup>6</sup> Previous studies on mice have shown that AQPs are involved in urine concentration, skin moisturization, and fat metabolism, along with having been implicated in tumorigenesis.<sup>7</sup>

AQP3s are structurally homotetramers, with each monomer comprising 6 transmembrane domains coupled by 5 loops spanning the whole cell membrane. Two highly conserved sequence motifs, asparagine-proline-alanine (NPA), are located on the opposite sides of the monomer. The NPA motifs bend into the AQP3 molecule and form a water pore.<sup>8,9</sup> The polypeptide in the structure is formed by a chain of 292 amino acids (AA), and both the amino (-NH<sub>2</sub>) and carboxy (-COOH) termini are cytoplasmic.<sup>9,10</sup> Figure 1 shows the structure of the human AQP3 monomer and AQP3 AA sequence adapted from Marlar et al,<sup>8</sup> GlobPlot2.3 (<http://globplot.embl.de>), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases at GenomeNet ([www.genome.jp](http://www.genome.jp)).<sup>11,12</sup> AQP3 is expressed in the human epithelium, particularly in the cell membranes of the kidney's collecting duct system, the urinary tract transitional epithelium, and the respiratory epithelium, along with the stratified squamous epithelial cells of the oral cavity, esophagus, and skin.<sup>8,13</sup> AQP3's major role is to provide

**TABLE 2.** Primary Antibodies of AQP3 Used in this Study

No.	Recognized Parts of AQP3	Antibody Host	Clone No.	Dilution	Antigen Retrieval	Incubation	Supplier
1	AA250-C terminal	PR	ab153694	1:1000	CB, 98°C, 40 min	4°C, ON	Abcam
2	AA180-228	PR	V214	1:100	Not performed	RT, 1 h	Bioworld Technology Inc.
3	N terminal-AA1-80	PR	sc-20811	1:100	CB, 98°C, 40 min	4°C, ON	Santa Cruz

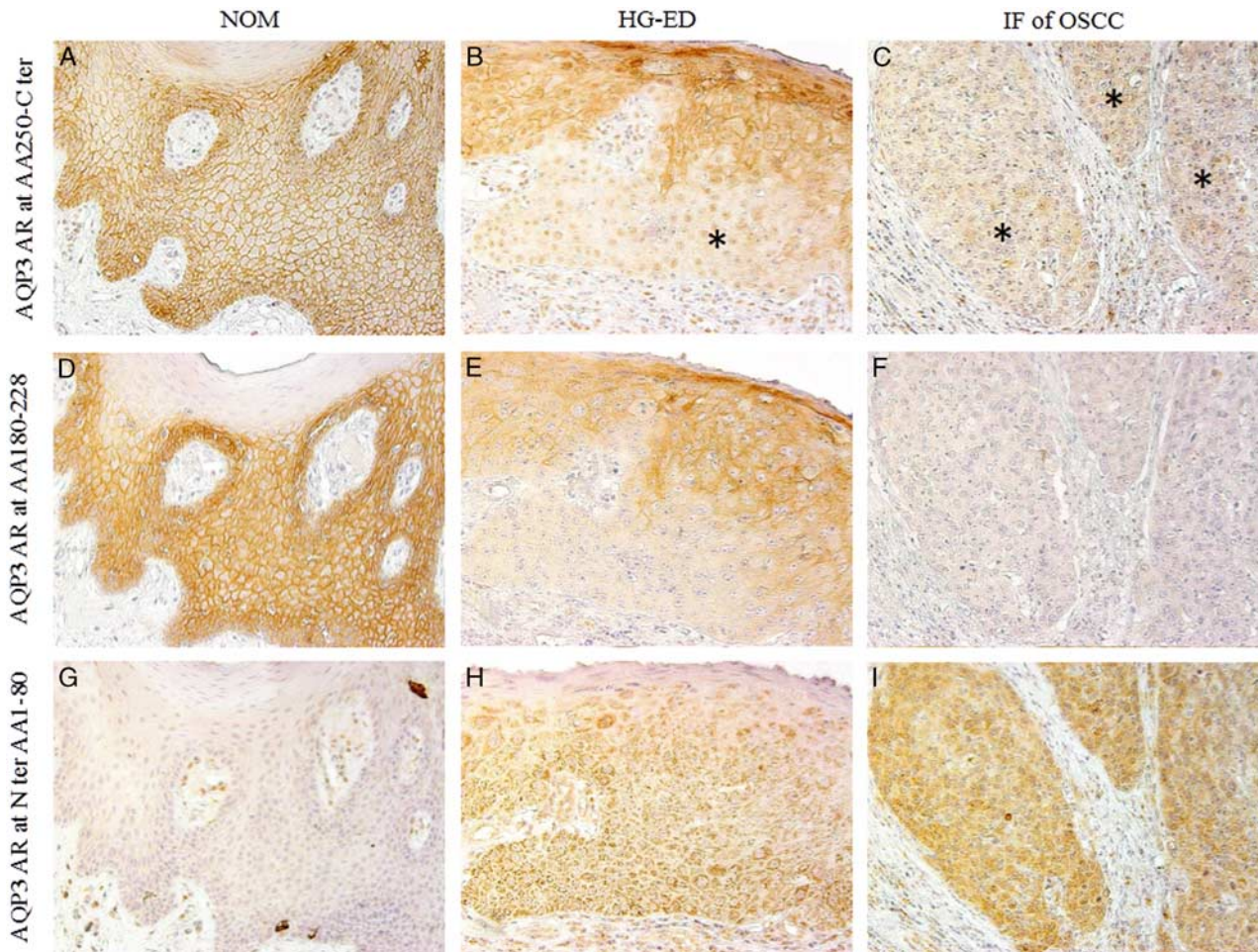
AQP3 indicates aquaporin 3; CB, citrate buffer (pH 6.0); ON, overnight; PR, rabbit polyclonal; RT, room temperature.

