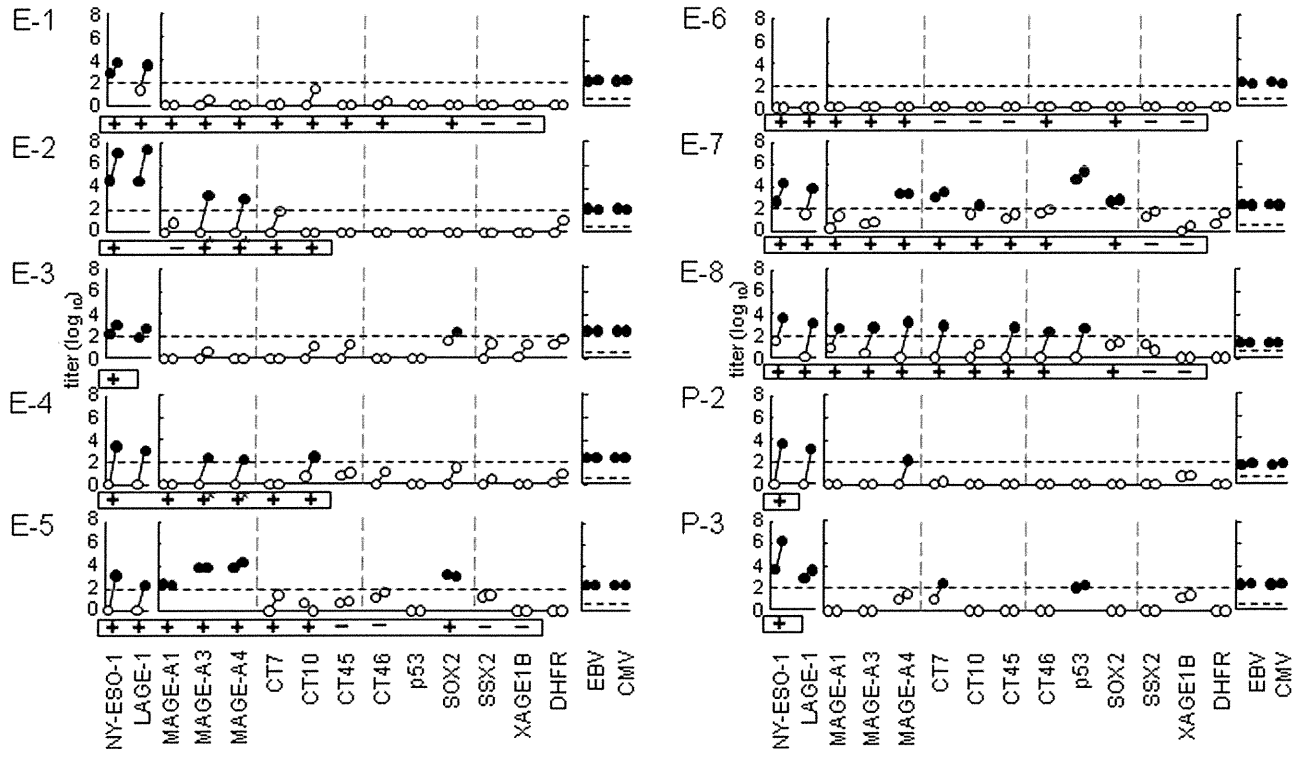

Figure 1. Reciprocal antibody titer against 13 tumor antigens in sera from patients vaccinated with CHP-NY-ESO-1 by ELISA. Serially diluted sera obtained before and after vaccination were assayed against N-His6-tagged recombinant proteins NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, p53, SOX2, SSX2, XAGE1B and DHFR. The reciprocal titer was the maximal dilution showing significant reaction (open and closed circles). Closed circles indicate reciprocal titers exceeding 100 (positive reaction). In each assay, antibody positive and negative sera were included as controls. Positive (+) and negative (−) expression of tumor antigens indicated in boxes under each panel was analyzed by RT-PCR and/or IHC when sample was available (see Supporting Information Table). Titer of EBV and CMV antibody in sera were measured by EBV and CMV kits, respectively. Values exceeding 4.0 were positive by manufacturer's indication. +*; 57B and M3H67 mAbs generated against MAGE-A3 and MAGE-A4 recombinant proteins, respectively, were both shown to recognize multiple MAGE-A family molecules.

comparing seroreactivity among the various antigens tested.[31] In each assay, positive and negative control sera were included. A positive result was defined as reciprocal titers >100. For conventional ELISA, peroxidase-conjugated goat anti-human IgG or IgM (Jackson Immuno Research Laboratory, West Grove, PA) was added to the wells for second antibody. After washing, signals were developed with *o*-phenylene diamine dihydrochloride, and absorbance at 490 nm was read using an ELISA reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA). Positivity was defined as sample optical density (OD) greater than three times that of the value for irrelevant control protein. Titers of Epstein–Barr virus (EBV) and Cytomegalovirus (CMV) antibody in sera were measured by EBV VCA kit (Denka Seiken, Tokyo, Japan) and CMV kit (Denka Seiken), respectively.

Western blot

Recombinant protein (20 ng) or cell lysate (20 μg) in sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS and 1 mM dithiothreitol) was boiled for 5 min and subjected to SDS-PAGE with 10–20% polyacrylamide BioRad Ready-Gels (Bio-Rad). After electrophoresis, the membrane (Hybond-P membrane, Amersham Pharmacia Biotech, Buckinghamshire, UK) was blocked with 5% FCS/PBS and then incubated with patients' sera diluted 1:1,000 for recombinant protein or 1:200 for cell lysate for 1 hr at room temperature. After washing, alkaline phosphatase-conjugated goat anti-human IgG (Jackson Immuno Research Laboratory) was added to the membrane. Signals were developed with a 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chromogenic substrate kit (Bio-Rad). Polyclonal rabbit anti-MAGE-A1 serum (Abcam, Cambridge, UK) and monoclonal anti-MAGE-A4 (clone 3D12; Abnova, Taipei, Taiwan), anti-p53 (clone PAb421; Enzo) and anti-His6-tag (clone OGHis; MBL, Nagoya, Japan) antibodies were used for positive controls at 1:1,000 dilution.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using formalin-fixed paraffin-embedded specimens. Monoclonal antibodies

used were anti-MAGE-A1 (clone MA454), anti-MAGE-A3 (clone M3H67), anti-MAGE-A4 (clone 57B), anti-CT7/MAGEC1 (clone CT7-33) and anti-CT10/MAGEC2 (clone LX-CT10.5). For cancer-testis (CT) antigens, only strong nuclear and/or cytoplasmic staining as observed in testicular tissue (positive control) in at least 5% of cells was scored as positive. 57B and M3H67 mAbs generated against MAGE-A3 and MAGE-A4 recombinant proteins, respectively, were both shown to recognize multiple MAGE-A family molecules.[35,36]

Table 1. Heteroclitic antibody response and clinical response after CHP-NY-ESO-1 vaccination

ID	Heteroclitic response No. of antigens	Weeks (the No.)	Clinical response
E-1	0	89 (31)	Regression
E-2	2	14 (7)	Partial regression
E-3	1	28 (12)	Stable
E-4	3	12 (6)	Progressive
E-5	2	22 (11)	Partial regression
E-6	0	4 (3)	N.E.
E-7	4	2 (2)	N.E.
E-8	7	54 (27)	Stable
P-2	1	28 (10)	PSA stabilization
P-3	2	29 (13)	PSA stabilization

Abbreviations: Weeks (the No.): weeks after the start of vaccination and the number of vaccinations given; N.E.: not evaluable.

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted from frozen tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). Conventional reverse transcription-polymerase chain reaction (RT-PCR) was performed against NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SOX2, SSX2 and XAGE1B.[30,31]

Results

Antibody response against 13 tumor antigens in CHP-NY-ESO-1-vaccinated patients

We analyzed antibody responses against NY-ESO-1, NY-ESO-1-related antigen LAGE-1, other CT antigens MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SSX2 and XAGE1B, SOX2 and p53 in esophageal cancer patients E-1, E-2, E-3, E-4, E-5, E-6, E-7 and E-8 and prostate cancer patients P-2 and P-3 before and after a cycle of CHP-NY-ESO-1 vaccination (Fig. 1 and Table 1). Before vaccination, strong antibody responses against NY-ESO-1 and/or LAGE-1 were observed in E-2 and P-3 and defined as baseline seropositive. Additionally,

