Ferredoxin Reductase Is Useful for Predicting the Effect of Chemoradiation Therapy on Esophageal Squamous Cell Carcinoma

HIROSHI OKUMURA[1], YASUTO UCHIKADO[1], ITARU OMOTO[1], MAKI MOTOMURA[1], YOSHIAKI KITA[1], KEN SASAKI[1], MASAHIRO NODA[1], TAKAAKI ARIGAMI[1], YOSHIKAZU UENOSONO[1], KENJI BABA[1], SINICHIRO MORI[1], YUKO KIJIMA[1], AKIHIRO NAKAJO[1], HIROSHI KURAHARA[1], KOSEI MAEMURA[1], MASAHIKO SAKODA[1], TETSUHIRO OWAKI[2], SUMIYA ISHIGAMI[1] and SHOJI NATSUGOE[1]

Departments of [1]Digestive Surgery, Breast and Thyroid Surgery and [2]Education Center for Doctors in Remote Islands and Rural Areas, Graduate School of Medical and Dental Sciences, Graduate School of Medical Sciences, Kagoshima University, Sakuragaoka, Kagoshima, Japan

Abstract. *Background: Ferredoxin reductase (Fdxr) is the mitochondrial cytochrome P-450 NADPH reductase. Fdxr overexpression increases the sensitivity of tumor cells to apoptosis in response to chemotherapy through reactive oxygen species (ROS) production. The aim of the present study was to examine the Fdxr expression in esophageal squamous cell carcinoma (ESCC) and determine if the expression is useful for predicting response to chemoradiation therapy (CRT). Materials and Methods: Fdxr expression in biopsy specimens from 50 patients before neoadjuvant CRT were immunohistochemically examined. Then, the correlation between Fdxr expression and response to CRT were analyzed. Results: Both clinically and histologically, significant correlations were found between positive Fdxr expression and favorable response to CRT. Furthermore, Fdxr was significantly correlated with postoperative outcomes and was found to be an independent prognostic factor. Conclusion: Fdxr expression was found to be closely related to the effect of CRT and could predict the CRT outcome in patients with ESCC.*

Prognosis of patients with esophageal squamous cell carcinoma (ESCC) remains poor, because postoperative relapse often occurs even when patients with ESCC undergo curative resection (1). Aggressive therapies, such as extended lymphadenectomy, radiotherapy, and chemotherapy, are currently used to improve patient prognosis (2-4). Chemoradiation therapy (CRT) for ESCC began in the late 1960s with bleomycin; cisplatin was introduced in the 1980s, and it is currently one of the most useful treatments (5). ESCC patients who respond to CRT survive longer than any other patients; therefore, it would be useful to pre-select responders (6, 7).

Ferredoxin reductase (Fdxr), the 54-kDa mammalian mitochondrial cytochrome *P-450* NADPH reductase, is located on the matrix side of the inner mitochondrial membrane, and it transfers electrons from NADPH *via* the single electron shuttle ferredoxin-cytochrome *P-450* to substrates during steroidogenesis (8). Under substrate-limiting conditions, electrons can leak from this shuttle system and generate reactive oxygen species (ROS) (8). Fdxr mediates 5-fluorouracil-induced apoptosis in colorectal cancer cells through generation of ROS, critical regulators of apoptosis (9, 10). Over-expression of Fdxr increases the sensitivity of tumor cells to apoptosis on H_2O_2 treatment through ROS production (11, 12).

The aim of the present retrospective study was to examine the expression of Fdxr in biopsy specimens of ESCC and to evaluate any usefulness in predicting response to CRT.

Patients and Methods

Study group. The present study involved 50 consecutive patients with advanced ESCC who underwent CRT at the Department of Surgical Oncology and Digestive Surgery of the Kagoshima University Hospital between 1997 and 2010. They underwent CRT followed by esophagectomy with lymph node dissection 4-6 weeks after completing CRT. After patients gave their informed consent, biopsy specimens of primary tumors were endoscopically collected.