**TABLE 3.** Expression of AQP3 in the 3 Different AQP3 Antigen Recognitions

		No. Cases (%)			
AQP3 Recognition		NOM (N = 51)	HG-ED (N = 12)	SP OSCC (N = 51)	IF OSCC (N = 51)
AA250-C terminal	HM	51 (100)	0 (0)	43 (84.3)	9 (17.6)
	LM	0 (0)	12 (100)	8 (15.7)	42 (82.4)
AA180-228	HM	51 (100)	0 (0)	16 (31.4)	4 (7.8)
	LM	0 (0)	12 (100)	35 (68.6)	47 (92.2)
N terminal-AA1-80	HC	0 (0)	12 (100)	11 (21.6)	46 (90.2)
	LC	51 (100)	0 (0)	40 (78.4)	5 (9.8)

AQP3 indicates aquaporin 3; HC, high cytoplasmic expression, labeling index > 50%; HG-ED, high-grade epithelial dysplasia; HM, high membranous expression, labeling index > 50%; IF, invasive front; LC, low cytoplasmic expression, labeling index ≤ 50%; LM, low membranous expression, labeling index ≤ 50%; NOM, normal oral mucosa; OSCC, oral squamous cell carcinoma; SP, superficial part.

water to water-deprived cells.<sup>13</sup> Although previous studies have implicated AQP3 in cancer, immunohistochemical expression of AQP3 in carcinomas remains controversial.<sup>7,8</sup> Several studies have indicated that overexpression of AQP3 may contribute to tumor cell proliferation in various solid tumors such as gastric adenocarcinoma (GC) and esophageal squamous cell carcinomas (SCCs).<sup>14-16</sup> In contrast, growing evidence shows that AQP3 expression decreases in urothelial carcinomas (UCs) and SCCs of the skin, with the molecular mechanism being unclear.<sup>17,18</sup> To the best of our knowledge, a few studies have reported the immunohistochemical expressions and possible roles of AQP3 in OSCC, the results of which are controversial.<sup>16,19,20</sup> Kusayama et al<sup>16</sup> and Ishimoto et al<sup>19</sup> used anti-AQP3 antibody prepared from the N terminus AA1-80 peptide of AQP3 in their immunohistochemical studies and reported that AQP3 immunostaining was overexpressed in the OSCC samples, when compared with the normal oral mucosa (NOM) samples. The authors supposed that AQP3 may be involved in the focal adhesion kinase-mitogen-activated protein kinase pathway, which regulates tumor progression and growth in the human OSCC cell lines.<sup>16,19</sup> In contrast, in our previous study (2014), we used anti-AQP3 antibody prepared from the AA180-228 peptide of AQP3 in our study and showed that AQP3 immunostaining in OSCC tissues was weaker than that in NOM tissues.<sup>20</sup> We suggested that decreased AQP3 expression may be associated with more aggressive tumor behavior and that it increased the incidence of lymphatic metastasis.<sup>20</sup> To solve the discrepancy of AQP3 expression in



**FIGURE 2.** Expression of aquaporin 3 (AQP3) in the representative case of moderately differentiated oral squamous cell carcinoma (OSCC) with its adjacent high-grade epithelial dysplasia (HG-ED) and normal oral mucosa (NOM). NOM (A, D, G); HG-ED (B, E, H); Invasive front (IF) of OSCC (C, F, I). Immunostaining for AQP3 antigen recognition (AR) at AA250-C terminus (A–C), AQP3 AR at AA180-228 (D–F), and AQP3 AR at N terminus AA1-80 (G–I). Original magnification:  $\times 200$ . AQP3 AR at AA250-C terminus and AA180-228 showed similar staining trend. In the NOM (A, D), strong membranous positive staining with faint cytoplasmic staining of the epithelial cells was observed. For HG-ED and IF of OSCC (B, C, E, F), AQP3 expression was decreased. Asterisks are marking abnormal nuclear and/or weak cytoplasmic AQP3 staining in the AQP3 AR site at the AA250-C terminus. AQP3 AR at N terminus AA1-80 showed different staining patterns. For NOM (G), AQP3 showed negative or slightly positive staining in the cytoplasm of the lower layers. In HG-ED and IF of OSCC (H, I), AQP3 expression was increased. [full color online](#)

carcinomas, accurate information about aquaporin 3 antigen-recognition (AQP3 AR) sites by anti-AQP3 antibodies is crucial. We hypothesized that differences in AQP3 AR may be indicative of their expression. We investigated the immunostaining patterns of the 3 different AQP3 AR sites in OSCC, comparing the adjacent areas of high-grade (moderate to severe) epithelial dysplasia (HG-ED) and NOM.

