Correlation between TXNRD1/HO-1 Expression and the Histological Response to Neoadjuvant Chemoradiation Therapy in patients with Esophageal Squamous Cell Carcinoma

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Research article

Keywords: Esophageal squamous cell carcinoma, Immunohistochemistry, Nrf2, TXNRD1, HO-1, Reactive oxygen species, Chemoradiation therapy, Chemoresistance, Radioresistance

Posted Date: October 14th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-89481/v1>

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Version of Record: A version of this preprint was published at Esophagus on January 8th, 2022. See the published version at <https://doi.org/10.1007/s10388-021-00904-3>.

Abstract

Background

Nrf2 signaling plays a pivotal role in antioxidant response, and its expression has been reported to increase in various human malignancies, including esophageal squamous cell carcinoma (ESCC). This also leads to resistance against chemotherapy and radiotherapy in patients. Thioredoxin reductase 1 (TXNRD1) and heme oxygenase-1 (HO-1) are proteins involved in this pathway that play key roles in antioxidant responses. However, the correlation between the expression of these two proteins and the therapeutic response to neoadjuvant chemoradiation therapy (NACRT), and the changes before and after chemoradiation therapy in patients with ESCC, remain unknown.

Methods

The proteins involved in the Nrf2 signaling pathway were immunolocalized in carcinoma cells in patients with ESCC undergoing NACRT with 5-fluorouracil and cisplatin followed by esophagectomy. The 8-OHdG levels were used to determine ROS levels in individual carcinoma cells. Fifty-two pre-operative endoscopic biopsy and fifty post-operative resected specimens were available for this study. Among these, 39 specimens were available for comparison of the results before and after NACRT. The changes in immunoreactivity before and after NACRT (Δ) were assessed.

Results

Significant histological resistance to NACRT was observed in patients with high levels of Nrf2, TXNRD1, and HO-1 expression. Among pre-therapeutic biopsy specimens, the tumor reduction effect was significantly attenuated in those with high levels of Nrf2, TXNRD1, and HO-1 expression. TXNRD1 Δ and HO-1 Δ were both significantly higher, while 8-OHdG Δ was significantly lower in the ineffective (poor response) groups. In resected specimens, the overall survival was significantly lower in groups with high Nrf2, TXNRD1, HO-1, and HO-1 Δ values. Disease-free survival was significantly lower in the groups with high expression of Nrf2, TXNRD1, HO-1, and Ki-67 and large values of HO-1 Δ , and Ki-67 Δ .

Conclusions

The results of this study indicate that high Nrf2, TXNRD1, and HO-1 expression in pre-therapeutic biopsy specimens could predict the histological response to NACRT, and their status in surgically resected specimens could predict clinical outcomes in patients with ESCC.

Background

Esophageal cancer is the eighth most common human malignancy and the sixth most common cause of cancer deaths worldwide [1]. Esophageal squamous cell carcinoma (ESCC) constitutes the majority of esophageal cancer cases in Japan and other Asian countries. The standard therapeutic strategy for patients with locally advanced ESCC that is currently in use is neoadjuvant chemotherapy (NAC) followed

by radical resection with extensive lymph node dissection [2]. However, therapeutic strategies for patients who develop resistance against NAC are yet to be established [3, 4]. Clinical trials of neoadjuvant chemoradiation therapy (NACRT) using 5-fluorouracil/cisplatin with concurrent irradiation are currently underway for patients with locally advanced ESCC [5–7], which may provide novel insights into the therapeutic algorithm for patients.

