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3   carcinoma

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40   **Abstract**

41   Aquaporin3 (AQP3) serves as a water and glycerol transporter facilitating epithelial

42   cells hydration. Recently, involvement of AQP3 in cancers has been reported. However,

43   immunohistochemical expression of AQP3 in carcinomas remains controversial. We

44   hypothesized that differences in AQP3 antigen recognition (AQP3 AR) may influence their

45   expressions. Thus, our study aimed to assess the immunostaining patterns of three AQP3 AR

46   sites in oral squamous cell carcinoma (OSCC) and comparing the adjacent areas of high-

47   grade epithelial dysplasia (HG-ED) and normal oral mucosa (NOM). Study group included

48   formalin-fixed OSCC samples (n=51) with adjacent regions of HG-ED (n=12) and NOM

49   (n=51). The tissues were stained with anti-AQP3 antibodies (AR sites at amino-acid (AA)

50   250-C terminus, AA180-228, and N terminus AA1-80) by immunohistochemistry. Our

51   results showed that strong membranous immunostaining was observed for AQP3 AR sites at

52   AA250-C terminus and AA180-228 in all the samples for NOM and weak AQP3

53   immunostaining for both the AR sites in all the 12 samples for HG-ED. The invasive front

54   (IF) of OSCC samples showed that AQP3 AR at AA250-C terminus decreased in 42/51

55   samples (82.4%) and AA180-228 in 47/51 samples (92.2%). Conversely, in AQP3 AR site at

56   N terminus AA1-80, all samples of the NOM showed negative or slightly positive staining in

57   the cytoplasm of the lower layers. AQP3 expression was increased in 12/12 cases (100%) and

58   46/51 cases (90.2%) in the HG-ED and IF of OSCC, respectively. AQP3 may be used as a

59   biomarker for detecting malignant transformations. AQP3 AR site differences influence their

60   immunohistochemical expression in OSCC.

61

62   **Keywords:** Oral cavity; Squamous cell carcinoma; Epithelial dysplasia; Aquaporin 3;

63   Immunohistochemistry

64   **Introduction**

65   Oral and oropharyngeal cancers together comprise the sixth most common form of

66   cancer in the world.[1] More than 90% of oral cancers are oral squamous cell carcinomas

67   (OSCCs).[1] OSCC is often preceded by oral potentially malignant disorders (OPMDs) such as

68   leukoplakia, which is defined as a white plaque of questionable risk, once other known

69   diseases or disorders that carry no increased risk for cancer are ruled out.[2] The presence of

70   epithelial dysplasia in OPMDs is an important prognostic indicator of malignant

71   transformation.[2] At present, surgery is the preferred treatment for OSCC.[3] However, the five-

72   year survival rates (28%–50%) remain unimproved despite progress in the treatment of

73   OSCC over the past few decades.[1, 4, 5] Therefore, a number of biomarkers have been studied

74   as potential prognostic factors and as therapeutic targets for treating OSCC.[1]

75   Aquaporins (AQPs) are water channel proteins that facilitate transepithelial water

76   movement across the cell membrane. In humans, thirteen isoforms (AQP0–AQP12) have

77   been identified. AQPs are categorized as aquaporins (AQP0, AQP1, AQP2, AQP4, AQP5,

78   AQP6, and AQP8), which exclusively transport water; aquaglyceroporins (AQP3, AQP7,

79   AQP9, and AQP10), which can transport water, glycerol, and other small molecules; and

80   super-aquaporins (AQP11 and AQP12), whose physiological roles remain unclear.[6] Previous

81   studies on mice have shown that AQPs are involved in urine concentration, skin

82   moisturization, and fat metabolism, along with having been implicated in tumorigenesis.[7]

83   AQP3s are structurally homotetramers, with each monomer comprising six

84   transmembrane domains coupled by five loops spanning the whole cell membrane. Two

85   highly conserved sequence motifs, asparagine-proline-alanine (NPA), are located on the

86   opposite sides of the monomer. The NPA motifs bend into the AQP3 molecule and form a

87   water pore.[8, 9] The polypeptide in the structure is formed by a chain of 292 amino acids (AA),

88 and both the amino (-NH$_2$) and carboxy (-COOH) termini are cytoplasmic.[9, 10] Figure 1 shows

89 the structure of the human AQP3 monomer and AQP3 AA sequence adapted from Marlar S,

90 et al., GlobPlot2.3 (http://globplot.embl.de)., and the Kyoto Encyclopedia of Genes and

91 Genomes (KEGG) databases at GenomeNet (https://www.genome.jp).[8, 11, 12] AQP3 is

92 expressed in the human epithelium, particularly in the cell membranes of the kidney

93 collecting duct system, urinary tract transitional epithelium, and respiratory epithelium, along

94 with the stratified squamous epithelial cells of the oral cavity, esophagus, and skin.[8, 13]

95 AQP3's major role is to provide water to water-deprived cells.[13] Although previous studies

96 have implicated AQP3 in cancer, immunohistochemical expression of AQP3 in carcinomas

97 remains controversial.[7, 8] Several studies have indicated that overexpression of AQP3 may

98 contribute to tumor-cell proliferation in various solid tumors such as gastric adenocarcinoma

99 and esophageal squamous cell carcinomas (SCCs).[14-16] On the other hand, growing evidence

100 shows that AQP3 expression decreases in urothelial carcinomas and SCCs of the skin, with

101 the molecular mechanism being unclear.[17, 18] To the best of our knowledge, a few studies

102 have reported the immunohistochemical expressions and possible roles of AQP3 in OSCC,

103 which the results are controversial.[16, 19, 20] Kusayama M, et al (2011) and Ishimoto S, et al

104 (2012) used anti-AQP3 antibody prepared from N terminus AA1-80 peptide of AQP3 in their

105 immunohistochemical studies and reported that AQP3 immunostaining was overexpressed in

106 the OSCC samples, when compared to the normal oral mucosa (NOM) samples.[16, 19] The

107 authors supposed that AQP3 may be involved in the focal adhesion kinase (FAK)-mitogen-

108 activated protein kinase (MAPK) pathway, which regulates tumor progression and growth in

109 the human OSCC cell lines.[16, 19] On the contrary, in our previous study (2014), we used anti-

110 AQP3 antibody prepared from AA180-228 peptide of AQP3 in our study and showed that

111 AQP3 immunostaining in OSCC tissues was weaker than that in NOM tissues.[20] We

112 suggested that decreased AQP3 expression is associated with more aggressive tumor

113   behavior and increased the incidence of lymphatic metastasis.[20] To solve the discrepancy of

114   AQP3 expression in carcinomas, accurate information about AQP3 antigen recognition

115   (AQP3 AR) sites by anti-AQP3 antibodies is crucial. We hypothesized that differences in

116   AQP3 AR may be indicative of their expression. We investigated the immunostaining

117   patterns of the three different AQP3 AR sites in OSCC, comparing the adjacent areas of high-

118   grade (moderate to severe) epithelial dysplasia (HG-ED) and NOM.