## MATERIALS AND METHODS

### Samples

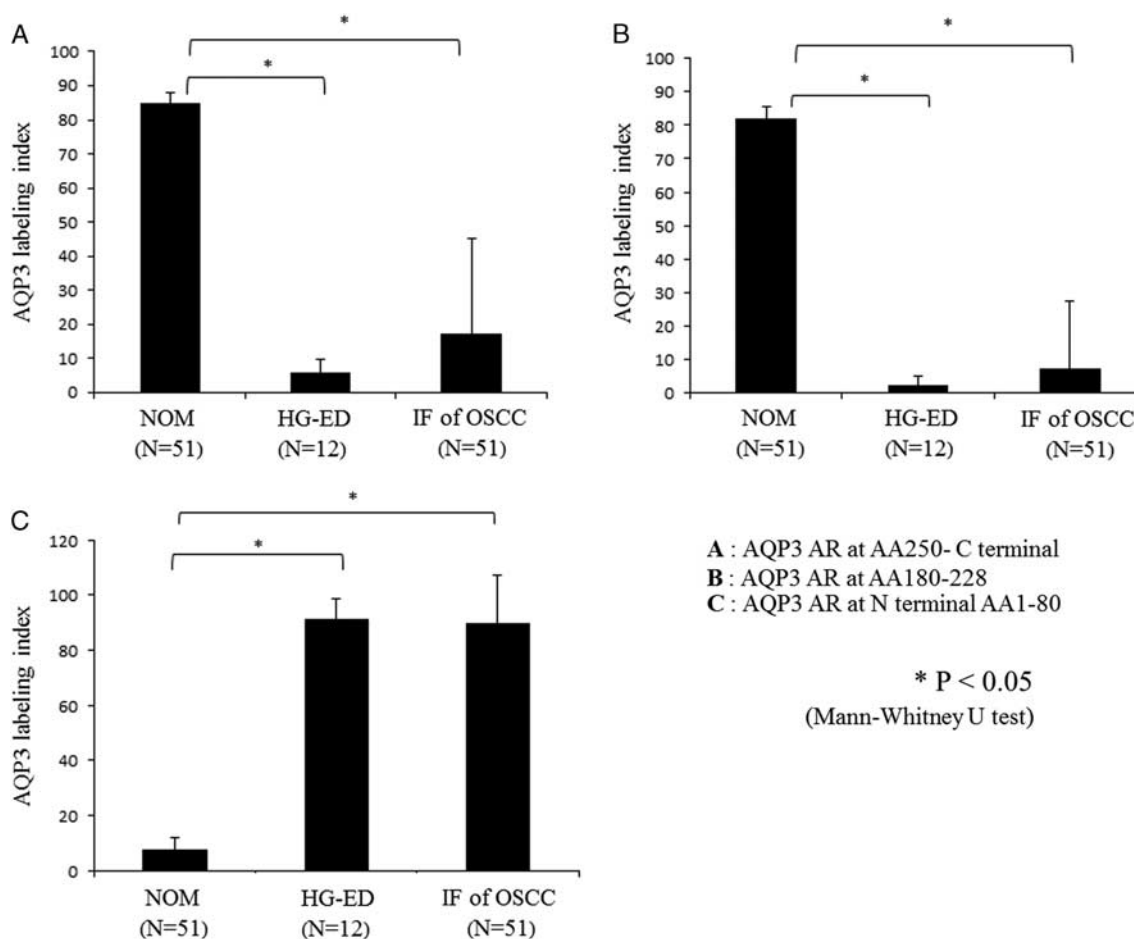
In total, 51 formalin-fixed, paraffin-embedded biopsy and resection specimens of OSCC, containing simultaneous areas of NOM and/or HG-ED were chosen for this study. The histopathologic diagnoses were confirmed by 2 oral

pathologists (N.Y. and K.M.). Clinical data on the patients, such as sex, age, and location, were also included. In addition, pathologic reports were used to assess the histologic grade, T status of the tumors, and lymphatic metastasis (Table 1). Each specimen was categorized as invasive front (IF) of OSCC ( $n=51$ ), superficial part (SP) of OSCC ( $n=51$ ), NOM ( $n=51$ ), and/or HG-ED ( $n=12$ ). This study was approved by the Kyushu Dental University Ethics Committee (approved number: 16-8).

### Immunohistochemical Study

Between February 2004 and November 2017 at the Department of Oral Pathology, Kyushu Dental University, all the specimens were fixed with 10% formalin and were embedded in paraffin. Four-micrometer-thick sections were