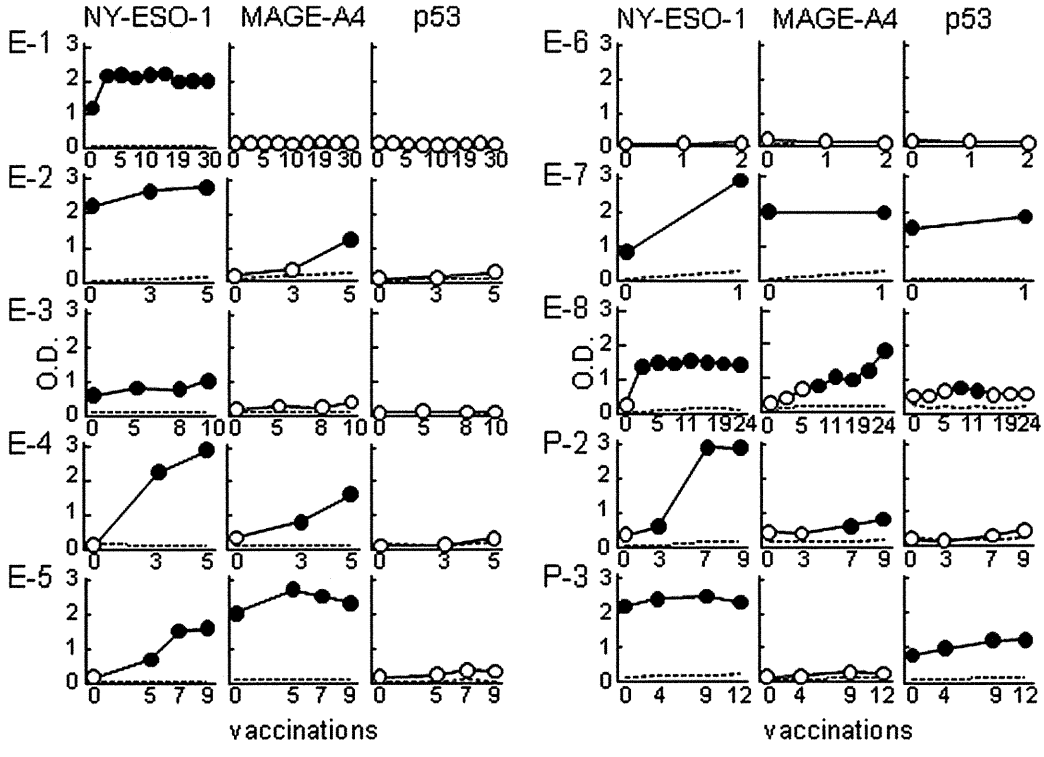

Figure 2. IgG antibody response against NY-ESO-1, MAGE-A4 and p53 in sera from patients before and after CHP-NY-ESO-1 vaccination by ELISA. Sera diluted at 1:100 were assayed against N-His6-tagged recombinant proteins NY-ESO-1, MAGE-A4 and Akt produced in *E. coli* and recombinant proteins p53 and CCDC-62 produced in *Baculovirus*. Akt and CCDC-62 were included as negative control (dotted line). Positive reaction (closed circles) represented the OD values exceeding three times the control OD value.

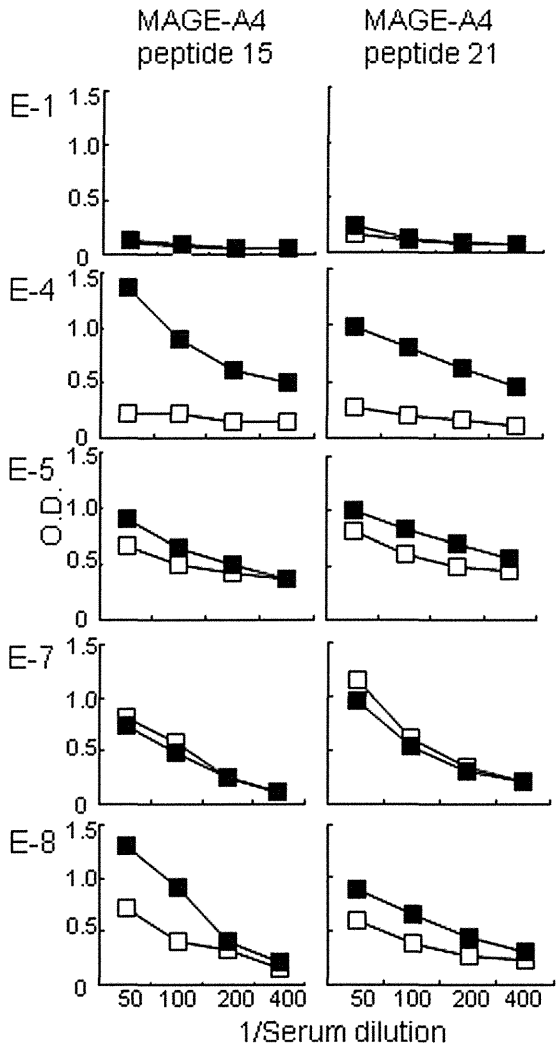

Figure 3. Serially diluted sera from patients before (open squares) and after (closed squares) CHP-NY-ESO-1 vaccination were assayed against MAGE-A4 peptide 15 and peptide 21 by IgG ELISA.

marginal antibody responses were observed in E-1, E-3 and E-7. In E-5, antibody against MAGE-A1, MAGE-A3, MAGE-A4 and SOX2 was observed. In E-7, antibody against MAGE-A4, CT7/MAGEC1, p53 and SOX2 was observed. In P-3, antibody against p53 was observed. After vaccination, in all patients except E-6, antibody response against NY-ESO-1 and LAGE-1 was increased or induced. In E-2, antibody responses against MAGE-A3 and MAGE-A4 were induced. In E-3, antibody response against SOX2 was induced. In E-4, antibody responses against MAGE-A3, MAGE-A4 and CT10/MAGEC2 were induced. In E-5, antibody responses against MAGE-A3 and MAGE-A4 were increased. In E-7, antibody responses against CT7/MAGEC1, p53 and SOX2 were increased and that against CT10/MAGEC2 was induced. In E-8, antibody responses against MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT45, CT46/HORMAD1 and p53 were induced. In P-2, antibody response against MAGE-A4 was induced. In P-3, antibody response against CT7/MAGEC1 was induced and that against p53 was increased. No antibody against DHFR included as a control was detected in any patient. Furthermore, no increase of antibody response was observed against EBV and CMV after CHP-NY-ESO-1 vaccination.

Expression of 13 tumor antigens in tumor specimens

Expression of NY-ESO-1 was detected by RT-PCR and IHC in tumors from all patients before vaccination. Expression of other tumor antigens except p53 was analyzed by RT-PCR in E-1, E-5, E-6, E-7 and E-8, and expression of MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1 and CT10/MAGEC2 was also analyzed by IHC in E-1, E-2, E-4, E-5, E-6, E-7 and E-8 (Fig. 1 and Supporting Information Table). Mutation of p53 was not determined in our study. Expression of corresponding antigen was confirmed with tumor specimens in patients who showed antibody against tumor antigens.

Antibody response against tumor antigens in CHP-NY-ESO-1-vaccinated patients: No involvement of antibody against His6-tag and the product of E. coli present in the vaccine

Antibody responses against selected tumor antigens were further confirmed in sera obtained at each time during multiple vaccinations. As shown in Figure 2, IgG antibody against MAGE-A4 was detected in sera from E-5 and E-7 before vaccination, and the response was increased or induced in E-2, E-4, E-5, E-8 and P-2 after vaccination. IgG antibody against p53 was detected in sera from E-7 and P-3 before vaccination, and the response was increased or induced in E-7, E-8 and P-3.

Induction of IgM antibody against MAGE-A4 was detected in sera from E-8 after vaccination (Supporting Information Fig. 1). IgM antibody against p53 was detected in sera from E-7 before vaccination. Increase or induction of IgM antibody against p53 was detected in E-7 and E-8 after vaccination. Interestingly, in E-8, transient IgM response against MAGE-A4 and p53 was followed by IgG response.

Recombinant NY-ESO-1 protein used for vaccination has His6-tag in the N-terminus and was produced in *E. coli* as the host cells. All antigens shown in Figure 1 also have His6-tag and were produced in *E. coli*. To exclude the possibility of detecting antibody against His6-tag and/or the product of *E. coli* in the assay that might be raised by vaccination, DHFR was tested as control. No antibody against DHFR was detected (see above). To further exclude the possibility, the antibody response against control antigens was examined by IgG ELISA using serum samples obtained in each time during multiple vaccinations. As shown in Figure 2, antibody against Akt protein with His6-tag and produced in *E. coli* was within a background level (<0.2 OD value). p53 used in the experiments shown in Figure 2 and Supporting Information Figure 1, but not in Figure 1, and CCDC-62 protein

