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Original Article

Prognostic significance of NQO1 expression in esophageal squamous cell carcinoma after preoperative chemotherapy with cisplatin and 5-fluorouracil followed by curative esophagectomy

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Abstract: NAD(P)H:quinone oxidoreductase-1 (NQO1) confers resistance to anticancer agents, particularly to oxidative stress inducers such as cisplatin or 5-fluorouracil in malignant tumors. Here we evaluated the association between NQO1 expression in esophageal squamous cell carcinoma (ESCC) cells and the patients' responses to preoperative chemotherapy with cisplatin and 5-fluorouracil (CF), and we elucidated the prognostic significance of NQO1 expression in ESCC patients. We retrospectively analyzed the cases of 40 patients who underwent preoperative CF therapy followed by curative esophagectomy with lymphadenectomy. Immunohistochemistry of the surgically resected specimens was conducted using the primary monoclonal antibody against NQO1. Eighteen of the 40 patients (45%) had tumors that showed high NQO1 expression (NQO1-high group). The poorer histological response to preoperative CF therapy was dominant in the NQO1-high group compared to the NQO1-low group (72% and 45%, respectively) but the difference was not significant (P=0.09). The 3-year recurrence-free survival rate after esophagectomy in the NQO1-high group was significantly lower compared to the NQO1-low group (39% vs. 76%; P<0.01). A Cox proportional hazards model revealed that high NQO1 expression was an independent unfavorable prognostic factor (HR=3.53; P=0.02) as was pN3 (HR=14.7; P<0.01). The immunohistochemical evaluation of NQO1 expression has potential to predict the treatment response and prognosis in patients who undergo preoperative CF therapy followed by esophagectomy for ESCC.

Keywords: NQO1, esophageal squamous cell carcinoma, preoperative chemotherapy, cisplatin, 5-fluorouracil

Introduction

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer death worldwide [1]. In East Asian countries, esophageal squamous cell carcinoma (ESCC) is a predominant histological subtype. Cisplatin and 5-fluorouracil are key drugs in chemotherapy for gastrointestinal cancers including ESCC. Preoperative chemotherapy with cisplatin and 5-fluorouracil (CF) followed by curative esophagectomy was shown to improve the survival of patients with resectable locally advanced ESCC, and is regarded as the standard treatment for ESCC in Japan [2]. However, approx. 50% of the patients who underwent preoperative CF therapy suffered tumor recurrence even after curative resection. The tumor response to preoperative CF therapy is a significant prognostic factor [3], and the molecular background underlying the resistance to the CF therapy may adversely affect patients' outcome and have potential as a prognostic indicator.

Oxidative stress promotes the nuclear accumulation of nuclear factor erythroid 2-related factor 2 (Nrf2), and it activates the transcription of

Table 1. Associations between clinicopathological characteristics and NQO1 expression

Variable		No. of patients (%)			*P*-value
		Total (n=40)	NQO1-high (n=18)	NQO1-low (n=22)	
Age (yrs)	Median (range)	63 (49-79)	62 (49-73)	63 (50-79)	0.68
Gender	Male	34	17 (94)	17 (77)	0.20
	Female	6	1 (6)	5 (23)	
Location	Ce/Ut	3	2 (11)	1 (5)	0.21
	Mt	22	7 (39)	15 (68)	
	Lt	15	9 (50)	6 (27)	
pT (UICC)	T1	7	1 (6)	6 (27)	0.12
	T2	2	1 (6)	1 (5)	
	T3	31	16 (89)	15 (68)	
pN (UICC)	N0	15	7 (39)	8 (36)	0.36
	N1	16	6 (33)	10 (45)	
	N2	5	4 (22)	1 (5)	
	N3	4	1 (6)	3 (14)	
Histological differentiation	Well	8	2 (11)	6 (27)	0.26
	Mod	25	11 (61)	14 (64)	
	Poor	7	5 (6)	2 (9)	
No. of CF courses	1	6	1 (6)	5 (23)	0.20
	2	34	17 (94)	17 (77)	
Histological response	Grade 0/1a	23	13 (72)	10 (45)	0.09
	Grade 1b/2	17	5 (28)	12 (55)	