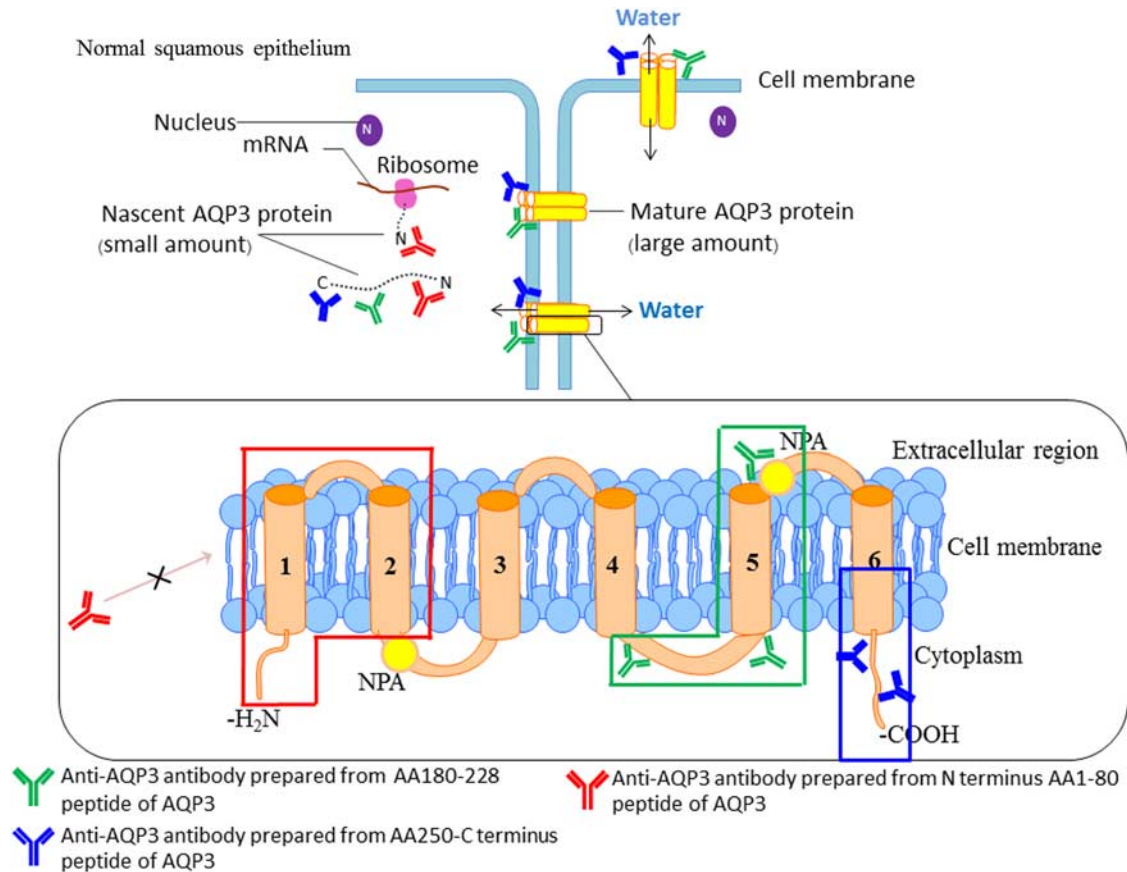
**FIGURE 3.** Averages of aquaporin 3 (AQP3) labeling index of the 3 different AQP3 antigen-recognition (AR) sites. The mean labeling index of AQP3 AR at AA250-C terminus (A) and AA180-228 (B) was significantly higher in normal oral mucosa (NOM) than that in high-grade epithelial dysplasia (HG-ED) and invasive front of oral squamous cell carcinoma (IF of OSCC) ( $P < 0.05$ ). Conversely, the mean labeling index of AQP3 AR at N terminus AA1-80 (C) was significantly higher in HG-ED and IF of OSCC than that in NOM ( $P < 0.05$ ).

deparaffinized in xylene and were serially rehydrated in ethanol. Endogenous peroxidase activity was then quenched with 3% hydrogen peroxide for 20 minutes. For antigen retrieval, if necessary, the sections were heated in 10 mM citrate buffer (pH 6.0) at 98°C for 40 minutes. Nonspecific protein binding was blocked by incubation in 10% normal goat serum for 10 minutes. Thereafter, the specimens were incubated with rabbit polyclonal anti-AQP3 antibodies (AR at AA250-C terminus, AA180-228, and N terminus AA1-80 parts of AQP3) for 1 hour at room temperature or overnight at 4°C. The recognized epitopes and other conditions are summarized in Table 2 and Figure 1B. The tissue sections were then incubated with the secondary antibody for 30 minutes at room temperature. Counterstaining was performed using Mayer's hematoxylin stain for 90 seconds, after which the sections were dehydrated serially in ethanol, cleared with xylene, and mounted on slides with a coverslip.

### Evaluation of Immunohistochemistry

Localization of staining was recorded, and the labeling index (LI) was calculated by dividing the number of

AQP3-positive epithelial cells by the total number of cells, and it was expressed in percentage. Expression of AQP3 localized at the basolateral membranes in the kidney's collecting duct in normal human tissue microarrays was used as the positive control.<sup>13</sup> For the AQP3 AR sites at AA250-C terminus and AA180-228, the criteria used to define AQP3-positive cells included complete membranous staining. Abnormal staining included absent membranous staining, and cytoplasmic and/or nuclear staining was considered as negative. For the AQP3 AR site at N terminus AA1-80, the epithelial cells were considered as positive when clear cytoplasmic staining was observed. A minimum of 500 cells was counted manually for each study group (NOM, HG-ED, SP, and IF of OSCC). Subsequently, the staining of AR sites at AA250-C terminus and AA180-228 was categorized as high membranous expression (HM: LI > 50%) and low membranous expression (LM: LI ≤ 50%). Staining of AR site at N terminus AA1-80 was categorized as high cytoplasmic expression (HC: LI > 50%) and low cytoplasmic expression (LC: LI ≤ 50%).



