

Immunogenic Tumor Cell Death Induced by Chemoradiotherapy in Patients with Esophageal Squamous Cell Carcinoma

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Abstract

Although it has been shown that chemoradiotherapy may induce immunogenic cell death, which could trigger T-cell immunity mediated by high-mobility group box 1 protein (HMGB1) and calreticulin, there is still limited information to support this theory directly in a clinical setting. In the present study, we evaluated antigen-specific T-cell responses against six cancer-testis antigens in peripheral blood lymphocytes from patients with esophageal squamous cell carcinoma (ESCC) receiving chemoradiation. Expression of HMGB1 and calreticulin within tumor microenvironment was also analyzed in resected samples with and without chemoradiotherapy in relation to patients survival. Tumor antigen-specific T-cell responses were confirmed in six (38%) of 16 patients with ESCC after chemoradiotherapy coexisting with elevated serum HMGB1. In addition, HMGB1 within tumor microenvironment was significantly upregulated in patients with ESCC with preoperative chemoradiotherapy, but not in those without chemoradiotherapy, and the degree of HMGB1 positively correlated with patient survival ($n = 88$). Both irradiation and chemotherapeutic drugs induced upregulation of HMGB1 and calreticulin in nine ESCC cell lines. Furthermore, HMGB1 was able to induce maturation of dendritic cells. Together, our findings indicate that chemoradiation induces tumor antigen-specific T-cell responses, and HMGB1 production is related to clinical outcome after chemoradiation. *Cancer Res*; 72(16); 3967–76. ©2012 AACR.

Introduction

Esophageal squamous cell carcinoma (ESCC) is well known to be sensitive to radiotherapy and its combination with chemotherapy was proven to have clinical benefits (1, 2). It is generally accepted that irradiation is able to directly induce apoptosis or necrosis against ESCC and various strategies of radiotherapy, such as hyperfractionated irradiation (3) and image-guided radiotherapy (4), to enhance direct cytotoxic and cytostatic effects against ESCC through radiation oncology (5, 6). However, there is accumulating evidence to support the novel concept that radiotherapy with/without chemotherapy may induce immunogenic cell death, which could trigger uptake of antigenic components by dendritic cells (DC) and

transfer antigenic signals to T-cell-mediated immunity, resulting in the expansion of antigen-specific CTLs and production of tumor-specific monoclonal antibodies (mAb) in murine models (7–14). It has been shown that the collaboration between the direct effects of irradiation and enhanced tumor-specific immunity induced by the irradiation could reject inoculated live tumors in murine models, where irradiation alone was not able to reject tumors (7, 8). Important mediators that induce immunogenic cell death include high-mobility group box 1 protein (HMGB1) and calreticulin induced by chemoradiation. For example, HMGB1 released from tumor cells treated with chemoradiation could enhance engulfment of antigenic components by DCs through Toll-like receptor 4 (TLR4) and mediate cross-presentation of tumor antigens into CD4 and CD8 T cells (7–13). Also, early membrane exposure of calreticulin induced by irradiation could enhance phagocytosis of dying tumor cells by DCs (13, 15, 16), and calreticulin is related to autophagy-dependent anticancer immune responses induced by chemotherapeutic agents (17).

However, there is limited information describing whether immunogenic cell death could be induced by radiotherapy and/or chemotherapy in clinical settings, probably due to the lack of accurate assay systems to evaluate antigen-specific T-cell responses in patients with cancer receiving chemoradiotherapy. We have recently identified several novel HLA class I-restricted epitopes presented on ESCC and recognized by tumor-specific T cells through the genome-wide approach (18–20). Our gene expression profile data indicated that

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cancer-testis antigens such as TTK protein kinase (*TTK*), lymphocyte antigen 6 complex locus K (*LY6K*), cell division cycle associated 1 (*CDCA1*), 34 kDa translocase of the outer mitochondrial membrane (*TOMM34*), hypoxia-inducible protein 2 (*HIG2*), and insulin-like growth factor (IGF)-II mRNA-binding protein 3 (*IMP3*) were highly expressed in ESCC (18–25). Peptides derived from these cancer-testis antigens could stimulate CTL that recognize and kill ESCC cells endogenously expressing these antigens (18, 19, 23). Furthermore, we have established a reliable *in vitro* assay system using peripheral blood lymphocytes (PBL) to detect tumor-specific CTL responses against the panels of HLA class I epitopes derived from these cancer-testis antigens (18, 19). Therefore, it is possible to analyze antigen-specific T-cell responses induced by chemoradiotherapy and evaluate the phenomenon immunogenic cell death in patients with ESCC receiving chemoradiotherapy. There is, to our knowledge, no previous report to directly prove antigen-specific T-cell responses following chemoradiotherapy in clinical settings.

In the present study, we evaluated (i) whether chemoradiotherapy could induce antigen-specific T-cell responses in patients with ESCC, (ii) whether chemoradiotherapy could upregulate HMGB1 and calreticulin in *in vivo* and *in vitro* settings, and (iii) whether expression of HMGB1 and calreticulin correlate to clinical outcomes.

Patients and Methods

Patients and samples

Study group 1. Sixteen *HLA-A2402*(+) or *HLA-A0201*(+) patients with histologically diagnosed primary ESCC who were treated at the Department of Radiation Oncology, Gunma University and the First Department of Surgery, University of Yamanashi Hospital were enrolled in this study. Patients with ESCC receiving chemoradiotherapy (15 men and 1 woman, 64.5 ± 10.2 years old) were 4 patients in stage II, 6 patients in stage III, and 6 patients in stage IV according to the tumor-node-metastasis (TNM) classification for esophageal cancers (UICC, 5th edition). None of the patients had received any treatment before this study. Enrolled patients received definitive concurrent chemoradiotherapy [2 Gy \times 30–33 = total 60–66 Gy and 5-fluorouracil (5FU)/CDGP or CDDP/docetaxel-based chemotherapy]. To evaluate the tumor antigen-specific T-cell response after chemoradiation, PBLs were taken from patients during prechemoradiotherapy periods (within 1 week before chemoradiotherapy), during chemoradiotherapy (21 ± 3 days after the initiation of chemoradiotherapy), postchemoradiotherapy day 3 (within 3 days after the termination of chemoradiotherapy), and postchemoradiotherapy day 30 (30 ± 3 days after the termination of chemoradiotherapy). PBLs were isolated with Ficoll-Paque Plus (GE Healthcare Bio-sciences), density gradient solution, and frozen in aliquots. This study and the use of all clinical materials were approved by the individual Institutional Ethical Committees, and written informed consent was obtained from all participants.