119   **Materials and Methods**

120   Samples

121   In total, 51 formalin fixed, paraffin-embedded biopsy and resection specimens of

122   OSCC, containing simultaneous areas of NOM and/or HG-ED were chosen for this study.

123   The histopathological diagnoses were confirmed by two oral pathologists (NY and KM).

124   Clinical data on the patients, such as sex, age, and location, were also included. In addition,

125   pathological reports were used to assess the histological grade, T status of the tumors, and

126   lymphatic metastasis (Table 1). Each specimen was categorized as invasive front (IF) of

127   OSCC (n = 51), superficial part (SP) of OSCC (n = 51), NOM (n = 51), and/or HG-ED (n =

128   12). This study was approved by the Kyushu Dental University Ethic Committee (approved

129   number: 16-8).

130   Immunohistochemical study

131   Between February 2004 and November 2017 at the Department of Oral Pathology,

132   Kyushu Dental University, all the specimens were fixed with 10% formalin and were

133   embedded in paraffin. Four-micrometer-thick sections were deparaffinized in xylene and

134   were serially rehydrated in ethanol. Endogenous peroxidase activity was then quenched with

135   3% hydrogen peroxide for 20 min. For antigen retrieval, if necessary, the sections were

136 heated in 10-mM citrate buffer (pH 6.0) at 98°C for 40 min. Non-specific protein binding was

137 blocked by incubation in 10% normal goat serum for 10 min. Then, the specimens were

138 incubated with rabbit polyclonal anti-AQP3 antibodies (AR at AA250-C terminus, AA180-

139 228, and N terminus AA1-80 parts of AQP3) for 1 h at room temperature or overnight at 4°C.

140 The recognized epitopes and other conditions are summarized in Table 2 and Figure 1B. The

141 tissue sections were then incubated with the secondary antibody for 30 min at room

142 temperature. Counterstaining was performed using Mayer's hematoxylin stain for 90 s, after

143 which the sections were dehydrated serially in ethanol, cleared with xylene, and mounted on

144 slide with a coverslip.

145 Evaluation of immunohistochemistry

146 Localization of staining was recorded and the labeling index (LI) was calculated by

147 dividing the number of AQP3 positive epithelial cells by the total number of cells, and was

148 expressed in percentage. Expression of AQP3 localized at basolateral membranes in the

149 kidney collecting duct in normal human tissue microarrays was used as the positive control.[13]

150 For the AQP3 AR sites at AA250- C terminus and AA180-228, the criteria used to define

151 AQP3 positive cells included complete membranous staining. Abnormal staining included

152 absent membranous staining, and cytoplasmic and/or nuclear staining was considered as

153 negative. For the AQP3 AR site at N terminus AA1-80, the epithelial cells were considered

154 as positive when clear cytoplasmic staining was observed. A minimum of 500 cells was

155 counted manually for each study group (NOM, HG-ED, SP and IF of OSCC). Subsequently,

156 the staining of AR sites at AA250- C terminus and AA180-228 was categorized as high

157 membranous expression (HM: LI > 50%) and low membranous expression (LM: LI $\leq$ 50%).

158 Staining of AR site at N terminus AA1-80 was categorized as high cytoplasmic expression

159 (HC: LI > 50%) and low cytoplasmic expression (LC: LI $\leq$ 50%).

160   Statistical analysis

161   Yate's Chi-square test was used to examine the association between AQP3 expression

162   and clinicopathological information. Mean labeling indices among the study groups were

163   compared using the Mann-Whitney U test. A p-value of <0.05 was considered significant.

164   **Results**

165   Clinical and histopathological data on the 51 OSCC samples are summarized in Table

166   1. No correlation between AQP3 expression and clinicopathological information was

167   observed (data not shown). The overall expression of AQP3 is summarized in Table 3.

168   Immunostaining of AQP3 AR at AA250- C terminus

169   For NOM, all 51 samples showed diffuse, homogeneous, and strong immunostaining

170   in the cell membrane, with faint in the cytoplasm, of basal, suprabasal, and spinous layers

171   (HM: 100% samples). AQP3 immunostaining was decreased in all the 12 samples of HG-ED

172   (LM: 100% samples). In the SP of OSCC, 43/51 samples retained a considerable

173   membranous expression (HM: 84.3% samples), whereas reduced expression of AQP3 was

174   observed in 42/51 samples in the IF of OSCC (LM: 82.4% samples) (Figure 2A-2C).

175   The mean LI values of NOM, HG-ED, and IF of OSCC were 84.9 ± 3.1, 5.9 ± 3.9,

176   and 17.4 ± 27.8, respectively. There was a statistically significant decrease in the mean LI of

177   AQP3 AR at the AA250-C terminus in HG-ED and IF of OSCC compared with that of NOM

178   (P < 0.05) (Figure 3A).

179   Immunostaining of AQP3 AR at AA180-228

180   For NOM, all 51 samples showed diffuse and strong membranous with faint

181   cytoplasmic immunostaining in suprabasal and spinous cell layers. The basal cells showed

182 trace staining (HM: 100% samples). For HG-ED, SP, and IF of OSCC, AQP3

183 immunostaining was often decreased, respectively, for 12/12 samples (LM: 100% samples),

184 35/51 samples (LM: 68.6% samples), and 47/51 samples (LM: 92.2% samples) (Figure 2D-

185 2F).

186   The mean LI values for NOM, HG-ED, and IF of OSCC were 82.0 ± 3.7, 2.4 ± 2.6,

187 and 7.3 ± 19.9, respectively. There was a statistically significant decrease in the mean LI of

188 AQP3 AR at AA180-228 in HG-ED and IF of OSCC compared with that of NOM (P < 0.05)

189 (Figure 3B).

190 Immunostaining of AQP3 AR at N terminus AA1-80

191   For the NOM, all 51/51 samples showed absent or slightly positive staining of

192 cytoplasm of basal and suprabasal layers (LC: 100% samples). For HG-ED, cytoplasmic

193 AQP3 immunostaining increased to intermediate and upper portions in all 12 samples (HC:

194 100% samples). In the SP of OSCC, 40/51 samples showed cytoplasmic AQP3 positivity at

195 the peripheral of tumor nests, with weaker or almost negative in center (LC: 78.4% samples).

196 More diffuse with moderate to strong cytoplasmic AQP3 immunostaining was observed in

197 46/51 samples of the IF of OSCC (HC: 90.2% samples) (Figure 2G-2I).

198   The mean LI values for NOM, HG-ED, and IF of OSCC were 7.4 ± 4.7, 91.1 ± 7.5,

199 89.9 ± 17.5, respectively. There was a statistically significant increase in the mean LI of

200 AQP3 AR at N terminus AA1-80 in HG-ED and IF of OSCC compared with that of NOM (P

201 < 0.05) (Figure 3C).

202 **Discussion**

203   Recently, AQP3 has been reported to be involved in several types of cancers.

204 However, immunohistochemical expression of AQP3 in carcinomas remains controversial.[7, 8]

205   Differences in AQP3 AR sites may influence the immunohistochemical expression patterns.

206   To our knowledge, this is the first attempt to evaluate the immunostaining patterns of three

207   different AQP3 AR sites in NOM, HG-ED, SP, and IF of OSCC, which would improve our

208   understanding of the role of AQP3 in oral carcinogenesis.