UICC, International Union Against Cancer; CF, cisplatin and 5-fluorouracil; Ce, cervical esophagus; Ut, upper thoracic esophagus; Mt, middle thoracic esophagus; Lt, lower thoracic esophagus; Well, well differentiated; Mod, moderately differentiated; Poor, poorly differentiated.

downstream genes coding for antioxidant enzymes, such as NAD(P)H:quinone oxidoreductase-1 (NQO1) [4]. NQO1 is a flavoprotein that catalyzes the two-electron reduction of quinones and related compounds [5]. In normal cells, NQO1 protects the cells against redox cycling and oxidative stress, as well as against carcinogenesis by stabilization of the p53 tumor suppressor [6-8]. In cancer cells, NQO1 confers resistance to anticancer agents, particularly to oxidative stress inducers such as cisplatin or 5-fluorouracil [9, 10]. However, the NQO1 expression in tumor cells of ESCC and its clinical significance in relation to preoperative CF therapy have not been examined.

In the present study, we hypothesized that high NQO1 expression in ESCC tumor cells may play a role in the resistance to preoperative CF therapy, and that this high expression may function as an adverse prognostic factor. We evaluated the immunohistochemical expression of NQO1 in surgically resected specimens of ESCC following CF therapy. Our goals were to determine the association between the NQO1 expression and the response to preoperative CF therapy, and to elucidate the prognostic significance of NQO1 expression in patients with ESCC.

Patients and methods

Patients and clinical information

A total of 43 patients underwent preoperative CF therapy followed by curative esophagectomy with lymphadenectomy for ESCC at Niigata University Medical and Dental Hospital between 2000 and 2012. Among these, 40 patients were enrolled in this study, and the remaining three patients with no residual viable tumor cells in the surgical specimen were excluded. There were 34 men and six women, with a median age of 63 years (range; 49-79 years). The clinicopathological data for the individual patients are summarized in **Table 1**. This study was approved by the institutional review board of the Niigata University Medical and Dental Hospital.