Study group 2. Formalin-embedded tumor samples from a total of 88 patients with ESCC were evaluated in study

group 2. The clinical background of patients receiving preoperative chemoradiotherapy ($n = 45$, 40 Gy irradiation + concurrent 5Fu/CDDP/docetaxel chemotherapy) and patients without preoperative chemoradiotherapy ($n = 43$) were shown in Supplementary Table S1. This study and the use of all clinical materials were approved by the Institutional Ethical Committee, University of Yamanashi (Yamanashi, Japan), and written informed consent was obtained from all participants.

Cell lines and transfection

ESCC cell lines, TE-1, TE-2, TE-3, TE-4, and TE-5 were obtained from the University of Tohoku cell bank (Institute of Development, Aging and Cancer, University of Tohoku, Sendai, Japan). ESCC cell lines, KYSE-30, KYSE-50, KYSE-70, and KYSE110 were purchased from the Health Science Research Resources Bank (Osaka, Japan). COS-7 cells were obtained from the American Type Culture Collection. The experiments were carried out within less than 6 months after receipt from the cell bank. Cell lines were regularly authenticated and matched short tandem repeat DNA profiles of the original cell lines in each cell bank. All cell lines were maintained in RPMI-1640 with 5% fetal calf serum (FCS), 50 U/mL penicillin, and 2 mmol/L L-glutamine.

COS-7 cells were cotransfected with vectors expressing *TTK*, *LY6K*, *IMP3*, *CDCA1*, *TOMM34*, and *HIG2* in the combination of *HLA-A2402* or *HLA-A0201* using FuGENE6 (Roche) reagents (18). Thereafter, 3×10^5 cells per well were incubated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) with 10% FCS and 1% anti-mycobiotics (Sigma-Aldrich) in 6-well flat bottom plates (Becton Dickinson Labware). Two days after culture, cells were used as target cells in enzyme-linked immunospot (ELISPOT) assays as described later.

In vitro T-cell culture and ELISPOT assay

In study group 1, PBLs (5×10^5 cells per well) obtained from different time points were simultaneously thawed and cultured in X-VIVO15 medium with 100 U/mL rIL-2 (Peprotech) in 48-well flat bottom plates (Becton Dickinson Labware) for 14 days without any antigen stimulation. On day 15, cultured cells were subjected to ELISPOT assays as responder cells.

To detect the antigen-specific T-cell response, an ELISPOT assay was conducted with the human IFN- γ ELISPOT kit (Mabtech). Ninety-six-well plates with nitrocellulose membranes (Millipore) were precoated with primary anti-IFN- γ antibody (1-D1K) at 4°C overnight. Plates were then prereacted with X-VIVO15 containing 1% human serum albumin (Sigma-Aldrich). COS-7 cells (2×10^3 per well) transfected with individual genes (see earlier) and responder cells (2×10^3 per well) were mixed and incubated in a final volume of 200 μ L per well of X-VIVO15 for 24 hours in triplicate. Cell mixtures were treated with biotinylated secondary anti-IFN- γ antibody (7-B6-1) and incubated for 2 hours. Plates were then incubated with horseradish peroxidase reagent and stained with TMB (Mabtech). Spots were quantified with the autoanalyzing system, KS ELISPOT Compact (ZEISS). Antigen-specific spots were the average of triplicates by subtracting target wells transfected with HLA-A molecule alone from target wells transfected with HLA-A molecule + tumor antigen molecules.

Positivity of the tumor antigen-specific T-cell response was defined when the number of antigen-specific spots was ≥ 200 . We defined the assessable lower limit of spots per well as ≥ 20 spots.

Immunohistochemistry

In study group 2, HMGB1-, calreticulin-, CD8- and Foxp3-immunostainings were conducted using the avidin-biotin-peroxidase complex method. For HMGB1 and calreticulin, each paraffin section was dewaxed, followed by antigen retrieval with Target Retrieval Solution (10 mmol citrate buffer at pH 6.0; Dako) in an autoclave (121°C, 15 minutes). Sections were cooled at room temperature for 30 minutes and endogenous peroxidase was blocked with 3% hydrogen peroxide. Thereafter, sections were incubated with diluted normal blocking serum for 20 minutes and incubated with rabbit anti-human HMGB1 mAb (Clone 2F6, Sigma-Aldrich, 3 $\mu\text{g}/\text{mL}$) overnight at 4°C or with mouse anti-human calreticulin mAb (Abcam, 5 $\mu\text{g}/\text{mL}$) for 2 hours at 37°C. Thereafter, peroxidase-linked secondary antibodies (Dako) and 3,3'-diaminobenzidine (DAB) were used to detect specific binding.

For Foxp3 staining, each paraffin section was dewaxed, followed by antigen retrieval with Epitope Retrieval Solution (10 mmol citrate buffer at pH 6.0, Dako) in a preheated water bath (98°C, 40 minutes), and endogenous peroxidase was blocked by Chemmate Peroxidase Blocking Solution (Dako). Then, biotinylated anti-human Foxp3 mAb (diluted by PBS at 1:20; eBioscience) was applied for 40 minutes at room temperature. Thereafter, peroxidase-linked secondary antibodies and DAB were used to detect specific binding.

