Acknowledgements

The present study was supported by a grant in aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant no. 23791553).

References

1. Sugimachi K, Matsuura H, Kai H, Kanematsu T, Inokuchi K and Jingu K: Prognostic factors of esophageal carcinoma: univariate and multivariate analyses. J Surg Oncol 31: 108-112, 1986.
2. Natsugoe S, Matsumoto M, Okumura H, *et al*: Prognostic factors in patients with submucosal esophageal cancer. J Gastrointest Surg 8: 631-635, 2004.
3. Richardson HE, Stueland CS, Thomas J, Russell P and Reed SI: Human cDNAs encoding homologs of the small $p34^{Cdc28/Cdc2}$-associated protein of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Genes Dev 4: 1332-1344, 1990.
4. Bourne Y, Watson MH, Hickey MJ, *et al*: Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1. Cell 84: 863-874, 1996.
5. Ganoth D, Bornstein G, Ko TK, *et al*: The cell-cycle regulatory protein Cks1 is required for SCF^{Skp2}-mediated ubiquitinylation of p27. Nat Cell Biol 3: 321-324, 2001.
6. Spruck CH, de Miguel MP, Smith AP, *et al*: Requirement of Cks2 for the first metaphase/anaphase transition of mammalian meiosis. Science 300: 647-650, 2003.
7. Spruck C, Strohmaier H, Watson M, *et al*: A CDK-independent function of mammalian Cks1: targeting of SCF^{Skp2} to the CDK inhibitor $p27^{Kip1}$. Mol Cell 7: 639-650, 2001.
8. Tedesco D, Lukas J and Reed SI: The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF^{Skp2}. Genes Dev 16: 2946-2957, 2002.
9. Masuda TA, Inoue H, Sonoda H, *et al*: Clinical and biological significance of *S-phase kinase-associated protein 2 (Skp2)* gene expression in gastric carcinoma: modulation of malignant phenotype by Skp2 overexpression, possibly via p27 proteolysis. Cancer Res 62: 3819-3825, 2002.
10. Welcker M, Orian A, Jin J, *et al*: The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. Proc Natl Acad Sci USA 101: 9085-9090, 2004.
11. Inui N, Kitagawa K, Miwa S, *et al*: High expression of Cks1 in human non-small cell lung carcinomas. Biochem Biophys Res Commun 303: 978-984, 2003.
12. Wong YF, Cheung TH, Tsao GS, *et al*: Genome-wide gene expression profiling of cervical cancer in Hong Kong women by oligonucleotide microarray. Int J Cancer 118: 2461-2469, 2006.
13. Lan Y, Zhang Y, Wang J, Lin C, Ittmann MM and Wang F: Aberrant expression of Cks1 and Cks2 contributes to prostate tumorigenesis by promoting proliferation and inhibiting programmed cell death. Int J Cancer 123: 543-551, 2008.
14. Wang XC, Tian J, Tian LL, *et al*: Role of Cks1 amplification and overexpression in breast cancer. Biochem Biophys Res Commun 379: 1107-1113, 2009.
15. Sobin LH and Fleming ID: TNM Classification of Malignant Tumors, fifth edition (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. Cancer 80: 1803-1804, 1997.
16. Arigami T, Natsugoe S, Uenosono Y, *et al*: Lymphatic invasion using D2-40 monoclonal antibody and its relationship to lymph node micrometastasis in pN0 gastric cancer. Br J Cancer 93: 688-693, 2005.
17. Ogawa K, Utsunomiya T, Mimori K, *et al*: Clinical significance of human kallikrein gene 6 messenger RNA expression in colorectal cancer. Clin Cancer Res 11: 2889-2893, 2005.
18. Uchikado Y, Inoue H, Haraguchi N, *et al*: Gene expression profiling of lymph node metastasis by oligomicroarray analysis using laser microdissection in esophageal squamous cell carcinoma. Int J Oncol 29: 1337-1347, 2006.
19. Kita Y, Mimori K, Iwatsuki M, *et al*: *STC2*: a predictive marker for lymph node metastasis in esophageal squamous-cell carcinoma. Ann Surg Oncol 18: 261-272, 2011.
20. Tanaka F, Matsuzaki S, Mimori K, Kita Y, Inoue H and Mori M: Clinicopathological and biological significance of *CDC28 protein kinase regulatory subunit 2* overexpression in human gastric cancer. Int J Oncol 39: 361-372, 2011.
21. Li M, Lin YM, Hasegawa S, *et al*: Genes associated with liver metastasis of colon cancer, identified by genome-wide cDNA microarray. Int J Oncol 24: 305-312, 2004.
22. Kawakami K, Enokida H, Tachiwada T, *et al*: Identification of differentially expressed genes in human bladder cancer through genome-wide gene expression profiling. Oncol Rep 16: 521-531, 2006.
23. Chen R, Feng C and Xu Y: Cyclin-dependent kinase-associated protein Cks2 is associated with bladder cancer progression. J Int Med Res 39: 533-540, 2011.
24. Wang JJ, Fang ZX, Ye HM, *et al*: Clinical significance of overexpressed cyclin-dependent kinase subunits 1 and 2 in esophageal carcinoma. Dis Esophagus 26: 729-736, 2013.
25. de Wit NJ, Rijntjes J, Diepstra JH, *et al*: Analysis of differential gene expression in human melanocytic tumour lesions by custom made oligonucleotide arrays. Br J Cancer 92: 2249-2261, 2005.
26. Rickman DS, Bobek MP, Misek DE, *et al*: Distinctive molecular profiles of high-grade and low-grade gliomas based on oligonucleotide microarray analysis. Cancer Res 61: 6885-6891, 2001.
27. Scrideli CA, Carlotti CG Jr, Okamoto OK, *et al*: Gene expression profile analysis of primary glioblastomas and non-neoplastic brain tissue: identification of potential target genes by oligonucleotide microarray and real-time quantitative PCR. J Neurooncol 88: 281-291, 2008.
28. Baylin SB and Jones PA: A decade of exploring the cancer epigenome - biological and translational implications. Nat Rev Cancer 11: 726-734, 2011.
29. He L and Hannon GJ: MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5: 522-531, 2004.
30. Fabbri M, Calore F, Paone A, Galli R and Calin GA: Epigenetic regulation of miRNAs in cancer. Adv Exp Med Biol 754: 137-148, 2013.
31. Lv M, Zhang X, Li M, *et al*: miR-26a and its target CKS2 modulate cell growth and tumorigenesis of papillary thyroid carcinoma. PLoS One 8: e67591, 2013.
32. Liberal V, Martinsson-Ahlzen HS, Liberal J, *et al*: Cyclin-dependent kinase subunit (Cks) 1 or Cks2 overexpression overrides the DNA damage response barrier triggered by activated oncoproteins. Proc Natl Acad Sci USA 109: 2754-2759, 2012.

