

***NRF2* Mutation Confers Malignant Potential and Resistance to Chemoradiation Therapy in Advanced Esophageal Squamous Cancer¹**

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Abstract

Esophageal squamous cancer (ESC) is one of the most aggressive tumors of the gastrointestinal tract. A combination of chemotherapy and radiation therapy (CRT) has improved the clinical outcome, but the molecular background determining the effectiveness of therapy remains unknown. NRF2 is a master transcriptional regulator of stress adaptation, and gain-of-function mutation of NRF2 in cancer confers resistance to stressors including anticancer therapy. Direct resequencing analysis revealed that *Nrf2* gain-of-function mutation occurred recurrently (18/82, 22%) in advanced ESC tumors and ESC cell lines (3/10). The presence of *Nrf2* mutation was associated with tumor recurrence and poor prognosis. Short hairpin RNA-mediated down-regulation of NRF2 in ESC cells that harbor only mutated *Nrf2* allele revealed that the mutant NRF2 conferred increased cell proliferation, attachment-independent survival, and resistance to 5-fluorouracil and γ -irradiation. Based on the *Nrf2* mutation status, gene expression signatures associated with *NRF2* mutation were extracted from ESC cell lines, and their potential utility for monitoring and prognosis was examined in a cohort of 33 pre-CRT cases of ESC. The molecular signatures of *NRF2* mutation were significantly predictive and prognostic for CRT response. In conclusion, recurrent *NRF2* mutation confers malignant potential and resistance to therapy in advanced ESC, resulting in a poorer outcome. Molecular signatures of *NRF2* mutation can be applied as predictive markers of response to CRT, and efficient inhibition of aberrant NRF2 activation could be a promising approach in combination with CRT.

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Introduction

Esophageal cancer (EC) is the sixth most common cause of cancer death worldwide [1]. The 5-year survival rate of patients with EC is reportedly less than 20%, making it one of the most aggressive tumors of the gastrointestinal tract [2]. Epidemiologically, alcohol consumption, cigarette smoking, and a preference for hot beverages, as well as the presence of Barrett esophagus resulting from gastroesophageal reflux, have been reported as risk factors for EC [3,4], and more than

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460,000 patients develop this cancer annually worldwide [1]. Esophageal squamous cancer (ESC) is the predominant type of EC, being prevalent in Africa and Eastern Asia including Japan [5].

Currently, only surgical resection can be regarded as a curative therapy for ESC, and adjuvant or neoadjuvant combination of chemotherapy with radiation therapy (CRT) has recently improved the clinical outcome [6–8]. However, responsiveness to CRT varies among patients, and the molecular backgrounds that determine therapeutic effectiveness remain largely unknown [6]. Therefore, the discovery of molecular markers for precise prediction of responsiveness to therapy is eagerly anticipated. It is also necessary to construct effective personalized therapeutic modalities based on the molecular characterization of ESC.

Oxidative stress has been shown to play important roles in the carcinogenesis and progression of many cancers including ESC [9–11]. In response to intrinsic (e.g., reactive oxygen species generated in mitochondria) and extrinsic (e.g., radiation) oxidative stresses, NRF2L2 (nuclear erythroid factor 2-like 2, or NRF2) functions as a master transcriptional regulator of cytoprotective genes [12]. Under physiological conditions, KEAP1, an E3 ubiquitin ligase, directly interacts with two amino-terminal motifs (DLG and ETGE) of NRF2 and negatively regulates its expression level through the proteasome [13]. Recently, it has been revealed that aberrant activation of the NRF2 pathway occurs frequently in cancer. We and other groups have reported that the *Keap1* gene is inactivated in a wide range of cancers [14–18]. Moreover, we have discovered that the *Nrf2* gene is a *bona fide* oncogene in lung and head/neck cancers [19]. In the present study, we discovered that *NRF2* mutation also occurs frequently in ESC and examined its clinical significance especially from the viewpoint of responsiveness to CRT.

Materials and Methods

Clinical Samples and DNA Extraction

All surgical specimens were obtained from patients who had been diagnosed and had undergone surgery at the National Cancer Center Hospital, Tokyo, Japan (pathologic diagnoses are presented in Table W1). Tumor cells and corresponding lymphocytes or non-cancerous tissues were dissected under a microscope from methanol-fixed or formalin-fixed (*in situ* carcinoma) paraffin-embedded tissues, and the DNA was extracted. High-molecular-weight DNA was also extracted from cell lines using DNAeasy (QIAGEN, Hamburg, Germany). Clinical data, including therapeutic response and prognosis, were collected from the medical charts. The protocol for analysis of the clinical samples was approved by the institutional review board of the National Cancer Center.

Polymerase Chain Reaction and Sequence Analysis

We amplified all exons of the *Nrf2* gene by polymerase chain reaction (PCR) using High-Fidelity Taq polymerase (Roche Diagnostic, Basel, Switzerland), as described [19]. The PCR products were then purified (QIAquick PCR Purification Kit; QIAGEN) and analyzed by sequencing (Big Dye Sequencing Kit; Applied Biosystems, Carlsbad, CA).

Biologic and Biochemical Experiments

Ten ESC cell lines (KYSE-30, -50, -70, -110, -140, -150, -170, -180, -220, and -270) were obtained from the Japanese Collection of Research Bioresources (<http://cellbank.nibio.go.jp>) and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Construction of the retroviral expression vector of

short hairpin RNA (shRNA) was carried out as described previously [20]. The shRNA-targeted sequences were as follows: NRF2–shRNA-1, ACTTGCTCAATGTCCTGTTGC; NRF2–shRNA-2, AGTTGAGCTTCATTGAACTGC. The control vector contains nontargeting shRNA sequence. The entry vectors were recombined with pDEST-CL-SI-MSCVpuro by LR reactions (Invitrogen, Carlsbad, CA), in accordance with the manufacturer's instructions. After infection (multiplicity of infection > 1), cells were selected and maintained in the presence of 1 µg/ml puromycin. NRF2 small interfering RNA (siRNA) was previously described [19]. NRF2-dependent luciferase activity was measured as described [15]. Cell proliferation was measured using the 96-well plate format by bromodeoxyuridine incorporation (Cell Proliferation ELISA; Roche Diagnostic). For attachment-free culture, 1×10^6 cells were seeded in ultra low-attachment dish (Ultra Low Cluster Plate, Corning, NY). 5-Fluorouracil (5-FU) was purchased from WAKO (Tokyo). Irradiation of cells was performed using the ^{60}Co source in the Gammacell 220 Research Irradiator (MDS Nordion, Ontario, Canada). The intracellular level of reduced glutathione was measured by GSH-Glo Glutathione Assay (Promega, Madison, WI). Protein extraction and immunoblot analysis were performed as described previously [15]. Antibodies used in this study are listed in Table W1.

Gene Expression Profiling and Quantitative Reverse Transcription–PCR

Ten micrograms of total RNA from ESC cell lines was reverse-transcribed by MMLV-RT, and a Cy3-labeled complementary RNA probe was synthesized using T7 RNA polymerase and hybridized with a microarray covering the whole human genome (Whole Human Genome Oligo Microarray, G4112F; Agilent Technologies, Santa Clara, CA). After washing, the microarray was scanned by the DNA microarray scanner (Agilent Technologies). Data were analyzed using GeneSpring software (Agilent Technologies). Quantitative reverse transcription (RT)–PCR was performed in triplicate and evaluated using universal probes for each amplicon and the LightCycler system (Roche). Primers designed by ProbeFinder (Version 2.45; Roche) and used in this study are shown in Table W2. The relative expression of each gene was determined by comparison with that of glyceraldehyde-3-phosphate dehydrogenase (GADPH).

Statistics

Statistical analysis of clinicopathologic data was performed using the StatView software package. Gene Set Enrichment Analysis (GSEA) was performed as follows. First, a gene list sorted in descending order based on fold change differences in expression between *NRF2*-mutated and wild-type ESC cell lines was calculated. Second, preranked GSEA was applied for the gene list to the C2 curated gene sets (MSigDB ver. 2.5) with default parameters (<http://www.broadinstitute.org/gsea/index.jsp>). Finally, significant gene sets were extracted if the false discovery rate (FDR) was less than 25%. To evaluate the predictive power of gene expression signatures for CRT and prognosis, biopsy samples with clinical information were classified into two groups by K-means clustering (R cluster package) using gene expression signatures for each significant gene set of GSEA, and then survival analysis was applied to the classified samples. The survival analysis was performed using the Kaplan-Meier method, and log-rank test was used to evaluate disease-free survival. To determine whether the *NRF2* target genes based on ChIP-seq (chromatin immunoprecipitation sequence) [21] were enriched in any gene sets, we calculated the *P* values using the hypergeometric distribution for each the C2 curated gene sets and estimated the FDR.