For CD8 staining, each paraffin section was dewaxed, followed by antigen retrieval with Target Retrieval Solution (10 mmol citrate buffer at pH 6.0; Dako) with an autoclave (121°C, 15 minutes). The sections were cooled at room temperature for 30 minutes and endogenous peroxidase was blocked with 3% hydrogen peroxide. Thereafter, the sections were incubated with diluted normal blocking serum for 20 minutes, and incubated with diluted anti-human-CD8-mAb, Clone C8/144B (Dako), overnight at 4°C. Thereafter, peroxidase-linked secondary antibodies and DAB were used to detect specific binding.

Quantitative evaluation of HMGB1- and calreticulin (membranous + intracellular)-positive cancer cells and tumor-infiltrating CD8- and Foxp3-positive cells was carried out in serial sections using 5 randomly selected areas at a magnification of $\times 400$ by 2 observers (S. Izawa and K. Kono) in a blinded manner. The grade of HMGB1 and calreticulin expression was classified into HMGB1-strong and HMGB1-weak or calreticulin-strong and calreticulin-weak groups based on the median value of the counts of positive cancer cells.

In vitro treatments of ESCC with irradiation and chemotherapeutic agents

Nine ESCC cell lines ($8 \times 10^4/\text{mL}$), TE-1, TE-2, TE-3, TE-4, TE-5, KYSE-30, KYSE-50, KYSE-70, and KYSE110 were incubated with 5% RPMI medium in 48-well plates and treated with 10 Gy irradiation on days 1, 2, and 3 (total = 30 Gy) or with combination chemotherapy of 5FU (2.5–10 ng/mL) + CDDP (5–20 ng/mL) + docetaxel (25–100 ng/mL) on day 1. Dying cells

were analyzed by Annexin-V (BD Pharmingen) and 7-aminoactinomycin D (7-AAD; BD Pharmingen) by flow cytometry, and the proportion of dying cells was determined by either Annexin-V (+) or 7-AAD (+) cells. Supernatants of treated ESCC cultures were measured for HMGB1 contents by ELISA (Shinotest) and cell surface expression of calreticulin was evaluated by flow cytometry with R-phycoerythrin (RPE)-conjugated anti-calreticulin mAb (Enzo life Sciences).

Treatment of DCs with HMGB1

Peripheral blood mononuclear cells (PBMC) from healthy volunteers ($n = 5$) were separated by a Ficoll-Paque Plus density gradient, and monocytes were separated from PBMCs by plastic adherences. Monocytes ($3.0 \times 10^5/\text{mL}$) were cultured in 6-well flat bottom plates (Becton Dickinson Biosciences) with 500 units/mL of granulocyte macrophage colony-stimulating factor (GM-CSF; Peprotech EC) and 500 units/mL of interleukin (IL)-4 (Peprotech) in X-VIVO. On day 5, immature DCs were treated with recombinant HMGB-1 (10 $\mu\text{g}/\text{mL}$, R&D Systems) or 10 $\mu\text{g}/\text{mL}$ of OK-432 (Chugai). On day 7, immature DCs, HMGB1-treated DCs, and OK-432-treated DCs were analyzed with fluorescence-activated cell sorting (FACS) using anti-human HLA-ABC fluorescein isothiocyanate (FITC) mAb (eBioscience), FITC-mouse anti-human HLA-DR, -DP, -DQ mAb (BD Pharmingen), FITC-mouse anti-human CD80 mAb (BD Pharmingen), APC-mouse anti-human CD83 mAb (BD Pharmingen), and PE-mouse anti-human CD86 mAb (BD Pharmingen).

Statistical analysis

Differences between values were determined using the paired and nonpaired Student *t* test. Overall survival (OS), which was measured in days from surgery to death or from chemoradiotherapy to death, was analyzed by the Kaplan-Meier method, and statistical differences were analyzed by the log-rank method. All statistical analyses were conducted with SPSS statistics 17.0 (SPSS Inc.). Significance was considered at $P < 0.05$.

Results

Antigen-specific T-cell response after chemoradiotherapy

In study group 1, PBLs obtained from before, during, and after chemoradiotherapy were cultured for 14 days *in vitro* and cultured T cells were subjected to an IFN- γ ELISPOT assay as responder cells.

As shown in Fig. 1A, a representative ELISPOT assay [case 10, HLA-A0201 (+) and HLA-A2402 (-)] indicated that *in vitro* cultured T cells from postchemoradiation day 3 significantly reacted with COS transfected with *IMP3* + *HLA-A0201* or *CDCA1* + *HLA-A0201* and did not in those transfected with *HLA-A0201* alone. In summarized data from case 10 at different time points, antigen-specific T-cell responses were significantly induced against COS transfected with *IMP3*, *CDCA1*, *TOMM34*, and *HIG2* in combination with *HLA-A0201* during chemoradiotherapy (Fig. 1B), whereas there was no specific T-cell response before chemoradiotherapy. These antigen-