Ann Surg Oncol (2014) 21:2347–2352
DOI 10.1245/s10434-014-3600-2

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – THORACIC ONCOLOGY

Nrf2 is Useful for Predicting the Effect of Chemoradiation Therapy on Esophageal Squamous Cell Carcinoma

Yota Kawasaki, MD, Hiroshi Okumura, MD, Yasuto Uchikado, MD, Yoshiaki Kita, MD, Ken Sasaki, MD, Tetsuhiro Owaki, MD, Sumiya Ishigami, MD, and Shoji Natsugoe, MD

Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical Sciences, Kagoshima University, Kagoshima, Japan

ABSTRACT

Background. The transcription factor NF-E2-related factor 2 (Nrf2) was originally identified to be a critical regulator of intracellular antioxidants and phase II detoxification enzymes. Recent studies have shown that high Nrf2 expression gives cancer cells an advantage for survival from anticancer chemotherapy and radiation therapy. The aims of this retrospective study were to examine the expression of Nrf2 in biopsy specimens of esophageal squamous cell carcinoma (ESCC) and to evaluate whether such expression is useful for predicting the response to chemoradiation therapy (CRT).

Methods. A total of 46 patients with ESCC who received curative surgery after CRT from 1997 to 2011 were enrolled in the current study. Nrf2 expression in the biopsy specimens before CRT was examined immunohistochemically using anti-Nrf2 antibody. The correlations between Nrf2 expression and clinical factors and histological and clinical response to CRT were analyzed.

Results. The rate of Nrf2-positive expression was 39 %. Both clinically and histologically, significant correlations were found between positive Nrf2 expression and unfavorable response to CRT. Furthermore, Nrf2 was significantly correlated with clinical lymph node metastases and patients' postoperative outcomes. Multivariate analysis showed that Nrf2 expression status was an independent prognostic factor.