Reactive oxygen species (ROS) are commonly known to mediate the damage caused to cancer cells during chemotherapy or irradiation therapy. Approximately two-thirds of the cytotoxic effects of X-ray irradiation were reported to be caused by ROS generated via the ionization of water molecules [8]. Cisplatin was also observed to induce apoptosis by ROS generation [9]. Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a transcription factor involved in the regulation of antioxidant protein expression in cells [10]. Nrf2 expression was reported to be stimulated in ESCC, which resulted in the development of therapeutic resistance to chemotherapy and radiotherapy in patients [11]. Nrf2 also promotes the expression of antioxidant enzymes, including thioredoxin reductase 1 (TXNRD1) [12, 13] and heme oxygenase-1 (HO-1) [14], which consequently increases cellular resistance against oxidative stress. TXNRD1 is one of the key enzymes that contribute to the defense of cancer cells against oxidative stress [15, 16], as well as to cell proliferation and viability [17]. Thioredoxin 1 (TXN1), a target of TXNRD1, directly binds to PTEN, a well-known tumor suppressor protein that deactivates the PI3K/Akt pathway [18]. The binding of TXN1 was observed to inhibit the phosphatase activity of PTEN, promote cell proliferation/tumor growth, and inhibit apoptosis [18]. HO-1 is also a target of Nrf2, and its involvement in cell proliferation and development in cancer has been studied extensively [19–21]. In addition, the cytoprotective function of HO-1 was reported to be mediated by its metabolites [22, 23]. Deletions in *PTEN* result in HO-1 overexpression, and subsequently lead to the formation of highly aggressive tumors, followed by metastasis owing to accelerated angiogenesis [24, 25]. HO-1 overexpression has also been reported to protect cancer cells from the antitumor effect of cisplatin [26–28].

Therefore, the upregulation of antioxidant enzymes in the Nrf2 signaling pathway in cancer cells is reasonably postulated to lead to the development of therapeutic resistance against chemoradiation therapy (CRT). In addition, low Nrf2 expression in pre-NACRT biopsy specimens was also reported to be correlated with a favorable histological response to NACRT in patients with ESCC [29, 30]. However, the correlation between the TXNRD1/HO-1 expression status and histological response in ESCC to NACRT remains virtually undetermined. Additionally, the prognostic significance of the proteins involved in these antioxidant pathways in human malignancies is yet to be established.

In the present study, we aimed to determine the following in patients with ESCC: (1) the expression status of antioxidant proteins and its correlation with the histological response to NACRT, (2) correlation between the expression status of the antioxidant proteins and clinical outcome/prognostic factors in corresponding patients with ESCC who underwent NACRT, and (3) the changes in antioxidant protein expression in pre- and post-NACRT specimens.

Methods

Patients and tumor specimens

Sixty-nine patients were diagnosed with ESCC between 2011 and 2015 at Tohoku University Hospital, Sendai, Japan, and underwent NACRT followed by esophagectomy with regional lymph node dissection. The pathological characteristics of the resected specimens, such as depth, lymph node metastasis, differentiation, and lymphovascular invasion, were independently reviewed by two pathologists (FF and HS). The histological criteria for therapeutic response to NACRT were tentatively determined as follows: Grade 0, ineffective: neither coagulative necrosis nor cellular/structural changes detected in the lesion; Grade 1: coagulative necrosis or histological disappearance of tumor cells in no more than two-thirds of the entire lesion; Grade 2: coagulative necrosis or disappearance of tumor cells in more than two-thirds of the entire lesion, though histologically viable tumor cells were still identified; Grade 3: entire lesion displaying coagulative necrosis and/or replacement by fibrosis, and no viable tumor cells detected histologically [31]. In this study, a Grade 0 or 1 response was interpreted as “ineffective,” whereas a Grade 2 or 3 response was considered “effective” [29, 30]. Among the 69 cases, surgically resected specimens were available for 50 cases for immunohistochemical analysis, in which residual primary lesions were detected histopathologically. Pre-therapeutic biopsy specimens were also available for evaluation in 52 cases, and viable tumor cells were detected in the surgically resected specimens in 42 out of 52 cases, while the primary lesions completely disappeared in 10 cases, as observed in the detailed histopathological evaluation. No residual viable carcinoma cells were identified in 3 out of 42 cases in the tissue slides for immunohistochemistry. Therefore, 39 cases were available for comparison of immunoreactivity before and after NACRT.

The therapeutic effects of NACRT were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 [32].