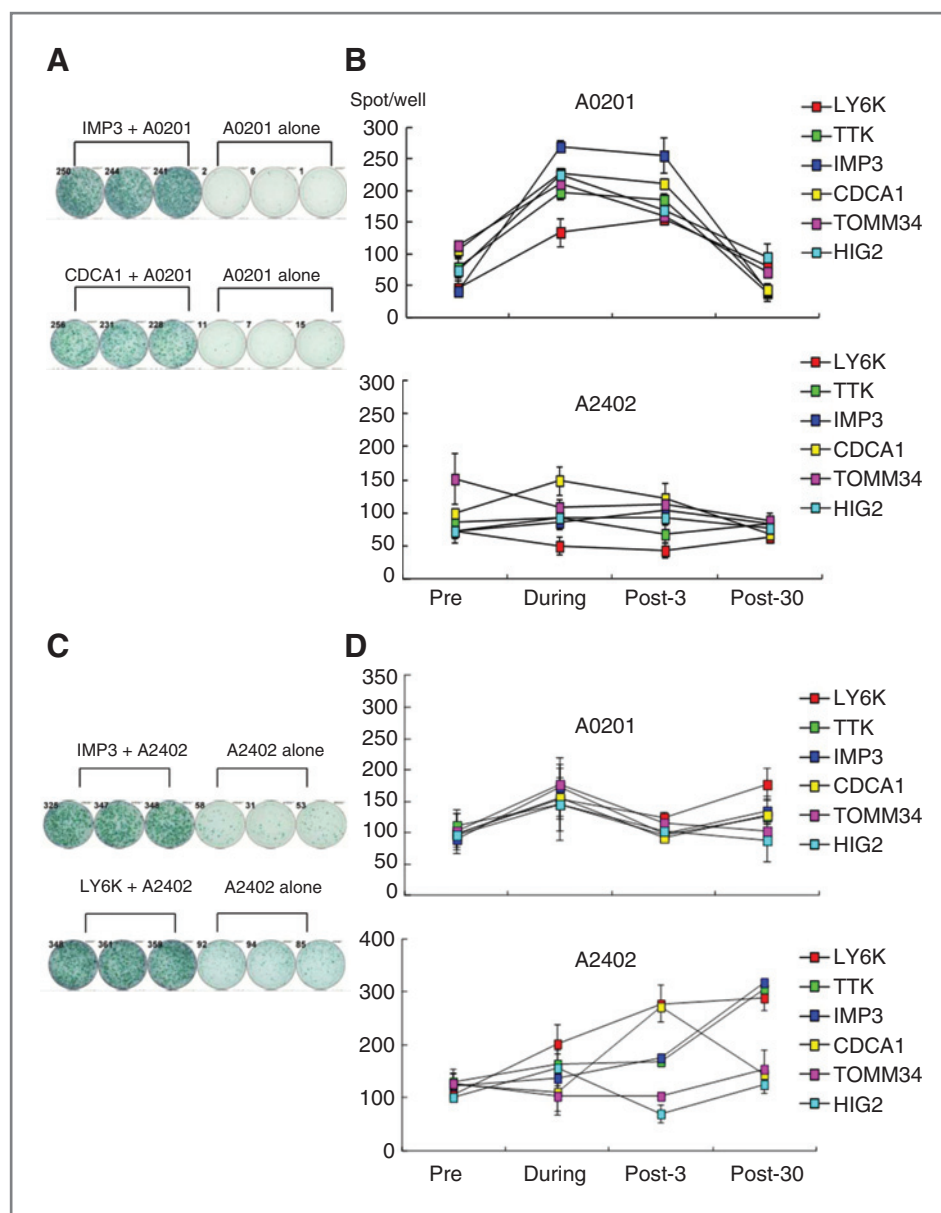


Figure 1. Tumor antigen-specific T-cell responses after chemoradiotherapy. In study group 1, PBLs obtained before (pre), during (during), and after (post-3 and post-30) chemoradiotherapy and COS-7 cells transfected with tumor antigens (TTK, LY6K, IMP-3, CDCA1, TOMM34, and HIG2) + HLA-A molecules (HLA-A2402 or HLA-A0201) were subjected to IFN- γ ELISPOT assays. A representative ELISPOT assay [case 10, HLA-A0201 (+) and HLA-A2402 (-)] is shown on postchemoradiotherapy day 3 (A) and summarized data from case 10 at different time points are shown (B). A representative ELISPOT assay [case 2, HLA-A0201 (-) and HLA-A2402 (+)] is shown on postchemoradiotherapy day 30 (C) and summarized data in case 2 at different time points are shown (D). Pre, prechemoradiation periods; during, during chemoradiation; post-3, postchemoradiation day 3; and post-30, postchemoradiation day 30.

specific T-cell responses tended to last until postchemoradiation day 3, although these specificities diminished postchemoradiation day 30 (Fig. 1B). Moreover, there was no specific T-cell response against COS transfected with target genes + HLA-A2402 before and after chemoradiation, in line with the patient being HLA-A2402 negative (Fig. 1B). Thus, the results indicated that the tumor antigen-specific T-cell response was induced by chemoradiotherapy in a patient with ESCC.

As shown in Fig. 1C, a representative ELISPOT assay [case 2, HLA-A0201 (-) and HLA-A2402 (+)] indicated that *in vitro* cultured T cells from postchemoradiation day 30 significantly reacted with COS transfected with IMP3 + HLA-A2402 or LY6K + HLA-A2402 and did not in those transfected with HLA-A2402 alone. In summarized data from case 2 at different time points, antigen-specific T-cell responses were significantly induced

against COS transfected with CDCA1 or LY6K in combination with HLA-A2402 at postchemoradiation day 3 and against COS transfected with IMP3, TTK, or LY6K in combination with HLA-A2402 at postchemoradiation day 30 (Fig. 1D), whereas there was no specific T-cell response before chemoradiation. Moreover, there was no specific T-cell response against COS transfected with target genes + HLA-A0201, in line with this patient being HLA-A0201-negative (Fig. 1D).

Taken together, the results of 6 (38%) of 16 patients in study group 1 revealed that antigen-specific T-cell responses were significantly induced by chemoradiotherapy (Table 1). When OS was compared between the patients with ($n = 6$) and without ($n = 10$) antigen-specific T-cell responses, there was no significant difference in the OS between them ($P = 0.212$, Supplementary Fig. S1).

Table 1. Antigen-specific T-cell responses following chemoradiotherapy

Patient	HLA-A		Tumor-specific antigens					
	A2402	A0201	LY6K	TTK	IMP3	CDCA1	TOMM34	HIG2
1	*							
2	*		+	+	+	+		
3		*	+		+			
4	*							
5	*		+					
6	*							
7		*						
8	*							
9	*							
10		*			+	+	+	+
11	*	*						
12		*						
13	*							
14	*							
15	*		+	+	+			
16	*		+	+				

NOTE: *, HLA-A allele type in the patients with ESCC receiving chemotherapy; +, the positive T-cell responses specific for the indicated antigens evaluated by ELISPOT assay in patients' peripheral blood leucocytes following chemoradiotherapy.