Correspondence to: Hiroshi Okumura MD, PhD, Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890-8520, Tel: +81-992-75-5361; Fax: +81-992-65-7426. E-mail: hokumura@m.kufm.kagoshima-u.ac.jp

Key Words: Ferredoxin reductase, esophageal squamous cell carcinoma, chemoradiation therapy, biomarker.

Table I. *Patients' characteristics.*

Characteristics	N
Mele/ Female	49/1
Mean age (range) (years)	62.0 (43-74)
Histological type (well/moderate/poor)	8/30/12
cT (T1/T2/T3/T4)	2/1/36/11
cN (N0/N1)	13/37
cM (M0/M1)	31/19

Table II. *Correlation between Fdxr expression and clinical response to CRT.*

	Clinical response to CRT (n=50)				
	CR	IR/SD	PD	Total	*p*-Value
Fdxr (+)	11	17	0	28	0.02
Fdxr (-)	2	15	1	18	

CR: Complete response, PD: progressive disease, IR/SD: incomplete response/stable disease.

Classifications of the specimen were determined according to the International Union against Cancer tumor-node-metastasis (TNM) classification system (13). No patients received adjuvant therapy until they developed recurrent disease. Follow-up data after surgery were available for all patients with a median follow-up period of 34 months (range=3-136 months). The clinicopathological features of the study group are summarized in Table I. The cT4 tumors in this study were resectable tumors that invaded the lung, pleura, or the recurrent nerve. All M1 tumors were due to distant lymph node metastases. The study was approved by the Institutional Review Board of the Kagoshima University and performed according to the Helsinki Declaration.

Chemoradiation therapy. A total radiation dose of 40 Gy were applied; 2-Gy fractions were delivered 5 days per week, for 4 weeks, to the mediastinum, neck and/or upper abdomen including clinical metastatic lymph node areas. In the same period, patients received intravenous chemotherapy with cisplatin (7 mg/m^2 over 2 h) and 5-fluorouracil (350 mg/m^2 over 24 h). The clinical criteria for the response to CRT against the primary ESCC site were evaluated by endoscopic examination (14, 15). A complete response (CR) was defined as the disappearance of tumor lesions, disappearance of ulceration, and absence of cancer cells in biopsy specimens. Existence of erosion, a granular protruded lesion, ulcer scar, and unstained lesions by iodine 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 patients whose clinical effect was CR were considered susceptible to CRT, whereas the patients with IR/SD or PD were considered not susceptible.

The histological criteria for the response to CRT were as follows (14, 15): 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 two-thirds of the whole lesion; Grade 2, necrosis or disappearance of the tumor is present in more than two-thirds of the whole lesion, but viable tumor cells are still remaining; and Grade 3, the whole lesion contains necrosis and/or is replaced by fibrosis, with or without granulomatous changes, and no viable tumor cells are observed. 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 0 or 1, the CRT was considered ineffective.

Table III. *Correlation between Fdxr expression and histological response to CRT (histological response judged as Grade 2 or 3 classified as effective)*

	Histological response to CRT (n=50)			
	Grade 1	Grade 2+3	Total	*p*-Value
Fdxr (+)	1	20	21	0.0001
Fdxr (-)	22	7	29	

Grade 1: Necrosis or disappearance of the tumor is present in no more than two-thirds of the whole lesion. Grade 2: Necrosis or disappearance of the tumor is present in more than two-thirds of the whole lesion, but viable tumor cells remain. 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.

Table IV. *Univariate and multivariable analyses of prognostic factors in ESCC.*

Clinical factors	Univariate analysis	Multivariate analysis		
	p-Value	*p*-Value	Hazard ratio	95% CI
Fdxr	0.012	0.0079	4.950	1.603-15.38
cT	0.5141	0.669	1.692	0.269-10.638
cN	0.0322	0.5749	1.703	0.151-19.210
cStage	0.0429	0.2677	3.788	0.359-40.0

ESCC: Esophageal squamous cell carcinoma, CI: confidence interval.