TNM staging was performed according to the guidelines of the eighth edition of the American Joint Committee on Cancer/Union for International Cancer Control TNM staging system for esophageal carcinoma [33]. The overall survival (OS) and disease-free survival (DFS) rates in patients were determined based on the time duration from surgery until death and until recurrence, respectively, or based on the last censor.

NACRT and esophagectomy

Chemotherapy was administered in conjunction with continuous intravenous infusion of 5-fluorouracil (400 mg/m²/day) for over 24 h on days 1–5 and 8–12, and infusion of cisplatin (40 mg/m²) for 2 h on days 1 and 8. Concurrent radiotherapy (total 30 Gy in 15 fractions over a period of 3 weeks) was performed. The irradiation field consisted of a long T-shaped space including the supraclavicular and the mediastinal lymph nodes in cases of cervical or upper and middle thoracic tumors, with additional irradiation administered to perigastric lymph nodes in patients with lower thoracic tumors. Thoracoscopic esophageal subtotal excision, gastric tube reconstruction by hand-assisted laparoscopic technique or

open laparotomy, and cervical esophagogastric anastomosis were performed subsequently with regional lymph node dissection.

Immunohistochemistry

The tissue specimens were fixed using 10% buffered formalin and embedded in paraffin to form tissue blocks. Information regarding immunohistochemical procedures, such as primary antibodies, antigen retrieval methods, and buffers used is summarized in Supplementary Table 1 (Additional file 1). 8-hydroxydeoxyguanosine (8-OHdG), a product of oxidative damage in DNA caused by hydroxyl radicals, was used to evaluate the ROS levels in the tumor cells [34, 35].

Briefly, 4 µm-thick sections were mounted on clean glass slides and deparaffinized by treating with xylene and ethanol. The sections were then heated for antigen retrieval, as described in Supplementary Table 1. To block nonspecific binding of the antibodies used, the treated sections were incubated in 10% normal rabbit serum (Histofine Kit; Nichirei Bioscience, Tokyo, Japan) for antibodies against Nrf2 and 8-OHdG, or 10% normal goat serum (Histofine Kit; Nichirei Bioscience) for antibodies against HO-1 at 24°C for 30 min. Subsequently, the sections were treated with primary antibodies overnight at 4 °C. For 8-OHdG immunohistochemistry, hydrogen peroxide processing was performed after overnight treatment with primary antibodies to prevent reaction between the primary antibodies and 8-OHdG produced by the direct reaction with hydroxyl radicals generated from hydrogen peroxide and normal dG present in the tissues studied. Endogenous peroxidase activity was inhibited by immersing the sections in 0.3% hydrogen peroxidase for 30 min at 24°C. The sections were subsequently treated with biotinylated rabbit anti-mouse or goat anti-rabbit immunoglobulin (Histofine Kit; Nichirei Bioscience) and peroxidase-labeled streptavidin (Histofine Kit; Nichirei Bioscience) at 24°C for 30 min. For TXNRD1 detection, the sections were subsequently treated with polymeric horseradish peroxidase immunoglobulin G reagent (EnVision FLEX Kit FLEX/HRP, Agilent Technologies, Santa Clara, CA, USA) for 10 min. The antigen-antibody complexes were then visualized using 1.0 mmol/L 3,3-diaminobenzidine in 50 mol/L Tris-HCl buffer (pH 7.6) with 0.006% H₂O₂, following which the sections were counterstained with hematoxylin.

Immunohistochemistry of Ki-67 was performed according to the manufacturer's instructions (EnVision FLEX Kit High pH, Agilent Technologies).

