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Decreased Expression of NRF2 Target Genes after Alcohol Exposure in the Background Esophageal Mucosa of Patients with Esophageal Squamous Cell Carcinoma

Shusuke Toda,[1] Waku Hatta,[1] Kiyotaka Asanuma,[1] Naoki Asano,[1] Yoshitaka Ono,[1] Hiroko Abe,[1] Yohei Ogata,[1] Masahiro Saito,[1] Takeshi Kanno,[1] Xiaoyi Jin,[1] Kaname Uno,[1] Tomoyuki Koike,[1] Akira Imatani,[1] Shin Hamada,[1] Tomohiro Nakamura,[2] Naoki Nakaya[3] and Atsushi Masamune[1]

[1]Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[2]Department of Health Record Informatics, Tohoku Medical Megabank Organization, Tohoku University, Sendai, Miyagi, Japan
[3]Department of Preventive Medicine and Epidemiology, Tohoku Medical Megabank Organization, Tohoku University, Sendai, Miyagi, Japan

Patients with esophageal squamous cell carcinoma (ESCC) might have a specific mechanism for the carcinogenesis by alcohol consumption in the background esophageal mucosa, and nuclear factor erythroid 2-related factor 2 (NRF2), which plays a protective role against esophageal carcinogenesis, and barrier dysfunction might be associated with this phenomenon. This study aimed to confirm this hypothesis. Twenty patients with superficial ESCCs (ESCC patients) and 20 age- and sex-matched patients without ESCC (non-ESCC patients) were enrolled. Biopsy samples were obtained from non-neoplastic esophageal mucosa: one for histological evaluation, one for quantitative real-time polymerase chain reaction (PCR), and two for the mini-Ussing chamber system to measure transepithelial electrical resistance (TEER) and, thereafter, for PCR. The TEER after acetaldehyde or both acetaldehyde and ethanol exposure did not differ significantly between ESCC and non-ESCC patients. Unlike non-ESCC patients, mRNA levels of NRF2 target genes and *claudin4* in ESCC patients tended to decrease after the exposure, with a significant difference between no exposure and both acetaldehyde and ethanol exposure in NRF2 target genes ($p < 0.05$). Furthermore, in ESCC patients, the decreased tendency of mRNA levels of NRF2 target genes after the exposure was more pronounced in high-risk states, such as *aldehyde dehydrogenase 2* (*ALDH2*) Lys alleles (Glu/Lys + Lys/Lys), Lugol-voiding lesion grade C, and drinking history. In conclusion, the protective role of NRF2 against carcinogenesis from alcohol exposure might be disrupted in the background esophageal mucosa of ESCC patients, which might lead to a high incidence of metachronous ESCC.

Keywords: acetaldehyde; esophageal mucosa; esophageal squamous cell carcinoma; ethanol; NRF2 target genes
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Introduction

Endoscopic resection is an established treatment method that is associated with a good long-term prognosis in patients with superficial esophageal squamous cell carcinoma (ESCC) without lymph node metastasis (Tsujii et al. 2015; Hatta et al. 2020, 2021; Ogata et al. 2021); however, the esophageal mucosa of these patients is at high-risk for metachronous ESCC. Indeed, the 5 year incidence rate of metachronous ESCC after endoscopic resection was found to be 25.7% (Oda et al. 2020). Moreover, alcohol consumption after endoscopic resection largely affects its incidence (Katada et al. 2016).

Ethanol (EtOH) and its metabolite, acetaldehyde (AcH), are considered as definite carcinogens for the esophagus (Secretan et al. 2009; Ohashi et al. 2015). AcH is a

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Correspondence: Waku Hatta, M.D., Ph.D., Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan.
e-mail: waku-style@festa.ocn.ne.jp

highly reactive compound that causes DNA damage, and its mutagenic and carcinogenic effect is supported by several studies (Seitz and Stickel 2007). Furthermore, high exposure of AcH to the esophageal epithelium, including the intrinsic and extrinsic pathways, is considered to increase the risk of ESCC (Ohashi et al. 2015; Yokoyama et al. 2016). However, it remains unclear why the effect of alcohol exposure to the esophageal mucosa for carcinogenesis is greater in patients with ESCC. The esophageal mucosa may have some protective mechanism against carcinogenesis due to alcohol exposure, and this mechanism might be disrupted in such patients.

Nuclear factor erythroid 2-related factor 2 (NRF2) is known to be a transcription factor that regulates the expression of enzymes involved in detoxification and antioxidative stress response, including NADPH quinone oxidoreductase 1 (NQO1) and glutathione S-transferases (GST) (Motohashi and Yamamoto 2004; Kensler et al. 2007; Nabeshima et al. 2020). Although NRF2 activity is a double-edged sword for the cancer-preventive function in ESCC (Shibata et al. 2011; Ohkoshi et al. 2013; Kawasaki et al. 2014), it has been reported to play a protective role against carcinogenesis in the tongue and esophagus of mice (Ohkoshi et al. 2013). Meanwhile, our previous study revealed that EtOH was still detectable in the esophageal epithelium even up to 60 min after alcohol consumption (Okata et al. 2018). Additionally, a previous study reported the synergistic effect of AcH and EtOH on barrier dysfunction in a human colon cancer cell line (Samak et al. 2016). Since tight junction disruption in premalignant neoplastic tissue can increase the likelihood of developing carcinoma (Singh et al. 2010), barrier dysfunction by sustained exposure of AcH and EtOH to such tissue may play a role in the pathogenesis of ESCC in high-risk patients, such as those with ESCC.

Hence, we hypothesized that patients with ESCC have a specific mechanism for the carcinogenesis of ESCC due to alcohol consumption in the background mucosa, for which NRF2 and impaired barrier function may play a role. This study aimed to test this hypothesis.

Materials and Methods

Study patients

Twenty patients with superficial ESCCs (ESCC patients) were enrolled in this study. As control subjects (low-risk for ESCC), 20 age- and sex-matched patients without ESCC (non-ESCC patients) were also enrolled in this study during the same period. The exclusion criteria were 1) the regular use of non-steroidal anti-inflammatory drugs, steroids, or immunosuppressive agents; 2) a history or a plan of esophageal, gastric, and duodenal surgery; 3) a history of chemotherapy or radiation therapy against head and neck or esophageal cancers; 4) the regular use of two or more anti-coagulant or anti-platelet medication; 5) severe systemic diseases; 6) pregnancy or lactation; 7) participation in other studies that involve esophageal biopsy; 8) severe mental disease; and 9) inappropriateness for participation in the study as determined by the investigators. This study was performed following the principles of the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the Tohoku University Hospital (2018-2-032-1), and written informed consent was acquired from all subjects before enrollment in this study.