Because the NRF2 ChIP-seq data were measured using a mouse cell line, we then converted the mouse genes to human genes using NCBI HomoloGene to apply them as a human gene set C2. We used a 10% FDR cutoff for this enrichment study.

Results

NRF2 Mutation in ESC

To evaluate the prevalence of NRF2 mutation in human cancer, we attempted resequencing analysis of the entire coding region of the NRF2 gene in a cohort (320 samples in total) of epithelial and non-epithelial tumors (pathologic diagnoses are shown in Table W2). We detected six nonsynonymous somatic mutations in the ECs (6/32, 18.8%) and one nonsynonymous somatic mutation in cervical cancer (1/18, 5.6%) and malignant melanoma (1/22, 4.5%), respectively (Figure 1A and Table 1). We additionally analyzed 50 cases of ESC and identified a total of 18 somatic mutations (18/82, 22%; Table 1). These mutations exclusively affected amino acid residues within and surrounding the KEAP1 binding motif, as reported previously in lung

cancers [19]. We then analyzed 10 ESC cell lines and found three NRF2-mutated ones (3/10; Figure 1B and Table 1), of which two (KYSE70 and KYSE180) harbored homozygous mutations. NRF2-mutated KYSE70 and KYSE110 cells were established from poorly differentiated ESC. No KEAP1 mutation was detected in ESC cases (data not shown). We additionally analyzed 36 cases of intraepithelial (*in situ*) ESC and detected no NRF2 mutation (0/36), suggesting that NRF2 mutation occurs predominantly at the advanced stage of esophageal carcinogenesis.

Association of NRF2 Mutation with Tumor Recurrence and Poor Prognosis of ESC

To further determine the clinical significance of NRF2 gene mutation in EC, we analyzed the association between NRF2 mutation status and clinicopathologic factors. The presence of NRF2 mutation was correlated with tumor recurrence ($P = .046$) and tended to be associated with lymph node metastasis ($P = .072$). Patients with NRF2-mutated tumors showed poorer survival ($P = .005$; Table 2 and Figure 1C). Clinical stage ($P = .038$) and lymph node metastasis ($P = .039$) were associated with the disease-free survival of the patient (Table 2).

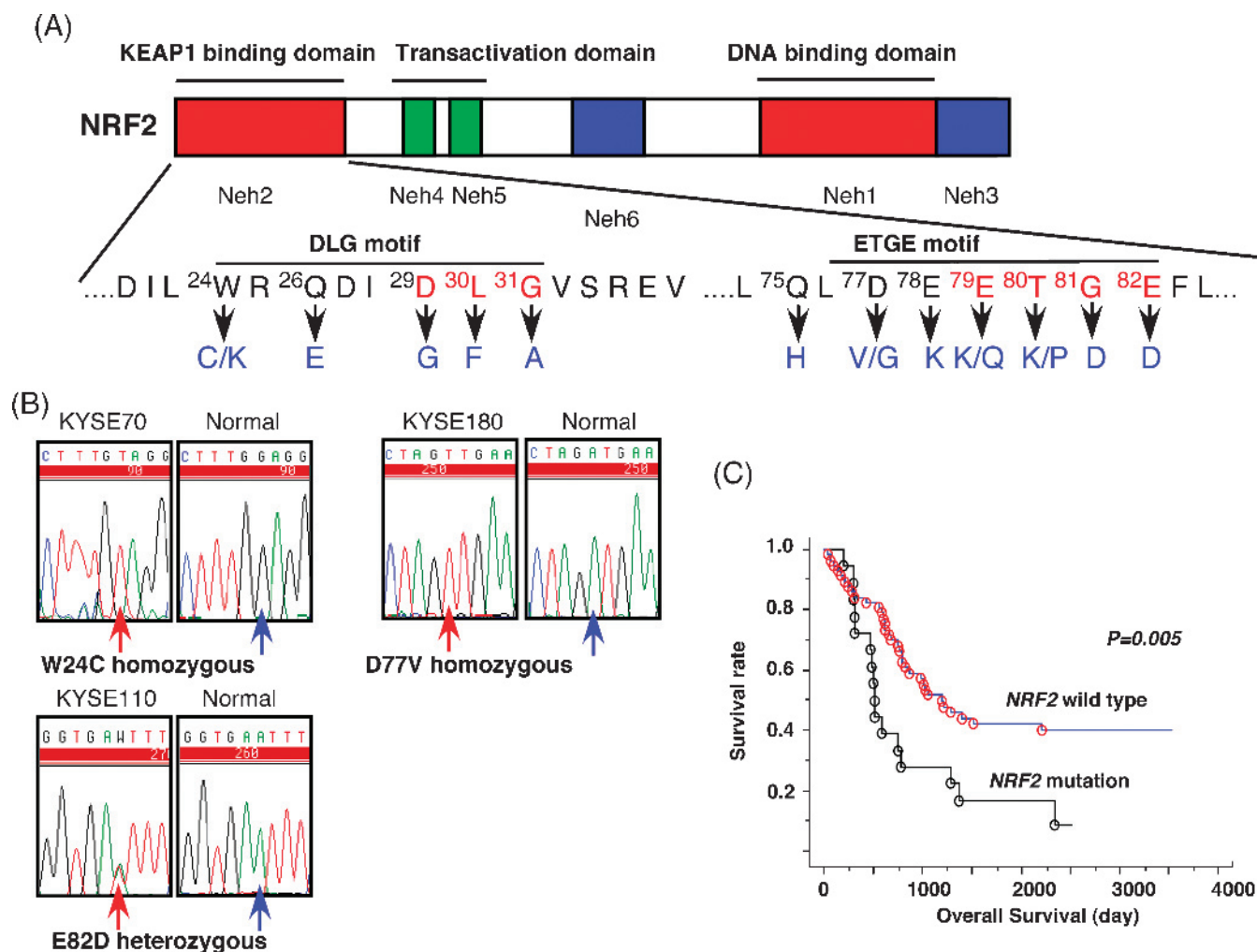


Figure 1. NRF2 mutation in EC. (A) Somatic NRF2 mutations are clustered in the KEAP1 binding domain. Schematic presentation of NRF2 protein indicates the location of Neh1~6 (Nrf2-ech homology) domains and functional annotations. Somatic mutations identified in this study (blue) affected normal amino acids in the DLG or ETGE motifs. (B) Sequence chromatography of NRF2 mutations in ESC cell lines with corresponding normal sequences in the bottom panel. KYSE70 and KYSE180 cells harbor homozygous mutations. (C) Kaplan-Meier plot showing overall survival of ESC patients segregated according to NRF2 mutation status.

Table 1. NRF2 Mutation in Human Cancers.

Sex	Tumor Type	Nucleotide Substitution	Amino Acid Change	Zygosity
M	Esophageal cancer (SCC)	c. 70T>C	p. W24K	Heterozygous
F	Esophageal cancer (SCC)	c. 72G>C	p. W24C	Heterozygous
M	Esophageal cancer (SCC)	c. 76C>G	p. Q26E	Heterozygous
M	Esophageal cancer (SCC)	c. 66A>G	p. D29G	Heterozygous
M	Esophageal cancer (SCC)	c. 66A>G	p. D29G	Allelic imbalance (mut > wt)
M	Esophageal cancer (SCC)	c. 88C>T	p. L30F	Heterozygous
M	Esophageal cancer (SCC)	c. 92G>C	p. G31A	Heterozygous
M	Esophageal cancer (SCC)	c. 225A>C	p. Q75H	Heterozygous
M	Esophageal cancer (SCC)	c. 230A>T	p. D77V	Heterozygous
M	Esophageal cancer (SCC)	c. 230A>G	p. D77G	Heterozygous
M	Esophageal cancer (SCC)	c. 232G>A	p. E78K	Heterozygous
M	Esophageal cancer (SCC)	c. 235G>A	p. E79K	Heterozygous
M	Esophageal cancer (SCC)	c. 235G>A	p. E79K	Heterozygous
M	Esophageal cancer (SCC)	c. 235G>A	p. E79K	Heterozygous
M	Esophageal cancer (SCC)	c. 239C>A	p. T80K	Heterozygous
F	Esophageal cancer (SCC)	c. 238A>C	p. T80P	Heterozygous
F	Esophageal cancer (SCC)	c. 242G>A	p. G81D	Heterozygous
M	Malignant melanoma	c. 88C>T	p. L30F	Heterozygous
F	Cervical cancer (SCC)	c. 235G>C	p. E79Q	Heterozygous
M	KYSE70: Esophageal cancer (SCC)	c. 72G>T	p. W24C	Homozygous
M	KYSE110: Esophageal cancer (SCC)	c. 246A>T	p. E82D	Heterozygous
M	KYSE180: Esophageal cancer (SCC)	c. 230A>T	p. D77V	Homozygous

Mutant NRF2 Has a Gain-of-Function Activity and Regulates Proliferation and Attachment-Independent Cell Survival in ESC Cells

We compared the transcriptional activities of ESC-associated NRF2 mutants (W24C and D77V) to that of wild NRF2 and determined that mutant NRF2 has a gain-of-function activity, which is partly resistant to KEAP1-mediated inhibition (Figure 2A). There was no significant difference in transcriptional activity between homozygous (KYSE30) and heterozygous (KYSE110) mutant cells (Figure W1).