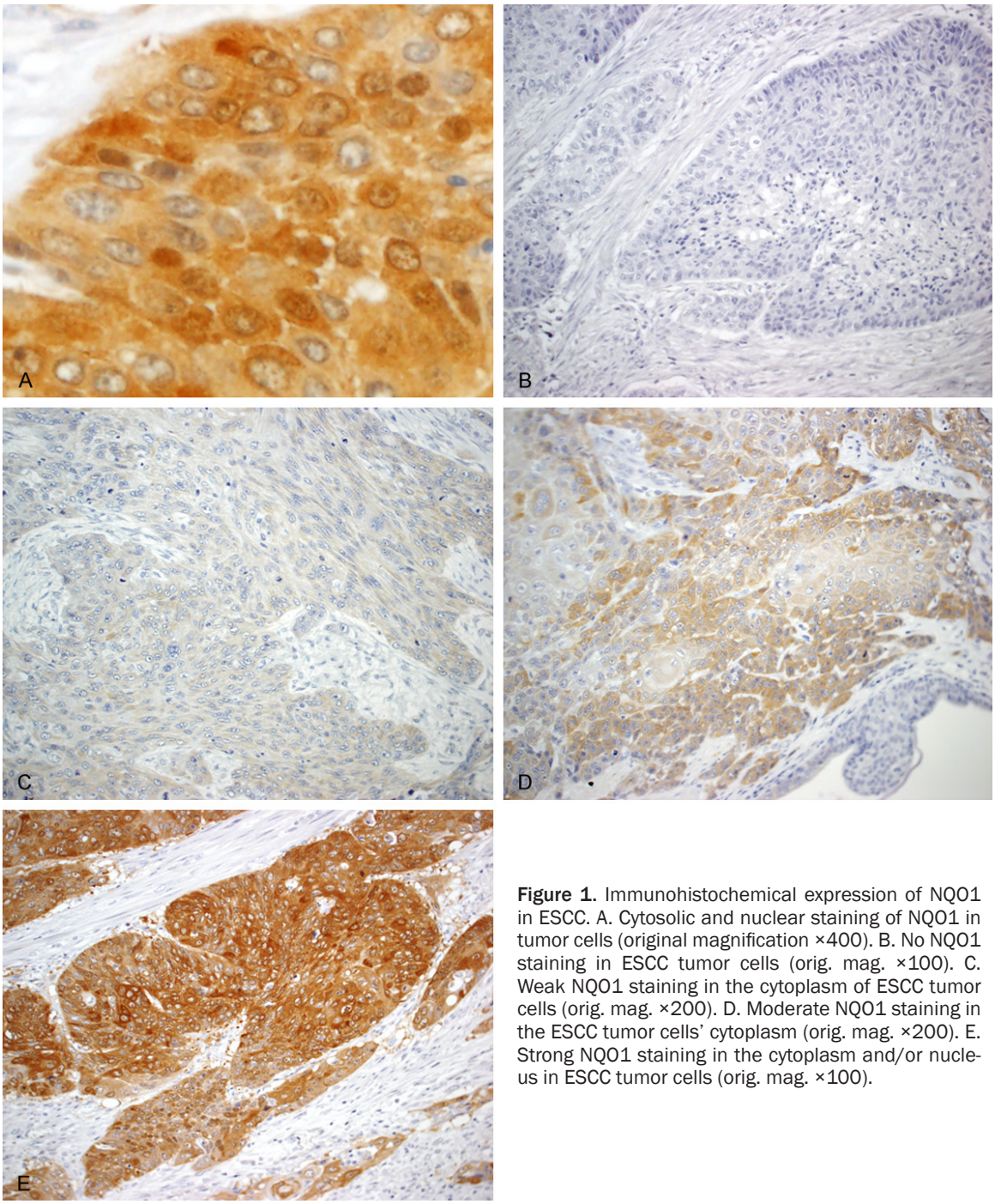

Figure 1. Immunohistochemical expression of NQO1 in ESCC. A. Cytosolic and nuclear staining of NQO1 in tumor cells (original magnification ×400). B. No NQO1 staining in ESCC tumor cells (orig. mag. ×100). C. Weak NQO1 staining in the cytoplasm of ESCC tumor cells (orig. mag. ×200). D. Moderate NQO1 staining in the ESCC tumor cells' cytoplasm (orig. mag. ×200). E. Strong NQO1 staining in the cytoplasm and/or nucleus in ESCC tumor cells (orig. mag. ×100).

Treatment protocols and histopathological examination

The patients' preoperative chemotherapy consisted of cisplatin and 5-fluorouracil, performed twice at a 3-week interval. A dose of 80 mg/m^2 cisplatin was administered by intravenous drip infusion on day 1; 5-fluorouracil was administered at 800 mg/m^2 by continuous infusion on days 1 through 5 [2]. Surgical resection was performed 4-6 weeks after the end of the chemotherapy.

Thirty-four and six patients underwent esophagectomy by the transthoracic and transhiatal approach, respectively. In the patients who underwent a transthoracic esophagectomy, two-field and three-field lymphadenectomies were performed in 13 and 21 patients, respectively. All of the patients achieved complete

resection with no residual tumor histologically. There were no patients who underwent postoperative adjuvant chemotherapy.

The histological status of the primary tumor (pT) and the resected lymph nodes (pN) were assessed according to the 7th edition of the International Union against Cancer tumor-node-metastasis (TNM) classification system [11]. The response of the primary tumor to the preoperative chemotherapy was evaluated according to the histological criteria of the Japanese Society for Esophageal Diseases as follows. Grade 0: ineffective (no recognizable cytological or histological therapeutic effect); Grade 1: slightly effective (Grade 1a: viable cancer cells account for 2/3 or more of the tumor tissue; Grade 1b: viable cancer cells account for 1/3 or more, but less than 2/3, of the tumor tissue); Grade 2: moderately effective (viable cancer cells account for less than 1/3 of the tumor tissue), and Grade 3: markedly effective (no viable cancer cells are evident) [12].

Follow-up

All of the patients underwent physical and blood biochemistry examinations every 3 months after the initial treatment as a regular checkup. Chest radiographs and computed tomography of the neck, chest, and abdomen were performed at least once a year. The recurrence-free survival (RFS) rate was calculated from the date of esophagectomy to that of first recurrence, censoring patients alive at the time of data collection and those who died without recurrence on the date of death. The median follow-up period after esophagectomy was 69 months (range 21-175 months) for surviving patients without recurrence.