Immunohistochemical staining and evaluation of Fdxr in ESCC. Tumor samples were fixed with 10% formaldehyde in phosphate-buffered saline (PBS), embedded in paraffin, and sectioned into 4-mm–thick slices. The sections were washed with PBS for 5 min three times and then blocked by PBS containing 3% skim milk for 10 min at room temperature. The blocked sections were incubated overnight at 4˚C with primary antibody against Fdxr (sc-365949, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted in PBS, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). Sections were counter-stained with hematoxylin, and mounted. Fdxr expression was determined by counting the number of cancer cells in which the cytoplasm was stained with the anti-Fdxr antibody. The Fdxr-expressing tumor tissues that we previously reported were used as positive controls, and negative controls were prepared by replacing the primary antibody with PBS (12). Fdxr expression was assessed by determining the proportion of positive cells and intensity. For cytoplasmic expression of Fdxr, immunostaining was scored on a 3-tiered scale for both intensity (absent/weak, 1; moderate, 2; strong,

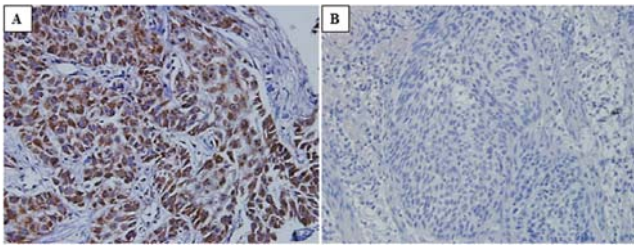

Figure 1. *Expression of Fdxr in clinical samples by immunostaining (original magnification, ×400). (A) Positive expression of Fdxr in ESCC. (B) Negative expression of ESCC. Positive staining was detected in the cytoplasm.*

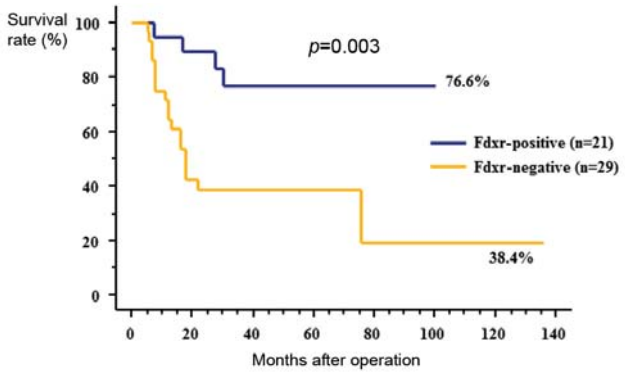

Figure 2. *Cause-specific survival curves for ESCC patients treated by CRT and surgery according to Fdxr expression (n=50). The 5-year survival rates are indicated for each curve. p-Values were calculated using log-rank tests.*

3) and percentage (<10%, 1; 10%-50%, 2; >50%, 3), and then the two scores were multiplied to give a combination score ranging from 1 to 9 for each section. Cytoplasmic protein expression was defined as positive when the combination scores were >6 and negative when the combination scores were <6. This evaluation method is an improved version of previous methods (12).

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 by the log-rank test. Prognostic factors were examined by univariate and multivariate analyses (Cox proportional hazards regression model). *p*-Values <0.05 were considered statistical significant. All statistical analyses were performed with StatView™ version 5.0 (Abacus Concepts, Berkeley, CA, USA).

Results

Expression of Fdxr in ESCC. Immunohistochemically, in human ESCC, Fdxr expression was identified mainly in the cytoplasm. According to the immunohistochemical evaluation, 21 of 50 patients (42.0%) were Fdxr-positive (Figure 1).

Relationship between Fdxr expression and clinical response to CRT. In the Fdxr-positive and Fdxr-negative 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 Fdxr-positive and -negative groups (p=0.002, Table II).

Relationship between Fdxr expression and histological response to CRT. In the Fdxr-positive and Fdxr-negative groups, there were 1 and 22 Grade 1 cases, 13 and 2 Grade 2 cases, and 7 and 5 Grade 3 cases, respectively. There was a significant difference in the histological effect of CRT between the Fdxr-positive and -negative groups (p=0.0001, Table III). The clinical and pathological responses to CRT were significantly correlated (data not shown) (p<0.01).