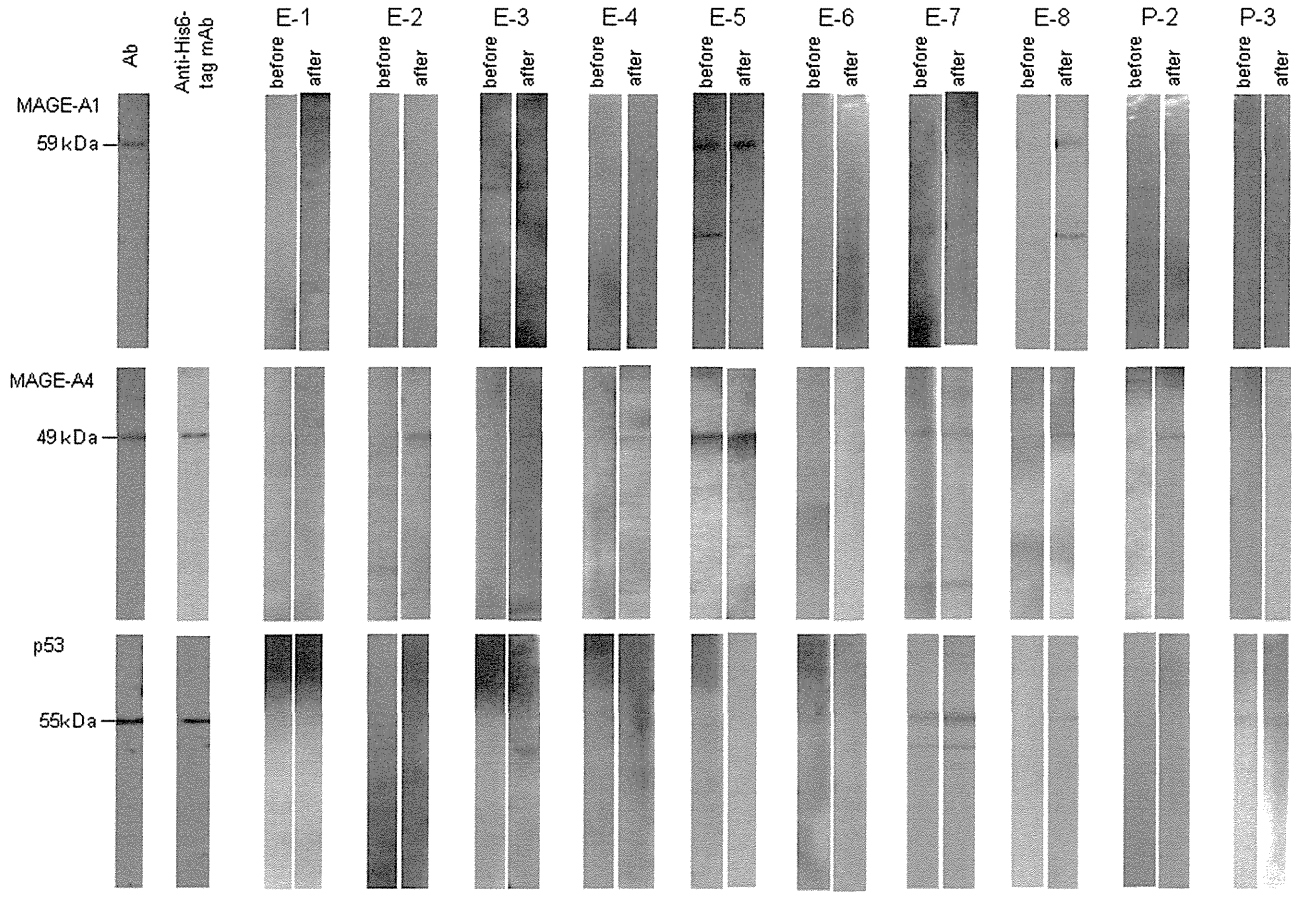

Figure 4. Western blot analysis. Reaction of sera against MAGE-A1, MAGE-A4 and p53 was investigated. Recombinant proteins (20 ng) were run by SDS-PAGE and transferred to a membrane by electrophoresis. Sera (1:1,000) from all patients obtained before and after vaccination were examined. Marker and control bands of each protein detected by monoclonal or polyclonal antibody (1:1,000) are also shown.

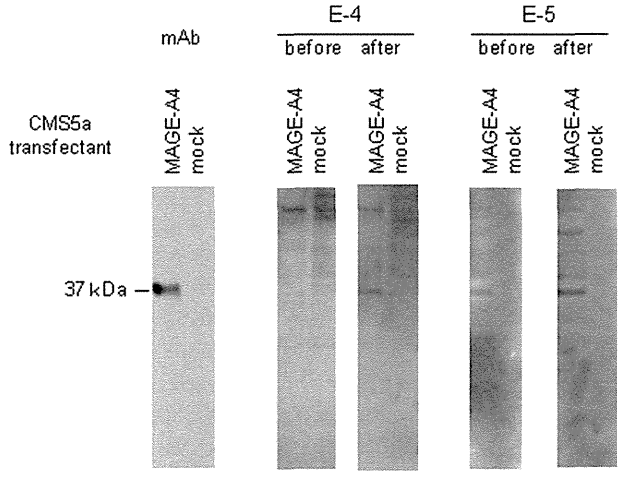

Figure 5. Western blot analysis of sera against MAGE-A4 in lysate of MAGE-A4-transfected CMS5a cells. Cell lysate (20 μg) was run by SDS-PAGE, transferred to a membrane by electrophoresis and sera (1:200) from E-4 and E-5 patients obtained before and after CHP-NY-ESO-1 vaccination were examined. Control band of the protein detected by monoclonal antibody (1:1,000) is shown.

share His6-tag and were produced by *Baculovirus*. Antibody against CCDC-62 was undetectable in sera from any patients.

Next, we synthesized MAGE-A4 OLPs and investigated antibody response by ELISA. Antibody response against MAGE-A4 peptides 15 and 21 was frequently observed in patients showing antibody response against MAGE-A4 protein (Supporting Information Fig. 2). Serially diluted sera from patients E-4, E-5, E-7 and E-8 obtained before and after CHP-NY-ESO-1 vaccination were examined against MAGE-A4 peptides 15 and 21 by IgG ELISA (Fig. 3). Increase or induction of antibody response was observed in E-4, E-5 and E-8, but not E-7 after vaccination. No antibody response was detected in E-1 included as negative control. These results were consistent with those by ELISA using recombinant MAGE-A4 protein in Figure 2.

Western blot analysis

The specificity of antibody against MAGE-A1, MAGE-A4 and p53 in sera from all patients vaccinated was further analyzed by Western blot (Fig. 4). Each antibody as positive control showed the representative band for MAGE-A1 protein at 59 kDa, for MAGE-A4 protein at 49 kDa and for p53 protein

at 55 kDa. Increase of reaction with the bands was observed with recombinant MAGE-A1 protein in sera from E-8, with recombinant MAGE-A4 protein in sera from E-2, E-4, E-5, E-8 and P-2 and with p53 in sera from E-7, E-8 and P-3 obtained after vaccination.

Specificity of the reaction was further confirmed using transfectants. As shown in Figure 5, sera from E-4 after vaccination and from E-5 before and after vaccination reacted to MAGE-A4 in lysate of MAGE-A4-transfected murine fibrosarcoma CMS5a cells. No reaction was observed with lysate of mock-transfected CMS5a cells.

Discussion

Efficient elicitation of host immune response is a prerequisite for successful immunotherapy using cancer vaccine, and immune monitoring of specific antibody, CD4 and CD8 T cell responses against tumor antigens after vaccination is crucial to evaluate the response. In our study, we investigated antibody response against 13 tumor antigens by ELISA using recombinant proteins to evaluate the immune response more precisely. Nine of ten patients analyzed except E-6 showed an increase or induction of antibody response against NY-ESO-1 and its related LAGE-1 antigen after CHP-NY-ESO-1 vaccination. Eight of these nine patients showed an increase or induction of antibody response to either of these antigens after vaccination. Previously, it was reported that sera from patients vaccinated with recombinant NY-ESO-1 protein and CpG in Montanide sometimes showed nonspecific production of antibody against other recombinant proteins used for control,[11,37] and some of these responses could be attributed to reactivity against bacterial components or His6-tag. To address this possibility, we performed specificity analysis of the antibody response using control recombinant proteins, synthetic peptides and by Western blot that showed heteroclitic responses were not against His6-tag and/or bacterial products included in a preparation of CHP-NY-ESO-1 used for vaccination.

We reported previously that those patients showed NY-ESO-1 specific antibody and CD4 and CD8 T cell responses during vaccination.[14,15] The findings suggest that increase or induction of antibody response against tumor antigens, *e.g.*, MAGE-A3 and MAGE-A4, as well as NY-ESO-1 after CHP-NY-ESO-1 vaccination may be caused by their release from tumor cells damaged by NY-ESO-1-specific immunity. Therefore, antibody response to multiple tumor antigens may suggest an intensity of the overall host immune response against the tumor, and detection of multiple heteroclitic serological responses using a panel of recombinant proteins would be a new tool of immunological monitoring for antitumor responses. A clear correlation between heteroclitic antibody responses and clinical outcomes could not be established in the limited number of patients analyzed in our study (Table 1). However, antibody response as well as CD4 and/or CD8 T cell responses to heteroclitic tumor antigens would be useful for evaluating overall immune response to tumor.

A number of studies have shown the relationship between heteroclitic immune response and clinical response. Germeau *et al.*[19] reported that the frequency of CTL precursor increased tenfold in some patients after vaccination using MAGE antigenic peptides, although they found no significant difference in the levels against immunizing antigens between the tumor-regressor and -progressor patients. They then analyzed CTL precursors against other tumor antigens than that utilized for vaccine and found that the immune responses elicited to those irrelevant antigens after vaccination might contribute to the whole immune response to a given tumor and was correlated to clinical responses. Similarly, Butterfield *et al.*[23,24] reported that peptide-specific T cell response was efficiently induced in most patients by immunization with MART-1/Melan-A peptide pulsed dendritic cells. However, cellular immune responses against not only MART-1/Melan-A but also gp100 and tyrosinase were detected only in a complete clinical responder. These findings suggest a relationship between heteroclitic CTL responses and clinical responses. Furthermore, Disis *et al.* reported induction of both cellular and humoral responses against other intramolecular determinants in patients immunized with HER-2/neu peptide vaccine, and of antibody response to p53 in patients immunized with HER-2/neu peptide vaccine.[17,22] They further studied the effect of HER-2/neu T-helper peptide-based vaccinated patients receiving trastuzumab therapy and observed prolonged immune responses against not only the vaccine antigen but also cryptic antigens.[38] Collectively, the presence of either humoral or cellular immune response to multiple tumor antigens appears to be indicative of the strength of overall response against the tumor and predictive of clinical response. In our study, we used a panel of 13 tumor antigens for the detection of the humoral response. Serological detection of responses to multiple tumor antigens that were shown to be highly immunogenic in cancer patients would be convenient and could be included in routine immune monitoring.

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Role of multidrug resistance protein 2 (MRP2) in chemoresistance and clinical outcome in oesophageal squamous cell carcinoma

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BACKGROUND: Although multidrug resistance protein 2 (MRP2) confers chemoresistance in some cancer types, its implication on oesophageal squamous cell carcinoma (ESCC) remains unclear.

METHODS: We evaluated MRP2 expression by immunohistochemistry and RT–PCR using 81 resected specimens from ESCC patients who did or did not receive neo-adjuvant chemotherapy (NACT), including 5-fluorouracil, doxorubicin, and cisplatin (CDDP). Correlation between MRP2 expression and response to chemotherapy was also examined in 42 pre-therapeutic biopsy samples and eight ESCC cell lines.