Immunohistochemistry

NQO1 expression was examined immunohistochemically using formalin-fixed paraffin-embedded blocks. Three serial 4-μm-thick sections were re-cut and prepared from each block; one for hematoxylin-eosin staining, one for immunohistochemical staining for NQO1, and one used as a negative control. For immunohistochemistry, the sections were deparaffinized and rehydrated before being microwaved at 700 W for 15 min in 10 mmol/l citrate buffer (pH 6.0) to retrieve antigenic activity. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxidase in methanol for 20 min. After blocking any nonspecific reactions with 10% normal goat serum, sections were incubated overnight at 4°C with the rabbit monoclonal antibody against NQO1 (1:100 dilution, Epitomics, Burlingame, CA, USA). Immunostaining was carried out by the streptavidin-biotin peroxidase method using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as the chromogen, and sections were counterstained with hematoxylin. As a negative control, normal rabbit immunoglobulin was substituted for the primary antibody.

Two independent surgical pathologists (H.I. and Y.H.), both blinded to the clinical data, reviewed the sections stained with anti-NQO1 antibody. NQO1 expression was defined as the presence of cytosolic and/or nuclear staining as similar to the previous our report [13]. The staining intensity was graded as follows: 0 for no staining, 1+ for weak staining in the cytoplasm, 2+ for moderate staining in the cytoplasm, and 3+ for strong staining in the cytoplasm and/or nucleus of ESCC tumor cells (**Figure 1**). High NQO1 expression was defined as 2+ or more intensity in 10% or more of viable tumor cells according to the modified criteria in a previous report [14], and low NQO1 expression was defined as the other staining patterns. In most cases, the difference was quite obvious and the two reviewers concurred as to the results.

Statistical analysis

Fisher's exact test and the Mann-Whitney *U*-test were used to compare the categorical and continuous variables, respectively. The RFS rate was estimated by the Kaplan-Meier method. Univariate survival analyses were performed using the log-rank test. The Cox proportional hazards model was applied to the multivariate survival analysis. The variables with a univariate *P*-value <0.10 were entered into the model. SPSS version 11.5 (SPSS, Chicago, IL) was used for all of the statistical analyses. All tests were two-tailed, and *P*-values <0.05 were regarded as significant.

Results

NQO1 expression and clinicopathological characteristics

According to the immunohistochemistry, 18 of the 40 patients (45%) had tumors that showed high NQO1 expression (NQO1-high group). The

Table 2. Prognostic factors for 3-year recurrence-free survival in 40 patients with ESCC

		Univariate		Multivariate		
Variable		3-year RFS rate (%)	*P*-value	HR	95% CI	*P*-value
Age (yrs)	>63	68	0.34			
	≤63	49				
Gender	Male	57	0.67			
	Female	67				
Location	Ce/Ut	0	0.32			
	Mt	62				
	Lt	67				
pT (UICC)	T1	83	0.14			
	T2	100				
	T3	51				
pN (UICC)	N0	73	0.05	1.00		
	N1	60		2.14	0.67-7.36	0.23
	N2	40		1.91	0.41-8.82	0.41
	N3	25		14.7	2.42-83.0	<0.01
Histological differentiation	Well	63	0.34			
	Mod	63				
	Poor	43				
Histological response	Grade 0/1a	47	0.04	1.96	0.60-6.36	0.27
	Grade 1b/2	75		1.00		
No. of CF courses	1	83	0.15			
	2	54				
NQO1 expression	Low	76	<0.01	1.00		
	High	39		3.53	1.24-10.1	0.02

UICC, International Union Against Cancer; CF, cisplatin and 5-fluorouracil; Ce, cervical esophagus; Ut, upper thoracic esophagus; Mt, middle thoracic esophagus; Lt, lower thoracic esophagus; Well, well differentiated; Mod, moderately differentiated; Poor, poorly differentiated; RFS, recurrence-free survival; HR, hazard ratio; CI, confidence interval.

details of clinicopathological characteristics according to NQO1 expression are shown in **Table 1**. Regarding the histological response to preoperative CF therapy, Grades 0 and 1a were more frequent in the NQO1-high group than in the NQO1-low group (72% and 45%, respectively). However, the association between the NQO1 expression and the histological response was not significant (P=0.09). There were no significant associations between NQO1 expression and other clinicopathological characteristics including, age, gender, tumor location, pT, pN, histological differentiation, and the number of CF courses.