Endoscopic examination

After a questionnaire on smoking and alcohol consumption, an endoscopic examination was performed. In this procedure, four biopsy tissue samples were obtained from non-neoplastic mucosa, which was confirmed by narrow band imaging (Matsuno et al. 2019), located in the middle part of the esophagus in each subject: one sample for histological evaluation, one for quantitative real-time polymerase chain reaction (qRT-PCR) experiments, and two for the mini-Ussing chamber system and, thereafter, for qRT-PCR experiments. In patients with ESCC, iodine staining (1.5%) was performed at more than one month before the endoscopic biopsy, so as to minimize the effect of iodine for the results in this study, and the Lugol-voiding lesion (LVL) grade [A, 0 lesions; B, 1-9 lesions; C, ≥ 10 lesions per endoscopic view (Katada et al. 2016)] was determined. The endoscopic procedure was performed by an expert endoscopist (W.H.).

Outcome measures

We compared transepithelial electrical resistance (TEER) measured using the mini-Ussing chamber system and the expressions of NRF2 target genes and tight junction protein between ESCC and non-ESCC patients. Since the risk for ESCC differs between the *aldehyde dehydrogenase2* (*ALDH2*) polymorphism (Cui et al. 2009; Tanaka et al. 2010; Abiko et al. 2018), we compared TEER and the expressions of these genes between *ALDH2* polymorphism [Glu/Glu vs. Lys alleles (Glu/Lys + Lys/Lys)]. These outcomes were evaluated in ESCC and non-ESCC patients, separately. The LVL grade reflects the risk of metachronous ESCC after endoscopic resection for the initial ESCC (Katada et al. 2016); thus, we compared TEER and the expression of the genes among the LVL grades in the ESCC patients.

Mini-Ussing chamber system and electrophysiological measurement of TEER

The barrier function of the esophageal mucosa after 200 μM AcH or both 200 μM AcH and 50 mM EtOH exposure was evaluated through electrophysiological measurements of TEER using a mini-Ussing chamber system (EM-CYSY-2 Ussing Chamber Systems, Physiologic Instruments, San Diego, CA, USA), the details of which were described previously (Norita et al. 2021). The TEERs of two samples were measured simultaneously every 15 min for a total of 120 min as the samples were stimulated with either 200 μM AcH or both 200 μM AcH and 50 mM

EtOH. The TEER value just before the exposure was recorded as the basal value (time = 0). Values were expressed as percentages of change in resistance from basal value. For the subsequent quantitative real-time polymerase chain reaction (qRT-PCR) experiment, mounted specimens were immersed in liquid nitrogen and stored at −80°C until RNA extraction.

RNA extraction and qRT-PCR

Regarding NRF2 target genes, previous studies revealed that decreased expressions of *NQO1* and *GSTM1* by polymorphisms are associated with an increased incidence of head and neck squamous cell carcinoma (Hashibe et al. 2003); hence, *NQO1* and *GSTM* are crucial among NRF2 target genes for preventing carcinogenesis. A key protein constituent of the tight junction, which is the most important structure for the mucosal barrier function (Blasig and Haseloff 2011), is transmembrane claudins (CLDNs) (Overgaard et al. 2011) and, of 24 family members, *CLDN4* is one of the dominant ones in normal esophageal epithelium (Jovov et al. 2007). Thus, we investigated *NQO1*, *GSTM*, and *CLDN4* mRNA expression levels in this study.

Total RNA was extracted from each biopsy tissue sample using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions, and first-strand cDNA was synthesized using a SuperScript® VILO™ cDNA Synthesis kit (Thermo Fisher Scientific). qRT-PCR was conducted using a StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) and SYBR green I (PowerSYBR Green PCR Master Mix; Applied Biosystems) with the following primer pairs (forward and reverse, respectively): *NQO1* (5′-AGCGTTCGGTATTACGATCC-3′ and 5′-AGTACAATCAGGGCTCTTCTCG-3') (Komatsu et al. 2010), *GSTM* (5′-CTACCTTGCCCGAAAGCAC-3′ and 5′-ATGTCTGCACGGATCCTCTC-3′) (Komatsu et al. 2010), *CLDN4* (5′-GTTGTCCCCACCCGGTGAGCATC-3′ and 5′-GGCCAACAAAGGCGTTCAGAGGC-3′), and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (5′-TCGACAGTC AGCCGCATCT-3′ and 5′-AGTTAAAAGCAGCCCTGGTGA-3′). mRNA expression levels were normalized to those of *GAPDH*. Fold changes in patients relative to controls were calculated on the basis of the delta-delta CT method.

Immunohistochemistry

Tissue sections were embedded in paraffin and cut into 4 μm sections and then deparaffinized and rehydrated with xylene and a graded alcohol series. To block endogenous peroxidase activity, tissue sections were immersed in 3% hydrogen peroxide in methanol for 10 min, followed by antigen retrieval with a target retrieval solution (Dako, Glostrup, Denmark) in a pressure cooking at 120°C for 10 min. A gout anti-Nqo1 antibody (Abcam, Cambridge, UK) and a rabbit anti-Claudin4 antibody (Abcam) were used in 4°C overnight incubation. Then, biotin-labeled anti-goat immunoglobulin (Dako) and peroxidase-conjugated streptavidin (Abcam) were used for NQO1 staining, and EnVision FLEX HRP Magenta (Dako) was applied for CLDN4 staining. Color development was obtained using diaminobenzidine (Dako).

Genotyping of ALDH2

DNA was extracted from each subject using DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genotyping for *ALDH2* (rs671) was conducted using TaqMan® Drug Metabolism Genotyping Assay (Thermo Fisher Scientific). *ALDH2* polymorphism was divided into *ALDH2* Glu/Glu and *ALDH2* Lys alleles.

Statistical analysis

Categorical data were expressed as frequencies and proportions (percentages). Comparisons between categorical variables were performed using Fisher's exact test. Continuous data were expressed as means with standard errors of the means. Student's *t*-test was used to compare continuous variables between the two groups. For multiple comparison, the one-way analysis of variance (ANOVA) test followed by Tukey-Kramer post hoc test was used. To compare TEER between the groups, the two-way ANOVA test and, if appropriate, Tukey-Kramer post hoc test were performed. Data were analyzed using SPSS version 25.0 for Windows software (IBM Corp., Armonk, NY, USA). A p value of < 0.05 was considered statistically significant.

Results

Baseline characteristics of the enrolled patients

A total of 20 ESCC patients and 20 non-ESCC patients, consisting of 14 patients with early gastric cancers and 6 with duodenal tumors who had a schedule to undergo endoscopic resection, were enrolled in this study between October 2018 and November 2020. Table 1 presents the details of the baseline characteristics of the study participants. There were no significant differences in the characteristics between the two groups. Gastroesophageal reflux disease was shown in one patient per each of the two groups.