209   AQP3 AR site at N terminus AA1-80

210   In AQP3 AR site at N terminus AA1-80, NOM stained negative or slightly positive in

211   the cytoplasm of basal and suprabasal layers. Normally, in human and rat tissues, AQP3 was

212   clearly expressed in the cell membranes of the squamous epithelia in the skin and oral

213   mucosa.[13, 21] It is probable that plenty of mature (membranous) AQP3 in the NOM may not

214   be recognized by anti-AQP3 antibody prepared from N terminus AA1-80 peptide of AQP3

215   while, this antibody may recognize nascent AQP3 protein, which was slightly produced in the

216   NOM (Figure 4). Cytoplasmic AQP3 immunostaining was increased in HG-ED and IF of

217   OSCC. It is possible that the dysplastic and tumor cells might generate a lot of nascent AQP3

218   protein. AA180-228 and AA250-C terminus regions of the nascent AQP3 protein might be

219   degraded (described later), whereas N terminus AA1-80 part of the nascent AQP3 protein

220   retained and could be detected by anti-AQP3 antibody prepared from N terminus AA1-80

221   peptide of AQP3 (Figure 5).

222   Our results were in agreement with previous studies using anti-AQP3 antibody

223   prepared from N terminus AA1-80 peptide of AQP3 in dysplastic squamous epithelium of the

224   cervix, SCC of the cervix, esophagus, and oral cavity, and gastric adenocarcinomas (GC)

225   (Table 4).[14-16, 19, 22, 23] Increased N terminus AA1-80 part of nascent AQP3 protein in GC is

226   associated with an increased in nuclear translocation of β-catenin, which leads to the

227   proliferation of tumor cells.[23] In addition, overexpression of N terminus AA1-80 part of

228   nascent AQP3 protein correlates with downregulation of E-cadherin and overexpression of

229   vimentin in poorly differentiated GC, thereby suggesting a role of AQP3 in the epithelial-to-

230   mesenchymal transition (EMT) process.[15] Moreover, in OSCC, overexpression of N terminus

231   AA1-80 part of nascent AQP3 protein may be related to the FAK-MAPK signaling pathway,

232   resulting in tumor cells proliferation.[16, 19]

233   AQP3 AR sites at AA180-228 and AA250- C terminus

234   In AQP3 AR sites at AA180-228 and AA250-C terminus, AQP3 staining was strong

235   in the cell membrane, but faint in the cytoplasm of the NOM, which is consistent with the

236   fact that AQP3 is a transmembrane protein.[13, 21, 24] A large amount of mature (membranous)

237   AQP3 with a small amount of nascent AQP3 protein in the NOM may be recognized by anti-

238   AQP3 antibody prepared from AA180-228 and AA250-C terminus peptide of AQP3 (Figure

239   4). AQP3 immunostaining was decreased in HG-ED and IF of OSCC. It is widely accepted

240   that tumor cells secrete proteases which can degrade the tumor barriers and thus facilitate

241   tumor progression and invasion.[25] Membrane-type 1 matrix metalloproteinase (MT1-MMP) is

242   one of the proteases that degrade extracellular matrix proteins, membrane proteins, and other

243   proteins.[26] Interestingly, Kjaergaard et al. (2015) have reported on unstructured or disordered

244   regions (DRs) in membrane proteins.[27] These DRs are important for signal transduction and

245   are extremely susceptible to proteolysis, thereby directly signaling for rapid degradation.[27, 28]

246   From GlobPlot2.3 (http://globplot.embl.de), there are four DRs in AQP3 protein: AA135-157,

247   AA182-188, AA208-218, and AA269-276.[11] MT1-MMP and other proteases might bind

248   these DRs of both mature (membranous) and nascent AQP3 protein in dysplastic epithelial

249   cells and tumor cells, resulting in degradation of the DRs and surrounding peptides.

250   Moreover, the disordered protein-NPA site (AA215-217) might degrade in the dysplastic and

251   cancer cells. Degradation of the NPA site may impair water transport across cell membranes

252   and cause water retention around the cancer cells, which might result in discohesion and

253   migration of the cancer cells (Figure 5).

254   Our results were similar to the studies using anti-AQP3 antibodies prepared from

255   AA180-228 and C terminus peptide of AQP3 in the SCCs of the lung, skin, and oral cavity,

256   urothelial carcinoma (UC), prostate adenocarcinoma, and bronchioloalveolar carcinoma

257   (Table 5).[17, 18, 20, 29-36] Degradation of AQP3 protein located near the C terminus part in UC

258   and AA180-228 part of AQP3 in OSCC is associated with higher grade of the tumors and

259   tumor cells invasion (muscle-invasive UC and OSCC with lymphatic metastasis cases),

260   which underlying molecular mechanism remains unclear.[17, 20, 33] However, our results were

261   contradictory to those of studies on medullary thyroid carcinoma, hepatocellular carcinoma,

262   pancreatic ductal adenocarcinoma, and triple-negative breast cancer, which suggested

263   increased expression of AQP3 in these carcinomas (Table 5).[37-40] Such contradictory

264   observations on AQP3 expression may be attributable to the differences in tumor cell types.

265   In addition, in the present study, in AQP3 AR sites at AA250- C terminus and AA180-228,

266   we found a discordance in the expression of OSCC at the SP. For AQP3 AR at AA250- C

267   terminus, tumor nests displayed a predominant membranous expression (84.3% samples),

268   while that at AA180-228, AQP3 immunostaining was reduced in most cases (68.6% samples).

269   In general, AQP3 AR at AA180-228 showed weaker staining in HG-ED and IF of OSCC

270   than AQP3 AR at AA250-C terminus. It is possible that at the HG-ED, SP, and IF of OSCC,

271   AQP3 AR at AA180-228 which detected of 18 DRs (AA182-188, 208-218) might result in

272   faster AQP3 protein degradation (by the proteases) when compared with AQP3 AR at

273   AA250-C terminus that detected of 8 DRs (AA269-276).[11, 27, 28]

274   **Conclusion**

275   To summarize, AQP3 could be used as a novel biomarker for detecting malignant

276   transformations in the squamous epithelium. Our findings show that the differences in AQP3

277   AR sites affected their immunohistochemical expression in OSCC. In AQP3 AR site at N

278   terminus AA1-80, AQP3 immunostaining was found to be increased in the dysplastic

279 squamous epithelium compared with the normal squamous epithelium, whereas in AQP3 AR

280 sites at AA180-228 and AA250-C terminus, AQP3 expression was weaker and irregular in

281 the dysplastic squamous epithelium than that in the normal squamous epithelium. Our data

282 suggest that understanding in AQP3 AR site of each anti-AQP3 antibody before performing

283 an immunohistochemistry is critical. A combination expression pattern of N terminus and C

284 terminus parts of AQP3 might be more accurate marker for detecting malignant

285 transformation. However, it is possible that, based on the field of cancerization concept, the

286 areas adjacent to carcinoma already harbor mutations that may not yet cause phenotypical

287 features. Further studies, with the use of additional molecular biology, are warranted to

288 confirm our results and precisely investigate the molecular mechanism underlying the role of

289 AQP3 in carcinogenesis.

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399   triple-negative breast cancer. *Oncol Lett* 2018;16:2661-2667.

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409   **Figure legends**

410   **Figure 1.** Diagrammatic representation of the structure and amino acids sequence of AQP3.

411   (A) Each AQP3 monomer has six transmembrane domains connected by five loops spanning

412   the cell membrane. Conserved motifs, asparagine-proline-alanine (NPA), bend into the

413   molecule and form the water channel. Both amino (-NH$_2$) and carboxy (-COOH) termini are

414   cytoplasmic.

415   (B) The polypeptide in the AQP3 structure is formed by a chain of 292 amino acids (AA).