Conclusions. Nrf2 expression was found to be closely related to the effect of CRT and could predict the CRT outcome in patients with ESCC.

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive tumors of the gastrointestinal tract. Since postoperative relapse often occurs even when patients with ESCC undergo curative resection, the prognosis of patients with ESCC remains poor.[1] Various types of aggressive therapy, such as extended lymphadenectomy, radiotherapy, and chemotherapy, are being used to improve patients' prognosis.[1–3] Chemoradiation therapy (CRT) for ESCC started in the late 1960s using bleomycin. In the 1980s CRT using cisplatin started, and it is currently considered to be one of the most useful treatments.[3] The most important fact is that ESCC patients who respond to CRT survive longer than any other patients; therefore, it would be useful to preselect responders.[4]

Oxidative stress has been shown to play important roles in the carcinogenesis and progression of many cancers, including ESCC.[5] The transcription factor NF-E2-related factor 2 (Nrf2), a basic redox-sensitive bZIP transcription factor, was originally identified to be a critical regulator of intracellular antioxidants and phase II detoxification enzymes by the transcriptional upregulation of many antioxidant response element (ARE)-containing genes.[4–6] Under basal conditions, Nrf2 is bound to the Kelch-like ECH-associated protein 1(Keap1), which is a cul3-based E3 ubiquitin ligase adapter that regulates Nrf2 ubiquitination and proteasome-dependent degradation.[7] On exposure of cells to oxidative stress or chemopreventive compounds, Nrf2 translocates to the nucleus, forms a heterodimer with its obligatory partner Maf, binds to the ARE DNA

Electronic supplementary material The online version of this article (doi:10.1245/s10434-014-3600-2) contains supplementary material, which is available to authorized users.

First Received: 7 October 2013;
Published Online: 6 March 2014

H. Okumura, MD
e-mail: hokumura@m.kufm.kagoshima-u.ac.jp

sequences, and activates the transcription of downstream genes, such as antioxidants and phase II detoxification enzymes.[4,8–10] Therefore, Nrf2 has been viewed as a good transcription factor that is essential in protecting us from oxidative stress-related disease and substances and therapies that produce reactive oxygen species.[11] Indeed, accumulating evidence has been provided recently indicating that Nrf2 has a protective role of against many human pathologic conditions.[12,13] However, new emerging data have revealed the dark side of Nrf2.[14] Recently, it has been revealed that aberrant activation of the Nrf2 pathway occurs frequently in cancer cells. Nrf2 protects not only normal cells, but also cancer cells from cellular stress and enhances cancer cell survival.[15] Many reports have shown that high Nrf2 expression gives cancer cells an advantage for survival from anticancer chemotherapy and radiation therapy.[13,14,16–23]

The aims of this retrospective study were to examine the expression of Nrf2 in biopsy specimens of ESCC and to evaluate whether such expression is useful for predicting the response to CRT.

MATERIALS AND METHODS

Study Groups

There were 46 patients diagnosed with ESCC (45 males and 1 female) who underwent CRT between 1997 and 2011 at Kagoshima University Hospital, Kagoshima, Japan. The median age of the patients was 61.1 years (range, 43–74 years).

They underwent CRT followed by esophagectomy with lymph node dissection 4–6 weeks after completing CRT. After all patients gave their informed consent, biopsy specimens of the primary tumors were endoscopically collected. Clinical factors were assessed by the International Union against Cancer tumor-node-metastasis (TNM) classification system.[24] According to this classification, 2 patients had cT1 tumors, 1 patient had cT2 tumor, 33 patients had cT3 tumors, and 10 patients had cT4 tumors (Supplemental Table 1). The cT4 tumors in this study were resectable tumors that invaded to lung, pleura, or the recurrent nerve. Follow-up data after surgery were available for all patients with a median follow-up period of 33 months (range, 3–136 months).

The study was approved by the Institutional Review Board of Kagoshima University and performed according to the Helsinki Declaration.

Chemoradiation Therapy

A total radiation dose of 40 Gy was applied; 2-Gy fractions were delivered 5 days per week for 4 weeks, to the mediastinum and neck. In the same period, chemotherapy was performed intravenously using two anticancer agents: cisplatin (7 mg/m^2 over 2 h) and 5-FU (350 mg/m^2 over 24 h). Basically, areas supraclavicular to lower mediastinal LN and cardiac LN areas were irradiated as a long T-shaped field for upper-to-lower thoracic tumors, and perigastric LN areas were additionally irradiated for lower tumors. The clinical response to CRT was evaluated by the findings of esophagography, esophagoscopy, endoscopic ultrasonography, and computed tomography.