TEER in the mini-Ussing chamber system

The basal TEER in esophageal biopsy tissue samples did not differ significantly between ESCC and non-ESCC patients ($p = 0.706$). Upon AcH exposure and both AcH and EtOH exposure, the difference in TEER between ESCC and non-ESCC patients was not statistically significant (Fig. 1A). In the subgroup comparisons, although the significantly decreased TEER in *ALDH2* Lys allele carriers appeared in the comparison of *ALDH2* polymorphism in ESCC patients after both AcH and EtOH exposure ($p < 0.05$), no significant difference appeared in the other comparisons (Fig. 1B, C).

Table 1. Baseline characteristics of the enrolled patients.

	Non-ESCC (n = 20)	ESCC (n = 20)	p value
Age (y), mean ± SE	68.5 ± 2.5	69.5 ± 2.3	0.778
Sex, *n* (%)			1.000
Male	16 (80.0)	16 (80.0)	
Female	4 (20.0)	4 (20.0)	
Smoking history, *n* (%)	12 (60.0)	16 (80.0)	0.301
Drinking history, *n* (%)	10 (50.0)	12 (60.0)	0.750
ALDH2 polymorphism, *n* (%)			1.000
Glu/Glu	12 (60.0)	11 (55.0)	
Lys alleles (Glu/Lys + Lys/Lys)	8 (40.0)	9 (45.0)	
LVL grade			
A	–	2 (10.0)	
B	–	11 (55.0)	
C	–	7 (35.0)	
Histological finding			1.000
Normal epithelium	20 (100.0)	20 (100.0)	
Squamous intraepithelial neoplasia	0 (0.0)	0 (0.0)	
Gastroesophageal reflux disease	1 (5.0)	1 (5.0)	1.000
Intake of acid-suppressive agent	8 (40.0)	9 (45.0)	1.000
Underlying disease			
Early gastric cancer	14 (70.0)	–	
Duodenal tumor	6 (30.0)	–	

ALDH2, *aldehyde dehydrogenase 2*; ESCC, esophageal squamous cell carcinoma; LVL, Lugol-voiding lesion; SE, standard error.

qRT-PCR of NRF2 target genes and CLDN4 in non-neoplastic esophageal mucosa ESCC and non-ESCC patients

In non-exposed samples, the *CLDN4* mRNA expression level in ESCC patients was significantly lower than that in non-ESCC patients ($p < 0.05$), but the *NQO1* and *GSTM* mRNA expression levels did not differ significantly between the ESCC and non-ESCC patients (Fig. 2). Unlike the results in non-ESCC patients, the mRNA expression levels of all three genes tended to decrease after AcH or both AcH and EtOH exposure in ESCC patients, and the differences between no exposure and both AcH and EtOH exposure were statistically significant in *NQO1* and *GSTM* (all, $p < 0.05$).

ALDH2 polymorphism in ESCC patients

In non-exposed samples of ESCC patients, the *NQO1* and *GSTM* mRNA expression levels in *ALDH2* Lys allele carriers were significantly lower than those in *ALDH2* Glu/Glu carriers (all, $p < 0.05$) (Fig. 3). The *NQO1*, *GSTM*, and *CLDN4* mRNA expression levels significantly decreased after AcH or both AcH and EtOH exposure in *ALDH2* Lys allele carriers (all, $p < 0.01$) (Fig. 3). A similar tendency was also shown in *ALDH2* Glu/Glu carriers, but the difference was not statistically significant in most comparisons.

ALDH2 polymorphism in non-ESCC patients

In the analysis of samples with no exposure from non-ESCC patients, the *GSTM* mRNA expression level in *ALDH2* Lys allele carriers was significantly higher than that in *ALDH2* Glu/Glu carriers ($p < 0.01$) (Fig. 4). After AcH or both AcH and EtOH exposure, the *NQO1*, *GSTM*, and *CLDN4* mRNA expression levels tended to increase in *ALDH2* Lys allele carriers, with significant difference between no exposure and AcH exposure in *CLDN4* ($p < 0.05$) (Fig. 4).

LVL grade in ESCC patients

The results of the three genes according to the LVL grade in ESCC patients are shown in Fig. 5. In non-exposed samples, the *NQO1* and *CLDN4* mRNA expression levels did not differ significantly among the LVL grades, but the *GSTM* mRNA expression level in LVL grade B was significantly lower than that in LVL grade C ($p < 0.01$). After AcH or both AcH and EtOH exposure, no significant difference in the mRNA expression levels of all three genes was shown in the LVL grade B. On the other hand, their mRNA expression levels tended to decrease after AcH or both AcH and EtOH exposure in LVL grade C, with a significant difference between no exposure and both AcH and EtOH exposure in *NQO1* ($p < 0.05$) and *GSTM* ($p < 0.01$).