**FIGURE 4.** Schematic illustration of possible results of aquaporin 3 (AQP3) antigen-recognition (AR) site differences in normal oral mucosa (NOM). A large amount of mature (membranous) AQP3 in the NOM may not be recognized by anti-AQP3 antibody prepared from N terminus AA1-80 peptide of AQP3, whereas this antibody may recognize nascent AQP3 protein, which was scarcely generated in the NOM. Thus, in the AQP3 AR site at N terminus AA1-80, NOM was negative or slightly positive in the lower layers. In contrast, a large amount of mature (membranous) AQP3 and a small amount of nascent AQP3 protein in the NOM could be recognized by anti-AQP3 antibody prepared from AA180-228 and AA250-C terminus peptide of AQP3. Therefore, in the AQP3 AR sites, at AA180-228 and AA250-C terminus, NOM showed strong membranous immunostaining with faint cytoplasmic staining. full color online

### Statistical Analysis

Yate  $\chi^2$  test was used to examine the association between AQP3 expression and clinicopathologic information. Mean labeling indices among the study groups were compared using the Mann-Whitney *U* test. A *P*-value <0.05 was considered significant.

### RESULTS

Clinical and histopathologic data on the 51 OSCC samples are summarized in Table 1. No correlation between AQP3 expression and clinicopathologic information was observed (data not shown). The overall expression of AQP3 is summarized in Table 3.

#### Immunostaining of AQP3 AR at AA250-C Terminus

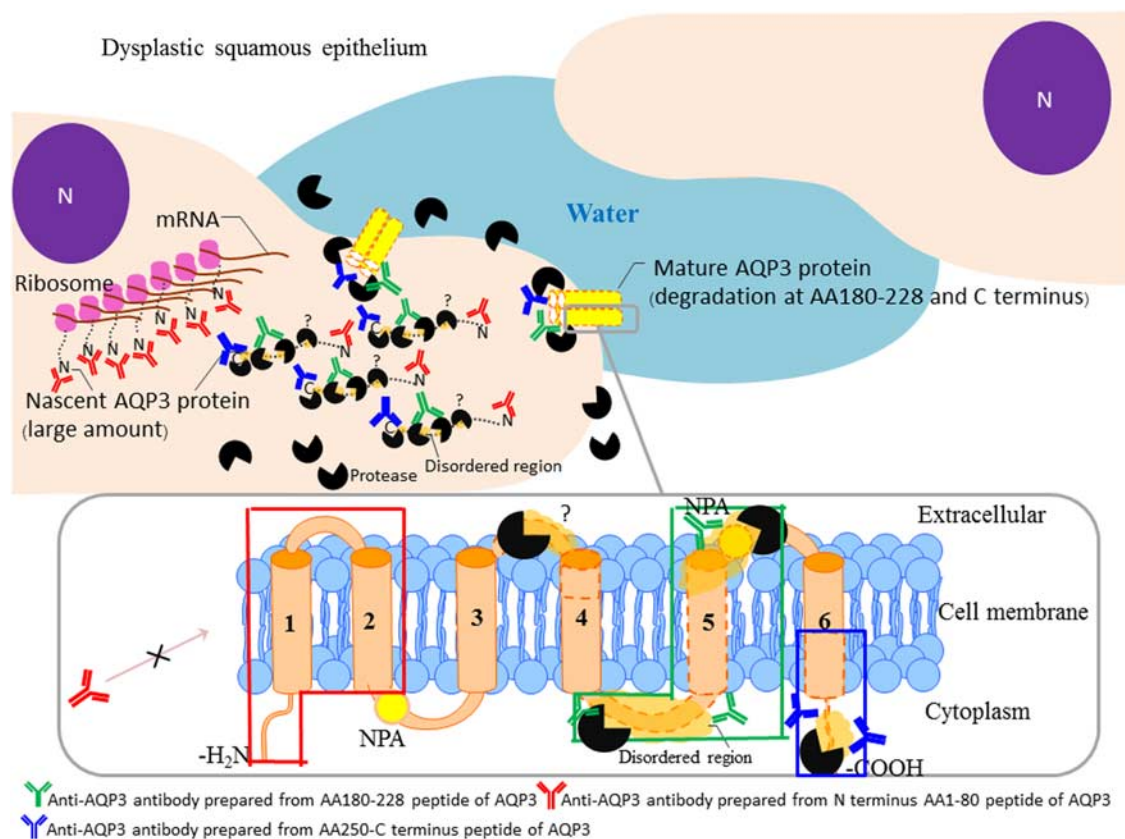
For NOM, all 51 samples showed diffuse, homogeneous, and strong immunostaining in the cell membrane, with faint immunostaining in the cytoplasm of cells of the

basal, suprabasal, and spinous layers (HM: 100% samples). AQP3 immunostaining was decreased in all the 12 samples of HG-ED (LM: 100% samples). In the SP of OSCC, 43/51 samples retained a considerable membranous expression (HM: 84.3% samples), whereas reduced expression of AQP3 was observed in 42/51 samples in the IF of OSCC (LM: 82.4% samples) (Figs. 2A–C).

The mean LI values of NOM, HG-ED, and IF of OSCC were  $84.9 \pm 3.1$ ,  $5.9 \pm 3.9$ , and  $17.4 \pm 27.8$ , respectively. There was a statistically significant decrease in the mean LI of AQP3 AR at the AA250-C terminus in HG-ED and IF of OSCC compared with that of NOM (*P* < 0.05) (Fig. 3A).