Each stained section was independently evaluated at the hot spots (×200 magnification) by two authors (RA and FF) who did not have prior knowledge of the clinicopathological variables of the patients. Nrf2 and Ki-67 immunoreactivities were evaluated in the nuclei of tumor cells, while TXNRD1, HO-1, and 8-OHdG immunoreactivities were evaluated in the cytoplasm of tumor cells. The immunoreactivities of Nrf2, TXNRD1, HO-1, and 8-OHdG were assessed semi-quantitatively assessed using modified H-score, by calculating the percentage of immunostained tumor cells multiplied by the relative immunointensity (0, negative; 1, weak; 2, moderate; 3, marked) ranging from 0 to 300 [36, 37]. Ki-67 immunoreactivity was scored by counting the proportion of positively-stained cells [38]. We tentatively determined the optimal cutoff values for the histological response of the patients using the receiver operating characteristic curve

method [36]. Thresholds were independently established for pre-NACRT biopsy specimens and post-NACRT surgically resected specimens as follows: thresholds for pre-NACRT biopsy specimens; Nrf2: 82%, TXNRD1: 82%, HO-1: 104%, 8-OHdG: 78%, and Ki-67: 39%; thresholds for post-NACRT resected specimens; Nrf2: 150%, TXNRD1: 86%, HO-1: 137%, 8-OHdG: 30%, and Ki-67: 40%. Specimens with an H-score or labeling index for Ki-67 below the cutoff value were tentatively classified as low expression specimens, while those with scores greater than the cutoff value were considered as high expression specimens in this study. We also calculated the differences in the H-score and Ki-67 labeling index (Δ : post-NACRT–pre-NACRT values) for 39 specimens, which were available for comparison of immunoreactivity before and after NACRT. Values with Δ above and below the median value were tentatively classified under high Δ and low Δ groups, respectively. Reactive stromal cells, lymphocytes, and/or non-neoplastic epithelial cells were used as positive internal controls [39].

Statistical analysis

JMP® Pro version 14.2.0 (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses. Continuous data were analyzed using Student's *t*-test or the Mann–Whitney U test. The relationship and correlation between two variables were determined using Pearson's chi-square test, Fisher's exact test, Mann–Whitney U test, or Wilcoxon test, as appropriate. OS and DFS curves were constructed according to the Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazards model was used for both univariate and multivariate analyses. $P < 0.05$ was considered statistically significant.

Results

Clinicopathological features of patients with ESCC

The clinicopathological characteristics of 69 patients are summarized in Supplementary Table 2 (Additional file 2). Among these, 30 cases were tentatively classified as ineffective (Grade 0 or 1), while the other 39 cases that exhibited a Grade 2 or 3 response to NACRT in the pathological analysis of the resected specimens were classified as effective. There were significant differences in RECIST, micro lymph-vascular invasion, and pathological (p) T/N grade between the ineffective and effective groups.

Expression status of antioxidant proteins and its correlation with the histological response to NACRT

Representative histopathological findings of Nrf2, TXNRD1, HO-1, 8-OHdG, and Ki-67 are presented in Figure 1. The proportions of resected specimen with high levels of Nrf2, TXNRD1, HO-1, 8-OHdG, and Ki-67 were 64.0%, 62.0%, 64.0%, 48.0%, and 68.0% of the cases examined, respectively (Table 1).

Therapeutic resistance to NACRT was significant in the groups with high expression of Nrf2 ($P = 0.004$), TXNRD1 ($P < 0.001$), HO-1 ($P < 0.001$), and Ki-67 ($P = 0.026$), and low levels of 8-OHdG ($P = 0.011$)

(Table 1). In addition, a statistically significant positive correlation was detected between TXNRD1 and HO-1 levels and pT (TXNRD1: $P = 0.029$, HO-1: $P = 0.023$) and pN (TXNRD1: $P = 0.018$, HO-1: $P = 0.026$) (Table 1).

Among the biopsy specimens examined, 75.0%, 57.7%, 82.7%, 55.8%, and 28.8% specimens exhibited high expression of Nrf2, TXNRD1, HO-1, 8-OHdG, and Ki-67, respectively (Table 2). An ineffective response to NACRT was observed in endoscopic biopsy specimens with high Nrf2 ($P = 0.01$), TXNRD1 ($P = 0.01$), and HO-1 immunoreactivity ($P = 0.025$) prior to therapy (Table 2). A significant positive correlation was also observed between Nrf2 expression and pT ($P = 0.023$), as well as between HO-1 expression and microlymphatic invasion ($P = 0.023$) (Table 2).