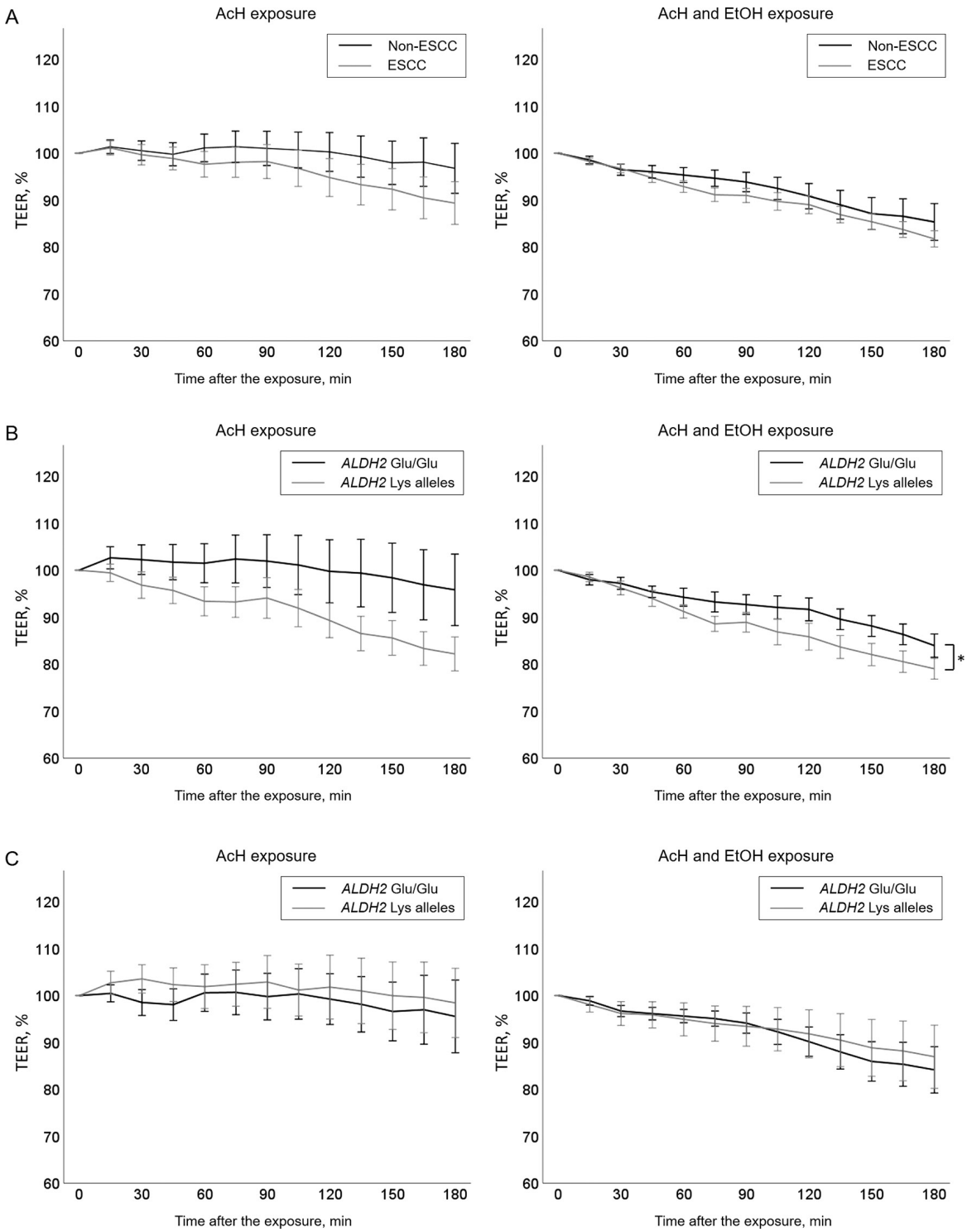

Fig. 1. Transepithelial electrical resistance (TEER) of the background esophageal mucosa after acetaldehyde (AcH) and both AcH and ethanol (EtOH) exposure.
(A) Esophageal squamous cell carcinoma (ESCC) vs. non-ESCC patients (n = 20 per group; AcH exposure, p = 0.387; both AcH and EtOH exposure, p = 0.884). (B) *ALDH2* Glu/Glu carriers (n = 11) vs. *ALDH2* Lys allele carriers (n = 9) in ESCC patients (AcH exposure, p = 0.105; both AcH and EtOH exposure, $p < 0.05$). (C) *ALDH2* Glu/Glu carriers (n = 12) vs. *ALDH2* Lys allele carriers (n = 8) in non-ESCC patients (AcH exposure, p = 1.000; both AcH and EtOH exposure, p = 0.996).
ALDH2, *aldehyde dehydrogenase 2.*

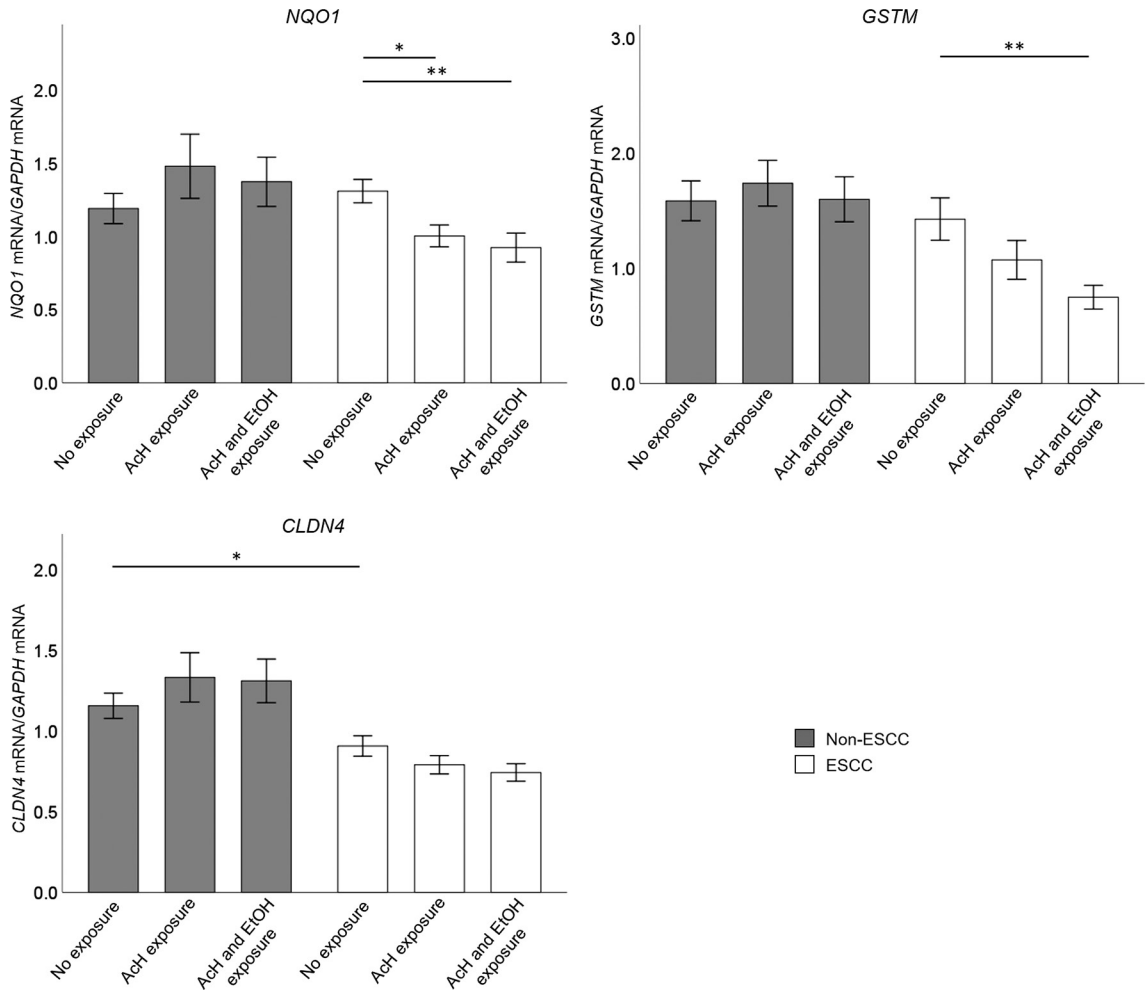

Fig. 2. Gene expression profiles of the background esophageal mucosa in esophageal squamous cell carcinoma (ESCC) and non-ESCC patients.
NQO1, *GSTM*, and *CLDN4* mRNA expression levels ($n = 20$ per group) [mean ± standard error (SE)]. In non-exposed samples, the *CLDN4* mRNA expression level in ESCC patients was significantly lower than that in non-ESCC patients ($p < 0.05$). In ESCC patients, the mRNA expression levels of all three genes tended to decrease after the exposure, and the differences between no exposure and both acetaldehyde (AcH) and ethanol (EtOH) exposure were significant in *NQO1* and *GSTM* (all, $p < 0.05$). *$p < 0.05$, **$p < 0.01$
CLDN4, *claudin 4*; *GSTM*, *glutathione S-transferases M*; *NQO1*, *NADPH quinone oxidoreductase 1*.