The previously mentioned analysis of clinical samples suggested that *NRF2* mutation plays a role in metastasis and tumor progression

of ESC. Exploiting our discovery of ESC cell lines with homozygous *NRF2* mutation, we established KYSE70 cells stably expressing shRNAs against *NRF2* to examine the biologic features solely regulated by mutant NRF2. We tested two different lentiviruses containing NRF2-targeted shRNA (shRNA-1 and -2) along with a control virus. Immunoblot analysis revealed that NRF2–shRNA-2 robustly (11.4% of control) reduced the expression of endogenous mutant NRF2 protein compared with the NRF2–shRNA-1 clone (71.2% of control) (Figure 2C). Similarly, NRF2-dependent transcriptional activity markedly decreased (8.9% of control) in shRNA-2 clone (Figure 2D).

Table 2. Associations among NRF2 Mutation Status, Clinicopathologic Factors, and Prognosis.

Clinicopathologic Factor	No. Patients	NRF2 Mutation, <i>P</i>	Overall Survival, <i>P</i>	Disease-free Survival, <i>P</i>
Sex	Male	.914	.119	.656
	Female			
NRF2 mutation	+	na*	.005	.288
	–			
Smoking	+	.302	.572	.557
	–			
Alcohol intake	+	.268	.366	.143
	–			
Stage	I-II	.709	.895	.038
	III-IV			
Invasion depth	>a1	.186	.453	.565
	<mp			
Lymph node metastasis	+	.072	.285	.039
	–			
Histologic differentiation	Well	.134	.7	.074
	Mod			
	Poor			
Lymphatic infiltration	+	.48	.746	.336
	–			
Venous involvement	+	.631	.215	.124
	–			
Intramucosal metastasis	+	.873	.376	.654
	–			
Tumor recurrence	+	.046	.587	.775
	–			

“+” indicates positive; “–,” negative; >a1, invade beyond muscularis propria; <mp invade within muscularis propria; well, well differentiated; mod, moderately differentiated; poor, poorly differentiated.
*Not applicable.

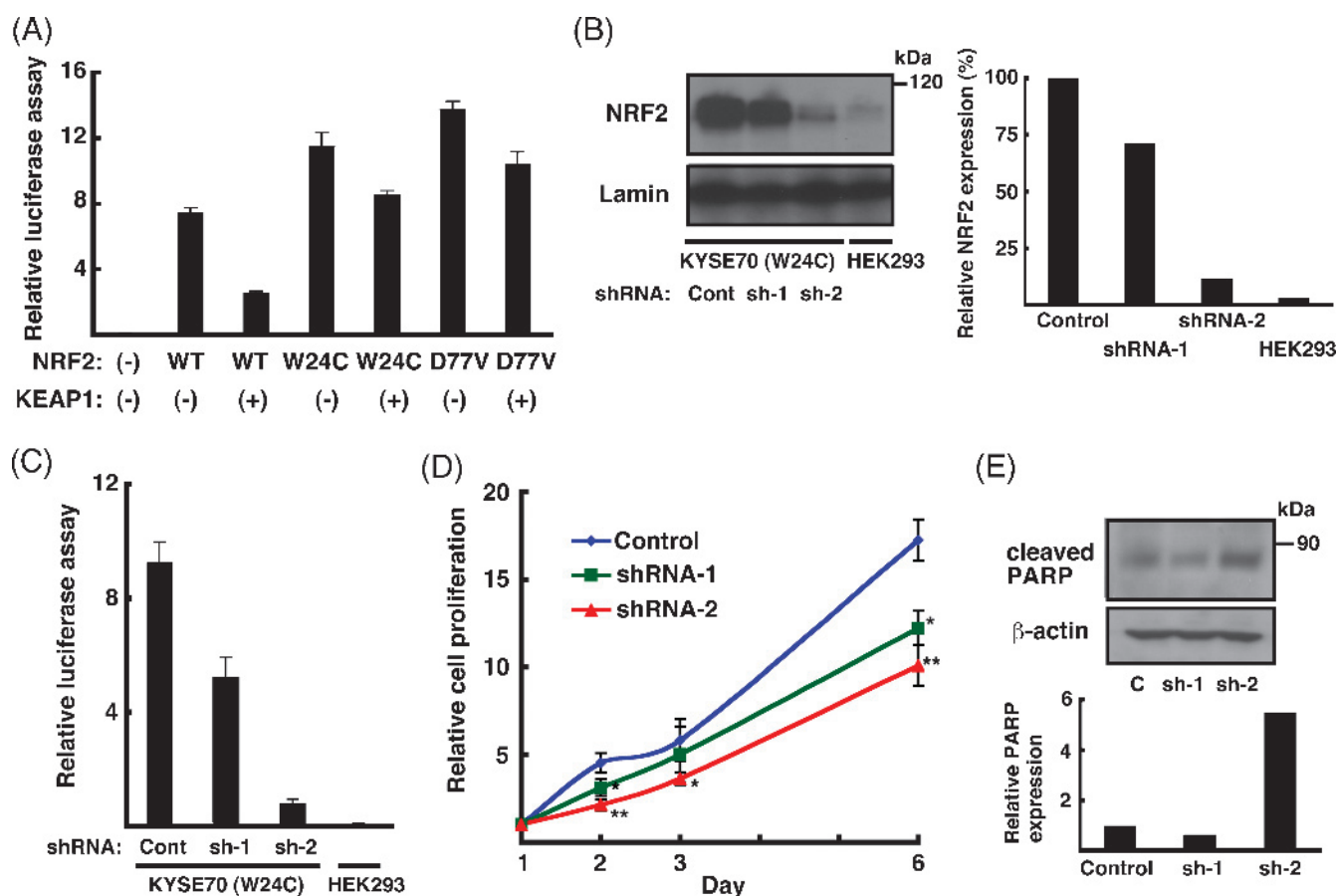


Figure 2. Down-regulation of gain-of-function NRF2 mutant in KYSE70 cells by shRNA. (A) Transcriptional activities of wild-type and ESC-associated mutant NRF2 were measured in the absence or presence of the *Keap1* gene. (B) Down-regulation of NRF2 protein in KYSE70 cells treated with NRF2-targeting shRNAs. Left, Immunoblot analysis of NRF2 protein in the nuclear fraction of KYSE70 cells infected with GFP shRNA (cont) or two different NRF2-shRNAs (sh-1 and sh-2). HEK293 cells lack NRF2 accumulation and were loaded as a negative control. Lamin B1 was used as a loading control. Right, Relative NRF2 expression in each clone was presented. (C) NRF2-dependent transcriptional activities of KYSE clones and HEK293. (D) Cell proliferation plot of KYSE70 clones infected with control or NRF2-shRNAs (shRNA-1 and shRNA-2). Data are mean \pm SD of three independent experiments. * $P < .05$, ** $P < .01$. (E) KYSE70 clones were cultured on an ultra low-attachment plate for 72 hours, and whole proteins were then extracted. Expression of cleaved PARP was examined by immunoblot analysis. β -Actin was used as a loading control. Relative PARP1 expression compared with the control shRNA was shown below. Molecular marker is indicated on the right (kDa).