416   The AA highlighted in grey are indicating the transmembrane domains of AQP3 protein.

417   **Figure 2.** Expression of AQP3 in the representative case of moderately differentiated oral

418   squamous cell carcinoma (OSCC) with its adjacent high-grade epithelial dysplasia (HG-ED)

419   and normal oral mucosa (NOM).

420   NOM (A, D, G); HG-ED (B, E, H); Invasive front (IF) of OSCC (C, F, I). Immunostaining

421   for AQP3 AR at AA250- C terminus (A-C), AQP3 AR at AA180-228 (D-F), and AQP3 AR

422   at N terminus AA1-80 (G-I). Original magnification: 200×. AQP3 AR at AA250- C terminus

423   and AA180-228 showed similar staining trend. In the NOM (A, D), strong membranous

424   positive staining with faint cytoplasmic staining of the epithelial cells was observed. For HG-

425   ED and IF of OSCC (B, C, E, F), AQP3 expression was decreased. Asterisks are marking

426   abnormal nuclear and/or weak cytoplasmic AQP3 staining in AQP3 AR site at AA250-C

427   terminus. AQP3 AR at N terminus AA1-80 showed different staining patterns. For NOM (G),

428   AQP3 showed negative or slightly positive staining in the cytoplasm of the lower layers. In

429   HG-ED and IF of OSCC (H, I), AQP3 expression was increased.

430   **Figure 3.** Averages of AQP3 labeling index (LI) of the three different AQP3 antigen

431   recognition sites.

432   The mean LI of AQP3 AR at AA250-C terminus (Figure 3A) and AA180-228 (Figure 3B)

433   were significantly higher in normal oral mucosa (NOM) than that in high-grade epithelial

434   dysplasia (HG-ED) and invasive front of oral squamous cell carcinoma (IF of OSCC) ($P <$

435   0.05). Conversely, the mean LI of AQP3 AR at N terminus AA1-80 (Figure 3C) was

436   significantly higher in HG-ED and IF of OSCC than that in NOM ($P < 0.05$).

437   **Figure 4.** Schematic illustration of possible results of AQP3 antigen recognition site

438   differences in normal oral mucosa (NOM).

439   A large amount of mature (membranous) AQP3 in the NOM may not be recognized by anti-

440   AQP3 antibody prepared from N terminus AA1-80 peptide of AQP3 while, this antibody may

441   recognize nascent AQP3 protein, which was scarcely generated in the NOM. Thus, in AQP3

442   AR site at N terminus AA1-80, NOM was negative or slightly positive in the lower layers.

443   On the contrary, a large amount of mature (membranous) AQP3 and a small amount of

444   nascent AQP3 protein in the NOM could be recognized by anti-AQP3 antibody prepared

445   from AA180-228 and AA250-C terminus peptide of AQP3. Therefore, in AQP3 AR sites at

446   AA180-228 and AA250-C terminus, NOM stained strong membranous immunostaining with

447   faint cytoplasmic staining.

448   **Figure 5.** Schematic illustration of possible results of AQP3 antigen recognition site

449   differences in high-grade epithelial dysplasia (HG-ED) and oral squamous cell carcinoma

450   (OSCC).

451   The dysplastic and tumor cells might produce a lot of nascent AQP3 protein. AA135-157,

452   AA180-228, and AA250-C terminus parts of the nascent AQP3 protein might be degraded

453   (described later), whereas N terminus AA1-80 part of the nascent AQP3 protein retained and

454   could be detected by anti-AQP3 antibody prepared from N terminus AA1-80 peptide of

455   AQP3. Consequently, in AQP3 AR site at N terminus AA1-80, cytoplasmic AQP3

456   immunostaining increased in HG-ED and invasive front (IF) of OSCC. On the other hand,

457   Membrane-type 1 matrix metalloproteinase (MT1-MMP) and other proteases, which were

458   secreted from the tumor cells, might bind the disordered regions (DRs) (AA135-157, AA182-

459   188, AA208-218, and AA269-276) of both mature (membranous) and nascent AQP3 protein,

460   resulting in degradation of these DRs and surrounding peptides. Thus, in AQP3 AR sites at

461   AA180-228 and AA250-C terminus, AQP3 immunostaining was decreased in HG-ED and IF

462   of OSCC. Moreover, degradation of the disordered protein-NPA site (AA215-217) may

463   impair water movement across cell membranes and cause water retention around the

464   dysplastic epithelium, which might result in discohesion and migration of the cancer cells.

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477 **Tables**

478 **Table 1.** Clinicopathologic features of fifty-one 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)
Histological 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)

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489   **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, O.N.	Abcam
2.	AA180-228	PR	V214	1:100	Not performed	RT, 1 h	Bioworld Tech.
3.	N terminal AA1-80	PR	sc-20811	1:100	CB, 98°C, 40 min	4°C, O.N.	Santa Cruz

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491   PR, Rabbit polyclonal; CB, Citrate buffer pH6.0; RT, Room temperature; O.N., Overnight

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514   **Table 3.** Expression of AQP3 in the three different AQP3 antigen recognitions

AQP3 Recognition	Score*	Number of cases			
		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)

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516   NOM, Normal oral mucosa; HG-ED, High-grade epithelial dysplasia; SP, Superficial part; IF,
517   Invasive front; OSCC, Oral squamous cell carcinoma

518   * HM, High membranous expression, Labeling index > 50%

519   LM, Low membranous expression, Labeling index ≤ 50%

520   HC, High cytoplasmic expression, Labeling index > 50%

521   LC, Low cytoplasmic expression, Labeling index ≤ 50%

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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	Organ	Normal tissues		Dysplastic tissues	Carcinomas		Authors and references
				Type	Expression pattern	Expression pattern	Type	Expression pattern	
1	N terminal	PR	Stomach	Gastric mucosa	Almost negative	NP	GC	Generally increased CP	Shen, et al 2010[14]
2	N terminal	PR	Esophagus	Squamous epithelium	Almost negative	NP	SCC	63% cases showed increased CP	Kusayama, et al 2011[16]
3	N terminal	PR	Oral cavity	Squamous epithelium	Almost negative	NP	SCC	73% cases showed increased CP	Kusayama, et al 2011[16]
4	N terminal	PR	Oral cavity	Squamous epithelium	Almost negative	NP	SCC	83% cases showed increased CP	Ishimoto, et al 2012[19]
5	N terminal	PR	Cervix	Squamous epithelium	Almost negative	Increased of CP	SCC	44% cases showed increased CP	Shi, et al 2012[22]
6	N terminal	PR	Stomach	Gastric mucosa	Almost negative	NP	GC	73% cases showed increased CP	Chen, et al 2014[15]
7	N terminal	PR	Stomach	Gastric mucosa	Almost negative	NP	GC	79% cases showed increased CP	Zhou, et al 2016[23]
8	N terminal	PR	Oral cavity	Squamous epithelium	Almost negative	Increased of CP	SCC	90% cases of IF part showed increased CP	The present study

AQP3, Aquaporin3; *AA*, Amino acid; *PR*, Rabbit polyclonal; *NP*, Not performed; *CP*, Cytoplasmic expression; *GC*, Gastric adenocarcinoma;