Drinking history in ESCC and non-ESCC patients

We further evaluated the mRNA expression levels of three genes after AcH or both AcH and EtOH exposure in ESCC and non-ESCC patients with and without drinking history. No significant results were obtained in non-ESCC patients, regardless of the status of drinking history. In ESCC patients without drinking history, all comparisons also showed no statistical significance. On the other hand, the expression levels of all three genes tended to decrease after AcH or both AcH and EtOH exposure in ESCC patients with drinking history, and the difference between no exposure and both AcH and EtOH exposure was statistically significant in *GSTM* ($p < 0.01$).

Immunohistochemistry of NRF2 target gene and CLDN4 in nonneoplastic esophageal mucosa

The expressions of NQO1 and CLDN4 were verified by immunohistochemistry. Immunostaining showed cytoplasm localization of NQO1 in esophageal cells, and immunostaining for CLDN4 showed positive staining on the cell membrane (Fig. 6A). Although the number of NQO1-positive cells did not differ significantly between ESCC and non-ESCC patients, the number of CLDN4-positive cells in ESCC patients was significantly smaller than that in non-ESCC patients ($p < 0.01$) (Fig. 6B).

Discussion

One of the critical issues in patients with endoscopic resection for ESCC is the high incidence of metachronous ESCC, and this risk is higher in patients with an alcohol-drinking habit after endoscopic resection, especially in LVL grade C (Katada et al. 2016). Nrf2 is related to the protective mechanism against carcinogenesis in the esophagus of mice (Ohkoshi et al. 2013). In addition, tight junction dis-

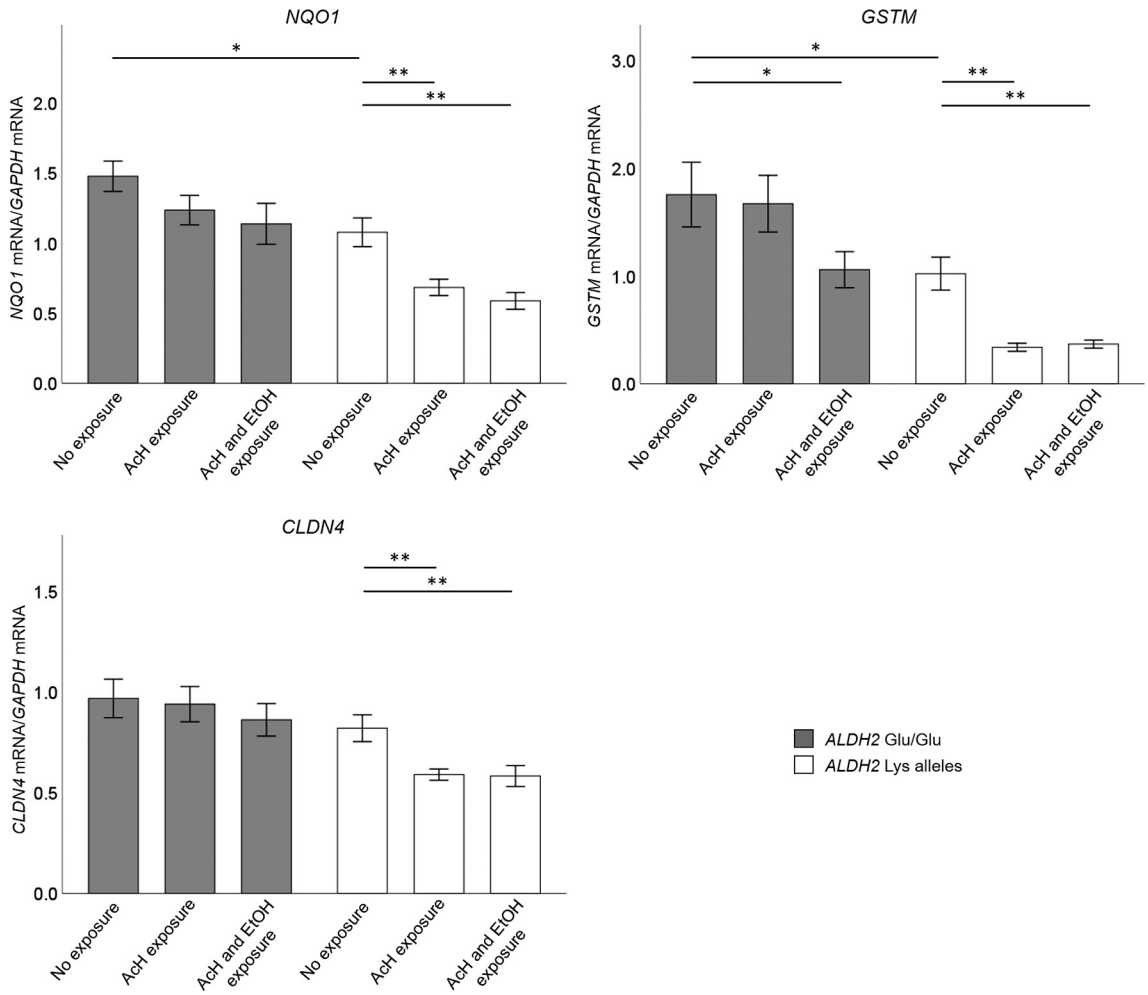

Fig. 3. Gene expression profiles of the background esophageal mucosa in esophageal squamous cell carcinoma (ESCC) patients, according to *ALDH2* polymorphism.
NQO1, *GSTM*, and *CLDN4* mRNA expression levels in *ALDH2* Glu/Glu carriers (n = 11) and *ALDH2* Lys allele carriers (n = 9) (mean ± SE). In non-exposed samples, the *NQO1* and *GSTM* mRNA expression levels in *ALDH2* Lys allele carriers were significantly lower than those in *ALDH2* Glu/Glu carriers (all, $p < 0.05$). The *NQO1*, *GSTM*, and *CLDN4* mRNA expression levels significantly decreased after acetaldehyde (AcH) or both AcH and ethanol (EtOH) exposure in *ALDH2* Lys allele carriers (all, $p < 0.01$). $*p < 0.05$, $**p < 0.01$
ALDH2, *aldehyde dehydrogenase 2*; *CLDN4*, *claudin 4*; *GSTM*, *glutathione S-transferases M*; *NQO1*, *NADPH quinone oxidoreductase 1*.

ruption in premalignant neoplastic tissue can increase the likelihood of developing carcinoma (Singh et al. 2010). Thus, we hypothesized that some protective mechanism against carcinogenesis due to alcohol exposure might be disrupted in such patients and NRF2 and barrier dysfunction might be associated with such a disruption.