Both shRNAs reduced cell proliferation in KYSE70 cells (Figures 2E and W2). We then measured attachment-independent cell survival in liquid culture, which mimics the antiapoptotic activity of floating cancer cells in tumor metastasis or recurrence. As shown in Figure 2C, only shRNA-2 clones exhibited more apoptosis in culture conditions where adhesion to a substrate was inhibited.

Down-regulation of Mutant NRF2 Increases the Sensitivity of ESC Cells to 5-FU

Previous studies have shown that activation of NRF2 in lung and gallbladder cancers confers resistance to chemotherapeutic drugs [14,15]. To determine whether NRF2 activation has any association with drug resistance in ESC cells, we examined the sensitivity to 5-FU, a commonly used chemotherapeutic reagent for ESC, in ESC cells. We found that NRF2-mutated cells were relatively resistant to 5-FU treatment compared with an NRF2 wild-type cell (Figure W3A) and that NRF2-downregulated KYSE70 cells (both shRNA-1 and shRNA-2 clones) showed increased sensitivity to 5-FU (the IC_{50} of each clone was 125.3, 81.3, and 13.1 μ M respectively; Figure 3A). We also

reduced the expression of NRF2 in other *NRF2*-mutated ESC cells using NRF2 siRNA and tested their sensitivity to 5-FU. This yielded results similar to that for KYSE70 cell (Figure 3B).

Robust Down-regulation of Mutant NRF2 Is Required for Enhancing Radiation Sensitivity in ESC Cells

Radiation therapy induces oxidative stress by producing reactive oxygen species in cancer cells. Thus, it is very likely that *NRF2* mutation is associated with radiosensitivity. To determine whether mutant NRF2 has a role in acquisition of resistance to irradiation, we exposed control and NRF2 shRNA-infected KYSE70 cells to γ -irradiation (2 Gy) and examined their clonogenic activities (basal colony formation activity of ESC cells is shown in Figure W3B), which reflect the degree of irradiation-induced cellular damage. As shown in Figure 3C, NRF2-downregulated cells (both shRNA-1 and shRNA-2 clones) were more sensitive to irradiation than control clone. However, when the relative number of colonies was compared among three clones, only the shRNA-2 clone showed a significant reduction ($P = .0002$). This suggests that moderate (~70%) NRF2

down-regulation affected the colony formation activity but that a more complete reduction (~10%) of mutant NRF2 would be required to enhance radiation sensitivity.

It has been reported that the intracellular glutathione level is correlated with sensitivity to radiation [22,23]. To determine whether mutant NRF2 has any role in regulating the level of glutathione in irradiated ESC cells, we measured the expression of major enzymes (glutamate-cysteine ligase, catalytic subunit [GCLC] and glutathione reductase [GSR]), which regulate glutathione synthesis and modification and are direct targets of NRF2 [21,24], in NRF2-downregulated KYSE70 cells exposed to various doses of irradiation. Quantitative RT-PCR analysis revealed that down-regulation of mutant NRF2 (shRNA-2 clone) was significantly reduced not only for the inducible expressions of GCLC and GSR but also for their basal level (Figure 3D). Interestingly, this reduction was not obvious in the

shRNA-1 clone, which is consistent with the radiation sensitivity experiment previously mentioned and suggests that robust NRF2 down-regulation would be required for decreasing glutathione-regulatory enzymes (Figure 3D). Consistently, the basal level and radiation-induced increase of reduced glutathione were diminished only in the shRNA-2 clone (Figure W4).

Molecular Signatures Associated with NRF2 Mutation Are Prognostic Factors for ESC and Predictive of Response to CRT

These *in vitro* data strongly suggested that NRF2 activation by genetic alteration could be associated with the clinical response to CRT. However, the previously mentioned ESC cohort, whose NRF2 mutation status was determined, contained only eight cases that had received postoperative CRT. Despite the difficulty in obtaining molecular data from minute biopsy samples of tumors before treatment, we

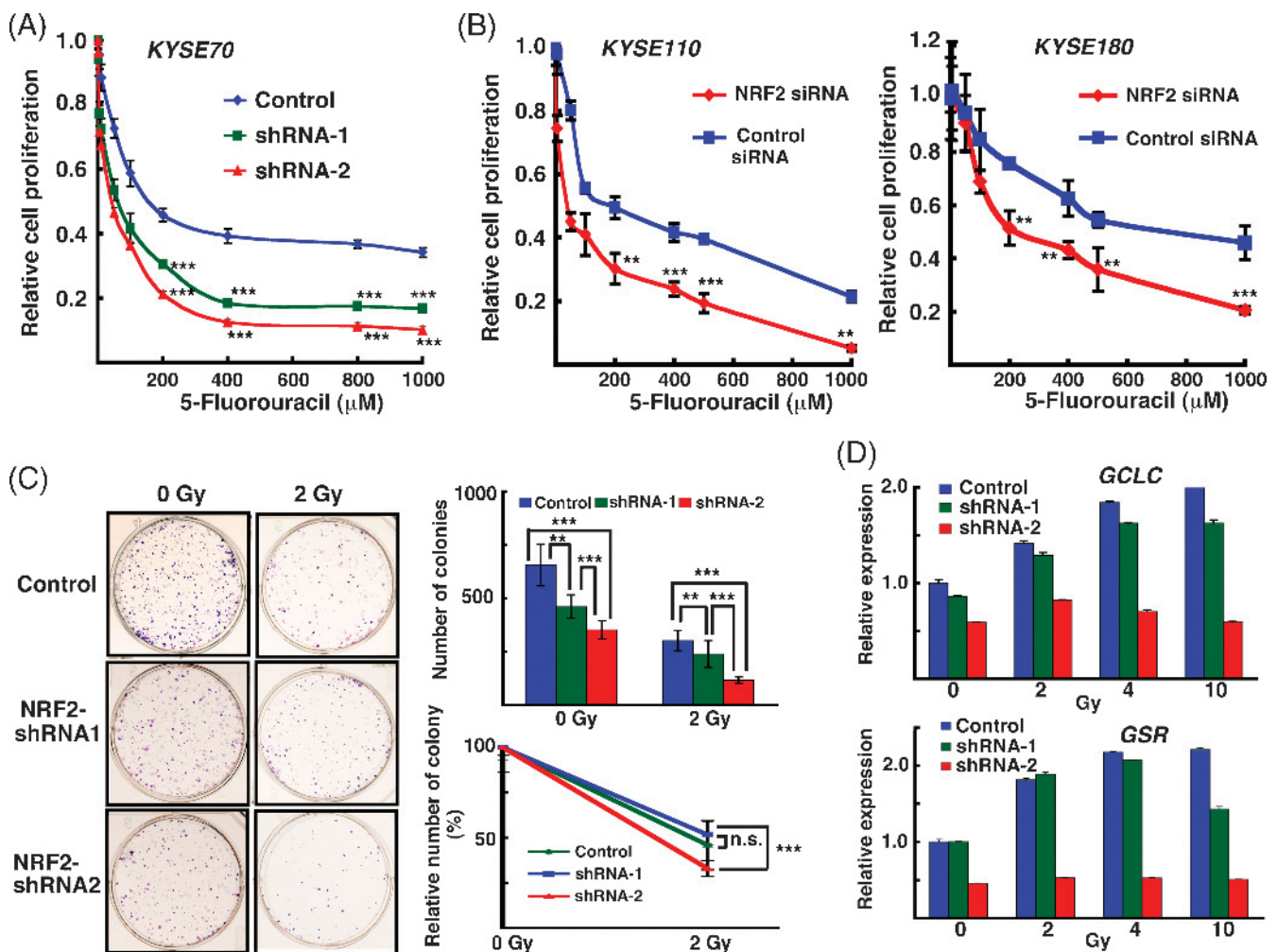


Figure 3. Mutant NRF2 confers resistance to chemotherapeutic reagent and radiation. (A) KYSE70 clones infected with control shRNA or NRF2-shRNAs were treated with various concentrations of 5-FU. (B) KYSE110 and KYSE180 cells were treated with control or NRF2 siRNA and various concentrations of 5-FU, as in A. Data represent cell viability after 48 hours relative to vehicle (DMSO)-treated control. Data are mean \pm SD of three independent experiments; statistical difference to control siRNA or shRNA, ** $P < .01$, *** $P < .001$. (C) Left, A representative plate showing colony formation by each clone with control (0 Gy) and 2-Gy irradiation. Top right, Number of KYSE70 clones infected with control shRNA and NRF2-shRNAs that formed colonies after control (0 Gy) and 2-Gy irradiation. Data are mean \pm SD of eight independent plates. Bottom right, The relative number of colonies after 2-Gy irradiation compared with the control (0 Gy). Note that the difference between control shRNA and shRNA-1 clones was not significant (n.s.). (D) KYSE70 clones were irradiated with different doses, and the expression of GCLC and GSR mRNAs was then examined. Data are mean \pm SD of three independent experiments, ** $P < .01$, *** $P < .001$.