Correlation between the expression status of antioxidant proteins and clinical outcomes/prognostic factors in patients

The 5-year OS rate was significantly lower in the high Nrf2 ($P = 0.019$), TXNRD1 ($P = 0.017$), and HO-1 ($P = 0.005$) expression groups of patients with ESCC determined using surgically resected specimens. The 5-year DFS rate was also significantly lower in the high Nrf2 ($P = 0.04$), TXNRD1 ($P = 0.046$), HO-1 ($P = 0.008$), and Ki-67 ($P = 0.021$) expression groups (Figure 2). Univariate analysis revealed that the OS rate was significantly associated with pT ($P = 0.001$), lymphatic invasion ($P = 0.013$), high Nrf2 expression ($P = 0.024$), high TXNRD1 expression ($P = 0.022$), and high HO-1 expression ($P = 0.008$) (Table 3). However, multivariate analysis showed that pT was an independent prognostic factor among the variables examined ($P = 0.008$) (Table 3). The factors significantly associated with DFS were pT ($P = 0.005$), lymphatic invasion ($P = 0.015$), vascular invasion ($P = 0.014$), high HO-1 expression ($P = 0.013$), and high Ki-67 expression ($P = 0.031$) (Table 3). Multivariate analysis revealed that pT was the only independent prognostic factor ($P = 0.045$) (Table 3).

The 5-year OS rates was significantly lower in the high Nrf2 ($P = 0.007$) and TXNRD1 ($P = 0.025$) expression groups determined using pre-NACRT biopsy specimens. In contrast, no significant differences were detected in the 5-year DFS rates of the patients examined (Figure 3).

Changes in the immunoreactivities of antioxidant proteins in pre- and post-NACRT specimens

The immunoreactivities of Nrf2 ($P < 0.001$) and Ki-67 ($P = 0.002$) were significantly higher, while that of 8-OHdG ($P < 0.001$) was significantly lower in surgically resected specimens compared to that in pre-NACRT endoscopic biopsy specimens (Figure 4). In contrast, no significant differences were observed between the H-score values of TXNRD1 and HO-1 in the pre- and post-NACRT specimens examined. TXNRD1 Δ ($P = 0.048$) and HO-1 Δ ($P = 0.021$) were significantly higher, while 8-OHdG Δ ($P = 0.048$) was significantly

lower in the NACRT-ineffective groups than in the NACRT-effective groups (Figure 5). The 5-year OS rate of the patients was significantly lower in the high HO-1 Δ group ($P = 0.025$). The 5-year DFS rate was significantly lower in the high HO-1 Δ ($P = 0.011$) and high Ki-67 expression ($P = 0.024$) groups (Figure 6). Univariate analysis revealed that OS was significantly associated with pT ($P = 0.005$), lymphatic invasion ($P = 0.013$), and high HO-1 expression ($P = 0.032$) (Table 4), and DFS was significantly associated with pT ($P = 0.008$), lymphatic invasion ($P = 0.021$), high HO-1 expression ($P = 0.017$), and high Ki-67 expression ($P = 0.034$) (Table 4). However, multivariate analysis revealed that only pT was an independent prognostic factor among the variables examined in this study ($P = 0.044$) (Table 4).

Discussion

Significant histological therapeutic resistance to NACRT was detected in groups with high expression of Nrf2, TXNRD1, and HO-1 among the ESCC cases examined in this study. In the pre-therapeutic biopsy specimens, the tumor reduction effect was significantly attenuated in the high Nrf2, TXNRD1, and HO-1 expression groups. TXNRD1 Δ and HO-1 Δ were significantly higher, while 8-OHdG Δ was significantly lower in the NACRT-ineffective groups. In surgical specimens, the OS was significantly lower in the high Nrf2, TXNRD1, and HO-1 expression groups. DFS was also significantly lower in the high Nrf2, TXNRD1, HO-1, and Ki-67 expression groups.