SCC, Squamous cell carcinoma; *IF*, Invasive front

543 **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		Authors and references
				Type	Expression pattern	Expression pattern	Type	Expression pattern	
1	C terminal	PR	Lung	BSE	MB	NP	SCC	64% cases showed loss of MB	Liu, et al 2007[29]
2	C terminal	PR	Lung	BSE	MB	NP	BAC with invasive ADC	Invasive ADC showed loss of MB	Machida, et al 2011[30]
3	C terminal	PR	Skin	Squamous epithelium	MB	NP	SCC	100% cases showed loss of MB	Voss, et al 2011[18]
4	C terminal	PG	Urinary bladder	Urothelium	MB	NP	pT2 UC	100% cases showed loss of MB	Rubenwolf, et al 2012 and 2009[31,32]
5	C terminal	PR	Urinary bladder	Urothelium	MB	NP	pT1 UC	41% cases showed loss of MB	Otto, et al 2012[17]
6	C terminal	PR	Thyroid gland	C cells and follicular cells	Almost negative	NP	MTC	90% cases showed increased MB	Niu, et al 2012[37]
7	C terminal	PG	Urinary bladder	Urothelium	MB	NP	pT2 UC	67% cases showed loss of MB	Rubenwolf, et al 2014[33]
8	AA180-228	PR	Oral cavity	Squamous epithelium	MB	Loss of MB	SCC	Generally loss of MB	Our previous study 2014[20]
9	C terminal	PR	Skin	Squamous epithelium	MB	NP	SCC	Generally loss of MB	Seleit, et al 2015[34]
10	C terminal	PR	Liver	Hepatocyte	Almost negative	NP	HCC	93% cases showed increased MB	Peng, et al 2016[38]
11	C terminal	PR	Pancreas	Ductal cells	Almost negative	NP	PDA	Generally increased MB	Direito, et al 2017[39]
12	C terminal	PR	Urinary bladder	Urothelium	NP	MB	pT2 UC	100% cases showed loss of MB	Breyer, et al 2017[35]
13	C terminal	PR	Prostate gland	Glandular epithelial cells	MB	NP	High-risk PC	Generally loss of MB	Brundl, et al 2018[36]

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No	Recognized part of AQP3	Antibody host	Normal tissues			Dysplastic tissues	Carcinomas		Authors and references
			Organ	Type	Expression pattern	Expression pattern	Type	Expression pattern	
14	C terminal	PR	Breast	Ductal cells	Almost negative	NP	TNBC	61% cases showed increased MB	Zhu, et al 2018[40]
15	C terminal	PR	Oral cavity	Squamous epithelium	MB	Loss of MB	SCC	84% cases of IF part showed loss of MB	The present study
16	AA180-228	PR	Oral cavity	Squamous epithelium	MB	Loss of MB	SCC	92% cases of IF part showed loss of MB	The present study

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548 *AQP3*, Aquaporin3; *AA*, Amino acid; *PR*, Rabbit polyclonal; *PG*, Goat polyclonal; *BSE*, Bronchial surface epithelium; *MB*, Membranous

549 expression; *NP*, Not performed; *SCC*, Squamous cell carcinoma; *BAC*, Bronchioloalveolar carcinoma; *ADC*, Pulmonary adenocarcinoma; *pT1*

550 *UC*, Urothelial carcinoma invades connective tissue; *pT2 UC*, Urothelial carcinoma invades muscle; *MTC*, Medullary thyroid carcinoma; *HCC*,

551 Hepatocellular carcinoma; *PDA*, Pancreatic ductal adenocarcinoma; *PC*, Prostate adenocarcinoma; *TNBC*, Triple-negative breast cancer; *IF*,

552 Invasive front

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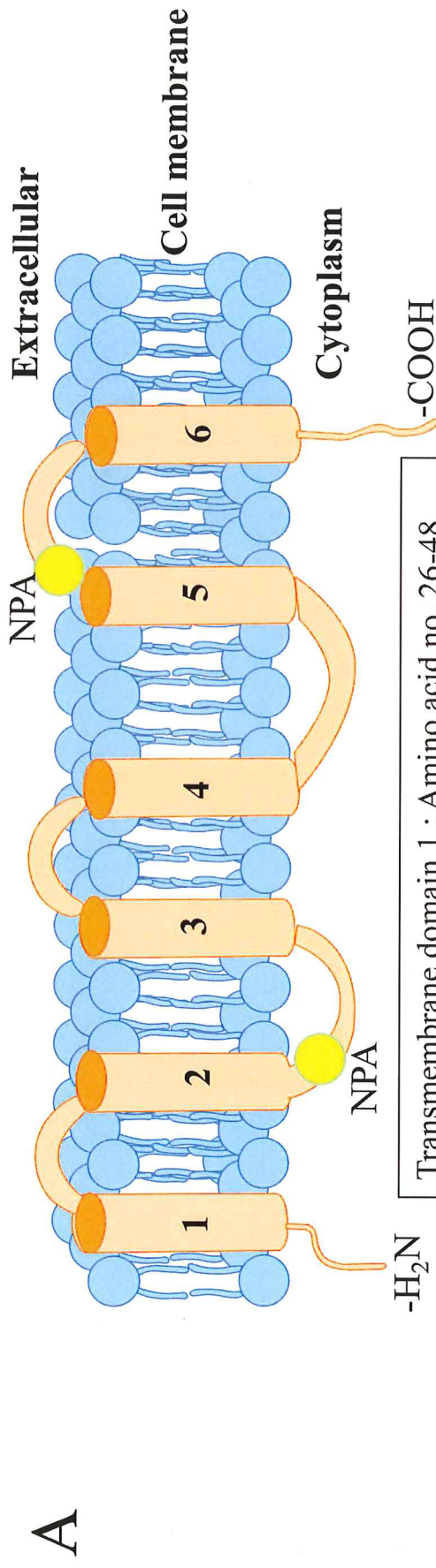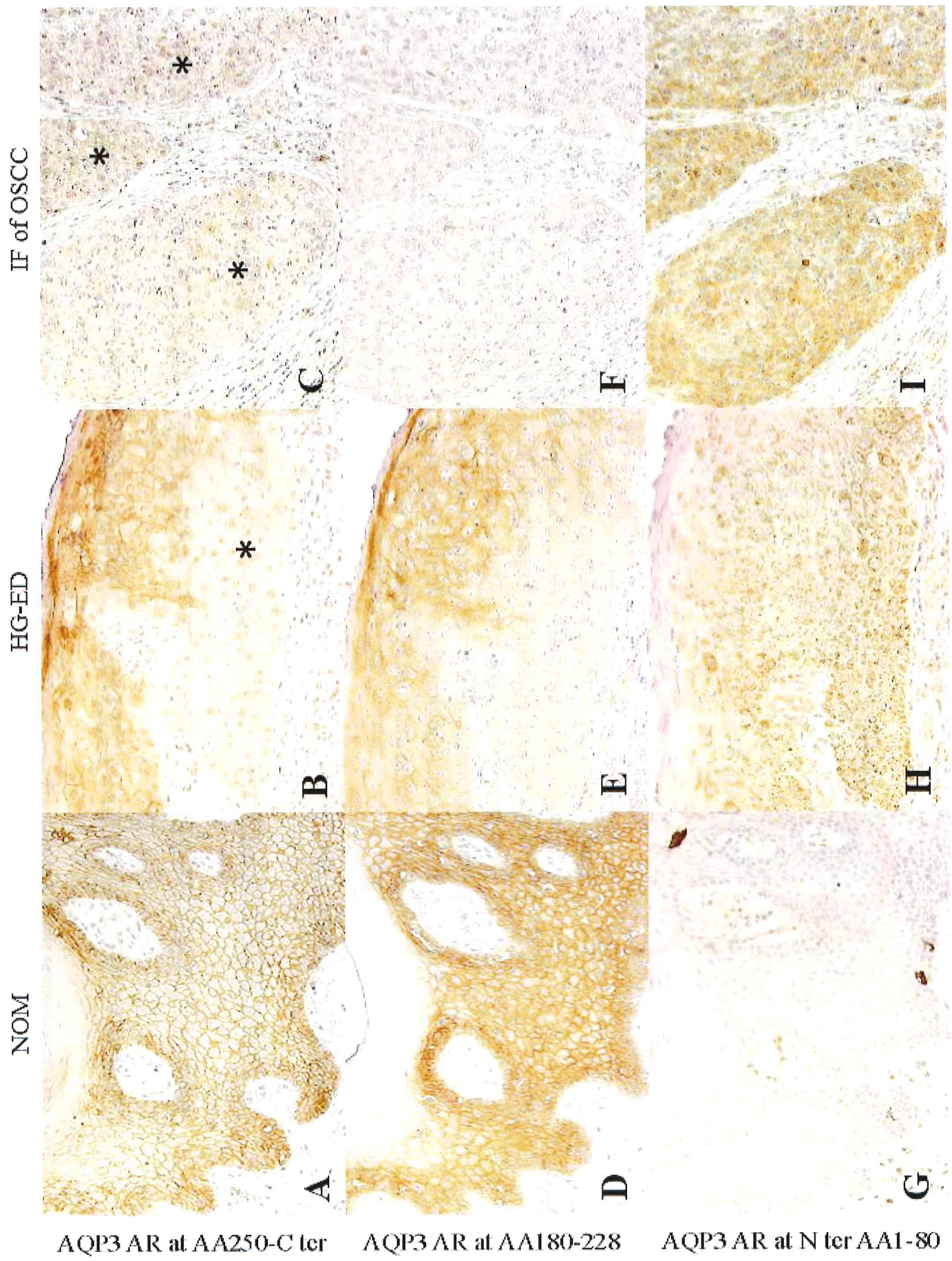554