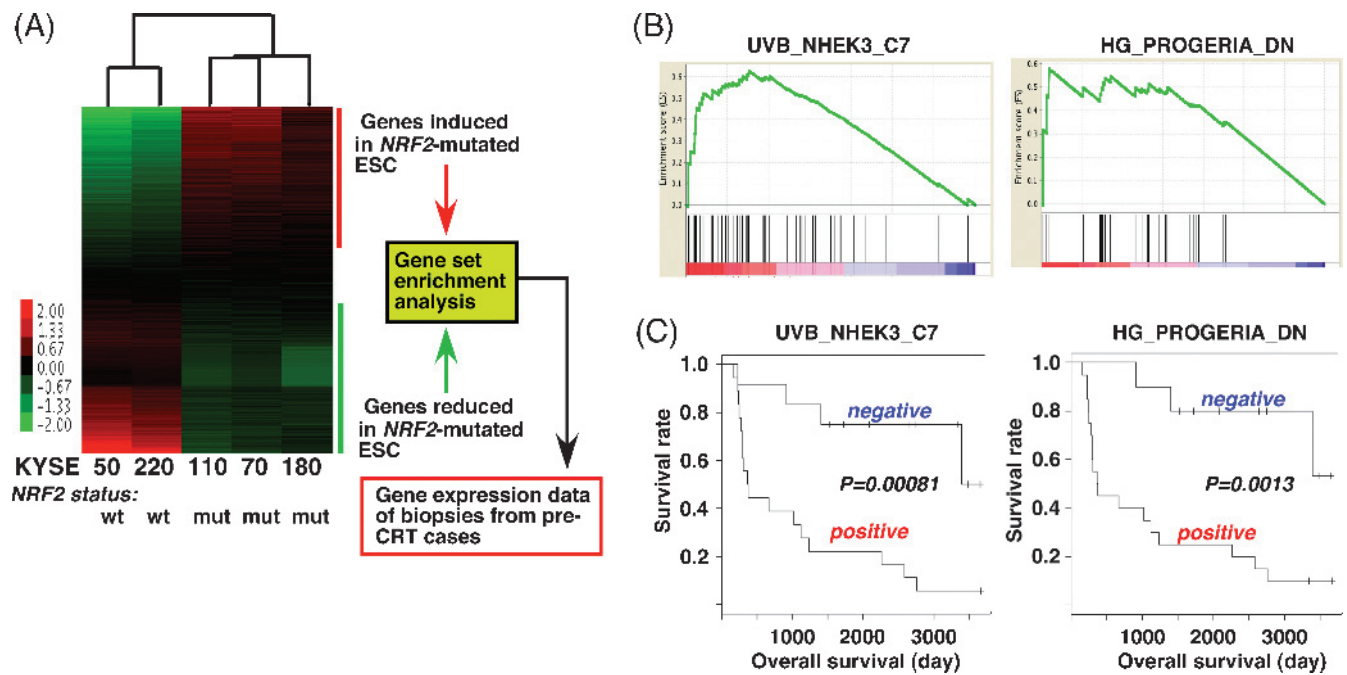


Figure 4. Pathway signatures of mutant NRF2 are associated with ESC patients' survival after CRT. (A) Two-dimensional hierarchal clustering of KYSE cells by genome-wide gene expression. NRF2 mutation status is shown at the bottom. GSEA extracted characteristic gene sets enriched in NRF2-mutated or wild-type ESC cells, and their prognostic significance was evaluated based on gene expression data for biopsies from pre-CRT cases. (B) Representative panels showing results of GSEA of genes enriched in NRF2-mutated ESC cell lines. (C) Kaplan-Meier plot showing overall survival of ESC patients after CRT segregated according to the NRF2 mutant signature.

previously collected the gene expression profiles of 33 pre-CRT samples of ESC for which the same therapeutic protocol had been used and for which the therapeutic response had been clinically evaluated [25]. Therefore, using this data set, we attempted to analyze whether the gene expression signatures associated with *NRF2* mutation could be predictive markers of response to CRT in ESC.

For this purpose, we first obtained the genome-wide gene expression data for five ESC cell lines and extracted the *NRF2*-mutated ESC signature based on the *NRF2* mutation status (Figure 4A). Using GSEA, we obtained a panel of molecular pathway signatures that were significantly associated with *NRF2* mutation status (representative pathways are shown in Figure 4B). The expressions of 703 gene sets were increased, and those of 14 gene sets were de-

creased in *NRF2*-mutated ESC cell lines (a complete list is shown in Table W3). We then tested whether changes in the expression of any of these signatures were associated with the clinical response to CRT and overall survival after treatment. We found that the increased expression of 15 gene sets was significantly ($P < .05$) associated with both poor response to CRT and a poor patient outcome after CRT (Table 3; representative Kaplan-Meier plots are shown in Figure 4C). All of the potentially predictive molecular signatures were increased in *NRF2*-mutated ESC cell lines and included pathways related to the oxidative stress response ($n = 4$), immune/cytokine response ($n = 4$), and hematopoietic disorder ($n = 3$) (Table 3). We further elucidated that direct NRF2 target genes confirmed by a combination of ChIP-seq and microarray analysis [21] were significantly

Table 3. Mutant NRF2 Signatures Associated with CRT Response.

Molecular Pathways	Prognosis, <i>P</i>	Clinical Response, <i>P</i>	NRF2 Target Enrichment, <i>P</i>	No. NRF2 Target Genes	Functional Annotation
ET743PT650_COLONCA_DN	.000103	.00404	.000917	7	Anticancer drug
GLYCOSPHINGOLIPID_METABOLISM	.000744	.00519	.585	1	Glycolipid metabolism
UVB_NHEK3_C7	.000813	.0235	.00279	7	Oxidative stress response
HG_PROGERIA_DN	.00132	.0109	.6	1	Aging
TAKEDA_NUP8_HOXA9_16D_UP	.00306	.0186	.318	8	Hematopoietic disorder
CARIES_PULP_HIGH_UP	.00466	.0186	.865	2	Immune/cytokine response
ABRAHAM_AL_VS_MM_UP	.0083	.00688	.254	2	Hematopoietic disorder
ABRAHAM_MM_VS_AL_DN	.0083	.00688	.24	2	Hematopoietic disorder
IFN_GAMMA_UP	.00847	.045	.165	3	Immune/cytokine response
AS3_FIBRO_C4	.00865	.0235	1	0	Oxidative stress response
TSADAC_RKOSILENT_UP	.0115	.045	.52	1	Methylated in colon cancer
BRCA1KO_MEF_DN	.0144	.0174	.0267	7	Oxidative stress response
UVB_SCC_DN	.016	.0235	.899	2	Oxidative stress response
CMV_HCMV_TIMECOURSE_24HRS_DN	.0196	.045	.0169	5	Immune/cytokine response
SANA_TNFA_ENDOTHELIAL_UP	.0286	.00669	.587	3	Immune/cytokine response

Table 4. NRF2 Target Genes Enriched in the CRT-predictive Pathways.