The clinical criteria for the response to CRT against the primary ESCC site were evaluated by endoscopic examination.[25] The criteria were as follows.[26,27] A complete response (CR) was defined as disappearance of tumor lesion, disappearance of ulceration, and absence of cancer cells in biopsy specimens. Existence of erosion, a granular protruded lesion, ulcer scar, and a Lugol-voiding lesion did not prevent a CR evaluation. Progressive disease (PD) was defined as obvious enlargement of the tumor lesion or progression of esophageal stenosis by tumor enlargement. Incomplete response/stable disease (IR/SD) was defined as not satisfying CR criteria without obvious enlargement of the tumor lesion.

The histological criteria for the response to CRT were: grade 0, neither necrosis nor cellular or structural changes can be seen throughout the lesion; grade 1, necrosis or disappearance of the tumor is present in no more than 2/3 of the whole lesion; grade 2, necrosis or disappearance of the tumor is present in more than 2/3 of the whole lesion, but viable tumor cells still remain; and grade 3, the whole lesion falls into necrosis and/or is replaced by fibrosis, with or without granulomatous changes, and no viable tumor cells are observed.[26,27] In patients whose histological response was grade 2 or 3, the CRT was considered effective. On the other hand, in patients whose histological response was grade 1, the CRT was considered ineffective.

Immunohistochemical Staining and Evaluation of Nrf2 in ESCC

Paraffin-embedded sections (4 μm), including tumor, were deparaffinized and soaked in PBS prior to immunohistochemical analysis. Sections were treated with 3 % H_2O_2 for 10 min in order to block endogenous tissue peroxidase. For staining with Nrf2 antibodies, sections were pretreated with citrate buffer for 10 min at 121 °C in a microwave oven. The sections were washed with PBS and then blocked by treatment with PBS containing 3 % skim milk. The blocked sections were incubated with the diluted primary antibody: Nrf2 (sc-365949, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), with PBS at 4 °C overnight, followed by staining with a streptavidin–biotin-peroxidase kit (Nichirei, Tokyo, Japan). The sections were washed in PBS, and the immune complex was visualized by incubating the sections with diaminobenzidine tetrahydrochloride. They were rinsed briefly in

water, counterstained with hematoxylin, and mounted. Nrf2 expression was determined by counting the number of cancer cells in which the nucleus was stained with the anti-Nrf2 antibody. Normal human placenta tissue was used as positive control of Nrf2, and the primary antibody was replaced with PBS for negative control. Evaluation of immunohistochemistry was independently carried out by 2 investigators (Y.K. and H.O.). To evaluate this, 10 fields within the tumor were selected, and expression in 1,000 cancer cells (100 cells per field) was evaluated using high-power (200×) microscopy. The average Nrf2 labeling index was assessed according to the proportion of positive cells in each field. Nrf2 expression was assessed using the proportion of positive cells and intensity. Nuclear Nrf2 expressions were quantified using a 3-value intensity score (0, 1+, or 2+) and the percentage (0–100 %) of the extent of reactivity. An immunohistochemical score was obtained by multiplying the intensity and reactivity extent values (range, 0–200), and these expression scores were used to determine expression levels. Positive nuclear Nrf2 expression was defined as a score >50, which represents the median expression for gastric cancer evaluated using whole tissue sections. The evaluation method used was an improved version of the method of Solis et al.[28]

Statistical Analysis

Statistical analysis of group differences was performed using the χ^2 test or the Mann–Whitney U test. The Kaplan–Meier method was used for survival analysis, and differences in survival were estimated using the log-rank test. Prognostic factors were examined by univariate and multivariate analyses (Cox proportional hazards regression model). A p value <0.05 was considered to indicate significance. All statistical analyses were performed using the StatFlex version 6.0 for Windows software (StatFlex version 6.0; Artec Inc., Osaka, Japan).

RESULTS

Expression of Nrf2 in ESCC

Immunohistochemically, in human ESCC, Nrf2 expression was identified mainly in cellular nuclei. According to the immunohistochemical evaluation, 18 of 46 patients (39.1 %) were placed in the Nrf2-positive expression group (Fig. 1).