Serum HMGB1 levels after chemoradiotherapy

Because HMGB1 is thought to be one of the mediators linking chemoradiation-induced cell death to antigen-specific T-cell responses, serum HMGB1 levels ($n = 14$) were evaluated before and after chemoradiotherapy (postchemoradiation day 3) in study group 1. As shown in Fig. 2A, increased levels of HMGB1 were significantly observed after chemoradiotherapy,

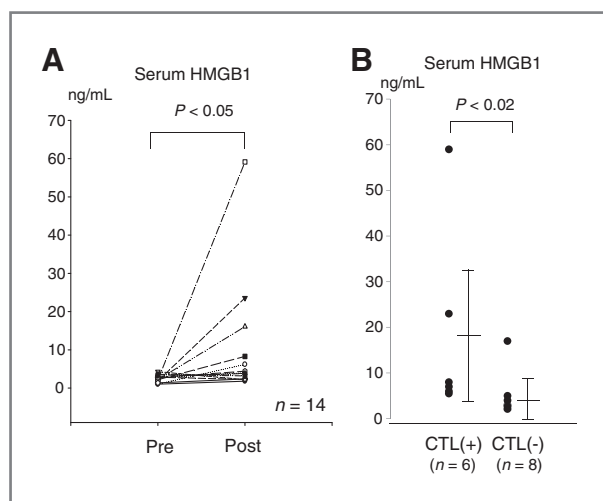


Figure 2. Serum HMGB1 level in the patients receiving chemoradiotherapy. A, serum from patients ($n = 14$) at prechemoradiation periods (pre) and postchemoradiation third day (post) were analyzed by ELISA assays specific for HMGB1. B, serum levels of HMGB1 at postchemoradiation third day are compared between the patients with [CTL(+)] and without antigen-specific T-cell responses [CTL(-)] evaluated by ELISPOT assay.

whereas there was no detectable level of HMGB1 before chemoradiotherapy. There was no significant correlation between the amount of serum HMGB1 at postchemoradiation day 3 and number of spot-forming cells in ELISPOT assay against each antigen, which is corresponding to the grade of tumor antigen-specific T-cell responses (Supplementary Fig. S2). However, when the amount of serum HMGB1 was compared between the patients with and without antigen-specific T-cell responses following chemoradiation, the level of HMGB1 in the patients with antigen-specific T-cell responses was significantly elevated in comparison to that in the patients without antigen-specific T-cell responses (18.1 ± 14.1 vs. 5.1 ± 4.9 ng/mL, Fig. 2B), suggesting that HMGB1 produced by chemoradiation play an important role in inducing systemic tumor antigen-specific T-cell responses.

HMGB1 and calreticulin expression induced by chemoradiotherapy

To evaluate HMGB1 and calreticulin expression within tumor microenvironments induced by chemoradiotherapy, immunohistochemical analysis was conducted in resected tumor samples from study group 2 between patients with ($n = 45$) and without preoperative chemoradiotherapy ($n = 43$). There was no difference in the background of patients except for the grade of lymph node dissection (Supplementary Table S1).

Representative immunostainings using anti-HMGB1 and anti-calreticulin mAbs in serial sections are shown in Fig. 3. The grade of HMGB1 and calreticulin expression was classified into HMGB1-strong (Fig. 3A) and HMGB1-weak (Fig. 3B) or calreticulin-strong (Fig. 3B) and calreticulin-weak (Fig. 3A)

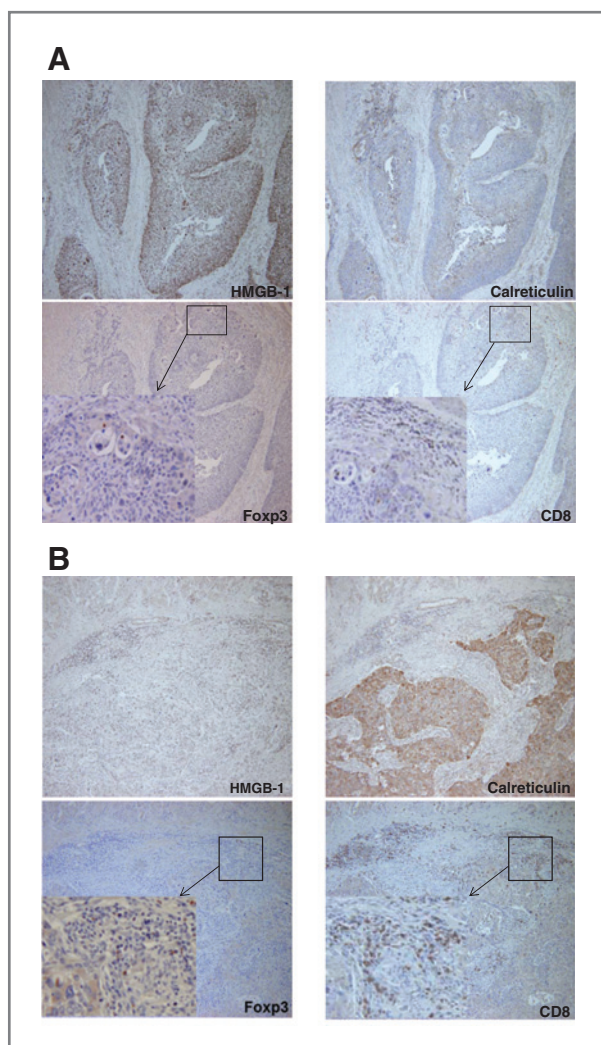


Figure 3. Representative immunostainings for HMGB1, calreticulin, CD8 (+) cells, and regulatory T cells. Representative immunostainings using anti-HMGB1, anti-calreticulin, anti-CD8, and anti-Foxp3 mAbs in serial sections from 2 different patients are shown in A and B.

groups based on the median value of the counts of positive tumor cells. Of note, the proportion of HMGB1-strong patients was significantly more predominant in the group with preoperative chemoradiotherapy than that without preoperative chemotherapy (Table 2). On the other hand, the distribution of calreticulin-strong and calreticulin-weak did not differ between groups with and without preoperative chemoradiotherapy (Table 2). Thus, it is suggested that chemoradiation upregulated HMGB1 expression, but not calreticulin expression, more progressively in tumor microenvironments with ESCC.