Pathway	Gene Symbol	Gene Name
ET743PT650_COLONCA_DN	<i>MGMT</i>	O ⁶ -methylguanine-DNA methyltransferase
	<i>LRBA</i>	LPS-responsive vesicle trafficking, beach, and anchor containing
	<i>KIFAP3</i>	Kinesin-associated protein 3
	<i>IRS1</i>	Insulin receptor substrate 1
	<i>GBE1</i>	Glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV)
UVB_NHEK3_C7	<i>ADK</i>	Adenosine kinase
	<i>BRE</i>	Brain and reproductive organ-expressed (TNFRSF1A modulator)
	<i>PGD</i>	Phosphogluconate dehydrogenase
	<i>ESD</i>	Esterase D/formylglutathione hydrolase
	<i>ADK</i>	Adenosine kinase
	<i>PAM</i>	Peptidylglycine alpha-amidating monooxygenase
	<i>AKR1A1</i>	Aldo-keto reductase family 1, member A1 (aldehyde reductase)
BRCA1KO_MEF_DN	<i>CDKN3</i>	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual-specificity phosphatase)
	<i>PLA2G4A</i>	Phospholipase A2, group IVA (cytosolic, calcium-dependent)
	<i>PDIA4</i>	Protein disulfide isomerase family A, member 4
	<i>GPT2</i>	Glutamic pyruvate transaminase (alanine aminotransferase) 2
	<i>MAN1A1</i>	Mannosidase, alpha, class 1A, member 1
	<i>LAMC1</i>	Laminin, gamma 1 (formerly LAMB2)
	<i>ADD3</i>	Adducin 3 (gamma)
	<i>CDC2L6</i>	Cell division cycle 2-like 6 (CDK8-like)
	<i>KLF9</i>	Kruppel-like factor 9
	CMV_HCMV_TIMECOURSE_24HRS_DN	<i>WNT5A</i>
<i>SERPINE1</i>		Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
<i>ANXA1</i>		Annexin A1
<i>VEGFC</i>		Vascular endothelial growth factor C
<i>ADD3</i>		Adducin 3 (gamma)

enriched in part of these pathways (Table 2; enriched genes are listed in Table 4).

Discussion

NRF2 Mutation in ESC

In the present study, we conducted mutation screening of the *Nrf2* gene in a wide range of tumor types including sarcoma and found that *NRF2* mutation is specifically frequent in EC and rarely occurs in cervical cancer and melanoma. These ESC-associated mutations harbor gain-of-function activity. Because *Keap1* recognizes two distinct regions of *NRF2*, single amino acid change may not completely inhibit *Keap1*-mediated degradation or sequestration [19]. Recently, Kim et al. [26] performed a mutation screening of hot spots in the *Nrf2* gene in more than 1000 tumors and reported that *NRF2* mutation is recurrent in tumors of the lung, skin, esophagus, and larynx, which is concordant with our previous and current observations. We previously reported that *NRF2* mutation was more common in smoking-associated lung cancer [19]; however, we failed to demonstrate any significant association between the presence of *NRF2* mutation and smoking history ($P = .302$) in ESC, probably because most of the affected patients in our cohort (64/82, 78%) were smokers. Also, *NRF2* mutation status was not associated with the habitual alcohol intake ($P = .268$). Previous genetic analyses have shown that polymorphisms of the *GSTP1* and *NQO1* genes, both of which are known direct targets of *NRF2*, are associated with susceptibility to ESC in Asian and white populations [27–29]. It would be interesting to examine the frequency of *NRF2* mutation in other ESC cohorts with different genetic, epidemiological, or ethnic backgrounds.

Because *Keap1*-deleted mice show hyperkeratosis in the forestomach [30], which is analogous to the esophagus, *NRF2* activation could affect differentiation process of esophageal epithelium, which might

be associated with malignant features of ESC. Our analysis of a cohort of ESC cases has further revealed the clinical significance of *NRF2* mutation. Our mutation analysis revealed that *NRF2* mutation was only detected in the late stage ESC. The presence of *NRF2* mutation was associated with tumor recurrence and was shown to be a significant prognostic factor. A recent study aimed at identifying *NRF2* target genes by ChIP-seq has revealed that *NRF2* directly regulates the expression of gene groups that are related to cell proliferation and survival, in addition to the detoxification response [21]. Using ESC cell lines containing *NRF2* mutation, we found that mutant *NRF2* promotes ESC cell proliferation. We further revealed that mutant *NRF2* confers attachment-independent cell survival, which is intimately associated with lymph node metastasis and tumor progression. Collectively, our results demonstrate that aberrant *NRF2* activation resulting from its genetic alteration occurs at a relatively advanced stage of ESC and that it induces cell proliferation and survival signals, resulting in acquisition of malignant features and poor prognosis.

Molecular Signature of NRF2 Mutation and Chemoradiation Therapy in ESC

In addition to these malignant characters of cancer cells, resistance to current therapies would also be associated with a poorer survival rate. Previous studies have shown that *NRF2* activation by loss-of-function mutations or down-regulation of the *Keap1* gene confers resistance to chemotherapeutic drugs and radiation [14,15,18,31]. As has been reported in lung cancer [19], down-regulation of mutant *NRF2* enhanced the sensitivity of ESC cells to a chemotherapeutic agent. Furthermore, mutant *NRF2* regulates resistance to irradiation partly through induction of enzymes related to glutathione synthesis. Consequently, effective inhibition of aberrant *NRF2* signaling could offer a novel therapeutic approach for ESC in combination with CRT. Interestingly, robust reduction of mutant *NRF2* was required to inhibit

resistance to radiation and induction of glutathione-regulating enzymes. Combined with other analyses, it could be possible that there are distinct groups of target genes regulated by mutant NRF2 in ESC cells. One group is sensitive to the amount of NRF2 and includes genes associated with cell proliferation, colony formation, and 5-FU resistance. The other is relatively resistant to NRF2 reduction and is responsible for attachment-independent survival and radiation resistance.

Our *in vitro* analyses also suggest that NRF2 activation could be associated with a poorer response to CRT in clinical cases of ESC. To examine this hypothesis, we carried out gene expression profiling, in view of the difficulty in collecting sufficient samples of pre-CRT tumors for mutation analysis. A number of previous studies have reported the identification of biomarkers that are predictive of the response of ESC to 5-FU or 5-FU-based neoadjuvant CRT [32–34]. Previously reported molecular markers have been categorized into several groups, including those related to 5-FU metabolism (e.g., thymidylate synthase and thymidine phosphorylase), the DNA repair pathway (e.g., excision repair cross-complementing rodent repair deficiency, complementation group 1 [ERCC1] and TP53), hypoxia (hypoxia-inducible factor 1 α and vascular endothelial growth factor), and apoptosis/survival signaling (e.g., survivin, Bax, epidermal growth factor receptor, and Cox2) [32]. We tested the association between the expression level of ERCC1 or survivin and the clinical response to CRT in our cohort, but found no significant relationship (ERCC1, $P = .209$; survivin, $P = .327$). In contrast to these studies focusing on molecules with functional associations, only a few studies have explored microarray data in an unbiased way to search for biomarkers predictive of CRT efficacy, and only a limited number of cases ($n = 13, 19, \text{ or } 20$) were examined [35–37].

In the present study, we adopted a different approach because NRF2 regulates a network of genes related to various molecular pathways at a different dose-response interaction. Instead of focusing on the expression of individual genes, we carried out “pathway analysis,” which seems to be more relevant to biologic phenotypes, including therapeutic response [38]. Based on the gene expression data for ESC cell lines, we extracted a number of functionally annotated pathways whose expression was associated with the presence of NRF2 mutation, which was also supported by ChIP-seq data of NRF2. We then used microarray data for the largest pre-CTR biopsy cohort ever reported (33 cases) and evaluated these pathways for predictive and prognostic utility. In total, up-regulation of 15 pathways was found to be significantly associated with a poor response to CRT and unfavorable prognosis, and our analysis revealed that expression of the gene sets related to oxidative stress and the immune response is associated with the clinical response of ESC to CRT and patient prognosis.