The results indicate that the therapeutic efficacy of NACRT could be predicted by examining the expression status of antioxidant proteins in pre-therapeutic endoscopic biopsy specimens. In addition, the results of recent studies indicate that the elimination of ROS is related to the development of radioresistance in ESCC [40-42]. Generally, ROS generation is considered an essential mediator of the cytotoxic effects of cisplatin [9]. A significant correlation between unfavorable histological responses to NACRT and high Nrf2, TXNRD1, and HO-1 expression in pre-NACRT endoscopic biopsy specimens indicated that the cytotoxic effects of NACRT could be impaired significantly in this group. Kawasaki et al. reported the possibility of the conversion of non-responder patients to responders by Nrf2 knockdown before subjecting patients with ESCC with high-Nrf2 expression to CRT, which may lead to a more favorable prognosis [29]. In contrast, targeting the transcription factor Nrf2 was considered significantly challenging in clinical settings [43]. To include curative resection as a viable treatment option for ESCC, surgical resection without NAC or NACRT might be a potential treatment strategy for patients with ESCC who exhibit high Nrf2, TXNRD1, and HO-1 expression, considering the highly aggressive malignant tendencies of tumors and the probable ineffectiveness of neoadjuvant therapy [17, 18, 24, 25, 44]. The expression of antioxidant proteins in the endoscopic biopsy specimens could also predict the effects of definitive CRT (dCRT) and NACRT, which could help avoid esophagectomy, a potentially invasive procedure, in patients with ESCC. In previously reported studies on dCRT, high Nrf2 expression status in biopsy specimens was reported to be correlated with the development of therapeutic resistance to dCRT [37, 45]. Therefore, patients with ESCC exhibiting low expression of antioxidant proteins in the Nrf2 signaling pathway in pre-therapeutic endoscopic biopsy specimens are considered reasonably suitable candidates for dCRT. In addition, results of several studies have also suggested that Nrf2 and TXNRD1 are useful markers for predicting the efficacy of chemotherapy or irradiation therapy in other neoplasms,

such as non-small cell lung cancer or ovarian cancer [46, 47]. However, further investigation is necessary for confirming these propositions.

The status of Nrf2, TXNRD1, and HO-1 expression in the surgically resected specimens correlated significantly with the 5-year OS and DFS rates of patients with ESCC participating in this study. In addition, increased HO-1 Δ correlated significantly with unfavorable 5-year OS and DFS rates, as well as with a high Ki-67 Δ value and DFS rate in the patients examined. HO-1 is well known to be associated with therapeutic resistance to cisplatin-based therapy [26-28]. Therefore, increased HO-1 Δ could predict the clinical outcomes in patients undergoing cisplatin-based chemotherapy or CRT. However, none of these markers were found to be independent predictive factors, as shown in the multivariate analysis in this study. This discrepancy is considered to result from the intrinsic correlation among the multiple factors examined. For instance, the upregulation of the Nrf2 pathway is commonly known to enhance antioxidant response and cell proliferation, which would result in increased expression of TXNRD1, HO-1, and Ki-67 [12, 13, 19-21, 38]. In addition, TXNRD1 and HO-1 expression were significantly correlated with established clinicopathological factors such as pT and pN in the present study.

The significant upregulation of the Nrf2 pathway has also been reported to contribute to increased tumor cell proliferation and [angiogenesis](#) in cancer [18, 24, 25, 38], which possibly led to a significantly lower DFS in high Nrf2, TXNRD1, HO-1, and Ki-67 expression groups in this study. Therefore, the abundance of these proteins could help predict the recurrence of ESCC in patients. In addition, upregulation of the Nrf2 pathway was reported to reduce the clinical benefits obtained from cisplatin-based adjuvant chemotherapy in lung squamous cell carcinoma [48]. As in lung carcinoma, the increased expression of Nrf2, TXNRD1, and HO-1 could potentially suppress the effects of NACRT in patients with ESCC; however, further clinical studies are warranted.