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Extracellular

Cell membrane

Cytoplasm

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NPA

NPA

-H₂N

-COOH

Transmembrane domain 1 : Amino acid no. 26-48
Transmembrane domain 2 : Amino acid no. 58-80
Transmembrane domain 3 : Amino acid no. 101-123
Transmembrane domain 4 : Amino acid no. 157-179
Transmembrane domain 5 : Amino acid no. 192-214
Transmembrane domain 6 : Amino acid no. 242-261
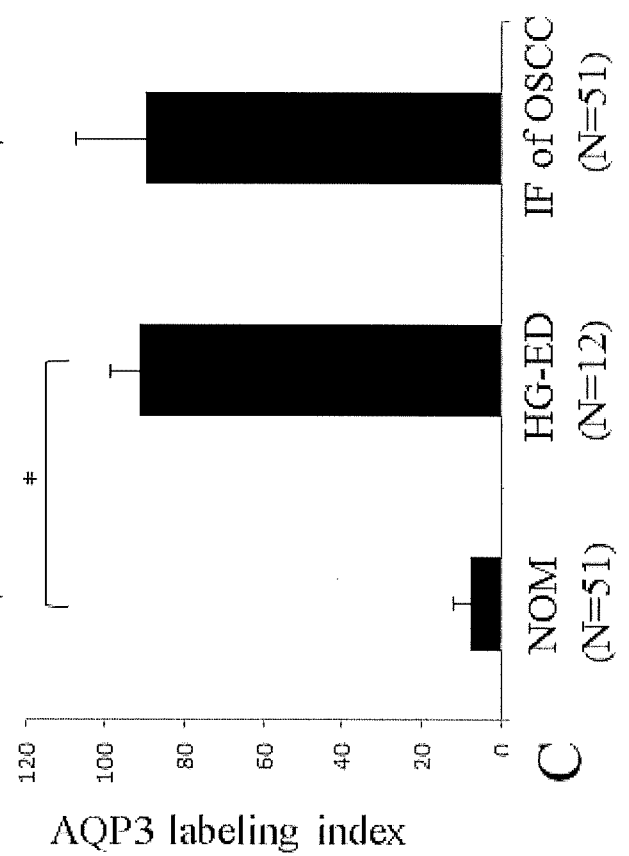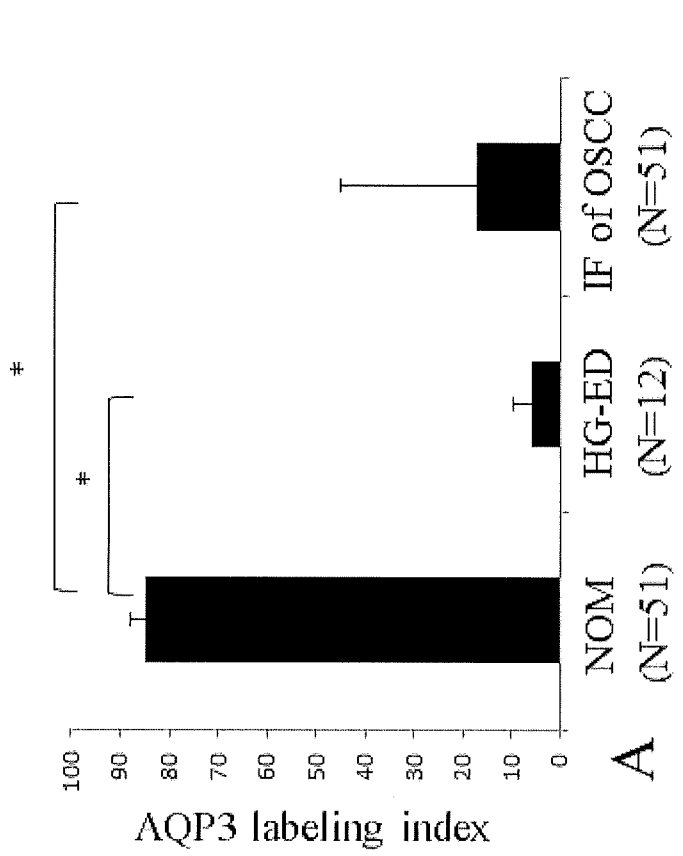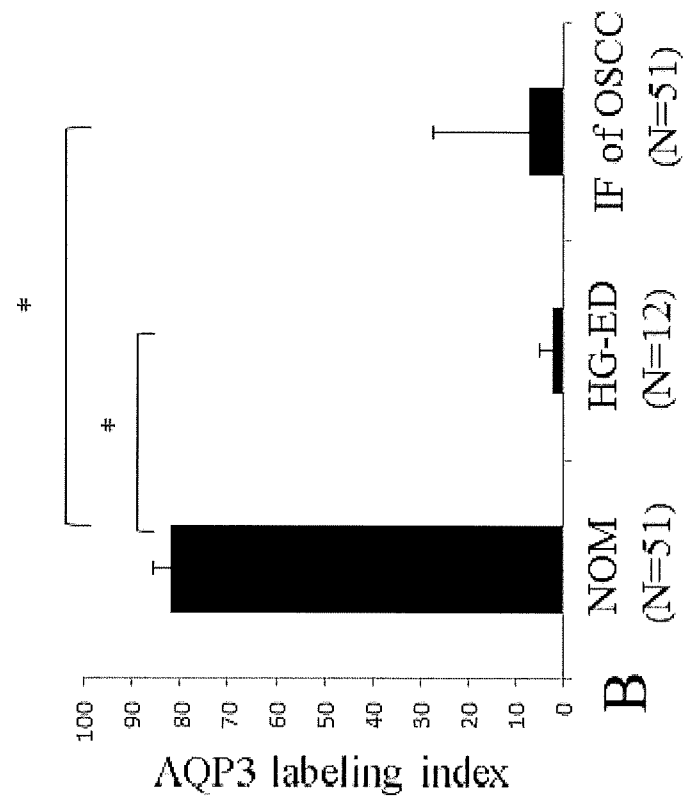NPA: Amino acid no. 83-85 and 215-217