Prognostic significance of NQO1 expression

The Kaplan-Meier survival analysis and log-rank test showed that the 3-year RFS rate in the NQO1-high group was significantly lower than that in the NQO1-low group (39% vs. 76%; P<0.01) (**Table 2**; **Figure 2**). The histological response to preoperative chemotherapy was also significantly associated with RFS in the univariate analysis (P=0.04). The association between the pN and RFS was statistically marginal (P=0.05). We therefore entered the above-mentioned three variables into a Cox proportional hazards model for the multivariate analysis, which revealed that high NQO1 expression was an independent unfavorable prognostic factor (HR=3.53; P=0.02), as was pN3 (HR=14.7; P<0.01) (**Table 2**).

Discussion

Preoperative chemotherapy with CF followed by curative esophagectomy is the standard treatment for ESCC in Japan [15]. The postoperative prognosis in non-responders to CF therapy is worse than that in responders [3]. Understanding the molecular backgrounds underly-

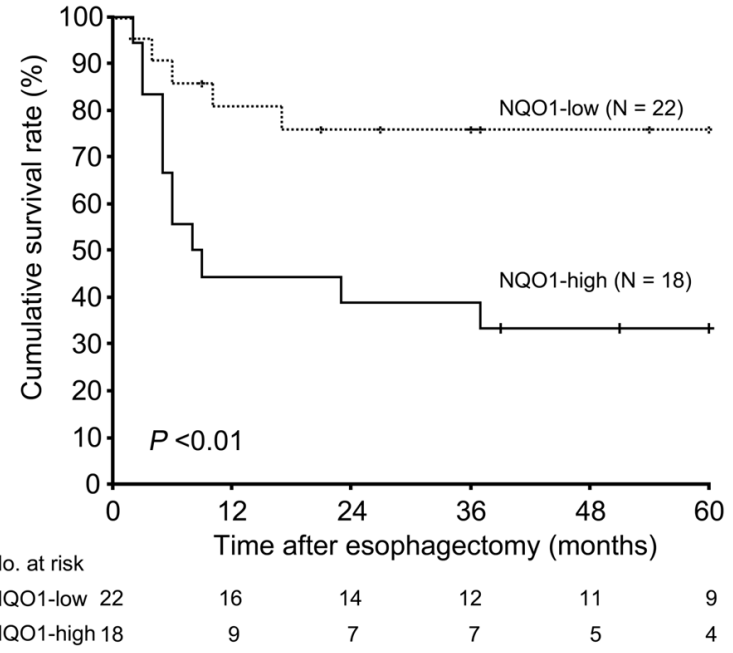

Figure 2. Kaplan-Meier survival curve for recurrence-free survival (RFS) after preoperative CF therapy followed by esophagectomy in ESCC patients, according to NQO1 expression. The 3-year RFS rate in the patients who had tumors with high NQO1 expression was significantly lower than that in the patients who had tumors with low NQO1 expression (39% vs. 76%; P<0.01).

ing the resistance to CF therapy is necessary to improve the clinical outcome of ESCC patients. NQO1 functions to protect normal cells against oxidative stress, and it confers resistance to the anticancer agents (4, 9, 10). We hypothesized that high NQO1 expression in ESCC tumor cells may play a role in the resistance to preoperative CF therapy and function as an adverse prognostic factor, and herein we evaluated the NQO1 expression in ESCC tumor cells by immunohistochemistry. To the best of our knowledge, this is the first study to evaluate the NQO1 expression in tumor specimens after preoperative CF therapy and the first to demonstrate prognostic utility in ESCC.

A redox-sensitive transcription factor, NFE2-related factor 2 (NRF2), regulates the expression of NQO1 [16]. Under physiological conditions, NRF2 is kept transcriptionally inactive through binding to its inhibitor, KEAP1, which targets NRF2 for proteasomal degradation. The oxidative stress induces a KEAP1 inactive modification, thereby reversing the proteasomal degradation of NRF2, which leads to the nuclear localization and transcription of anti-oxidative genes including NQO1 [17]. It was reported that KEAP1 or NRF2 mutation leads to an aberrant activation of NRF2 in a wide range of cancers including ESCC [18-20]. In addition to the KEAP1 or NRF2 mutation, the down-regulation of some microRNAs was reported to be associated with the increased expression of NRF2 in ESCC [21]. Thus, multiple alterations in the KEAP1-NRF2-NQO1 pathway confer the NQO1 expression level. The immunohistochemical evaluation of NQO1 expression might reflect the overall aberrant activation of this pathway.