Significant differences were observed in the expression status of Nrf2 and Ki-67 and the levels of 8-OHdG between endoscopic biopsy and surgical specimens. In addition, TXNRD1 Δ , HO-1 Δ , and 8-OHdG Δ were significantly associated with the histological therapeutic efficacy in the cases examined in this study. The significant increase in Nrf2 expression in ESCC was considered to be attributable to the antioxidant response, which is consistent with results of a previous study by Kawasaki et al. [29]. In addition, Ki-67 was also reported to be upregulated in specimens with increased Nrf2 expression, which is indicative of high levels of cell proliferation [38]. However, the expression of TXNRD1 and HO-1 did not increase significantly in response to NACRT, possibly because the expression levels of TXNRD1 and HO-1 were high even before NACRT was administered. Increased Nrf2 expression owing to genetic alterations in the *NRF2* (known as *NFE2L2*) have been reported [49]. Therefore, high levels of TXNRD1 and HO-1 expression could be associated with these genetic abnormalities upstream of the Nrf2 pathway. Furthermore, the activation of *Kras*, *Braf*, and *Myc* oncogene activation and *PTEN* anti-oncogene disruption, which are detected frequently in ESCC, could also lead to the upregulation of the transcription of *Nrf2* [50-52]. Therefore, TXNRD1 and HO-1 overexpression could be induced because of diverse Nrf2 upregulation patterns in carcinogenesis. A significant positive correlation was also observed between Nrf2 and TXNRD1 expression in pre-NACRT endoscopic biopsy specimens in the present study (Additional file 3). In

addition, TXNRD1 expression was reported to be inhibited efficiently by both cisplatin and its monohydrated complex [53] therefore, the upregulation of TXNRD1 expression by Nrf2 can be opposed. An alteration in HO-1 expression in response to chemotherapy or irradiation therapy has not been reported yet; however, the upregulation of HO-1 as well as TXNRD1 expression by Nrf2 could be countered by treatment with cisplatin or by irradiation. The significant difference in TXNRD1 Δ , HO-1 Δ , and 8-OHdG Δ values between the ineffective and effective groups in the present study also suggested that the ineffective group elicited a greater antioxidant response. However, no significant differences were observed in Nrf2 Δ values between the two groups. These results indicate that patients with ESCC in the NACRT-ineffective group could exhibit an antioxidant response that involves the selective and stronger upregulation of TXNRD1 and HO-1 expression, which contributes to ROS detoxification by Nrf2 pathway proteins [15, 16, 54]. In addition, in the effective group, TXNRD1 expression was significantly suppressed upon cisplatin treatment, which could occur independent of the Nrf2 pathway. The difference in TXNRD1 expression between the ineffective and effective groups suggested that the suppression mechanism undertaken by cisplatin could vary.

It is also important to note the limitations of this study. First, ESCC is characterized by intertumoral heterogeneity; therefore, the sites of endoscopic biopsy could have considerably influenced the results of the expression of antioxidant proteins in these specimens. Second, as carcinoma cells completely disappeared after NACRT in some cases, the changes in protein expression in these cells remained unevaluated. Third, besides pT, no other factor was determined to be an independent prognostic factor, possibly owing to the intrinsic correlation among the factors studied. Further investigations are necessary to elucidate the clinicopathological significance of the results of our present study.

Conclusions

The therapeutic efficacy of NACRT, and the clinical outcomes in patients with ESCC could be predicted by examining the status of Nrf2, TXNRD1, and HO-1 expression in carcinoma cells in pre-therapeutic endoscopic biopsy and surgically resected specimens. Further investigation of the findings could lead to the establishment of potential prognostic factors in the treatment of patients with ESCC.