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MGRQKELVSRCGEMLHIRYRLLRQALAECLGTLILVMFGCGSVAQVVLSRGTHGGFLTIN
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LAFGFAVTLGILIAGQVSGAHL**NPA**VTFAMCFLAREPWIKLPIYTLAQTLGAFLGAGIVF
120

GLYYDAIWHFADNQLFVSGPNGTAGIFATYPSGHLDMINGFFDQFIGTASLIVCVLAIVD
180

PYNNPVPRGLEAFTVGLVVLVIGTSMGFNSGYAV**NPA**RDFGPRLFTALAGWGSAVFTTGQ
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HWWWPIVSPLLGSIAGVFVYQLMIGCHLEQPPPSNEEENVKLAHVKHKEQI
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NOM   HG-ED   IF of OSCC

A   B   C   AQP3 AR at AA250-C ter

D   E   F   AQP3 AR at AA180-228

G   H   I   AQP3 AR at N ter AA1-80

B

AQP3 labeling index

NOM (N=51)　　HG-ED (N=12)　　IF of OSCC (N=51)

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A : AQP3 AR at AA250- C terminal
B : AQP3 AR at AA180-228
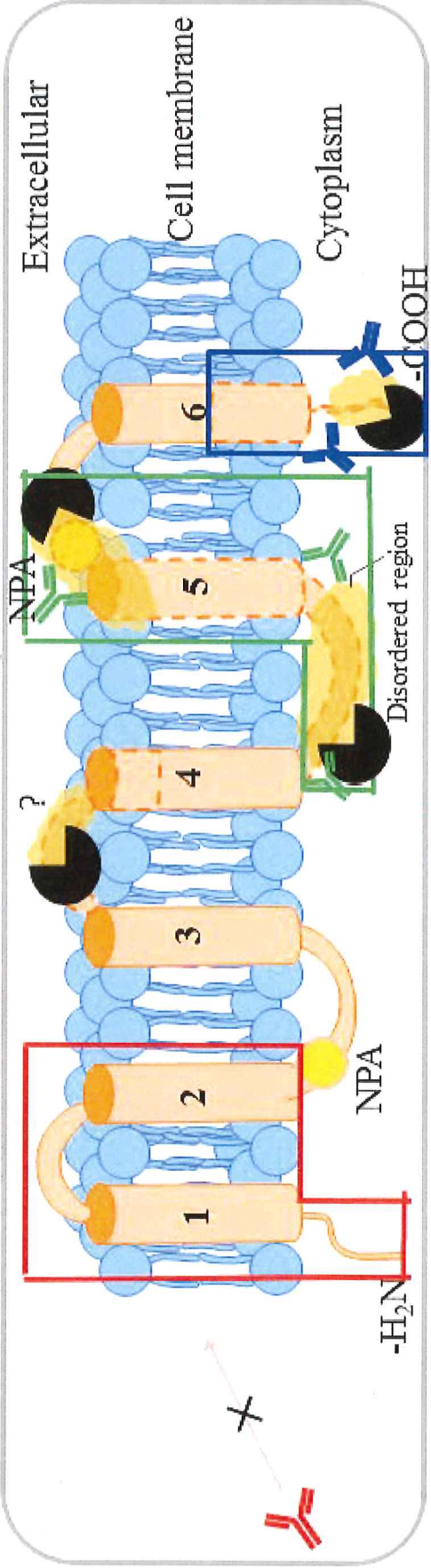C : AQP3 AR at N terminal AA1-80

* P < 0.05

(Mann-Whitney U test)
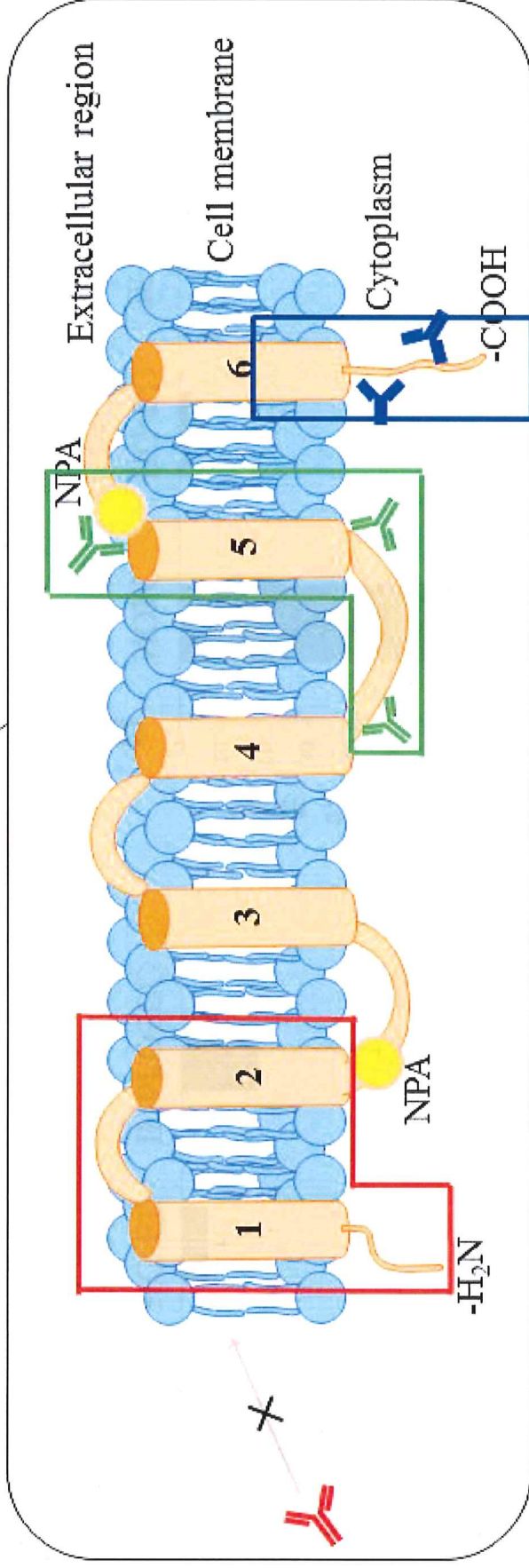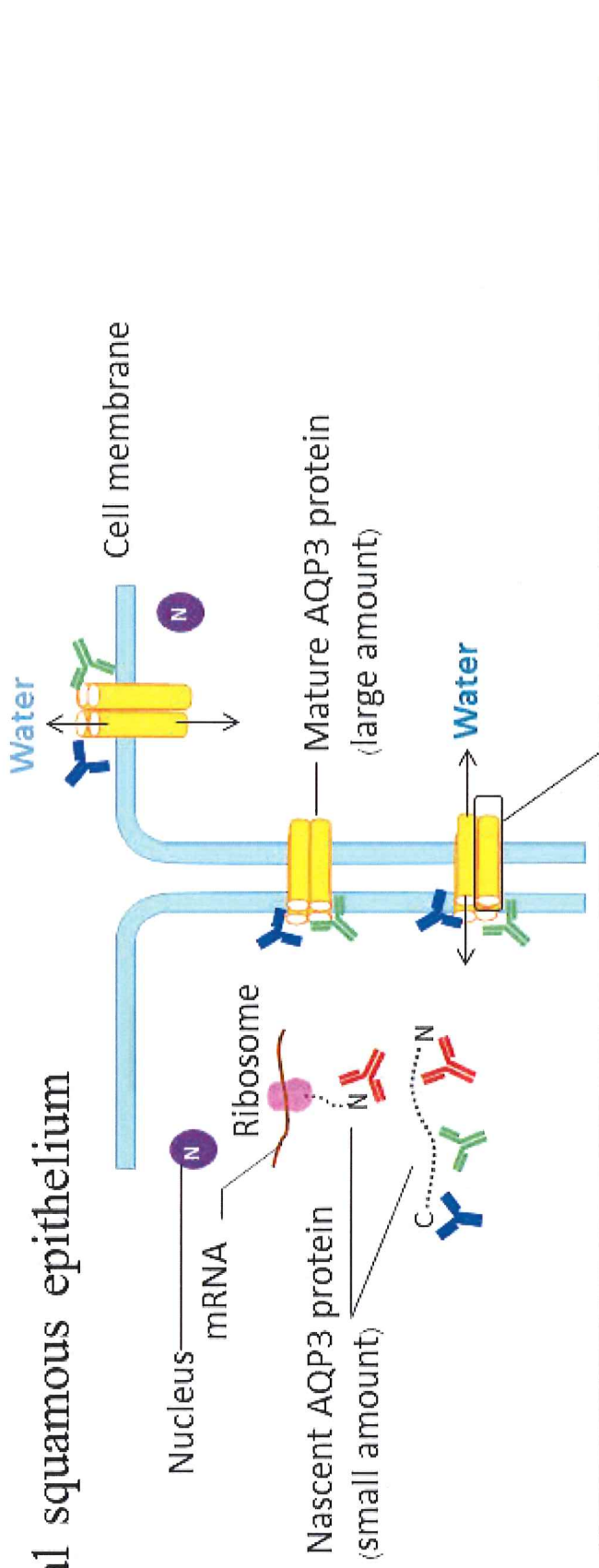
A