Clinical outcomes according to Fdxr expression or CRT response. In analyzing clinical outcomes according to Fdxr expression and pathological response to CRT in 50 patients who underwent surgery, the 5-year survival rates were 76.6% in the Fdxr-positive group and 38.4% in the Fdxr-negative group (p=0.003, Figure 2). On univariate regression analyses, clinical lymph node metastasis (cN) and Fdxr expression significantly affected postoperative outcome. On multivariate analysis, Fdxr expression was a significant prognostic factor (Table IV).

Discussion

Generation of intracellular ROS is an early event in the apoptosis of cancer cells induced by treatment with chemotherapy or radiotherapy (16-18). The anti-oxidant system plays an important role in the development of resistance to chemotherapy and radiation therapy. A cisplatin-based regimen and radiation therapy, which are common major therapies for ESCC, are effective by inducing ROS (17, 19). This mechanism may explain the difference in the efficacy of CRT for the treatment of ESCC between the Fdxr-positive and the Fdxr-negative expression group. In the present study, the expression of Fdxr protein was examined in biopsy specimens of ESCC to determine whether such expression was useful for predicting response to CRT. As shown in Table II, there was a significant correlation between Fdxr expression and the clinical effects of CRT. Furthermore, as shown in Table III, not only the clinical effect, but also the histological effects of CRT showed significant correlations with Fdxr expression. These data imply that a tumor with Fdxr expression leads to production of ROS, resulting in greater apoptosis than in tumors with negative expression of Fdxr, leading to high sensitivity to CRT. Taken together, these results suggest that evaluation of the Fdxr expression level in ESCC can be useful to predict the effectiveness of CRT. Although there was one previous report on the relationship between Fdxr expression and the clinical efficacy of colorectal chemotherapy (20), this is the first report on the predictive value of Fdxr for the effect of CRT on ESCC.

Concerning the survival analysis, Fdxr expression was a good prognostic factor in patients of this study, and Fdxr expression was an independent prognostic factor. Thus, the Fdxr expression level could be used as a useful prognostic parameter for predicting survival of patients who underwent radical resection after CRT. In patients treated with neoadjuvant CRT, the patients who responded to CRT survived longer than any other patients, as previously reported (6). On the other hand, Fdxr protein is fragile and stabilized by binding to the tumor suppressor protein Fhit and heat shock proteins Hsp60 and Hsp10 (12, 17). Therefore, in ESCC patients with Fdxr-negative expression, the stabilization of Fdxr in cancer cells before CRT should contribute to converting non-responders to responders, resulting in a more favorable prognosis. From this perspective, Fdxr could be a new therapeutic target molecule.

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

Conclusion

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

Acknowledgments

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 15K10107).

References

1 Natsugoe S, Matsumoto M, Okumura H, Uchikado Y, Setoyama T, Sasaki K, Sakurai T, Omoto I, Owaki T, Shinchi H, Ueno S and Ishigami S: Clinical course and outcome after esophagectomy with three-field lymphadenectomy in esophageal cancer. Langenbecks Arch Surg *395*: 341-346, 2010.

2 Baba M, Aikou T, Yoshinaka H, Natsugoe S, Fukumoto T, Shimazu H and Akazawa K: Long-term results of subtotal esophagectomy with three-field lymphadenectomy for carcinoma of the thoracic esophagus. Ann Surg *219*: 310-316, 1994.

3 John MJ, Flam MS, Mowry PA, Podolsky WJ, Xavier AM, Wittlinger PS and Padmanabhan A: Radiotherapy alone and chemoradiation for nonmetastatic esophageal carcinoma. A critical review of chemoradiation. Cancer *632*: 397-403, 1989.

4 Naunheim KS, Petruska P, Roy TS, Andrus CH, Johnson FE, Schlueter JM and Baue AE: Preoperative chemotherapy and radiotherapy for esophageal carcinoma. J Thorac Cardiovasc Surg *103*: 887-893, 1992.

5 Natsugoe S, Okumura H, Matsumoto M, Uchikado Y, Setoyama T, Yokomakura N, Ishigami S, Owaki T and Aikou T: Randomized controlled study on preoperative chemoradiotherapy followed by surgery versus surgery alone for esophageal squamous cell cancer in a single institution. Dis Esophagus *19*: 468-472, 2006.