RESULTS: MRP2-positive immunostaining was more frequently observed in ESCCs with NACT than in those without NACT (27.3 *vs* 5.4%). The MRP2-positive patients showed poorer prognosis than MRP2-negative patients (5-year survival rate, 25.6 *vs* 55.7%). Concordantly, ESCC with NACT showed 2.1-fold higher mRNA expression of MRP2 than those without NACT ($P = 0.0350$). In pre-therapeutic biopsy samples of patients with NACT, non-responders showed 2.9-fold higher mRNA expression of MRP2 than responders ($P = 0.0035$). Among the panel of ESCC cell lines, TE14 showed the highest MRP2 mRNA expression along with the strongest resistance to CDDP. Inhibition of MRP2 expression by small-interfering RNA reduced chemoresistance to CDDP.

CONCLUSION: Our data suggested that MRP2 is one of molecules, which regulate the sensitivity to chemotherapy including CDDP in advanced ESCC patients.

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Oesophageal squamous cell carcinoma (ESCC) is the major histological form of oesophageal cancer in East Asian countries. It is one of the most lethal malignancies of the digestive tract and in most cases the initial diagnosis is established only once the malignancy is in the advanced stage (Shimada *et al*, 2003). Multimodal therapies are therefore necessary to prolong the survival of ESCC patients. Chemotherapy has become the standard first-line therapy for advanced ESCC patients, especially neo-adjuvant chemotherapy (NACT) (Tamoto *et al*, 2004). However, the initial response rate for NACT remains at 35–66% (Ajani *et al*, 1992; Iizuka *et al*, 1992; Hilgenberg *et al*, 1988; Ilson *et al*, 1998, 1999; Millar *et al*, 2005) and non-responders risk serious adverse effects without achieving any survival benefit.

The effectiveness of chemotherapy is often limited by drug-resistance factors in the tumours themselves. In fact, some tumours are intrinsically resistant to many kinds of chemotherapeutic agents, whereas other tumours, initially sensitive, often recur or become resistant not only to the initial agents used but also to those used subsequently. These two types of chemoresistance, intrinsic and acquired, are clinically serious problems in many types of cancer including ESCC; however, the molecular mechanisms underlying this resistance are not fully understood. More investigation into the mechanisms of chemoresistance in ESCC is needed with the goal of identifying novel predictive markers that can accurately identify non-responders before the administration of chemotherapy, thus enabling personalised therapies in ESCC patients.

Several members of the ATP-binding cassette (ABC) transporter superfamily have an important role in drug resistance in tumour cell models as well as in the clinic (Lage, 2003). These transporters mediate the ATP-dependent cellular efflux of chemotherapeutic drugs. Of the 48 human ABC transporters, multidrug resistance protein 2 (MRP2; also designated as ABCC2 or cMOAT) is expressed in the hepatocyte canalicular membrane (Kool *et al*, 1997), in which it functions as the major exporter of organic anions from the liver into the bile (Wada *et al*, 1998). Multidrug resistance protein 2 is also expressed in the kidney, gall bladder, small intestine, colon, and lung (Surowiak *et al*, 2006). Interestingly, several cisplatin (CDDP)-resistant human cancer cell lines overexpress MRP2, including ovarian cancer, hepatocellular carcinoma, bladder cancer, and colon cancer (Taniguchi *et al*, 1996; Kool *et al*, 1997; Liedert *et al*, 2003; Materna *et al*, 2005). *In vitro* data also implicated MRP2 in multidrug resistance (MDR) mechanisms during chemotherapy in some cancer cell lines

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(Koike *et al*, 1997; Materna *et al*, 2006; Ma *et al*, 2009). However, few studies have investigated MRP2 expression in ESCC (Gan *et al*, 2010; Tanaka *et al*, 2010), and thus the relationship between MRP2 expression and chemoresistance in ESCC remains unclear. The present study examined the clinical significance of MRP2 expression and its role in intrinsic and acquired resistance to chemotherapy in ESCC patients.

PATIENTS AND METHODS

Patients and treatments

The present study examined samples from 81 patients with histopathologically confirmed primary thoracic oesophageal cancer who underwent surgical resection at our hospital from 1988 to 2007. Table 1 details the patient characteristics. The cohort comprised 9 female and 72 male patients, aged from 42 to 80 years (median 62 years). Sub-total oesophagectomy by right thoracotomy with two or three-field lymphadenectomy was performed in all patients. Curative resection (R0) was achieved in 75 patients (92.6%), whereas the remaining 6 (7.4%) patients underwent a non-curative resection (R1, 2). None of the patients died of post-operative complications. A total of 44 patients (54.3%) with lymph node metastasis at initial diagnosis received NACT comprising two courses of 5-fluorouracil (5-FU), CDDP, and doxorubicin (DXR) (Akita *et al*, 2006; Yano *et al*, 2006; Matsuyama *et al*, 2007; Makino *et al*, 2008, 2010). Only a few patients who showed multiple metastatic lymph nodes in the surgical specimen received a regimen of docetaxel or CDDP plus 5-FU after operation (Ando *et al*, 2003).

Table 1 Correlation between MRP2 expression by immunohistochemistry and various clinico-pathological parameters

	MRP2 expression			
Parameter	**Positive**	**Negative**	**Total**	***P*-value**
Age (years)				
<65	8 (16.3)	41 (83.7)	49	0.7731
⩾65	6 (18.8)	26 (81.2)	32	
Gender				
Male	10 (13.9)	62 (86.1)	72	0.0435
Female	4 (44.4)	5 (55.6)	9	
Histopathology				
Well-, moderately differentiated	10 (16.7)	50 (83.3)	60	0.7500
Poorly differentiated	4 (19.0)	17 (81.0)	21	
Location				
Upper, middle thoracic oesophagus	6 (11.5)	46 (88.5)	52	0.1227
Lower thoracic oesophagus	8 (27.6)	21 (72.4)	29	
Neo-adjuvant chemotherapy				
Yes	12 (27.3)	32 (72.7)	44	0.0161
No	2 (5.4)	35 (94.6)	37	
pT				
T0–2	4 (16.7)	20 (83.3)	24	>0.9999
T3–4	10 (17.5)	47 (82.5)	57	
Number of pN				
<4	6 (11.1)	48 (88.9)	54	0.0594
⩾4	8 (29.6)	19 (70.4)	27	
pStage				
Stages 0–2	4 (12.1)	29 (87.9)	33	0.3800
Stages 3–4	10 (20.8)	38 (79.2)	48	

pT, pN, pStage (pathological classification) according to TNM classification.

After surgery, the patients were surveyed every 3 months by physical examination and measurement of serum tumour markers, every 6 months by CT scan and abdominal ultrasonography, and every year by endoscopy until tumour recurrence was evident. Patients with tumour recurrence received chemotherapy or chemoradiotherapy as long as their systemic condition permitted. The mean overall survival (OS) was 31.6 months and mean disease-free survival was 28.3 months. The mean follow-up period after surgery was 42.9 months.

Immunohistochemical analysis

MRP2 protein accumulation was examined by immunohistochemical (IHC) staining of formalin-fixed and paraffin-embedded ESCC tissue sections (Makino *et al*, 2009). Briefly, after deparaffinization in xylenes and dehydration through graded ethanol solutions; endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 20 min. The tissue sections were then heated at 95°C for 40 min in citrate buffer (0.05 mol l^{-1}, pH 6.0) for antigen retrieval. The sections were then incubated with mouse monoclonal antibody to MRP2 (Clone: M_2III-6, ALEXIS Biochemicals, dilution 1 : 10) for 2 h at room temperature, and antibody binding was visualised using the labeled-streptavidin biotin method. Negative controls for the IHC included omission of the primary antibody. Normal human liver tissue was used as a positive control. MRP2 staining for each ESCC sample was judged 'positive' when more than 10% of the cancer cells in the section were immunoreactive to MRP2, and 'negative' when 10% or less of the cells were positive. All slides were assessed by two observers, independently and then in conference; both were blinded to the clinico-pathological parameters.

Quantitative RT–PCR analysis

Total RNA was extracted from fresh frozen resected tumours or endoscopic biopsy samples from ESCCs patients, and from cancer cell lines using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was generated from 1 μg RNA in a final volume of 20 μl containing oligo-(dT)-15 primer and avian myeloblastosis virus transcriptase, using the Reverse Transcription System (Promega, Madison, WI, USA). Analysis by PCR was performed using a LightCycler, real-time monitoring thermal cycler. Reaction mixture for PCR was prepared containing 2 μl of cDNA template, 3 mmol l^{-1} $MgCl_2$, and 250 nmol l^{-1} of primer pairs, using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The amount of each transcript was normalised against the expression of the housekeeping gene porphobilinogen deaminase (PBGD). Standard curves were constructed with 10-fold serial dilutions of cDNA obtained from non-cancerous oesophageal mucosal cell layers of tissue samples from 10 cases as a standard mixture. The sequences of PCR primers for PBGD, MRP2 were as follows: forward primer 5′-TGTCTGGTAACGGCAATGCGGCTGCAAC-3′, reverse primer 5′-TCAATGTTGCCACCACACTGTCCGTCT-3′ used for amplification of PBGD, forward primer 5′-TAATGGTCCTAGACAACGGG-3′, reverse primer 5′-GGGCCTTCTGCTAGAATTT-3′ for MRP2. The PCR cycling condition was set as follows: an initial denaturing step at 95°C for 10 min and 40 cycles at 95°C for 15 s, 58°C for 10 s, and 72°C for 25 s. The relative amount of cDNA in each sample was measured by interpolation on the standard curve, and then the relative ratio of MRP2/PBGD mRNA expression in log2 scale was calculated for each ESCC sample.