Regarding the response of NRF2 target genes for AcH or both AcH and EtOH exposure in the non-neoplastic esophageal mucosa, the mRNA expression levels of these genes decreased after the exposure only in ESCC patients. Furthermore, in ESCC patients, the decreased expression levels of these genes after the exposure were consistently shown at high-risk states for metachronous ESCC, such as *ALDH2* Lys allele, LVL grade C, and drinking history. In particular, the decreased tendency of these genes after the exposure was pronounced as the LVL grade increased from A to C. These findings suggest that, as the risk for metachronous ESCC is higher, the expression of NRF2 target genes after alcohol exposure in the non-neoplastic esophageal mucosa might decrease. Although the relationship between alcohol exposure and NRF2 remains complex (Odera et al. 2020), the activation of NRF2 is noted to prevent ethanol-induced oxidative stress, lipid accumulation, and accelerated AcH metabolism in the liver and lung (Wu et al. 2012; Jensen et al. 2013). Furthermore, Nrf2 plays a protective role against carcinogenesis in the esophagus of mice (Ohkoshi et al. 2013). These findings and our results suggest that the protective role of NRF2 against carcinogenesis due to alcohol exposure might be disrupted in the background esophageal mucosa of ESCC patients, which might lead to an increased risk of metachronous ESCC due to alcohol consumption in ESCC patients (Katada et al.

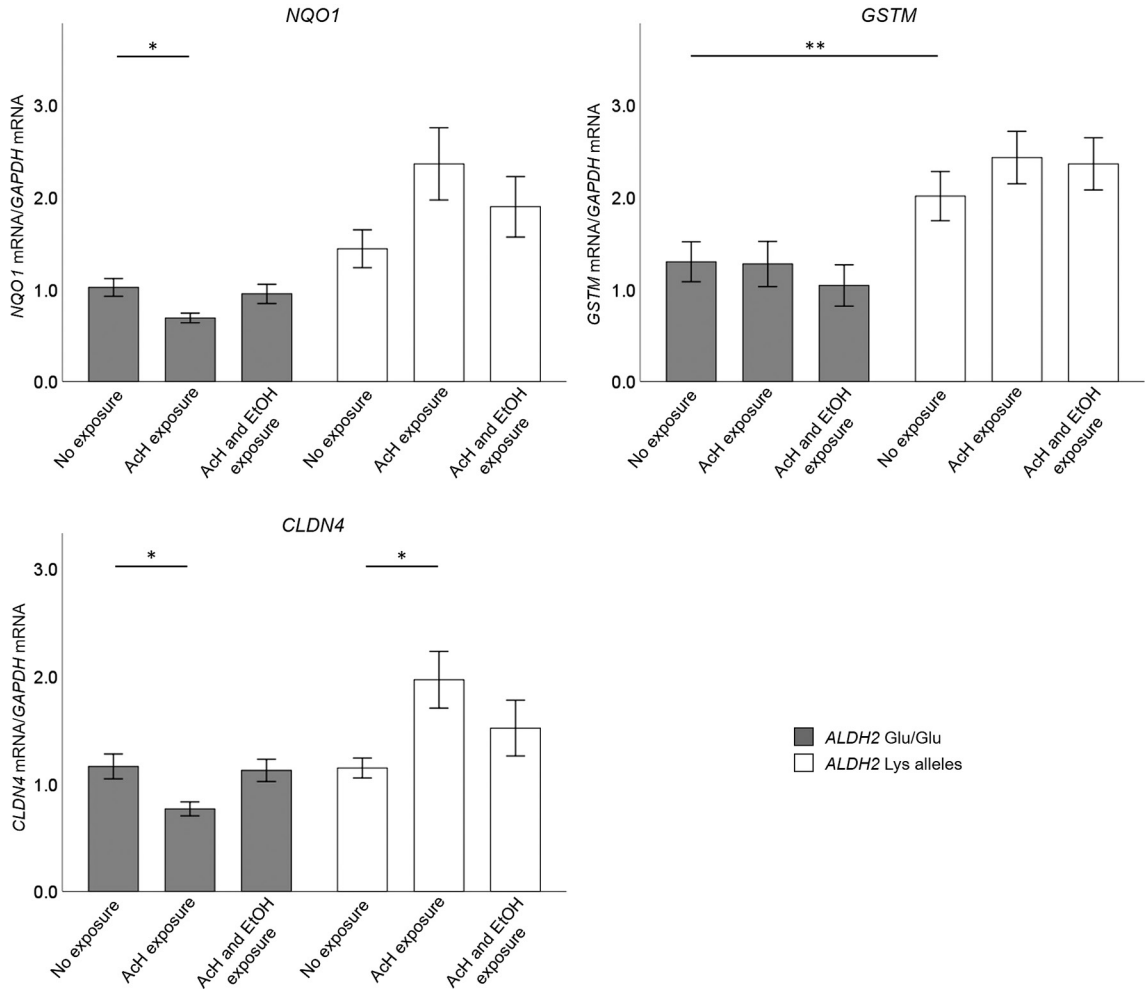

Fig. 4. Gene expression profiles of the background esophageal mucosa in non-ESCC patients, according to *ALDH2* polymorphism,
NQO1, *GSTM*, and *CLDN4* mRNA expression levels in *ALDH2* Glu/Glu carriers (n = 12) and *ALDH2* Lys allele carriers (n = 8) (mean ± SE). In non-exposed samples, the *GSTM* mRNA expression level in *ALDH2* Lys allele carriers was significantly higher than that in *ALDH2* Glu/Glu carriers ($p < 0.01$). The *NQO1*, *GSTM*, and *CLDN4* mRNA expression levels tended to increase after acetaldehyde (AcH) or both AcH and ethanol (EtOH) exposure in *ALDH2* Lys allele carriers, with a significant difference between no exposure and AcH exposure in *CLDN4* ($p < 0.05$). *$p < 0.05$, **$p < 0.01$
ALDH2, *aldehyde dehydrogenase 2*; *CLDN4*, *claudin 4*; *GSTM*, *glutathione S-transferases M*; *NQO1*, *NADPH quinone oxidoreductase 1*.

2016).