Relationship Between Nrf2 Expression and Clinicopathological Findings

About the correlations between Nrf2 expression and clinicopathological characteristics, there was a significant correlation between the Nrf2-positive group and clinical lymph node metastases ($p = 0.006$), while no significant differences were observed regarding age, sex, histology, tumor depth, and clinical stage (Supplemental Table 2).

Relationship Between Nrf2 Expression and Clinical Response to CRT

In the Nrf2-negative and Nrf2-positive groups, the clinical response was CR in 11 and 2 cases, respectively, IR/SD in 17 and 15 cases, respectively, and PD in 0 and 1 cases, respectively. There was a significant difference in the clinical effect of CRT between the Nrf2-negative and Nrf2-positive groups ($p = 0.02$, Table 1).

Relationship Between Nrf2 Expression and Histological Response to CRT

In the Nrf2-negative and Nrf2-positive groups, there were 9 and 12 grade 1 cases, respectively, and 19 and 6 grade 2 and 3 cases, respectively. There was a significant difference in the histological effect of CRT between the Nrf2-negative and Nrf2-positive groups ($p = 0.02$, Table 2). There is significant correlation between clinical and pathological response of the CRT ($p < 0.01$).

Clinical Outcomes According to Nrf2 Expression or CRT Response

In analyzing clinical outcomes according to Nrf2 and pathological response to CRT in 46 patients who underwent surgery, the 5-year survival rates were 65.4 % in the Nrf2-negative group and 23.2 % in the Nrf2-positive group ($p = 0.0037$, Fig. 2). On univariate regression analyses, clinical lymph node metastasis (cN) and Nrf2 expression significantly affected postoperative outcome. On multivariate analysis, Nrf2 expression was a significant prognostic factor (Table 3).

DISCUSSION

Cancer cell apoptosis is believed to occur as a result of the antitumor efficacy of chemotherapy and radiotherapy that induces reactive oxygen species (ROS) within cancer cells.[21,29,30] Genetic or functional inhibition of Nrf2 results in repressed cellular Nrf2-regulated antioxidant enzymes, including cellular glutathione, thioredoxin, and nonprotein thiols. Finally, these alterations can restore the sensitivity of human cancer cells to anticancer chemotherapy and radiation therapy.[11,14,20,23,28,31] These studies revealed that the antioxidant system plays an important role in the development of resistance to chemotherapy and radiation therapy. In fact, Cho et al.[23] reported that functional

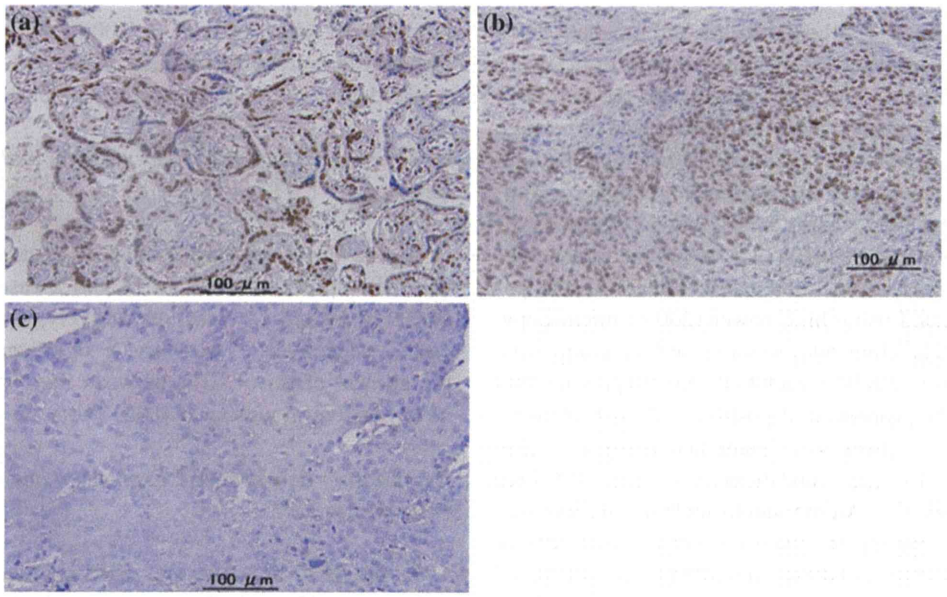

FIG. 1 Expression of Nrf2 in clinical samples. Immunostaining of Nrf2 (original magnification, ×400). **a** Example of noncancerous placental tissue as a positive control. **b** Nrf2-positive ESCC. **c** Nrf2-negative ESCC. Positive staining is detected in the cell nucleus