Knockdown analysis using MRP2-siRNAs

Two small-interfering RNA (siRNA-1, -2) of MRP2 (HSS102057, HSS174719) and negative control (NC) (Medium GC duplex of stealth RNAi NC duplexes) were purchased from Invitrogen.

Among the eight ESCC cell lines supplied by RIKEN cell bank (Tsukuba, Japan), TE14 cells showed the highest MRP2 mRNA expression and were subsequently transfected with 15 nmol l^{-1} siRNA using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM I Reduced Serum Medium (Invitrogen). After 24 h, the medium was replaced by standard medium, and then 96 h from the siRNA administration, cells were collected for the following growth inhibitory assay as described below.

Growth inhibitory assay

Cells (TE14, 1×10^4 cells per well) were added in triplicate to a 96-well microplate, and after overnight incubation, the medium was replaced with 100 μl of fresh medium containing various concentrations of DXR and CDDP, both of which chemoagents have been reported to be transported by MRP2 in some types of cell lines. The TE14 cells suspended in complete medium were used as a control for cell viability. After 4 h (DXR and CDDP) treatment, the cells were washed with fresh medium. The number of viable cells was assessed by the 3-(4-, 5-dimethylthiazol-2-yl)-2, 5-dyphenyl tetrazolium bromide (MTT) (Sigma, St Louis, MO, USA) assay. Briefly, 10 μl (50 μg) of MTT were added to each well after 48 h (DXR and CDDP) from the chemoadministration. The plate was incubated for 4 h at 37°C, followed by removal of medium and the addition of 100 μl of 2-propanol to each well to dissolve the resultant formazan crystals. Plate absorbance was measured in a microplate reader at a wavelength of 650 nm. After a pulsed exposure, the IC_{50} was calculated as percentage of control cultures that were not exposed to chemoagents using an interpolated logarithmic concentration curve. Results were derived from three independent sets of triplicate experiments.

Statistical analysis

Data are expressed as mean ± s.d. Correlations between MRP2 expression and various clinico-pathological parameters were each evaluated by the χ^2 test and Fisher's exact probability test. Differences in continuous parameters between two groups were evaluated by the Mann–Whitney's *U*-test. Prognostic variables were assessed by log-lank test, and OS was analysed by Kaplan–Meier method. These analyses were carried out using SPSS for Windows v10 (SPSS, Chicago, IL, USA). A *P*-value of less than 0.05 denoted the presence of statistical significance.

RESULTS

MRP2 protein expression by immunohistochemistry in ESCC and its correlation with clinico-pathological parameters

A total of 81 samples that contained both cancerous and non-cancerous lesions were evaluated for MRP2 protein expression by IHC analysis. As a positive control, liver tissue showed strong MRP2 immunostaining mainly in the hepatocyte plasma membrane (Figure 1A). No normal squamous epithelium showed significant levels of immunostaining (Figure 1B). Of all samples, 14 (17.3%) showed positive MRP2 expression, mainly in the cell membrane and cytoplasm of tumour cells (Figure 1C), whereas the remaining 67 (82.7%) were negative for MRP2 expression (Figure 1D). The positive staining was almost homogeneous in single-cancer nests and among different areas (surface, central, and deepest areas) of the cancer lesion.

Table 1 lists the correlations between MRP2 expression and various clinico-pathological parameters. Of note, MRP2 expression was exceptional in the ESCC patients without NACT (2 out of 37, 5.4%), but was significantly more frequent in patients after NACT (12 out of 44, 27.3%). Women tended to have a higher rate of MRP2 expression than men (44.4 *vs* 13.9%, respectively), although the difference was small. Other clinico-pathological parameters including age, histological type, tumour location, pT, pN, and pStage were not associated with MRP2 expression.

Disease recurrence after curative resection was diagnosed in 35 (46.7%) of 75 patients with curative resection (R0) and the mean

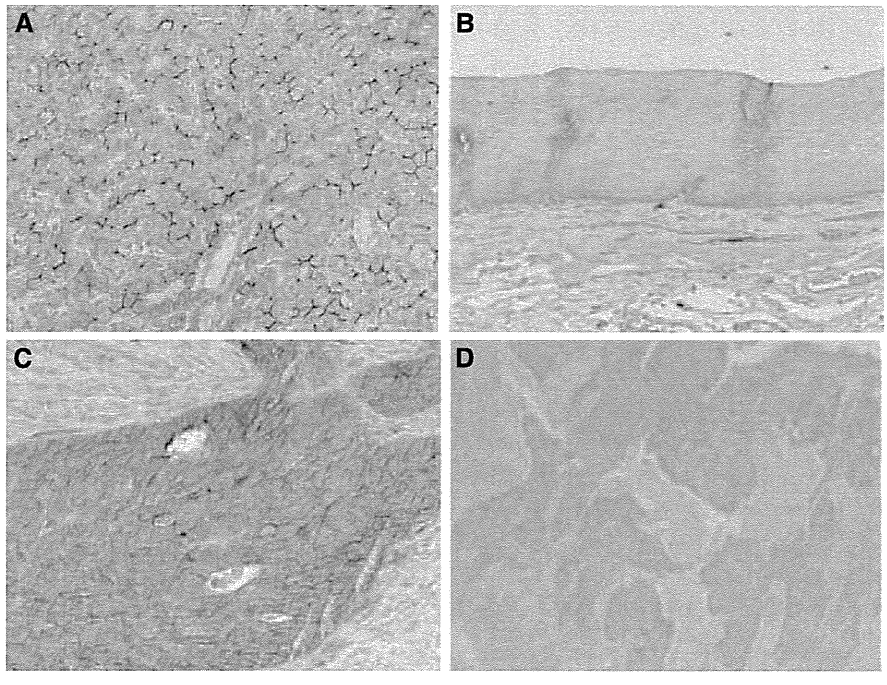

Figure 1 MRP2 expression by immunohistochemistry. (**A**) Strong MRP2 expression in liver tissue as a positive control (magnification, × 400). (**B**) Representative normal squamous epithelium negative for MRP2 expression (magnification, × 200). (**C**) Representative MRP2-positive ESCC showing staining mainly in the membrane and cytoplasm of tumour cells (magnification, × 200). (**D**) Representative MRP2-negative oesophageal squamous cell carcinoma with no appreciable staining of tumour cells (magnification, × 200).

time to recurrence was 10.5 months. A total of 35 (43.2%) patients died and their average survival time from diagnosis to death was 1.4 years (range 0.2–4.2 years). The total 5-year OS rate was 50.9% and MRP2-positive patients showed a significantly poorer prognosis than MRP2-negative patients (5-year OS 55.7 *vs* 25.6%) (Figure 2).

MRP2 mRNA expressions in resected specimens and endoscopy biopsy samples

RT–PCR analysis was performed to quantify the expression of MRP2 mRNA in surgically removed specimens from 26 representative cases, including 16 with NACT and 10 without NACT. MRP2 mRNA expression in tumours with NACT was 2.1-fold higher than in those without NACT, although there was no significant difference in TNM stage and other clinico-pathological parameters between the groups (data not shown) (Figure 3A).

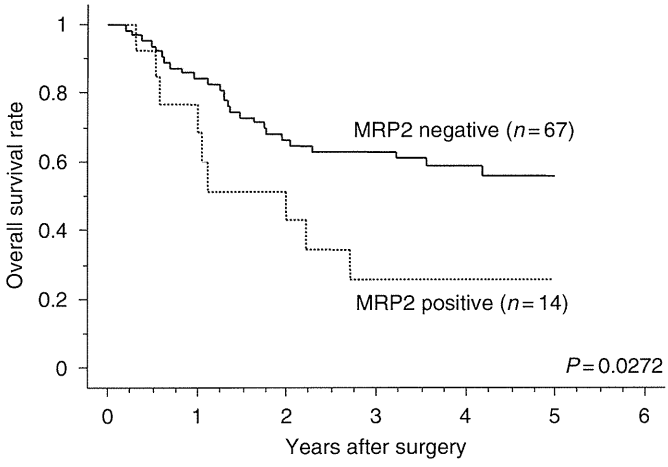

Figure 2 Survival curves according to MRP2 expression. Overall survival curve classified according to MRP2 expression for all patients were plotted by Kaplan–Meier method. Differences between two groups were evaluated by log–rank test. Ordinate: overall survival rate, abscissa: time after surgery (years).

The association between MRP2 mRNA expression and the effect of NACT was investigated in biopsy samples before NACT from 42 patients; the response of these patients to NACT was classified as non-responder in 22 and responder in 20. As shown in Figure 3B, MRP2 mRNA expression in non-responders was 2.9-fold higher than that in responders. Again, although these 42 samples were all advanced tumours with clinically positive lymph node metastases, there was no significant difference in clinical background parameters between the groups (data not shown).

Association between MRP2 mRNA expression and chemoresistance in ESCC cancer lines

To explore whether MRP2 expression functions specifically in chemoresistance to CDDP, we tested for a correlation between MRP2 mRNA expression and CDDP resistance (IC_{50}) in eight ESCC cell lines (Figure 4). Relatively high MRP2 expression was observed in TE14 and TE5 cell lines, both of which displayed strong resistance to CDDP. Regression analysis showed a significant correlation between MRP2 mRNA expression and IC_{50}

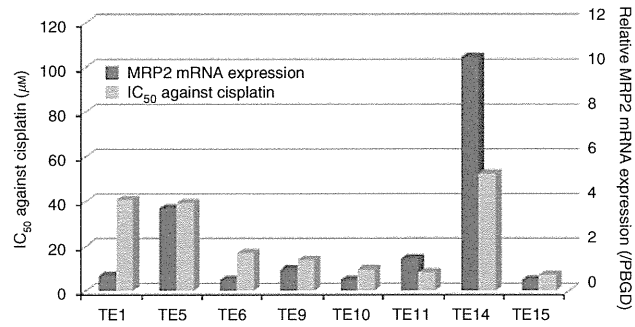

Figure 4 Correlation between MRP2 mRNA expression and CDDP-resistance (IC_{50}) in eight cell lines of ESCC. Relatively high MRP2 expression was observed in TE14 and TE5 cell lines, both of which displayed strong resistance to CDDP. Black bar: the relative ratio of MRP2 mRNA expression, grey bar: IC_{50} values against CDDP.