6 Okumura H, Uchikado Y, Matsumoto M, Owaki T, Kita Y, Omoto I, Sasaki K, Sakurai T, Setoyama T, Nabeki B, Matsushita D, Ishigami S, Hiraki Y, Nakajo M and Natsugoe S: Prognostic factors in esophageal squamous cell carcinoma patients treated with neoadjuvant chemoradiation therapy. Int J Clin Oncol1 *8*: 329-334, 2013.

7 Okumura H, Uchikado Y, Setoyama T, Matsumoto M, Owaki T, Ishigami S and Natsugoe S: Biomarkers for predicting the response of esophageal squamous cell carcinoma to neoadjuvant chemoradiation therapy. Surg Today *44*: 421-428, 2014.

8 Kimura T and Suzuki K. Components of the electron transport system in adrenal steroid hydroxylase. Isolation and properties of non-heme iron protein (adrenodoxin). J Biol Chem *242*: 485-491, 1967.

9 Hwang PM, Bunz F, Yu J, Rago C, Chan TA, Murphy MP, Kelso GF, Smith RA, Kinzler KW and Vogelstein B: Ferredoxin reductase affects p53-dependent, 5-fluorouracil-induced apoptosis in colorectal cancer cells. Nat Med *7*: 1111-1117, 2001.

10 Liu G and Chen X. The ferredoxin reductase gene is regulated by the p53 family and sensitizes cells to oxidative stress-induced apoptosis Oncogene *21*: 7195-7204, 2002.

11 Trapasso F, Pichiorri F, Gasparo M, Palumbo T, Aqeilan RI, Gaudio E, Okumura H, Iuliano R, Di Leva G, Fabbri M, Birk DE, Raso C, Green-Church K, Spagnoli LG, Venuta S, Huebner K and Croce CM: Fhit interaction with ferredoxin reductase triggers generation of reactive oxygen species and apoptosis. J Biol Chem *283*: 13736-13744, 2008.

12 Pichiorri F, Okumura H, Nakamura T, Garrison PN, Gasparini P, Suh SS, Druck T, McCorkell KA, Barnes LD, Croce CM and Huebner K: Correlation of Fhit structural features with effector interactions and biological functions. J Biol Chem *284*: 1040-1049, 2009.

13 Sobin LH: International Union Against Cancer (UICC) TNM Classification of Malignant Tumors, Sixth Edition: Wiley-Blackwell, 2002.

14 Japan Esophageal Society: Japanese Classification of Esophageal Cancer, Tenth Edition, part I: Esophagus *6*: 1-25, 2009.

15 Japan Esophageal Society: Japanese Classification of Esophageal Cancer, Tenth Edition, parts II and III: Esophagus *6*: 71-94, 2009.

16 Alexandre J, Batteux F, Nicco C, Chéreau C, Laurent A, Guillevin L, Weill B and Goldwasser F: Accumulation of hydrogen peroxide is an early and crucial step for paclitaxel-induced cancer cell death both *in vitro* and *in vivo*. Int J Cancer *119*: 41-48, 2006.

17 Okumura H, Ishii H, Pichiorri F, Croce CM, Mori M and Huebner K: Fragile gene product, Fhit, in oxidative and replicative stress responses. Cancer Sci *100*: 1145-1150, 2009.

18 Gupta SC, Hevia D, Patchva S, Park B, Koh W and Aggarwal BB: Upsides and downsides of reactive oxygen species for cancer: the roles of reactive oxygen species in tumorigenesis, prevention, and therapy. Antioxid Redox Signal *16*: 1295-1322, 2012.

19 Siddik ZH: Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene *22*: 7265-7279, 2003.

20 Nobili S, Napoli C, Landini I, Morganti M, Cianchi F, Valanzano R, Tonelli F, Cortesini C, Mazzei T and Mini E: Identification of potential pharmacogenomic markers of clinical efficacy of 5-fluorouracil in colorectal cancer. Int J Cancer *128*: 1935-1945, 2011.

Received August 19, 2015
Revised September 17, 2015
Accepted September 23, 2015