References

- Ferlay J, Shin HR, Bray F, Forman D, Mathers C, and Parkin DM (2010). Estimates of worldwide burden of cancer in 2008. *Int J Cancer* **127**, 2893–2917.
- Parkin DM, Bray F, Ferlay J, and Pisani P (2005). Global cancer statistics, 2002. *CA Cancer J Clin* **55**, 74–108.
- Morita M, Kumashiro R, Kubo N, Nakashima Y, Yoshida R, Yoshinaga K, Saeki H, Emi Y, Kakeji Y, Sakaguchi Y, et al. (2010). Alcohol drinking, cigarette smoking, and the development of squamous cell carcinoma of the esophagus: epidemiology, clinical findings, and prevention. *Int J Clin Oncol* **15**, 126–134.
- Falk GW (2009). Risk factors for esophageal cancer development. *Surg Oncol Clin N Am* **18**, 469–485.
- Hamilton SR and Aaltonen LA (2000). *Pathology and Genetics of Tumors of the Digestive Systems. World Health Organization Classification of Tumours*. IARC Press, Lyon, France.
- Kleinberg L and Forastiere AA (2007). Chemoradiation in the management of esophageal cancer. *J Clin Oncol* **25**, 4110–4117.
- Chirieac LR, Swisher SG, Ajani JA, Komaki RR, Correa AM, Morris JS, Roth JA, Rashid A, Hamilton SR, and Wu TT (2005). Posttherapy pathologic stage predicts survival in patients with esophageal carcinoma receiving preoperative chemoradiation. *Cancer* **103**, 1347–1355.
- Berger AC, Farma J, Scott WJ, Freedman G, Weiner L, Cheng JD, Wang H, and Goldberg M (2005). Complete response to neoadjuvant chemoradiotherapy in esophageal carcinoma is associated with significantly improved survival. *J Clin Oncol* **23**, 4330–4337.
- Martin RC, Liu Q, Wo JM, Ray MB, and Li Y (2007). Chemoprevention of carcinogenic progression to esophageal adenocarcinoma by the manganese superoxide dismutase supplementation. *Clin Cancer Res* **13**, 5176–5182.
- Cao L, Xu X, Cao LL, Wang RH, Coumoul X, Kim SS, and Deng CX (2007). Absence of full-length Brca1 sensitizes mice to oxidative stress and carcinogen-induced tumorigenesis in the esophagus and forestomach. *Carcinogenesis* **28**, 1401–1407.
- Chen X, Ding YW, Yang G, Bondoc F, Lee MJ, and Yang CS (2000). Oxidative damage in an esophageal adenocarcinoma model with rats. *Carcinogenesis* **21**, 257–263.
- Motohashi H and Yamamoto M (2004). Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med* **10**, 549–557.
- Tong KI, Kobayashi A, Katsuoka F, and Yamamoto M (2006). Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. *Biol Chem* **387**, 1311–1320.
- Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsui M, Suzuki T, Kobayashi A, Yokota J, Sakiyama T, et al. (2008). Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res* **68**, 1303–1309.
- Shibata T, Kokubu A, Gotoh M, Ojima H, Ohta T, Yamamoto M, and Hirohashi S (2008). Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology* **135**, 1358–1368.
- Lee DF, Kuo HP, Liu M, Chou CK, Xia W, Du Y, Shen J, Chen CT, Huo L, Hsu MC, et al. (2009). KEAP1 E3 ligase-mediated downregulation of NF- κ B signaling by targeting IKK β . *Mol Cell* **36**, 131–140.
- Takahashi T, Sonobe M, Menju T, Nakayama E, Mino N, Iwakiri S, Nagai S, Sato K, Miyahara R, Okubo K, et al. (2010). Mutations in Keap1 are a potential prognostic factor in resected non-small cell lung cancer. *J Surg Oncol* **101**, 500–506.
- Zhang P, Singh A, Yegnasubramanian S, Esopi D, Kombairaju P, Bodas M, Wu H, Bova SG, and Biswal S (2010). Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. *Mol Cancer Ther* **9**, 336–346.
- Shibata T, Ohta T, Tong KI, Kokubu A, Odogawa R, Tsuta K, Asamura H, Yamamoto M, and Hirohashi S (2008). Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc Natl Acad Sci USA* **105**, 13568–13573.
- Narisawa-Saito M, Handa K, Yugawa T, Ohno S, Fujita M, and Kiyono T (2007). HPV16 E6-mediated stabilization of ErbB2 in neoplastic transformation of human cervical keratinocytes. *Oncogene* **26**, 2988–2996.
- Malhotra D, Portales-Casamar E, Singh A, Srivastava S, Arenillas D, Happel C, Shyr C, Wakabayashi N, Kensler TW, Wasserman WW, et al. (2010). Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. *Nucleic Acids Res* **38**, 5718–5734.
- Duchesne GM (1994). Fundamental bases of combined therapy in lung cancer: cell resistance to chemotherapy and radiotherapy. *Lung Cancer* **10**(suppl 1), S67–S72.
- Coleman CN, Bump EA, and Kramer RA (1988). Chemical modifiers of cancer treatment. *J Clin Oncol* **6**, 709–733.
- Harvey CJ, Thimmulappa RK, Singh A, Blake DJ, Ling G, Wakabayashi N, Fujii J, Myers A, and Biswal S (2009). Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. *Free Radic Biol Med* **46**, 443–453.
- Ashida A, Boku N, Aoyagi K, Sato H, Tsubosa Y, Minashi K, Muto M, Ohtsu A, Ochiai A, Yoshida T, et al. (2006). Expression profiling of esophageal squamous cell carcinoma patients treated with definitive chemoradiotherapy: clinical implications. *Int J Oncol* **28**, 1345–1352.
- Kim YR, Oh JE, Kim MS, Kang MR, Park SW, Han JY, Eom HS, Yoo NJ, and Lee SH (2010). Oncogenic NRF2 mutations in squamous cell carcinomas of oesophagus and skin. *J Pathol* **220**, 446–451.

- [27] Morita S, Yano M, Tsujinaka T, Ogawa A, Taniguchi M, Kaneko K, Shiozaki H, Doki Y, Inoue M, and Monden M (1998). Association between genetic polymorphisms of glutathione *S*-transferase P1 and *N*-acetyltransferase 2 and susceptibility to squamous-cell carcinoma of the esophagus. *Int J Cancer* **79**, 517–520.
- [28] Rossini A, Rapozo DC, Soares Lima SC, Guimarães DP, Ferreira MA, Teixeira R, Krueel CD, Barros SG, Andreollo NA, Acatauassú R, et al. (2007). Polymorphisms of GSTP1 and GSTT1, but not of CYP2A6, CYP2E1 or GSTM1, modify the risk for esophageal cancer in a western population. *Carcinogenesis* **28**, 2537–2542.
- [29] Zhang J, Schulz WA, Li Y, Wang R, Zotz R, Wen D, Siegel D, Ross D, Gabbert HE, and Sarbia M (2003). Association of NAD(P)H: quinone oxidoreductase 1 (NQO1) C609T polymorphism with esophageal squamous cell carcinoma in a German Caucasian and a northern Chinese population. *Carcinogenesis* **24**, 905–909.
- [30] Wakabayashi N, Itoh K, Wakabayashi J, Motohashi H, Noda S, Takahashi S, Imakado S, Kotsuji T, Otsuka F, Roop DR, et al. (2003). Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat Genet* **35**, 238–245.
- [31] Singh A, Bodas M, Wakabayashi N, Bunz F, and Biswal S (2010). Gain of Nrf2 function in non-small-cell lung cancer cells confers radioresistance. *Antioxid Redox Signal* **13**, 1627–1637.
- [32] Fareed KR, Kaye P, Soomro IN, Ilyas M, Martin S, Parsons SL, and Madhusudan S (2009). Biomarkers of response to therapy in oesophago-gastric cancer. *Gut* **58**, 127–143.
- [33] Höfler H, Langer R, Ott K, and Keller G (2006). Prediction of response to neoadjuvant chemotherapy in carcinomas of the upper gastrointestinal tract. *Adv Exp Med Biol* **587**, 115–120.
- [34] Luthra R, Luthra MG, Izzo J, Wu TT, Lopez-Alvarez E, Malhotra U, Choi IS, Zhang L, and Ajani JA (2006). Biomarkers of response to preoperative chemoradiation in esophageal cancers. *Semin Oncol* **33**(6 suppl 11), S2–S5.
- [35] Kihara C, Tsunoda T, Tanaka T, Yamana H, Furukawa Y, Ono K, Kitahara O, Zembutsu H, Yanagawa R, Hirata K, et al. (2001). Prediction of sensitivity of esophageal tumors to adjuvant chemotherapy by cDNA microarray analysis of gene-expression profiles. *Cancer Res* **61**, 6474–6479.
- [36] Luthra R, Wu TT, Luthra MG, Izzo J, Lopez-Alvarez E, Zhang L, Bailey J, Lee JH, Bresalier R, Rashid A, et al. (2006). Gene expression profiling of localized esophageal carcinomas: association with pathologic response to preoperative chemoradiation. *J Clin Oncol* **24**, 259–267.
- [37] Maher SG, Gillham CM, Duggan SP, Smyth PC, Miller N, Muldoon C, O’Byrne KJ, Sheils OM, Hollywood D, and Reynolds JV (2009). Gene expression analysis of diagnostic biopsies predicts pathological response to neoadjuvant chemoradiotherapy of esophageal cancer. *Ann Surg* **250**, 729–737.
- [38] Murat A, Migliavacca E, Gorlia T, Lambiv WL, Shay T, Hamou MF, de Tribolet N, Regli L, Wick W, Kouwenhoven MC, et al. (2008). Stem cell-related “self-renewal” signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. *J Clin Oncol* **26**, 3015–3024.