#### Immunostaining of AQP3 AR at AA180-228

For NOM, all 51 samples showed diffuse and strong membranous with faint cytoplasmic immunostaining in the suprabasal and spinous cell layers. The basal cells showed trace staining (HM: 100% samples). For HG-ED, SP, and IF of OSCC, AQP3 immunostaining was often



**FIGURE 5.** Schematic illustration of possible results of aquaporin 3 (AQP3) antigen-recognition (AR) site differences in high-grade epithelial dysplasia (HG-ED) and oral squamous cell carcinoma (OSCC). The dysplastic and tumor cells might produce a lot of nascent AQP3 protein. AA135-157, AA180-228, and AA250-C terminus parts of the nascent AQP3 protein might be degraded (described later), whereas N terminus AA1-80 part of the nascent AQP3 protein was retained and could be detected by anti-AQP3 antibody prepared from N terminus AA1-80 peptide of AQP3. Consequently, in the AQP3 AR site at N terminus AA1-80, cytoplasmic AQP3 immunostaining increased in the HG-ED and invasive front of OSCC. In contrast, membrane-type 1 matrix metalloproteinase and other proteases, which were secreted from the tumor cells, might bind the disordered regions (AA135-157, AA182-188, AA208-218, and AA269-276) of both mature (membranous) and nascent AQP3 proteins, resulting in degradation of these disordered regions and surrounding peptides. Thus, in the AQP3 AR sites at AA180-228 and AA250-C terminus, AQP3 immunostaining was decreased in HG-ED and invasive front of OSCC. Moreover, degradation of the disordered protein-asparagine-proline-alanine (NPA) site (AA215-217) may impair water movement across cell membranes and cause water retention around the dysplastic epithelium, which might result in dis-cohesion and migration of the cancer cells. [full color online](#)

decreased, respectively, for 12/12 samples (LM: 100% samples), 35/51 samples (LM: 68.6% samples), and 47/51 samples (LM: 92.2% samples) (Figs. 2D–F).

The mean LI values for NOM, HG-ED, and IF of OSCC were  $82.0 \pm 3.7$ ,  $2.4 \pm 2.6$ , and  $7.3 \pm 19.9$ , respectively. There was a statistically significant decrease in the mean LI of AQP3 AR at AA180-228 in HG-ED and IF of OSCC compared with that of NOM ( $P < 0.05$ ) (Fig. 3B).

### Immunostaining of AQP3 AR at N Terminus AA1-80

For the NOM, all 51/51 samples showed absent or slightly positive staining of the cytoplasm of basal and suprabasal layers (LC: 100% samples). For HG-ED, cytoplasmic AQP3 immunostaining increased to intermediate and upper portions in all 12 samples (HC: 100% samples). In the SP of OSCC, 40/51 samples showed

cytoplasmic AQP3 positivity at the periphery of tumor nests, with weaker or almost negative staining in the center (LC: 78.4% samples). More diffuse with moderate to strong cytoplasmic AQP3 immunostaining was observed in 46/51 samples of the IF of OSCC (HC: 90.2% samples) (Figs. 2G–I).

The mean LI values for NOM, HG-ED, and IF of OSCC were  $7.4 \pm 4.7$ ,  $91.1 \pm 7.5$ , and  $89.9 \pm 17.5$ , respectively. There was a statistically significant increase in the mean LI of AQP3 AR at N terminus AA1-80 in HG-ED and IF of OSCC compared with that of NOM ( $P < 0.05$ ) (Fig. 3C).

### DISCUSSION

Recently, AQP3 has been reported to be involved in several types of cancers. However, the immunohistochemical expression of AQP3 in carcinomas remains controversial.<sup>7,8</sup>

TABLE 4. Summary of AQP3 Antigen Recognitions at N Terminus AA1-80 Expression Patterns in the Reported Carcinomas

No.	Recognized Part of AQP3	Antibody Host	Normal Tissues			Dysplastic Tissues			Carcinomas		
			Organ	Type	Expression Pattern	Expression Pattern	Type	Expression Pattern	Type	Expression Pattern	References
1	N terminal	PR	Stomach	Gastric mucosa	Almost negative	NP	NP	GC	Generally increasing CP	Shen et al <sup>14</sup>	
2	N terminal	PR	Esophagus	Squamous epithelium	Almost negative	NP	NP	SCC	63% of cases showed increased CP	Kusayama et al <sup>16</sup>	
3	N terminal	PR	Oral cavity	Squamous epithelium	Almost negative	NP	NP	SCC	73% of cases showed increased CP	Kusayama et al <sup>16</sup>	
4	N terminal	PR	Oral cavity	Squamous epithelium	Almost negative	NP	NP	SCC	83% of cases showed increased CP	Ishimoto et al <sup>19</sup>	
5	N terminal	PR	Cervix	Squamous epithelium	Almost negative	Increased CP	Increased CP	SCC	44% of cases showed increased CP	Shi et al <sup>22</sup>	
6	N terminal	PR	Stomach	Gastric mucosa	Almost negative	NP	NP	GC	73% of cases showed increased CP	Chen et al <sup>15</sup>	
7	N terminal	PR	Stomach	Gastric mucosa	Almost negative	NP	NP	GC	79% of cases showed increased CP	Zhou et al <sup>23</sup>	
8	N terminal	PR	Oral cavity	Squamous epithelium	Almost negative	Increased CP	Increased CP	SCC	90% of cases of the IF part showed increased CP	This study	

AA indicates amino acid; AQP3, aquaporin 3; CP, cytoplasmic expression; GC, gastric adenocarcinoma; IF, invasive front; NP, not performed; PR, rabbit polyclonal; SCC, squamous cell carcinoma.