To further evaluate the significance of HMGB1 in relation to T-cell immunity, the tumor-infiltrating grade of CD8- and regulatory T-cells were analyzed by immunohistochemistry in serial sections and compared between HMGB1-strong and -weak groups. Representative immunostainings using anti-CD8 and anti-Foxp3 mAbs are shown in Fig. 3A and B. As shown in Fig. 4B, summarized data indicated that there was a tendency of increased infiltrating CD8 (+) cells in the HMGB1-strong group (189 ± 92 cells per field) in comparison to those in the HMGB1-weak group (140 ± 62 cells per field), although the difference did not reach a significant level ($P = 0.065$). There was no difference in the infiltrating grade of regulatory T cells (Foxp3-positive cells) between HMGB1-strong and -weak groups (Fig. 4A). Moreover, when the infiltrating grade of CD8 (+) cells was compared between the calreticulin-strong and -weak groups, there was no significant difference (153 ± 79 vs. 149 ± 89 cells per field, respectively).

HMGB1 and calreticulin expression relating to the survival

As shown in Fig. 5A, OS in the HMGB1-strong group was significantly superior to that in the HMGB1-weak group in total patients ($n = 88$), patients without preoperative chemoradiotherapy ($n = 43$), and patients with preoperative chemoradiotherapy ($n = 45$).

On the other hand, there were no significant differences in OS between calreticulin-strong and calreticulin-weak groups in total patients, patients without preoperative chemoradiotherapy, and patients with preoperative chemoradiotherapy (Fig. 5B).

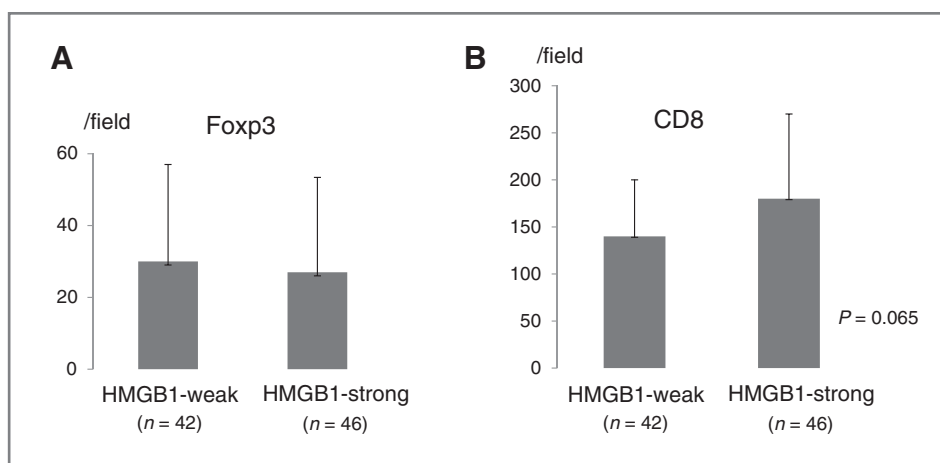


Figure 4. Immunohistochemical analysis for CD8 (+) cells and regulatory T cells. Immunohistochemical analysis was conducted in resected tumor samples from study group 2. The grade of and regulatory T cells (Foxp3, A) and tumor-infiltrating CD8 (B) are shown.

Table 2. HMGB1 and calreticulin expressions on tumors between patients with and without preoperative chemoradiotherapy

HMGB-1 (n = 88)	HMGB-1-weak (n = 42)	HMGB-1-strong (n = 46)	
Operation alone (n = 43)	26	17	
Operation + NeocRT (n = 45)	16	29	$P = 0.04$ (χ^2 test)
Calreticulin (n = 88)	Calreticulin-weak (n = 47)	Calreticulin-strong (n = 41)	
Operation alone (n = 43)	23	20	
Operation + NeocRT (n = 45)	24	21	$P = 0.85$ (χ^2 test)

NOTE: HMGB1 and calreticulin expressions on tumors between patients with preoperative chemotherapy (n = 45, operation + NeocRT) and without preoperative chemoradiotherapy (n = 43, operation alone) are shown.

Thus, upregulation of HMGB1 may be one of the phenotypes of ESCC with better survival.

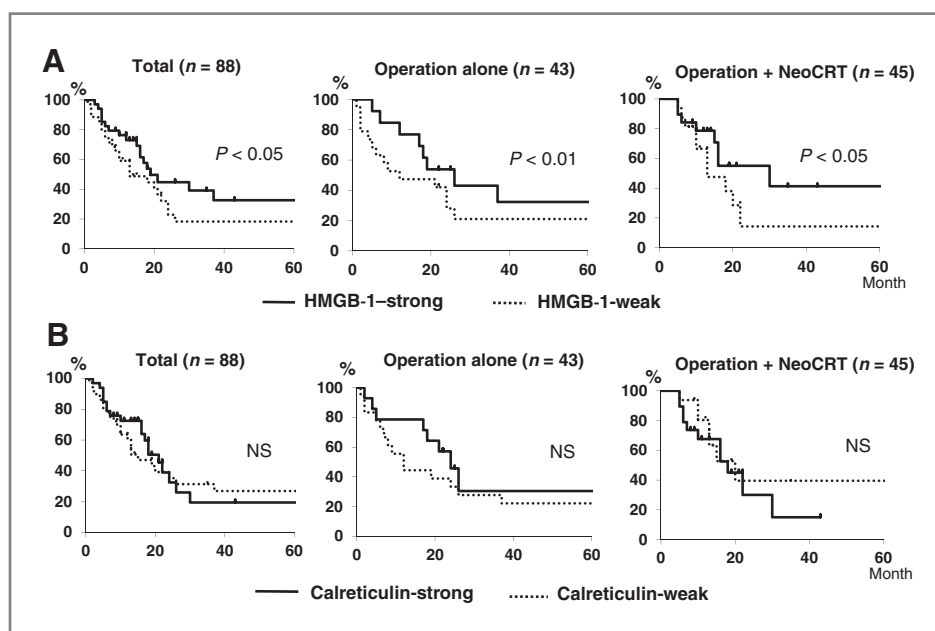
In vitro treatment of ESCC with anticancer drugs or irradiation