Table W1. Primers and Antibodies Used in This Study.

Gene Name	Forward PCR Primer	Reverse PCR Primer	Roche Universal, No.
<i>GCLC</i>	ggatgatgctaagtgtctgacc	tctactctccatcaatgtrctgag	25
<i>GSR</i>	aacaacatcccaactgtggctc	ccatattatgaatggcttcatctt	83
<i>GAPDH</i>	ccaaccgcgagaagatga	ccagaggcgtacaggatag	64

Antibodies Used in This Study			
Antigen	Vendor	Clone No.	Dilution for Immunoblot
NRF2	Santa Cruz	Polyclonal	×500
β-Actin	Sigma	Monoclonal	×1000
Lamin B1	Santa Cruz	Polyclonal	×500

Table W2. Pathologic Diagnosis of the Analyzed Sarcoma Cases.

Case No.	Histology
028-L	Leiomyosarcoma
029-L	Leiomyosarcoma
032-L	Leiomyosarcoma
034-L	Leiomyosarcoma
035-L	Leiomyosarcoma
037-L	Leiomyosarcoma
038-L	Leiomyosarcoma
039-L	Leiomyosarcoma
040-L	Leiomyosarcoma
093-mL	Liposarcoma, myxoid
094-mL	Liposarcoma, myxoid
095-mL	Liposarcoma, myxoid
096-mL	Liposarcoma, myxoid
097-mL	Liposarcoma, myxoid
098-mL	Liposarcoma, myxoid
099-mL	Liposarcoma, myxoid
100-mL	Liposarcoma, myxoid
101-mL	Liposarcoma, myxoid
102-mL	Liposarcoma, myxoid
103-mL	Liposarcoma, myxoid
105-mL	Liposarcoma, myxoid
106-mL	Liposarcoma, myxoid
108-mL	Liposarcoma, myxoid
020-M	MPNST
022-M	MPNST
023-M	MPNST
024-M	MPNST
025-M	MPNST
026-M	MPNST
056-mM	Myxoid MFH
057-mM	Myxoid MFH
060-mM	Myxoid MFH
062-mM	Myxoid MFH
063-mM	Myxoid MFH
065-mM	Myxoid MFH
066-mM	Myxoid MFH
067-mM	Myxoid MFH
068-mM	Myxoid MFH
069-mM	Myxoid MFH
070-mM	Myxoid MFH
073-pM	Pleomorphic MFH
074-pM	Pleomorphic MFH
075-pM	Pleomorphic MFH
077-pM	Pleomorphic MFH
078-pM	Pleomorphic MFH
079-pM	Pleomorphic MFH

Table W2. (continued)

Case No.	Histology
080-pM	Pleomorphic MFH
081-pM	Pleomorphic MFH
082-pM	Pleomorphic MFH
083-pM	Pleomorphic MFH
084-pM	Pleomorphic MFH
085-pM	Pleomorphic MFH
086-pM	Pleomorphic MFH
087-pM	Pleomorphic MFH
088-pM	Pleomorphic MFH
RMS-16	Rhabdomyosarcoma
RMS-17	Rhabdomyosarcoma
RMS-18	Rhabdomyosarcoma
043-S	Synovial sarcoma
044-S	Synovial sarcoma
045-S	Synovial sarcoma
046-S	Synovial sarcoma
049-S	Synovial sarcoma
051-S	Synovial sarcoma
052-S	Synovial sarcoma
054-S	Synovial sarcoma
055-S	Synovial sarcoma
SS1	Synovial sarcoma
SS11	Synovial sarcoma
SS12	Synovial sarcoma
SS13	Synovial sarcoma
SS14	Synovial sarcoma
SS2	Synovial sarcoma
SS3	Synovial sarcoma
SS4	Synovial sarcoma
SS5	Synovial sarcoma
SS6	Synovial sarcoma
SS7	Synovial sarcoma
SS8	Synovial sarcoma
SS9	Synovial sarcoma
OS-41	Osteosarcoma
OS-44	Osteosarcoma
OS-45	Osteosarcoma
OS-46	Osteosarcoma
OS-47	Osteosarcoma
OS-49	Osteosarcoma
ES-01	Epithelioid sarcoma
ES-02	Epithelioid sarcoma
ES-04	Epithelioid sarcoma
ES-05	Epithelioid sarcoma
ES-06	Epithelioid sarcoma
ES-10	Epithelioid sarcoma

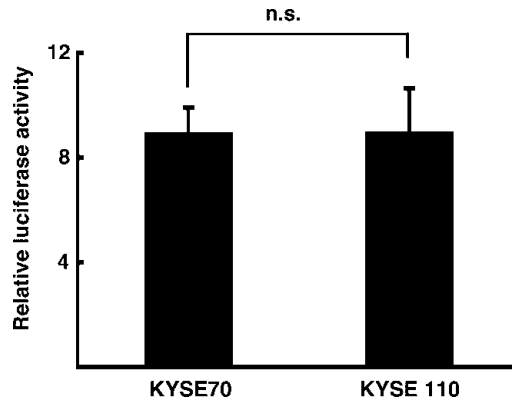


Figure W1. NRF2-dependent transcriptional activity in homozygous (KYSE70) and heterozygous (KYSE110) cells. n.s. indicates not significant.

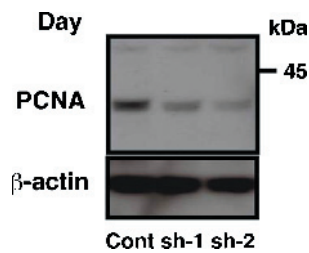


Figure W2. The expression of proliferating cell nuclear antigen (PCNA) in control (Cont) and *NRF2* (sh-1 and sh-2) shRNA expression clones. Protein extracted from each clone were immunoblotted with anti-PCNA mouse antibody (clone PC10; Cell Signaling Technologies, Danvers, MA). β -Actin was used as a loading control. Molecular marker is indicated on the right (kDa).

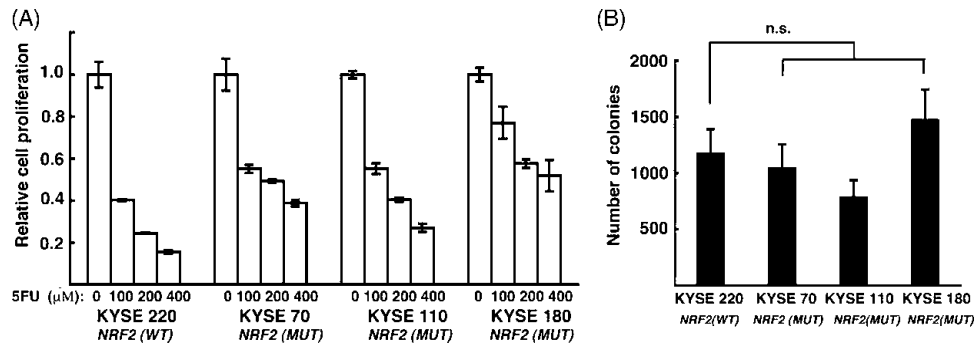


Figure W3. 5-FU sensitivity and basal colony formation capacity of *NRF2* wild-type (KYSE220) and mutated (KYSE70, KYSE110, KYSE180) ESC cell lines. (A) ESC cells were treated with various concentrations of 5-FU, and relative cell proliferation was shown. (B) Number of colony formation of each ESC cell was shown.

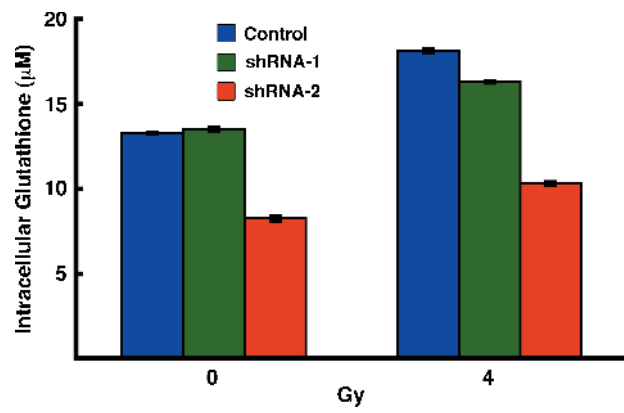


Figure W4. KYSE70 clones were irradiated (4 Gy), and the amount of intracellular reduced glutathione was measured after 24 hours.