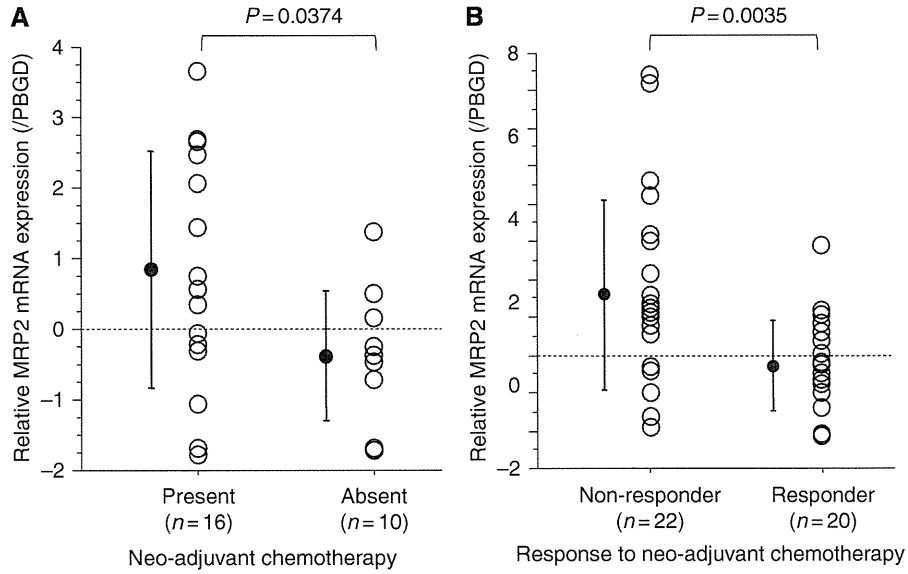

Figure 3 Differences in MRP2 mRNA expression between patients with and without neo-adjuvant chemotherapy in resected specimens (**A**), and between responders and non-responders at biopsy (**B**). (**A**) The relative ratio of MRP2 mRNA expression in resected tumours treated with neo-adjuvant chemotherapy ($n=16$) was significantly higher than in untreated cancers ($n=10$). (**B**) In endoscopy biopsy samples, the relative ratios of MRP2 mRNA expression in responders ($n=22$) were significantly higher than those in non-responders ($n=20$). Data are shown as mean ± s.d. (log2 values).

Table 2 Modulation of resistance against cisplatin and doxorubicin by MRP2 siRNA

	IC_{50}	
	Cisplatin (μM)	**Doxorubicin (μM)**
TE14 NC	32.4 (± 1.2)	6.2 (± 0.16)
TE14 siRNA-1	20.5 (± 1.4)[a]	5.8 (± 0.47)[b]
TE14 siRNA-2	17.8 (± 1.2)[c]	5.4 (± 0.54)[d]

Abbreviations: NC = negative control; IC_{50} = half maximal inhibitory concentration; siRNA = small-interfering RNA. [a]$P = 0.0003$, compared with NC. [b]$P = 0.2869$, compared with NC. [c]$P = 0.0005$, compared with NC. [d]$P = 0.2285$, compared with NC. Data are shown as mean ± s.d.

against CDDP ($R = 0.741$, $R2 = 0.549$), suggesting that ESCC cell lines with higher MRP2 mRNA expression were more resistant to CDDP compared with those showing lower MRP2 expression.

To confirm these findings by an alternative approach, we transfected MPR2 siRNAs into the TE14 line, which had the highest cellular MRP2 expression. The specific gene silencing started 48 h after the administration of siRNA (two siRNAs for MRP2 with different sequences were used: siRNA-1 and siRNA-2) and continued for 144 h, which was examined by quantitative PCR, resulting in 63.8% (siRNA-1) and 65.9% (siRNA-2) of peak MRP2 downregulation compared with NCs. The knockdown effect was stable during this period. As shown in Table 2, downregulation of MRP2 conferred increased sensitivity to CDDP, but not to DXR. IC_{50} values against CDDP were significantly lower in TE14 cell lines transfected with siRNA-1 and siRNA-2 compared with those transfected with NC (20.5 ± 1.4, 17.8 ± 1.2 *vs* 32.4 ± 1.2 μM, (siRNA-1 *vs* NC); $P = 0.0003$, (siRNA-2 *vs* NC); $P = 0.0005$). On the other hand, IC_{50} values of DXR were almost similar among TE14 cells transfected with siRNA-1, siRNA-2, and NC (5.8 ± 0.47, 5.4 ± 0.54 *vs* 6.2 ± 0.16 μM, (siRNA-1 *vs* NC); $P = 0.2869$, (siRNA-2 *vs* NC); $P = 0.2285$).

DISCUSSION

To our knowledge, this study is the first to identify the clinical significance of MRP2 expression in chemoresistance in ESCC. Such a relationship was strongly suggested by the findings that (1) MRP2 expression in the clinical biopsy samples before NACT was significantly negatively correlated with the effect of NACT, and (2) in the cultured cell line, artificial MRP2 downregulation resulted in increased resistance to the chemotherapy. Furthermore, the clinical samples of patients treated with NACT showed significantly higher expression of MRP2 at both the protein and mRNA levels than those without NACT, and the increased MRP2 expression was associated with poor prognosis. Although complicated, these clinical observations implicated MRP2 in the acquired resistance to chemotherapy commonly encountered in ESCC patients.

Intrinsic or acquired drug resistance is a major factor limiting the effectiveness of chemotherapy in various cancers including ESCC. Drug resistance by tumours occurs not only to a single cytotoxic agent but also in the form of cross-resistance to many agents called MDR. One of the major mechanisms of MDR is an increased ability of tumour cells to actively efflux drugs, decreasing the intracellular drug accumulation. This mechanism is mediated by ATP-dependent drug efflux pumps known as ABC transporters (Leonard *et al*, 2003; Ozben, 2006). To date, at least 48 human ABC transporters have been identified, and they have been divided into seven sub-families, ABC-A through ABC-G. The first ABC transporter identified in this context was P-glycoprotein (PgP, MDR1, ABCB1) (Kartner *et al*, 1983), and in the absence of overexpressed MDR1, the protein MRP1, ABCC1 was discovered because of the MDR phenotype (Cole *et al*, 1992).

Cisplatin resistance is not a feature of MDR phenotypes conferred by either MDR1 or MRP1 (Borst *et al*, 2000). The finding that ABC transporter MRP2 could mediate active efflux of CDDP conjugated to glutathione (Taniguchi *et al*, 1996), supported by evidence that intracellular glutathione levels were related to CDDP toxicity (Ozols, 1985), suggested a possible role for active efflux as a resistance mechanism. In addition, human carcinoma cell line studies showed increased levels of MRP2 mRNA associated with relative CDDP resistance, decreased intracellular accumulation of CDDP, and decreased DNA adduct formation (Kool *et al*, 1997; Liedert *et al*, 2003). In ESCC cell lines (TE2, TE13) Tanaka *et al* (2010), in their analysis of the intracellular localisation of CDDP by using in-air micro-particle induced X-ray emission, recently reported that TE2 cells, which express lower MRP2 than TE13, had higher intracellular CDDP concentrations and sensitivity than TE13 cells. This is also in agreement with our present *in vitro* data regarding CDDP. In human tissue samples, accumulating evidence indicates that MRP2 expression is also associated with intrinsic CDDP resistance in the clinical setting, using tissues obtained from patients with colorectal cancer (Hinoshita *et al*, 2000), small-cell lung carcinoma (Ushijima *et al*, 2007), and ovarian cancer (Surowiak *et al*, 2006). These results are also consistent with our data from cancer tissue samples, although our *in vitro* data involving each single agent could not necessarily be translated directly to a clinical response to combination chemotherapy because of possible synergistic effects. However, in contrast with these data, other studies failed to show a significant association between MRP2 expression and chemosensitivity in patients with ovarian cancer (Arts *et al*, 1999; Materna *et al*, 2004) or lung cancer (Filipits *et al*, 2007; Kim *et al*, 2009). It therefore seems likely that multiple factors such as drug accumulation, DNA repair capacity, and apoptotic sensitivity contribute to clinical tumour chemosensitivity, and a mechanistic relationship could be difficult to detect amongst an unselected patient cohort in which a number of other factors also affect clinical outcome. As clinical significance of MRP2 other than chemosensitivity, Gan *et al* (2010) reported that MRP2 expression was significantly higher in poorly differentiated ESCC tumours compared with moderate or well differentiated ones, which was not observed in our study.