To further evaluate HMGB1 and calreticulin expression following irradiation and chemotherapy, 9 ESCC tumor cell lines were treated with 30 Gy irradiation or a combination of 5FU + CDDP + docetaxel *in vitro* and, the production of HMGB1 and surface expression of calreticulin, along with the proportion of dying cells, were analyzed. There was no significant amount of HMGB1 in supernatants from any ESCC without irradiation or chemotherapy (Supplementary Fig. S3). As shown in Fig. 6 and Supplementary Fig. S3, irradiation could induce variable levels of HMGB1 production depending on the cell line, for example, TE2 and KYSE70 produced substantial amounts of HMGB1 after irradiation whereas TE4

and KYSE110 did not produce HMGB1 even though these cell lines underwent cell death by irradiation (the proportion of Annexin-V (+) or 7-AAD (+) cells were 75%–95%). Similarly, chemotherapeutic drugs induced different levels of HMGB1 production among 9 ESCCs, however, the production levels of HMGB1 were relatively less than those induced by irradiation regardless of almost the same amount of dying cells (Fig. 6 and Supplementary Fig. S3).

With respect to calreticulin expression, both irradiation and chemodrugs could upregulate surface calreticulin more than the nontreatment controls (Supplementary Figs. S3 and S4). As shown in Fig. 6 and Supplementary Fig. S3, irradiation could upregulate calreticulin depending on the cell line, for example, TE4 and KYSE30 revealed increased levels of calreticulin after irradiation, whereas the others did not show a dramatic upregulation. Again, chemodrugs could induce increased calreticulin expression among 9 ESCCs, however, upregulated

Figure 5. HMGB1 and calreticulin expression relating to survival. OS in study group 2 are shown in total patients (n = 88, total), patients without preoperative chemoradiotherapy (n = 43, operation alone), and patients with preoperative chemoradiotherapy (n = 45, operation + NeocRT) in terms of HMGB1 (A) and calreticulin (B) expression. The grade of HMGB1 and calreticulin expression was classified into HMGB1-strong and HMGB1-weak or calreticulin-strong and calreticulin-weak groups based on the median value of the counts of positive cells within tumors.



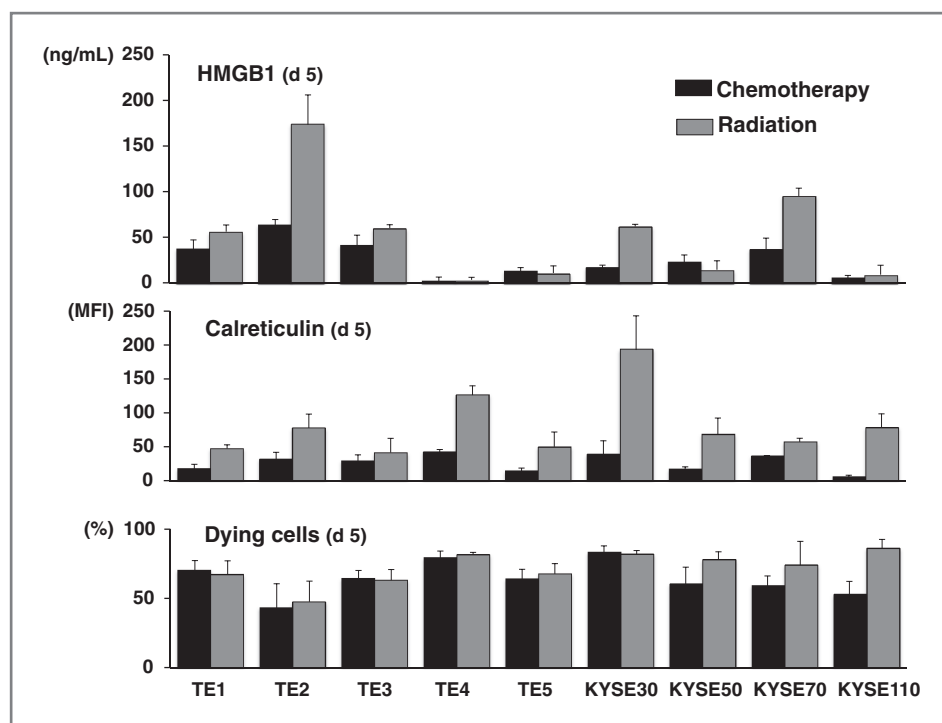


Figure 6. *In vitro* treatment of ESCC with chemodrugs or irradiation. Nine ESCC tumor cell lines were treated with 30 Gy (10 Gy irradiation on days 1, 2, and 3) radiation or a combination of 5FU + CDDP + docetaxel on day 1 and analyzed on day 5. Contents of HMGB1 in the supernatant were evaluated by ELISA, and surface expression of calreticulin along with the proportion of dying cells [Annexin-V (+) or 7-AAD (+)] were analyzed by flow cytometry.

levels of calreticulin were relatively less than those induced by irradiation regardless of almost the same amount of dying cells (Fig. 6 and Supplementary Fig. S3).

Taken together, although both irradiation and chemodrugs could induce upregulation of HMGB1 and calreticulin on ESCCs, there were remarkable variations of the amounts increased depending on cell lines. Interestingly, irradiation could upregulate HMGB1 and calreticulin more remarkably than chemotherapeutic drugs regardless of almost the same amount of dying cells.

Maturation of DCs treated by HMGB1

To confirm whether HMGB1 could mature DCs, which is a crucial step for induction of antigenic T-cell responses, monocyte-derived immature DCs were treated with HMGB1 and their maturation markers were evaluated by flow cytometry. Representative data from 5 different donors indicated that increased expression of maturation markers such as MHC class I, CD83, and CD86 on DCs was seen after treatment with HMGB1 in comparison to immature DCs, and the increased levels after treatment with HMGB1 were slightly less than mature DCs treated by OK-432 (ref. 26; Supplementary Fig. S5). These observations were confirmed in 5 independent experiments from different donors.