TABLE 1 Correlation between Nrf2 expression and clinical response to CRT

	Clinical response to CRT ($n = 46$)			Total	p value
	CR	IR/SD	PD		
Nrf2 (−)	11	17	0	28	0.02
Nrf2 (+)	2	15	1	18	

CR complete response, *PD* progressive disease, *IR/SD* incomplete response/stable disease

TABLE 2 Correlation between Nrf2 expression and histological response to CRT

	Histological response to CRT ($n = 46$)			p value
	Grade 1	Grade 2 and 3	Total	
Nrf2 (−)	9	19	28	0.02
Nrf2 (+)	12	6	18	

Grade 1 necrosis or disappearance of the tumor is present in no more than 2/3 of the whole lesion, *Grade 2* necrosis or disappearance of the tumor is present in more than 2/3 of the whole lesion, but viable tumor cells are still remaining, *Grade 3* the whole lesion falls into necrosis and/or is replaced by fibrosis, with or without granulomatous changes. No viable tumor cells are observed

inhibition of Nrf2 leads to sensitization of cancer cells to alkylating anticancer agents. Also, Ma et al.[20] reported that not only cisplatin treatment combined with Nrf2 knockdown, but also Nrf2 knockdown alone inhibited tumor growth significantly in vivo. Therefore, it was suggested that Nrf2, which is a critical regulator of intracellular

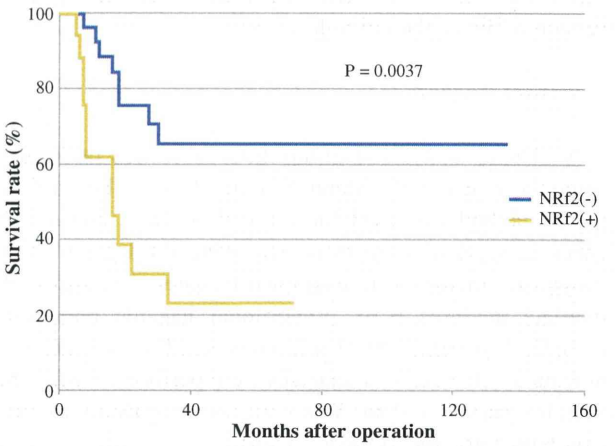

FIG. 2 Cause-specific survival curves for ESCC patients treated by CRT and surgery according to Nrf2 expression ($n = 46$).The 5-year survival rates are indicated for each curve. The p values were calculated using log-rank tests

TABLE 3 Univariate and multivariable analyses of prognostic factors in ESCC

Clinical factors	Univariate analysis p value	Multivariate analysis		
		p value	Hazard ratio	95 % CI
Nrf2	0.0037	0.044	2.674	1.024–6.979
cT	0.5293	0.487	2.068	0.266–16.065
cN	0.0087	0.073	3.998	0.875–18.253

antioxidants, is an important factor that affects antitumor efficacy.

A cisplatin-based regimen and radiation therapy, which are common major therapies for ESCC, show effectiveness by inducing ROS.[9] Thus, this may explain the difference in the efficacy of CRT for the treatment of ESCC between the high Nrf2 expression group and the low Nrf2 expression group. In the present study, the expression of the protein Nrf2 was examined in biopsy specimens of ESCC to determine whether such expression was useful for predicting the response to CRT. As shown in Table 1, there was a significant correlation between Nrf2 expression and the clinical effects of CRT. Furthermore, as shown in Table 2, not only the clinical effect, but also the histological effects of CRT showed significant correlations with Nrf2 expression. These data imply that, in a tumor with low expression of Nrf2, depression of antioxidants, which is the target gene of Nrf2, leads to depression of ROS scavenging ability, resulting in more apoptosis in tumors with low expression of Nrf2, leading to high sensitivity to CRT. With this viewpoint, we examined the Nrf2 expression in the surgical specimens after treating CRT. Although the percentage of Nrf2 positive in the biopsy specimens before CRT was 39.1, 80.4 % of surgical specimens had positive Nrf2 expression in their nuclei (data not shown). All cases with Nrf2 positive expression before CRT in the biopsy specimens never changed their positivity in the surgical specimens after surgery. This phenomenon implied that residual cancer cells might have a high antioxidant ability that is able to overcome ROS produced by CRT and survive (Supplemental Fig. 1). Taken together, it was suggested that evaluation of the Nrf2 expression level in ESCC can be useful to predict the effectiveness of CRT.