AQP3 labeling index

NOM (N=51)　　HG-ED (N=12)　　IF of OSCC (N=51)

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AQP3 labeling index

NOM (N=51)　　HG-ED (N=12)　　IF of OSCC (N=51)

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Normal squamous epithelium

Dysplastic squamous epithelium

Water

Mature AQP3 protein
(degradation at AA180-228 and C terminus)

mRNA

Ribosome

Nascent AQP3 protein
(large amount)

Protease

Disordered region

Extracellular

Cell membrane

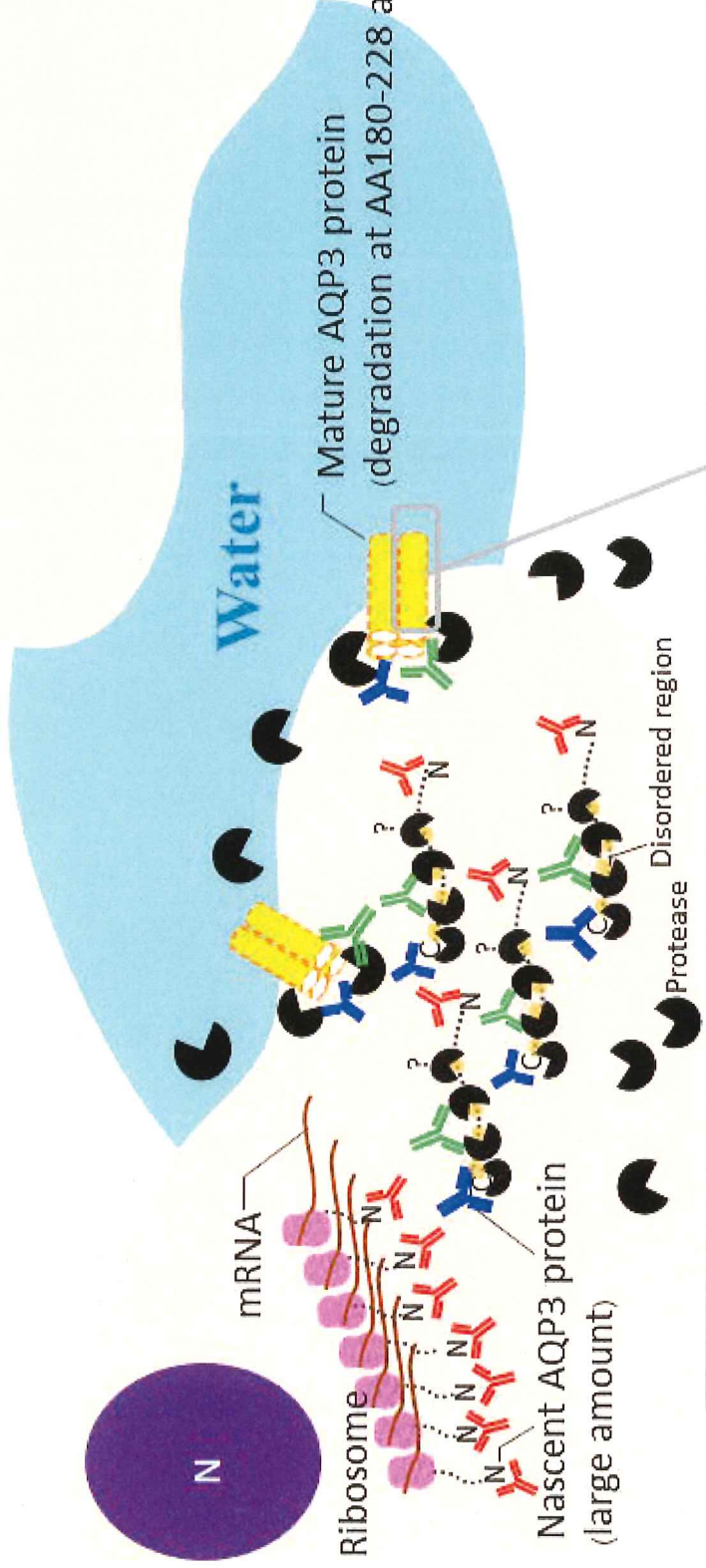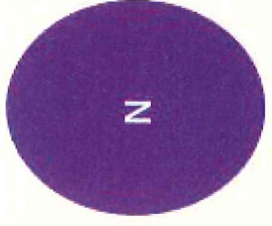Cytoplasm

-COOH

NPA

Disordered region

NPA

-H₂N

Y Anti-AQP3 antibody prepared from AA180-228 peptide of AQP3   Y Anti-AQP3 antibody prepared from N terminus AA1-80 peptide of AQP3

Y Anti-AQP3 antibody prepared from AA250-C terminus peptide of AQP3