In terms of the contribution to acquired chemoresistance, the present IHC and qRT-PCR data showed higher MRP2 expression in resected tumours with NACT compared with those without NACT, implying residual tumours after NACT acquired the feature of chemoresistance. Unfortunately, we could not compare MRP2 expression levels in cancer tissues from the same patient before and after NACT because no samples were available. Nooter *et al* (1998) reported significantly higher MRP expression, although not specific MRP2 expression, in ESCC tumours from non-responders to CDDP-based chemotherapy when comparing MRP levels in paired tumour samples before and after chemotherapy, suggesting that chemotherapy was selected for drug-resistant cell clones. Furthermore, other *in vitro* analyses by Noma *et al* (2008) established two CDDP-resistant pancreatic cancer cell lines (SUIT-2-CD3 and SUIT-2-CD4) by continuously administering 10 nM CDDP for 3 and 4 months, respectively. Results of RT-PCR indicated that induction of MRP2 mRNA expression was significantly increased by 1.5- and 2.5-fold in SUIT-2-CD3 and SUIT-2-CD4 cells, respectively, compared with parent cells, whereas MRP1 and MRP3 expression remained unchanged, implying a contribution of MRP2 to acquired resistance for CDDP in pancreatic cancer.

An important observation regarding the functional significance of MRP2 expressed in tumour cells could be the sub-cellular localisation. In normal tissues, MRP2 is expressed in functionally

polarised cells in which it specifically localises to the apical membrane of these cells. Apical localisation has also been described in tumours arising from these sites, a feature attributed to a targeting signal in the C-terminus of the MRP2 molecule (Harris *et al*, 2001). Single-nucleotide polymorphisms in MRP2 have been described that result in cytoplasmic localisation of the protein and that may reduce *in vivo* function (Hirouchi *et al*, 2004). Reduced CDDP sensitivity has also been reported in polarised mammalian kidney cells transfected with appropriately localised MRP2 (Cui *et al*, 1999). Furthermore, data of Surowiak *et al* (2006) indicated that MRP2 could confer resistance to CDDP in ovarian carcinoma only when expressed at the nuclear membrane, and this was supported by *in vitro* data (Materna *et al*, 2006). Although our IHC results showed MRP2-positive staining of both cytoplasm and membrane in tumour cells, MRP2 protein located in the cell cytoplasm might not function as an efflux pump (Evers *et al*, 1998). Further analysis focusing on the sub-cellular localisation of MRP2, and on the functional and clinical significance of such cellular location, is needed to elucidate the specific mechanism of chemoresistance induced by MRP2 in ESCC.

In conclusion, MRP2 expression seems to be associated with intrinsic resistance to chemotherapy in patients with ESCC, and is likely to also have a role in acquired chemoresistance. Further studies with larger cohorts are warranted to verify these results prospectively. The findings of this study open the door for exploration of efficacious treatment strategies and development of new therapeutic approaches for ESCC.

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ORIGINAL ARTICLE – THORACIC ONCOLOGY

Survival Factors in Patients with Recurrence After Curative Resection of Esophageal Squamous Cell Carcinomas

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ABSTRACT

Background. Approximately half of patients who undergo curative resection for esophageal cancers develop recurrence postoperatively. The factors affecting survival after such recurrence remain largely unknown.

Methods. To investigate factors affecting survival after recurrence in patients who had undergone curative resection for esophageal cancer, we retrospectively reviewed data for 461 patients who underwent curative esophagectomy with or without preoperative therapy for esophageal squamous cell carcinoma from January 1996 to December 2007. The correlations between several clinicopathological factors and survival after recurrence were examined.

Results. Recurrence occurred in 196 of 461 patients (42.5%), with a median survival time after recurrence of 8.2 months. Multivariate analysis identified advanced tumor stage, preoperative chemoradiotherapy (CRT), number of recurrent tumors, and the presence of recurrence at the local site and liver as associated with shortened survival after recurrence. The analysis also indicated that treatment of the recurrence prolonged survival regardless of the treatment type. Although the pattern of recurrence did not significantly differ according to type of preoperative therapy, patients who underwent preoperative CRT were less often treated with radiotherapy for recurrence. Patients with multiple recurrent tumors less often received radiotherapy or surgery than those with a solitary recurrence. Chemotherapy for recurrence was not associated with either preoperative therapy or the number of recurrences.

Conclusions. Our retrospective study showed that multiple recurrent tumors and preoperative CRT limit the available treatment for recurrence and thereby are associated with poor prognosis. Vigorous treatment for recurrence can extend survival after recurrence in patients who undergo esophagectomy.

Surgical resection remains the primary treatment for thoracic esophageal cancers as it offers the best chance of cure. However, patients who undergo curative tumor resection often develop recurrent disease within a few years after surgery; the 5-year survival rates range from 31 to 55%.[1–4] Surgical series of such patients have documented the pattern and timing of recurrent disease and showed that the recurrence rate after curative esophagectomy ranges from 36% to 56% and the median time to recurrence ranges from 10 to 12 months.[3–10] Significant difficulty is often encountered in treating recurrent disease after esophagectomy, and patient prognosis is generally poor.[5–9] Thus, although recurrent disease after esophagectomy is not uncommon, a recommended treatment strategy remains to be established.

Multimodal treatment combining surgery with other treatments such as preoperative chemotherapy or chemoradiotherapy (CRT) is now used widely to improve resection strategies for esophageal cancers, although the associated survival benefit remains controversial.[11–15] Recent advances in anticancer drug and radiation techniques may particularly benefit patients with recurrent disease after curative resection and open up many new treatment options not previously available. In fact, recurrent disease sometimes responds better to anticancer

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treatment, and patients with recurrence can achieve relatively long-term survival. Thus, the factors affecting this survival after recurrence in patients with thoracic esophageal carcinomas need to be fully explored. Moreover, most patients included in previous studies of recurrent disease after esophagectomy received surgery alone without preoperative treatment, despite preoperative chemotherapy or CRT followed by surgery achieving mainstream status as a curative therapy for advanced esophageal cancer. Whether preoperative treatments affect the pattern of recurrence, treatment for recurrent disease, and survival after recurrence remains to be elucidated.

In the present study, we investigated those factors that affect survival of patients who experienced recurrence after curative resection for esophageal squamous cell carcinoma. Moreover, we also determined the pattern of recurrence according to type of preoperative treatment and examined whether preoperative therapy affects treatment for recurrent disease and survival after recurrence.

MATERIALS AND METHODS

Patient Population

From January 1996 to December 2007, 538 patients with thoracic esophageal cancer underwent surgery at The Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University. Among them, 31 patients underwent surgical resection via a transhiatal approach, and 18 patients underwent incomplete curative resection (R1 or R2). Excluding these 49 patients, 489 patients underwent curative esophagectomy (R0) with systematic lymphadenectomy. The primary tumor was typed histopathologically as squamous cell carcinoma in 461 patients, adenocarcinoma in 15, carcinosarcoma in 4, basaloid in 4, undifferentiated in 3, and melanoma in the remaining 2 patients. This study analyzed the 461 patients with squamous cell carcinoma, of whom 240 patients underwent 2-field lymphadenectomy and the remaining 221 patients underwent 3-field lymphadenectomy. During this period, indication for cervical lymph node dissection was determined, based on intraoperative genetic diagnosis of micrometastasis in recurrent laryngeal nerve chain nodes.[16,17]

Among 461 patients, 120 patients received preoperative chemotherapy followed by surgery and 83 patients received preoperative CRT followed by surgery; the remaining 258 patients received surgery alone. According to the principles of our institute, preoperative CRT followed by surgery was performed for patients showing deeply invading thoracic esophageal cancers (T3–T4) without distant organ metastasis or for those with tumors in the upper third of the thoracic esophagus that had infiltrated into the cervical esophagus. Preoperative chemotherapy followed by surgery was performed for patients with any T (cT1–4) and lymph node involvement, including regional lymph nodes (N1) and distant lymph nodes (M1 lym) without distant organ metastasis.

The study protocol was approved by the Human Ethics Review Committee of Osaka University Graduate School of Medicine.

Preoperative Treatment

The preoperative CRT regimen followed in our hospital comprises simultaneous radiation with administration of 5-fluorouracil (5-FU) and cisplatin as described previously.[18] A single daily fraction of 2 Gy was administered for 4–6 weeks, for a total dose of 40–60 Gy, concurrently with cisplatin and 5-FU. Preoperative chemotherapy in our institution consisted of cisplatin, adriamycin, and 5-FU. Cisplatin was administered at 70 mg/m,^{2} adriamycin at 35 mg/m^2 by rapid intravenous infusion on day 1, and 5-FU at 700 mg/m^2 administered by continuous intravenous infusion on day 1 through day 7. Two courses of chemotherapy were used, separated by a 4-week interval.[19]

Surgical Procedures

Our standard procedures consisted of subtotal esophagectomy with mediastinal lymphadenectomy via right thoracotomy, upper abdominal lymphadenectomy, reconstruction of a gastric tube, and anastomosis in the cervical incision. Two-field lymphadenectomy involved resection of the following lymph nodes based on the International Society for Disease of the Esophagus (ISDE): bilateral recurrent nerve nodes, upper-middle-lower thoracic paraesophageal nodes, tracheobronchial nodes, bifurcation nodes, bilateral main bronchus nodes, posterior mediastinal nodes, supradiaphragmatic nodes, cardiac nodes, celiac artery nodes, left gastric artery nodes, splenic artery nodes, lesser curvature nodes, and common hepatic artery nodes.[10,20] In cases of 3-field lymphadenectomy, deep cervical nodes, supraclavicular nodes, and cervical paraesophageal nodes were additionally removed. Abdominal para-aortic nodes were not usually removed, with rare exceptions. In patients undergoing preoperative chemotherapy or CRT, surgical resection was performed 3–6 weeks after completion of the preoperative therapy.