As another finding in this study that involved patients who underwent radical resection after CRT, a significant relationship was seen between the expression level of Nrf2 and clinical lymph node metastases. In patients who underwent CRT before radical surgical resection, it has been proposed that Nrf2 expression status in biopsy specimens of ESCC could be used to help identify patients who are at high risk of developing lymph node metastasis.[32]

Concerning the survival analysis, Nrf2 was a good prognostic factor in the patients of this study, and Nrf2 expression was an independent prognostic factor. Thus, the Nrf2 expression level could be used as a useful prognostic parameter for predicting the survival of patients who underwent radical resection after CRT.

It was suggested that, in ESCC, high expression of Nrf2 is associated with high antioxidant ability, which is responsible for natural immunity against not only CRT, but also the ROS produced by macrophage or leukocyte as tumor immunity against cancer cells. In this study group, the patients who responded to CRT survived longer than any other patients, as in other reports.[33] Therefore, in ESCC patients with high Nrf2 expression, knockdown of Nrf2 in the cancer cells before CRT should contribute to converting nonresponders to responders, resulting in a more favorable prognosis. From this perspective, Nrf2 could be a new therapeutic target.

In conclusion, Nrf2-negative expression in biopsy specimens of primary tumors is associated with not only a favorable effect of CRT, but also the prognosis of ESCC. Patients with Nrf2-negative expression may be good candidates for CRT. Since immunohistochemical analysis of biopsy specimens for Nrf2 expression is a simple and inexpensive test, Nrf2 expression should be evaluated before treatment.

ACKNOWLEDGMENT This study is supported by a Grant in Aid for Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant Number 10398282).

DISCLOSURES There are no financial disclosures from any authors.

REFERENCES

1. Naunheim KS, Petruska P, Roy TS, Andrus CH, Johnson FE, Schlueter JM, et al. Preoperative chemotherapy and radiotherapy for esophageal carcinoma. *J Thorac Cardiovasc Surg.* 1992; 103:887–93; discussion 893–5.
2. Baba M, Aikou T, Yoshinaka H, Natsugoe S, Fukumoto T, Shimazu H, et al. Long-term results of subtotal esophagectomy with three-field lymphadenectomy for carcinoma of the thoracic esophagus. *Ann Surg.* 1994;219:310–6.
3. Natsugoe S, Okumura H, Matsumoto M, Uchikado Y, Setoyama T, Yokomakura N, et al. Randomized controlled study on preoperative chemoradiotherapy followed by surgery versus surgery alone for esophageal squamous cell cancer in a single institution. *Dis Esophagus.* 2006;19:468–72.
4. Zhang DD. Mechanistic studies of the Nrf2-Keap1 signaling pathway. *Drug Metab Rev.* 2006;38:769–89.
5. Shibata T, Saito S, Kokubu A, Suzuki T, Yamamoto M, Hirohashi S. Global downstream pathway analysis reveals a dependence of oncogenic NF-E2-related factor 2 mutation on the mTOR growth signaling pathway. *Cancer Res.* 2010;70:9095–105.
6. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc Natl Acad Sci U S A.* 1996;93:14960–5.
7. Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M. Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol Cell Biol.* 2004;24:10941–53.
8. Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, et al. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem.* 2000;275:16023–9.
9. Katoh Y, Itoh K, Yoshida E, Miyagishi M, Fukamizu A, Yamamoto M. Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes Cells.* 2001;6:857–68.
10. Banning A, Deubel S, Kluth D, Zhou Z, Brigelius-Flohe R. The GI-GPx gene is a target for Nrf2. *Mol Cell Biol.* 2005; 25:4914–23.