Differences in AQP3 AR sites may influence the immunohistochemical expression patterns. To our knowledge, this is the first attempt to evaluate the immunostaining patterns of 3 different AQP3 AR sites in NOM, HG-ED, SP, and IF of OSCC, which would improve our understanding of the role of AQP3 in oral carcinogenesis.

### AQP3 AR Site at N Terminus AA1-80

In AQP3 AR site at N terminus AA1-80, NOM stained negative or slightly positive in the cytoplasm of basal and suprabasal layers. Normally, in human and rat tissues, AQP3 was clearly expressed in the cell membranes of the squamous epithelia in the skin and oral mucosa.<sup>13,21</sup> It is probable that plenty of mature (membranous) AQP3s in the NOM may not be recognized by anti-AQP3 antibody prepared from the N terminus AA1-80 peptide of AQP3, while this antibody may recognize nascent AQP3 protein, which was slightly produced in the NOM (Fig. 4). Cytoplasmic AQP3 immunostaining was increased in HG-ED and IF of OSCC. It is possible that the dysplastic and tumor cells might generate a lot of nascent AQP3 protein. AA180-228 and AA250-C terminus regions of the nascent AQP3 protein might be degraded (described later), whereas the N terminus AA1-80 part of the nascent AQP3 protein was retained and could be detected by anti-AQP3 antibody prepared from N terminus AA1-80 peptide of AQP3 (Fig. 5).

Our results were in agreement with previous studies using anti-AQP3 antibody prepared from N terminus AA1-80 peptide of AQP3 in the dysplastic squamous epithelium of the cervix, SCC of the cervix, esophagus, and oral cavity, and GCs (Table 4).<sup>14-16,19,22,23</sup> Increased N terminus AA1-80 part of nascent AQP3 protein in GC is associated with an increase in nuclear translocation of  $\beta$ -catenin, which leads to the proliferation of tumor cells.<sup>23</sup> In addition, overexpression of N terminus AA1-80 part of nascent AQP3 protein correlates with downregulation of E-cadherin and overexpression of vimentin in poorly differentiated GC, thereby suggesting a role of AQP3 in the epithelial-to-mesenchymal transition process.<sup>15</sup> Moreover, in OSCC, overexpression of N terminus AA1-80 part of nascent AQP3 protein may be related to the focal adhesion kinase-mitogen-activated protein kinase signaling pathway, resulting in tumor cell proliferation.<sup>16,19</sup>

### AQP3 AR Sites at AA180-228 and AA250-C Terminus

In AQP3 AR sites at AA180-228 and AA250-C terminus, AQP3 staining was strong in the cell membrane, but it was faint in the cytoplasm of the NOM, which is consistent with the fact that AQP3 is a transmembrane protein.<sup>13,21,24</sup> A large amount of mature (membranous) AQP3 with a small amount of nascent AQP3 protein in the NOM may be recognized by anti-AQP3 antibody prepared from AA180-228 and AA250-C terminus peptide of AQP3 (Fig. 4). AQP3 immunostaining was decreased in HG-ED and IF of OSCC. It is widely accepted that tumor cells secrete proteases that can degrade the tumor barriers and thus facilitate tumor progression and invasion.<sup>25</sup> Membrane-type 1 matrix metalloproteinase is one of the proteases that degrade extracellular matrix proteins,

**TABLE 5.** Summary of AQP3 Antigen Recognitions at C Terminus and AA180-228 Expression Patterns in the Reported Carcinomas

No.	Recognized Part of AQP3	Antibody Host	Organ	Normal Tissues		Dysplastic Tissues		Carcinomas		References
				Type	Expression Pattern	Type	Expression Pattern	Type	Expression Pattern	
1	C terminal	PR	Lung	BSE	MB	NP	SCC	64% of cases showed loss of MB	Liu et al <sup>29</sup>	
2	C terminal	PR	Lung	BSE	MB	NP	BAC with invasive ADC	Invasive ADC showed loss of MB	Machida et al <sup>30</sup>	
3	C terminal	PR	Skin	Squamous epithelium	MB	NP	SCC	100% of cases showed loss of MB	Voss et al <sup>18</sup>	
4	C terminal	PG	Urinary bladder	Urothelium	MB	NP	pT2 UC	100% of cases showed loss of MB	Rubewolf and colleagues <sup>31,32</sup>	
5	C terminal	PR	Urinary bladder	Urothelium	MB	NP	pT1 UC	41% of cases showed loss of MB	Otto et al <sup>17</sup>	
6	C terminal	PR	Thyroid gland	C cells and follicular cells	Almost negative	NP	MTC	90% of cases showed increased MB	Niu et al <sup>33</sup>	
7	C terminal	PG	Urinary bladder	Urothelium	MB	NP	pT2 UC	67% of cases showed loss of MB	Rubewolf et al <sup>34</sup>	
8	AA180-228	PR	Oral cavity	Squamous epithelium	MB	Loss of MB	SCC	Generally the loss of MB	Matsuo and Kawano <sup>20</sup>	
9	C terminal	PR	Skin	Squamous epithelium	MB	NP	SCC	Generally the loss of MB	Seleit et al <sup>35</sup>	
10	C terminal	PR	Liver	Hepatocyte	Almost negative	NP	HCC	93% of cases showed increased MB	Peng et al <sup>36</sup>	
11	C terminal	PR	Pancreas	Ductal cells	Almost negative	NP	PDA	Generally increased MB	Direito et al <sup>37</sup>	
12	C terminal	PR	Urinary bladder	Urothelium	NP	MB	pT2 UC	100% of cases showed loss of MB	Breyer et al <sup>38</sup>	
13	C terminal	PR	Prostate gland	Glandular epithelial cells	MB	NP	High-risk PC	Generally the loss of MB	Brundl et al <sup>39</sup>	
14	C terminal	PR	Breast	Ductal cells	Almost negative	NP	TNBC	61% of cases showed increased MB	Zhu et al <sup>40</sup>	
15	C terminal	PR	Oral cavity	Squamous epithelium	MB	Loss of MB	SCC	84% of cases of the IF part showed loss of MB	This study	
16	AA180-228	PR	Oral cavity	Squamous epithelium	MB	Loss of MB	SCC	92% of cases of the IF part showed loss of MB	This study	