An association between NQO1 expression and chemoresistance was suggested in previous in vitro and in situ studies. Watanabe et al. revealed that NQO1 knockdown or inhibition of its enzymatic activity enhanced cisplatin-induced cytotoxicity in urogenital cancer cell lines [22]. Zeekpudsa et al. reported that the overexpression of NQO1 made cholangiocarcinoma cell lines more resistant to 5-FU, doxorubicin, and gemcitabine [10]. NQO1 might contribute to the resistance to the cisplatin and 5-FU in ESCC tumor cells as in these previous studies.

In the present study, the ESCC cases with high NQO1 expression tended to achieve lower histological responses to the preoperative CF therapy compared to the cases with low NQO1 expression. However, we did not observe a significant association between NQO1 expression and histological response. Further studies using a larger number of clinical samples and in vivo and/or in vitro assays are necessary to enhance the reliability of this finding.

There are few reports about the association between immunohistochemical NQO1 expression and the clinicopathological characteristics or prognoses of patients with malignant tumors. Cui et al. reported that a high-level expression of NQO1 was correlated with large tumor size, late pathologic stage and the presence of

lymph node metastasis, and was an independent adverse prognostic factor in small-cell lung cancer [23]. Similar contributions of high NQO1 expression to tumor characteristics and poor outcome were demonstrated in breast cancer [24], cervical cancer [25] and non-small-cell lung cancer [26]. In the present study, high NQO1 expression was an unfavorable prognostic factor after CF therapy followed by esophagectomy in ESCC patients, as in previous reports; however, there was no significant association between high NQO1 expression and the clinicopathological characteristics that represent tumor aggressiveness. These results imply that NQO1 expression might be both a predictive factor for the response to preoperative CF therapy and a prognostic indicator that is independent of the tumor characteristics in ESCC.

Mechanisms that affect NQO1 expression other than the KEAP1-NRF2-NQO1 pathway have been reported; for example, a single nucleotide polymorphism of NQO1 (C609T) was strongly associated with the NQO1 protein stability. The mutant NQO1 protein rapidly degraded via the ubiquitination and proteasomal degradation [27]. Therefore, the T/T genotype causes a complete lack of enzyme activity of NQO1 [28]. A 2013 meta-analysis demonstrated that the NQO1 T/T genotype increases susceptibility to ESCC [29]. However, the association between NQO1 C609T polymorphism and the clinical course and outcome of patients suffering from ESCC has not been elucidated. In the present study, we did not investigate whether patients with the T/T genotype (which causes a low expression of NQO1) had better responses to preoperative CF therapy and favorable outcomes after esophagectomy. Further studies are necessary to clarify the clinical significance of NQO1 C609T polymorphism in ESCC.

There are two main limitations in the present study. First, this was a single institutional retrospective analysis with a small number of patients. However, 40 (93%) of 43 consecutive patients who underwent CF therapy followed by esophagectomy in our institution between 2001 and 2012 were enrolled. This high inclusion rate thus led to a reduced selection bias. Second, the validity of immunohistochemical evaluations of NQO1 has not been demonstrated. Multi-institutional studies are necessary to identify a simple and reproducible immunohistochemical staining protocol and the evaluation criteria with a clinical application perspective. Nonetheless, we believe that the results of this study are informative for further prospective studies to clarify the clinical utility of the immunohistochemical evaluation of NQO1 in endoscopic biopsy specimens prior to CF therapy for ESCC patients.

In conclusion, NQO1 expression might play a role in the resistance to CF therapy in ESCC. High NQO1 expression in tumor cells is an unfavorable prognostic factor after preoperative CF therapy followed by esophagectomy in ESCC. The immunohistochemical evaluation of NQO1 expression has a potential to predict the treatment response and prognosis in ESCC patients.

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Disclosure of conflict of interest

None.

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