This study also revealed the decreased gene expression of *CLDN4* only in the background esophageal mucosa of ESCC patients. On the other hand, the difference in TEER after the exposure between ESCC and non-ESCC patients was not statistically significant. This discrepancy might be partially explained by the fact that TEER reflects not only the integrity of tight junctions but also that of the cell layer (Zucco et al. 2005). Furthermore, a limited number of cases may have also led to no significant results. However, since it was difficult to determine the relationship between tight junction disruption and the carcinogenesis of ESCC from this study, further studies are required.

ALDH2 Lys allele carriers are at higher risk for ESCC than *ALDH2* Glu/Glu carriers (Cui et al. 2009; Tanaka et al. 2010; Abiko et al. 2018), although the difference in risk between ESCC and non-ESCC patients is much larger than those with *ALDH2* polymorphism. In the stratified analysis of *ALDH2* polymorphism, the decreased mRNA expression of NRF2 target genes and *CLDN4* after AcH or both AcH and EtOH exposure was pronounced in patients with *ALDH2* Lys alleles among ESCC patients. Interestingly, the converse tendency, i.e., increased mRNA expression of these genes in *ALDH2* Lys allele carriers, was observed in non-ESCC patients. The reason for the converse results in this study is unclear. However, one possible explanation is the defense mechanism of the human body against carcinogenesis, based on the protective role of NRF2 (Ohkoshi et al. 2013). In the early stage of carcinogenesis such as in non-ESCC patients, NRF2 might react as a defense mechanism against carcinogenesis due to alcohol exposure in high-risk states such as in *ALDH2* Lys allele carriers.

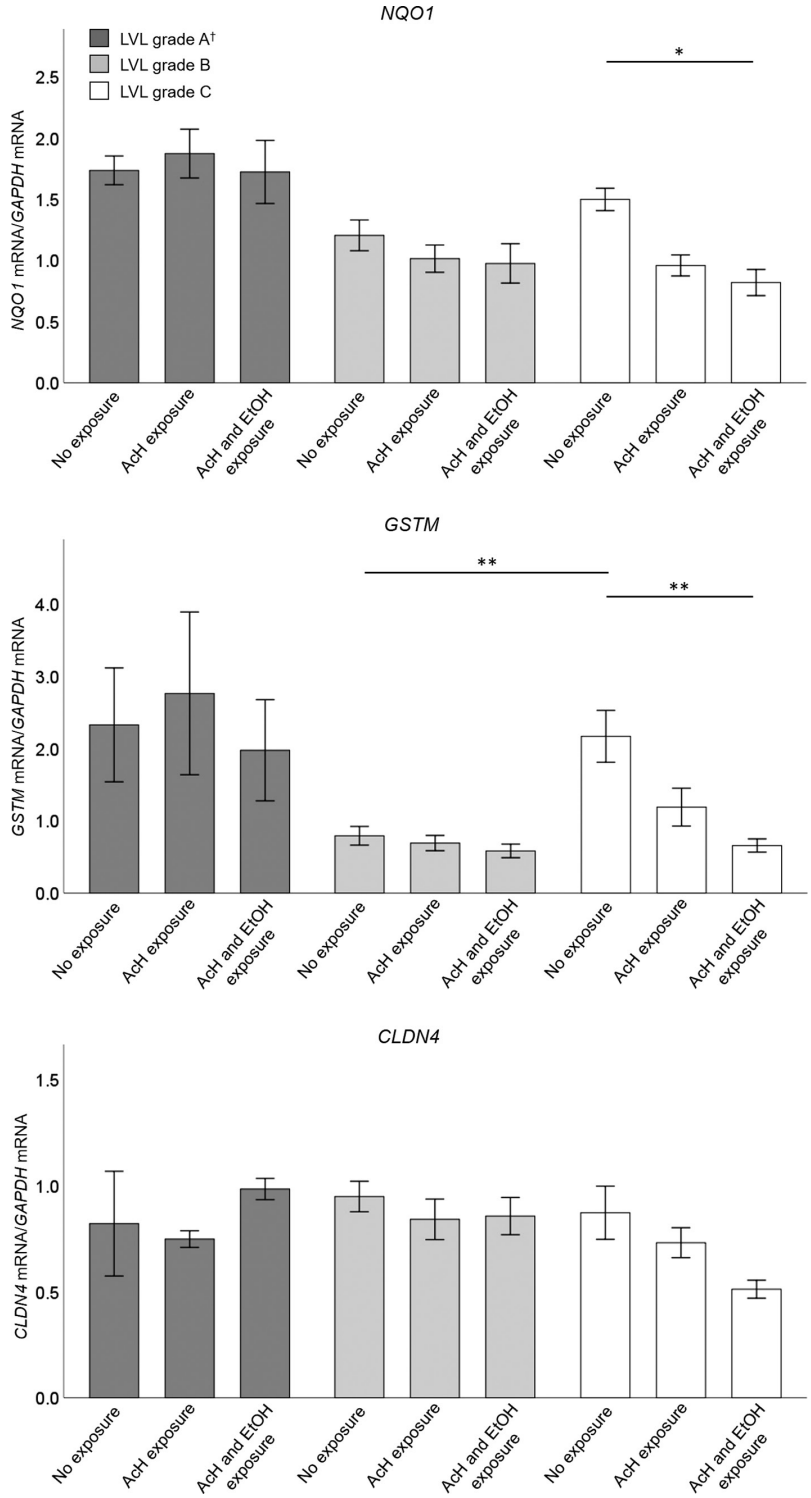

Fig. 5. Gene expression profiles of the background esophageal mucosa in esophageal squamous cell carcinoma (ESCC) patients, according to the Lugol-voiding lesion (LVL) grade.
NQO1, *GSTM*, and *CLDN4* mRNA expression levels in ESCC patients with LVL grade A ($n = 2$), B ($n = 11$), and C ($n = 7$) (mean ± SE). After acetaldehyde (AcH) or both AcH and ethanol (EtOH) exposure, no significant difference in the mRNA expression levels of all three genes was shown in LVL grade B, whereas their mRNA expression levels tended to decrease in LVL grade C, with a significant difference between no exposure and both AcH and EtOH exposure in *NQO1* ($p < 0.05$) and *GSTM* ($p < 0.01$). $^{*}p < 0.05$, $^{**}p < 0.01$
†The statistical analysis about comparisons including LVL grade A was not performed due to a small number of cases.
CLDN4, *claudin 4*; *GSTM*, *glutathione S-transferases M*; *NQO1*, *NADPH quinone oxidoreductase 1*.