Discussion

The present study contains novel findings to support the concept of immunogenic cell death induced by chemoradiotherapy in patients with ESCC. First, tumor antigen-specific T-cell responses were confirmed in 6 (38%) of 16 patients with ESCC following chemoradiotherapy. Second, the serum level of HMGB1 following chemoradiation in the patients with

antigen-specific T-cell responses was significantly elevated in comparison to that in the patients without antigen-specific T-cell responses. Third, upregulation of HMGB1 within tumor microenvironments was significantly related to preoperative chemoradiotherapy and the degree of HMGB1 positively correlated with patients' survival. Fourth, both irradiation and chemodrugs could induce upregulation of HMGB1 and calreticulin on ESCC cell lines *in vitro*. Finally, HMGB1 was able to induce maturation of DCs in an *in vitro* culture system.

In general, chemoradiotherapy is thought to induce an immunosuppressive state in both T-cell and natural killer-cell immunity and there has been a general consensus that systemic immunity in patients receiving chemoradiotherapy was severely impaired, leading to infectious complications and growth of residual tumors (27, 28). Also, many chemotherapeutic drugs or radiotherapy mediated their cytotoxic effects by the induction of apoptosis, and this apoptosis was generally considered to be noninflammatory and nonimmunogenic (29, 30). However, it has been recently shown that danger signals from dying cells treated by some chemotherapeutic drugs, such as oxaliplatin, or radiotherapy could induce TLR-dependent, antigen-specific T-cell immunity, as it has been shown that chemoradiation could not reject inoculated live tumors in *tlr4*^{-/-} mice, whereas chemoradiation could reject the tumors in wild-type mice (7, 8). Furthermore, among various danger signals released from dying cells in the tumor-bearing mouse model, HMGB1, but not other known TLR4 ligands, could be a mandatory factor to induce tumor antigen-specific T-cell immunity (7, 8). It has also been shown that HMGB1 released from radiation-induced tumor cell death could enhance engulfment of antigenic components by DCs through TLR4 and mediate cross-presentation of tumor antigens into CD4

and CD8 T cells, effectively leading to tumor antigen-specific T-cell responses in murine model (7, 8). In the present study, we showed for the first time in a human clinical study that tumor antigen-specific T-cell responses were induced in patients with ESCC following chemoradiation along with elevated HMGB1 in patients' serum. Furthermore, our immunohistochemical study clearly indicated that HMGB1 production within tumor microenvironments was significantly related to preoperative chemoradiotherapy and the degree of HMGB1 positively correlated with patients' survivals. Furthermore, infiltrating grade of CD8(+) T cells within tumor microenvironments tended to be upregulated in HMGB1 high-expressing group. Taken together, these observations in clinical settings strongly support the fact that immunogenic cell death is induced by chemoradiotherapy in patients with ESCC and HMGB1 is one of the important mediators linking chemoradiation-induced cell death to antigen-specific T-cell responses.

Moreover, it has been shown that early membrane exposure of calreticulin induced by radiation could enhance phagocytosis of dying tumor cells by DCs *in vitro* (13, 15, 16), and both HMGB1 release and calreticulin cell surface expression are required for antigen-specific T-cell response in murine model. However, we could not show any significant differences in calreticulin expression between patients with and without chemoradiation, and there was no survival difference between calreticulin-strong and -weak groups. Moreover, the infiltrating grade of CD8(+) T cells did not differ between calreticulin-strong and -weak groups. Thus, there is a discrepancy between murine model and clinical setting in terms of significance of calreticulin expression in relation to T-cell immunity or in relation to chemoradiotherapy. There is one possible explanation that we classified both membranous and intracellular staining as a positive case by immunohistochemistry in the present study, as differentiation of staining pattern between membranous and intracellular was difficult in the immunohistochemical setting of clinical samples. Obeid and colleagues previously reported that membranous expression of calreticulin was more relevant than intracellular global expression when immunogenic tumor cell death occurred in murine model (16). Further study with different methodology to evaluate the cell surface expression of calreticulin in clinical samples will be needed.

Of interest, we showed that chemoradiation could induce upregulation of HMGB1 with significant variations among patients with ESCC in study group 2, and patients with high HMGB1 expression had better OS than patients with weak HMGB1 expression. Also, the *in vitro* study indicated that there were substantial variations in HMGB1 production following chemoradiation depending on ESCC cell lines regardless of almost the same amount of dying cells. These observations suggest that immune reactions related to HMGB1 production

following chemoradiation may affect clinical outcomes in patients with ESCC. Apetoh and colleagues reported that patients with breast cancer with a *TLR4* loss-of-function allele relapse more quickly after chemotherapy and radiotherapy than those with a normal *TLR4* allele (8), indicating a clinically relevant immune reaction triggered by chemotherapy-induced cell death. Thus, these observations strongly indicated that the HMGB1-related immune response after chemoradiotherapy may play an important and critical role in clinical outcome of cancer treatments, and parameters such as HMGB1 expression and TLR polymorphism may be able to predict clinical outcome after chemoradiation.

There are some debates about the capability of HMGB1 to mature DCs. It has been shown that HMGB1 has an ability to promote DC activation and trigger DC migration in human DC *in vitro* (31), in line with our present study. On the contrary, Apetoh and colleagues reported there was no clear evidence that HMGB1 is required for the maturation of human DCs in immunogenic tumor cell death system (8). The discrepancy may be explained by the different protocol of DC-induction and -maturation including dose and timing of HMGB1. The exact mechanism by which HMGB1-TLR4 interactions affect the tumor antigen presentation on DCs in immunogenic tumor cell death has not been clarified yet.

In conclusion, the present study strongly indicated that tumor antigen-specific T-cell responses were induced following chemoradiation and HMGB1 production is related to clinical outcome after chemoradiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Immunogenic Tumor Cell Death Induced by Chemoradiotherapy in Patients with Esophageal Squamous Cell Carcinoma

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