11. Shibata T, Kokubu A, Gotoh M, Ojima H, Ohta T, Yamamoto M, et al. Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology.* 2008;135:1358–1368, e1–4.
12. Cho HY, Reddy SP, Kleeberger SR. Nrf2 defends the lung from oxidative stress. *Antioxid Redox Signal.* 2006;8:76–87.
13. Kensler TW, Wakabayash N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol.* 2007;47:89–116.
14. Lau A, Villeneuve NF, Sun Z, Wong PK, Zhang DD. Dual roles of Nrf2 in cancer. *Pharmacol Res.* 2008;58:262–70.
15. Stacy DR, Ely K, Massion PP, Yarbrough WG, Hallahan DE, Sekhar KR, et al. Increased expression of nuclear factor E2 p45-related factor 2 (NRF2) in head and neck squamous cell carcinomas. *Head Neck.* 2006;28:813–8.
16. Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsuji M, et al. Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res.* 2008;68:1303–9.
17. Homma S, Ishii Y, Morishima Y, Yamadori T, Matsuno Y, Haraguchi N, et al. Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. *Clin Cancer Res.* 2009;15:3423–32.
18. Li W, Kong AN. Molecular mechanisms of Nrf2-mediated antioxidant response. *Mol Carcinog.* 2009;48:91–104.
19. Zhang P, Singh A, Yegnasubramanian S, Esopi D, Kombairaju P, Bodas M, et al. Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. *Mol Cancer Ther.* 2010;9:336–46.
20. Ma X, Zhang J, Liu S, Huang Y, Chen B, Wang D. Nrf2 knockdown by shRNA inhibits tumor growth and increases efficacy of chemotherapy in cervical cancer. *Cancer Chemother Pharmacol.* 2012;69:485–94.
21. Chen Y, Jungsuwadee P, Vore M, Butterfield DA, Clair DKS. Collateral damage in cancer chemotherapy—Oxidative stress in nontargeted tissues. *Mol Interv.* 2007;7:147–56.
22. Kim JH, Bogner PN, Ramnath N, Park Y, Yu J, Park YM. Elevated peroxiredoxin 1, but not NF-E2-related factor 2, is an independent prognostic factor for disease recurrence and reduced survival in stage I non-small cell lung cancer. *Clin Cancer Res.* 2007;13:3875–82.
23. Cho JM, Manandhar S, Lee HR, Park HM, Kwak MK. Role of the Nrf2-antioxidant system in cytotoxicity mediated by anticancer cisplatin: implication to cancer cell resistance. *Cancer Lett.* 2008;260:96–108.
24. Sobin LH, Wittekind Ch, eds. *International union against cancer (UICC) TNM classification of malignant tumors* (ICD-O C15). 6th ed. New York: Wiley. 2002.
25. Tahara M, Ohtsu A, Hironaka S, Boku N, Ishikura S, Miyata Y, et al. Clinical impact of criteria for complete response (CR) of primary site to treatment of esophageal cancer. *Jpn J Clin Oncol.* 2005;35:316–23.
26. Takubo K, Makuuchi H, Fujita H, Aikou T, Kajiyama Y, Kawano T, et al. Japanese classification of esophageal cancer, 10th ed. Part I. *Esophagus.* 2009;6:1–25.
27. Takubo K, Makuuchi H, Fujita H, Aikou T, Kajiyama Y, Kawano T, et al. Japanese classification of esophageal cancer, 10th ed. Parts II and III. *Esophagus.* 2009;6:71–94.
28. Solis LM, Behrens C, Dong W, Suraokar M, Ozburn NC, Moran CA, et al. Nrf2 and Keap1 abnormalities in non-small cell lung carcinoma and association with clinicopathologic features. *Clin Cancer Res.* 2010;16:3743–53.
29. Boulikas T, Vougiouka M. Cisplatin and platinum drugs at the molecular level (review). *Oncol Rep.* 2003;10:1663–82.
30. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene.* 2003;22:7265–79.
31. Wang XJ, Sun Z, Villeneuve NF, Zhang S, Zhao F, Li Y, et al. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. *Carcinogenesis.* 2008;29:1235–43.
32. Wang J, Zhang M, Zhang L, Cai H, Zhou S, Zhang J, et al. Correlation of Nrf2, HO-1, and MRP3 in gallbladder cancer and their relationships to clinicopathologic features and survival. *J Surg Res.* 2010;164:e99–105.
33. Okumura H, Natsugoe S, Matsumoto M, Yokomakura N, Uchikado Y, Takatori H, et al. The predictive value of p53, p53R2, and p21 for the effect of chemoradiation therapy on oesophageal squamous cell carcinoma. *Br J Cancer.* 2005;92:284–9.