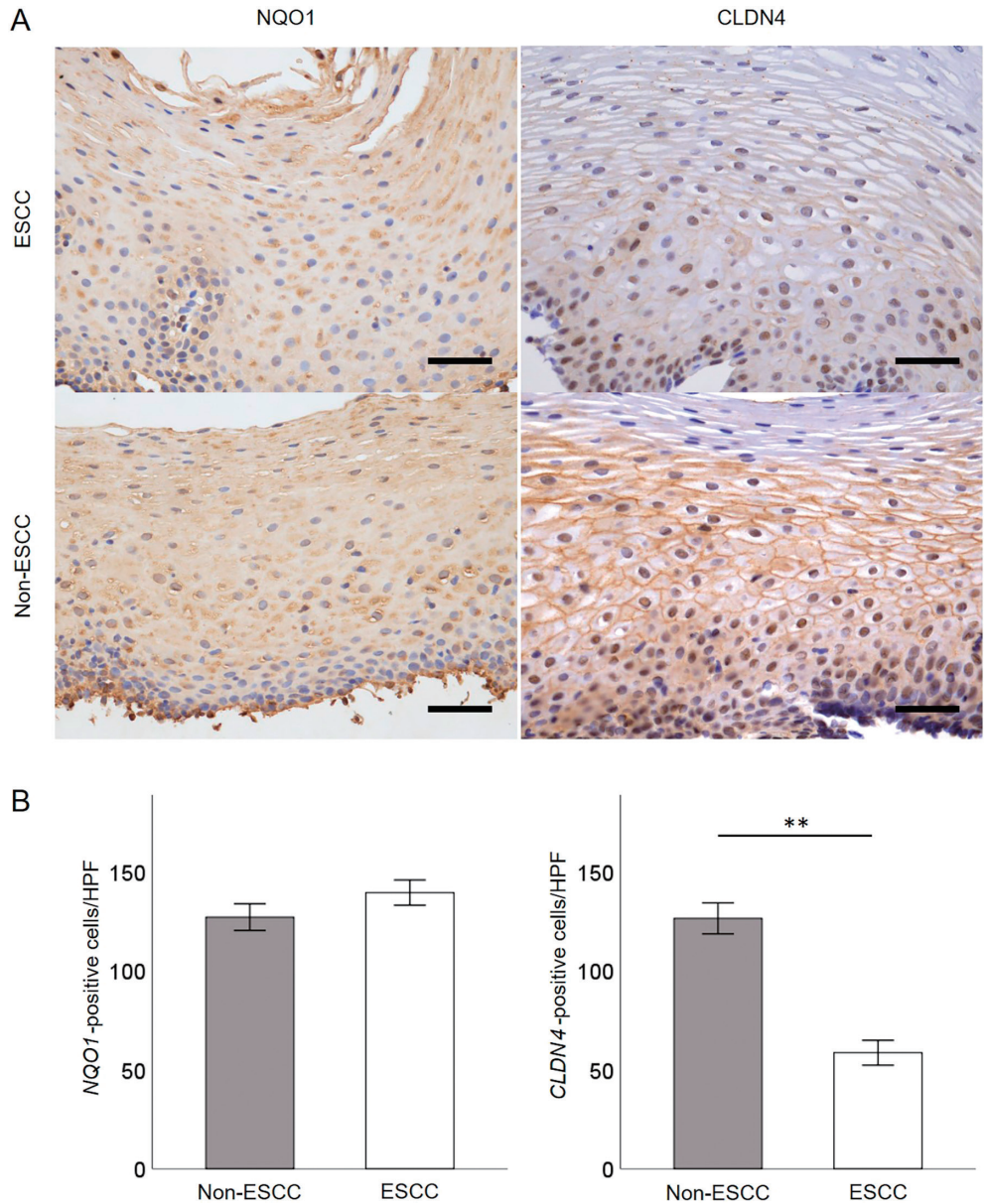

Fig. 6. Expression of NQO1 and CLDN4 in biopsy specimens of the background esophageal mucosa in esophageal squamous cell carcinoma (ESCC) and non-ESCC patients.
(A) Representative immunostaining for NQO1 and CLDN4 in biopsy specimens of the background esophageal mucosa in ESCC and non-ESCC patients. Black bars indicate 50 μm. (B) Quantification of total number of positive cells for NQO1 and CLDN4. The number of CLDN4-positive cells was significantly smaller in ESCC patients ($p < 0.01$).
**$p < 0.01$
CLDN4, claudin 4; NQO1, NADPH quinone oxidoreductase 1.

Conversely, the defense mechanism of NRF2 might be impaired in the late stage of carcinogenesis such as in ESCC patients, and this effect might be larger in high-risk states. Furthermore, these results may partially explain the relationship between the mRNA expression of NRF2 target genes and *CLDN4* after alcohol exposure. The *CLDN* mRNA expression level after the exposure was almost in parallel with the expression levels of NRF2 target genes, irrespective of the increased and decreased expression. Since Nrf2 deficiency impairs the esophageal barrier function through the disruption of energy-dependent tight junctions (Chen et al. 2014), *CLDN4* of the non-neoplastic esophageal mucosa after AcH or both AcH and EtOH exposure might be regulated by *NRF2*.

This study has several limitations. First, this study included a small number of patients, which may have affected the results. Second, despite evaluation of the expression of three genes, the activation of these genes was not investigated. Third, although the decreased expression of NRF2 target genes after alcohol exposure was pronounced in ESCC patients with *ALDH2* Lys allele and LVL grade C, confounding of *ALDH2* polymorphism and the

LVL grade was not investigated. Fourth, although the biopsy specimens were acquired from the non-neoplastic esophageal mucosa, it was difficult to identify whether the biopsy specimens were normal epithelium or LVL. However, we confirmed that there were no cases with squamous intraepithelial neoplasia in biopsy specimens for histological assessment. Fifth, patients at high risk for ESCC have heterogeneous accumulation of genetic alteration in the background esophageal mucosa. However, this study investigated only a few biopsy specimens; thus, it is difficult to conclude genetic alteration in the whole esophagus. Sixth, esophageal mucosa may have been injured by prior iodine staining, although iodine staining was performed at more than one month before the endoscopic biopsy. The exact time to mucosal healing from iodine staining is unclear. Lastly, the exact relationship between tight junction disruption and esophageal carcinogenesis has yet to be determined. Further studies, such as using *cldn4* deficient mice, are required for elucidating their precise relationship.

In conclusion, we first demonstrated the decreased expression of NRF2 target genes after alcohol exposure in the non-neoplastic esophageal mucosa of ESCC patients. Furthermore, the decreased expression levels of these genes after the exposure were more pronounced in high-risk patients for metachronous ESCC, such as ESCC patients with *ALDH2* Lys allele or LVL grade C. These findings suggest that the protective role of NRF2 against carcinogenesis due to alcohol exposure might be disrupted in the background esophageal mucosa of ESCC patients, which might lead to a high incidence of metachronous ESCC.

Conflict of Interest

The authors declare no conflict of interest.

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