AA indicates amino acid; ADC, pulmonary adenocarcinoma; AQP3, aquaporin 3; BAC, bronchioloalveolar carcinoma; BSE, bronchial surface epithelium; HCC, hepatocellular carcinoma; IF, invasive front; MB, membranous expression; MTC, medullary thyroid carcinoma; NP, not performed; PC, prostate adenocarcinoma; PDA, pancreatic ductal adenocarcinoma; PG, goat polyclonal; PR, rabbit polyclonal; pT1 UC, urothelial carcinoma invades connective tissue; pT2 UC, urothelial carcinoma invades muscle; SCC, squamous cell carcinoma; TNBC, triple-negative breast cancer.



membrane proteins, and other proteins.<sup>26</sup> Interestingly, Kjaergaard et al<sup>27</sup> have reported on unstructured or disordered regions (DRs) in membrane proteins. These DRs are important for signal transduction and are extremely susceptible to proteolysis, thereby directly signaling for rapid degradation.<sup>27,28</sup> From GlobPlot2.3 (<http://globplot.embl.de>), there are 4 DRs in the AQP3 protein: AA135-157, AA182-188, AA208-218, and AA269-276.<sup>11</sup> Membrane-type 1 matrix metalloproteinase and other proteases might bind these DRs of both mature (membranous) and nascent AQP3 proteins in dysplastic epithelial cells and tumor cells, resulting in degradation of the DRs and surrounding peptides. Moreover, the disordered protein-NPA site (AA215-217) might degrade in the dysplastic and cancer cells. Degradation of the NPA site may impair water transport across cell membranes and cause water retention around the cancer cells, which might result in discohesion and migration of the cancer cells (Fig. 5).

Our results were similar to the studies using anti-AQP3 antibodies prepared from AA180-228 and C terminus peptide of AQP3 in the SCCs of the lung, skin, and oral cavity, UC, prostate adenocarcinoma, and bronchioloalveolar carcinoma (Table 5).<sup>17,18,20,29-32,34,35,38,39</sup> Degradation of AQP3 protein located near the C terminus part in UC and AA180-228 part of AQP3 in OSCC is associated with a higher grade of the tumors and tumor cell invasion (muscle-invasive UC and OSCC with lymphatic metastasis cases), the underlying molecular mechanism of which remains unclear.<sup>17,20,34</sup> However, our results were contradictory to those of studies on medullary thyroid carcinoma, hepatocellular carcinoma, pancreatic ductal adenocarcinoma, and triple-negative breast cancer, which suggested increased expression of AQP3 in these carcinomas (Table 5).<sup>33,36,37,40</sup> Such contradictory observations on AQP3 expression may be attributable to the differences in tumor cell types. In addition, in the present study, in AQP3 AR sites at AA250-C terminus and AA180-228, we found a discordance in the expression of OSCC at the SP. For AQP3 AR at AA250-C terminus, tumor nests displayed a predominant membranous expression (84.3% samples), while for that at AA180-228, AQP3 immunostaining was reduced in most cases (68.6% samples). In general, AQP3 AR at AA180-228 showed weaker staining in HG-ED and IF of OSCC than AQP3 AR at AA250-C terminus. It is possible that, at the HG-ED, SP, and IF of OSCC, AQP3 AR at AA180-228, which detected 18 DRs (AA182-188 and AA208-218), might result in faster AQP3 protein degradation (by the proteases) when compared with AQP3 AR at the AA250-C terminus that detected 8 DRs (AA269-276).<sup>11,27,28</sup>

## CONCLUSIONS

To summarize, AQP3 could be used as a novel biomarker for detecting malignant transformations in the squamous epithelium. Our findings show that the differences in AQP3 AR sites affected their immunohistochemical expression in OSCC. In the AQP3 AR site at N terminus AA180, AQP3 immunostaining was found to be increased in the

dysplastic squamous epithelium compared with the normal squamous epithelium, whereas in AQP3 AR sites at AA180-228 and AA250-C terminus, AQP3 expression was weaker and irregular in the dysplastic squamous epithelium than that in the normal squamous epithelium. Our data suggest that understanding the AQP3 AR site of each anti-AQP3 antibody before performing an immunohistochemistry analysis is critical. A combination expression pattern of N terminus and C terminus parts of AQP3 might be a more accurate marker for detecting malignant transformation. However, it is possible that, on the basis of the field of cancerization concept, the areas adjacent to carcinoma already harbor mutations that may not yet cause phenotypical features. Further studies, with the use of additional molecular biology, are warranted to confirm our results and precisely investigate the molecular mechanism underlying the role of AQP3 in carcinogenesis.

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