Abbreviations

5-FU: 5-Fluorouracil

8-OHdG: 8-hydroxy-2'-deoxyguanosine

CRT: Chemoradiation therapy

dCRT: Definitive chemoradiation therapy

DFS: Disease-free survival

ESCC: Esophageal squamous cell carcinoma

HO-1: Heme oxygenase-1

NAC: Neoadjuvant chemotherapy

NACRT: Neoadjuvant chemoradiation therapy

Nrf2: Nuclear factor-erythroid 2-related factor 2

OS: Overall survival

RECIST: Response Evaluation Criteria in Solid Tumors

ROS: Reactive oxygen species

TXN1: Thioredoxin 1

TXNRD1: Thioredoxin reductase 1

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Tohoku University School of Medicine (Accession No. 2020-1-87), and informed consent was obtained from all participants prior to surgery. All participants signed forms providing informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contribution

RA and FF performed the immunohistochemical examinations. RA, FF, and HI performed the statistical analyses. RA, FF, HI, JT, TY, YG, SU, TF, HO, KT, CS, YT, TK, and HS conceived the study, participated in its design and coordination, and helped draft the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

We appreciate the technical assistance provided by Ms. Yayoi Aoyama (Department of Pathology, Tohoku University Hospital, Sendai, Japan) and Ms. Yasuko Furukawa (Department of Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan). We would like to thank Editage (www.editage.com) for English language editing.

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Tables

Due to technical limitations, table1, table2, table3 and table4 are only available as a download in the Supplemental Files section.

Figures

Figure 1

Representative illustration of immunohistochemical features. (a) Low Nrf2 expression, (b) high Nrf2 expression; representative specimen depicting immunoreactivity in the nuclei of carcinoma cells. (c) Low TXNRD1 expression, (d) high TXNRD1 expression; representative specimen depicting immunoreactivity in the cytoplasm of carcinoma cells. (e) Low HO-1 expression, (f) high HO-1 expression; representative specimen depicting immunoreactivity in the cytoplasm of carcinoma cells. (g) Low 8-OHdG levels, (h) high 8-OHdG levels; representative specimen depicting immunoreactivity in the cytoplasm of carcinoma cells. (i) Low Ki-67 expression, (j) high Ki-67; representative specimen depicting immunoreactivity in the nuclei of carcinoma cells.

Figure 2

Kaplan-Meier estimates of OS and DFS based on post-NACRT expression status of biomarkers. The 5-year OS was significantly lower in post-NACRT specimens with high (a) Nrf2, (b) TXNRD1, and (c) HO-1 expression. The 5-year DFS was significantly lower in post-NACRT specimens with high (f) Nrf2, (g) TXNRD1, (h) HO-1, and (j) Ki-67 expression. OS, overall survival; DFS, disease-free survival; NACRT, neoadjuvant chemoradiation therapy.

Figure 3

Kaplan-Meier estimates of OS and DFS based on pre-NACRT expression status of biomarkers. The 5-year OS was significantly lower in the pre-NACRT specimens with high (a) Nrf2 and (b) TXNRD1 expression. No significance was detected in the 5-year DFS. OS, overall survival; DFS, disease-free survival; NACRT, neoadjuvant chemoradiation therapy.

Figure 4

H-scores and Ki-67-positive rate in pre- and post-NACRT specimens. The difference in biomarker expression between pre- and post-NACRT specimens was evaluated using the Wilcoxon test. Significant differences were observed in (a) Nrf2 ($P < 0.001$), (d) 8-OHdG ($P < 0.001$), and (e) Ki-67 ($P = 0.002$) levels. NACRT, neoadjuvant chemoradiation therapy.

Figure 5

Correlation between the difference in expression (Δ) and histological response to NACRT. Correlation was determined using the Wilcoxon test. A significant difference was observed for (a) TXNRD1 Δ ($P = 0.048$), (c) HO-1 Δ ($P = 0.021$), and (d) 8-OHdG Δ ($P = 0.048$) between the NACRT-effective and -ineffective groups. NACRT, neoadjuvant chemoradiation therapy.

Figure 6

Kaplan-Meier estimates of OS and DFS based on Δ value. The value " Δ " represents the difference in biomarker expression between pre- and post-NACRT specimens. The 5-year OS was significantly lower in the high (a) HO-1 Δ group. The 5-year DFS was significantly low in the high (b) HO-1 Δ , and (d) Ki-67 Δ groups. OS, overall survival; DFS, disease-free survival; NACRT, neoadjuvant chemoradiation therapy.

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