Adjuvant Therapy

During the period of this study in our institute, postoperative adjuvant therapy was not willingly undertaken. However, selected cases received postoperative adjuvant

therapy based on pathological tumor stage, particularly nodal status, tumor depth, and patient willingness. Of 461 patients, 66 patients (14.3%) received postoperative adjuvant therapy; 3 patients received radiotherapy alone, 7 patients received CRT consisting of simultaneous radiotherapy and administration of 5-FU/cisplatin, and 56 patients received chemotherapy alone (28 cases with 5-FU/cisplatin, 23 cases with intravenous administration of docetaxel or paclitaxel, and 5 cases with oral administration of S-1).

Follow-Up Examination and Definition of Recurrence Pattern

Following hospital discharge, patients were seen every 2 months for the first 2 years, and every 3 months thereafter. Computed tomography of the neck, thorax, and upper abdomen was performed every 4 months for the first 2 years and every 6 months thereafter. Upper gastrointestinal endoscopy was performed annually. When recurrence was suspected by computed tomography scan, more selective investigations such as positron emission tomography (PET), bone scintigraphy, and magnetic resonance imaging were performed to confirm or refute recurrent disease. During follow-up periods, the first site recurrence was noted, and any additional recurrence found within 1 month was considered to have occurred simultaneously. The pattern of recurrence was classified as follows: local recurrence was defined by recurrence at the site of the primary tumors, lymphatic recurrence was defined by recurrence at cervical, mediastinal, and/or abdominal lymph nodes, distant recurrence was defined as recurrence in distant organs such as lung, liver, bone, pleura, or peritoneum. The number of recurrent tumors was defined by adding up the number of recurrent nodules in each recurrent site. All data were collected, entered prospectively into a database, and updated at regular intervals. The median follow-up period of all 461 patients was 55.7 months (range, 22.1–105.8 months). Complete follow-up information until death or December 2009 was available for all patients.

Treatment for Recurrence

During the period of this study, available treatment was recommended for patients showing recurrent disease, providing that their general status permitted such a strategy and that the patient was willing. Surgery, radiation therapy, and different chemotherapy regimens were regarded as different treatments for recurrent disease in this study. Radiotherapy for recurrent disease was defined as that delivered at a dose of more than 30 Gy, excluding radiotherapy against bone metastasis for the purpose of palliative treatment. Radiotherapy delivered at different sites was defined as separate treatments for the purposes of this analysis.

Statistical Analysis

The pattern of recurrence and treatment for recurrence according to preoperative therapy and the number of recurrent tumors were compared using the chi-square test or Mann–Whitney U test. Overall survival was calculated from the date of operation to the event or last known date of follow-up. Actual survival was calculated according to Kaplan–Meyer and statistically evaluated by the log-rank test. The Cox proportional hazards regression model was used to analyze the simultaneous influence of prognostic factors. In all analyses, a P value $< .05$ was accepted as statistically significant. These analyses were carried out using StatView J 5.0 software package (Abacus Concepts, Berkeley, CA).

RESULTS

Pattern of Recurrence

Of 461 patients in this study, recurrence was observed in 196 patients (42.5%); Table 1 details the characteristics of these patients. Among various clinicopathological factors, histology, tumor depth, number of lymph node metastasis, tumor stage, and operative complications were associated with occurrence of recurrent disease. There was no statistically significant difference in incidence of recurrence according to preoperative treatment.

Local recurrence was observed in 36 patients, lymphatic recurrence in 125 (cervical in 35, thoracic in 76, abdominal in 42), and distant metastasis in 113 patients (sites were lung in 44, liver in 43, pleura or peritoneum in 27, bone in 25, skin in 4, brain in 4, adrenal gland in 1, and kidney in 1). Of the 196 cases of recurrent disease, 142 (72.4%) were recognized within the 1st year after resection, and 179 (91.3%) were within 2 years. The median time to recurrence overall was 7.3 months.

Treatment for Recurrent Disease

Of 196 patients, 52 (26.5%) received only treatment for symptomatic relief, while 144 (73.5%) patients received some form of therapy for recurrent disease including 120 (61.2%) of chemotherapy, 59 (30.1%) of radiotherapy, and 18 (9.2%) cases of surgical treatment (Table 2). In total, 235 treatment protocols were performed on the 144 patients; 74 received only 1 treatment, 53 received 2

TABLE 1 Characteristics of patients

	Total	Recurrence		*P* value
		Negative	Positive	
Cases	461	265 (57)	196 (43)	
Age	62.5 ± 8.2	62.7 ± 8.7	62.5 ± 8.9	0.7920
Gender				0.4179
Male	405	230 (57)	175 (43)	
Female	56	35 (63)	21 (37)	
Location				0.7422
Upper third	69	40 (58)	29 (42)	
Middle third	227	134 (59)	93 (41)	
Lower third	165	91 (55)	74 (45)	
Histology				0.0003
Well SCC	116	80 (69)	36 (31)	
Mod SCC	208	124 (60)	84 (40)	
Poorly SCC	137	61 (45)	76 (55)	
Tumor depth				<0.0001
pT0–1	163	126 (77)	37 (27)	
pT2	87	53 (61)	34 (39)	
pT3	192	83 (43)	109 (57)	
pT4	19	3 (16)	16 (84)	
No. of involved LN				<0.0001
<4	338	239 (71)	99 (29)	
≥4	123	26 (21)	97 (79)	
pStage				<0.0001
Stage 0–I	103	92 (89)	11 (11)	
Stage II	162	106 (65)	56 (35)	
Stage III	103	47 (46)	56 (54)	
Stage IV	93	20 (22)	73 (78)	
Preoperative therapy				0.1253
Surgery alone	258	159 (61)	99 (39)	
Chemotherapy	120	62 (52)	58 (48)	
Chemoradiotherapy	83	44 (53)	39 (47)	
Complications				0.0165
Absent	274	170 (62)	104 (38)	
Present	187	95 (51)	92 (49)	
Postoperative therapy				0.0005
Performed	66	25 (38)	41 (62)	
Not performed	395	240 (61)	155 (39)	

Mod moderately, *SCC* squamous cell carcinoma

different treatments, and 17 received 3 or more different treatments.

The most commonly used chemotherapeutic regimen for recurrence in the current study was the combination therapy of cisplatin and 5-FU with or without adriamycin, administered to 42 patients. The second-most commonly used regimen was combination therapy with S-1 and docetaxel, administered to 27 patients. Other less commonly used regimens included combined S-1 and cisplatin, docetaxel alone, paclitaxel alone, or cisplatin alone. Of 59 patients treated with radiotherapy, 17 received radiotherapy alone, while the remaining 42 patients received CRT. There were 18 recurrent diseases removed by surgery at the following sites: 4 cervical lymph node, 3 upper mediastinal lymph node, 3 abdominal lymph node, 2 brain, 2 lung, 2 anastomotic site, 1 liver, and 1 adrenal gland. For 10 patients with recurrence in lymph node, lymph node resection was performed. Among them, 2 patients who had recurrence in upper medial lymph node underwent lymph node resection combined with partial resection of trachea and making entrance of the trachea in anterior chest wall. For patients who had recurrence in brain, lung, and liver, resection of brain metastasis, pulmonary metastasis, and liver metastasis was performed, respectively. For patients with anastomotic recurrence in the neck, partial resection of anastomotic site and reanastomosis was performed. A patient who had recurrence in adrenal gland underwent resection of right adrenal gland.

Recurrence Pattern and Treatment for Recurrence Based on Preoperative Therapy

We next examined whether preoperative therapy affected the pattern and timing of recurrence and treatment for recurrence (Table 2). The patterns of recurrence did not significantly differ according to type of preoperative therapy. Time to recurrence was significantly shorter in patients who underwent preoperative chemotherapy or CRT than in those cases not given preoperative therapy (median time to recurrence; surgery alone, 8.4 months versus preoperative chemotherapy, 6.1 months, preoperative CRT, 5.7 months). Although chemotherapy for recurrence was performed regardless of preoperative therapy, patients who underwent preoperative CRT were less often treated with radiotherapy for recurrence than those who underwent surgery alone (34.3 vs. 15.4%). Similarly, patients who underwent preoperative CRT tended to less often receive surgical resection for recurrence compared with those who underwent either preoperative chemotherapy or surgery alone, although this difference was not statistically significant.

Factors Affecting Survival After Recurrence

The median survival time after recurrence was 8.2 months. Univariate analysis of the factors affecting survival after recurrence identified clinical stage, preoperative therapy, pathological stage, recurrence within 1 year, number of recurrent tumors, and recurrence at a local site, liver, or bone as significantly associated (Table 3). Patients who had 4 or more recurrent tumors showed significantly

TABLE 2 Recurrence pattern and treatment for recurrence according to preoperative therapy

	Preoperative therapy			
	Surgery alone	CT	CRT	Total
Recurrent cases	99	58	39	196
Initial stage				
cStage I	10 (10)	0 (0)	0 (0)	10 (5)
cStage II	38 (38)	9 (16)	6 (15)	53 (27)
cStage III	36 (36)	30 (52)	26 (67)	92 (47)
cStage IV	15 (16)	19 (32)	7 (18)	41 (21)
Pathological stage				
pStage 0–I	8 (8)	1 (2)	3 (8)	12 (6)
pStage II	24 (24)	16 (28)	15 (38)	55 (28)
pStage III	36 (36)	14 (24)	6 (16)	56 (29)
pStage IV	31 (32)	27 (46)	15 (38)	73 (37)
Recurrence within 1 year*				
Present	64 (65)	46 (79)	32 (82)	142 (72)
Absent	35 (35)	12 (21)	7 (18)	54 (28)
First recurrence site				
Local	16 (16)	9 (16)	11 (28)	36 (18)
Lymphatic	62 (63)	42 (72)	21 (54)	125 (64)
Distant	55 (56)	35 (60)	23 (59)	113 (58)
Number of recurrent tumors				
1	43 (43)	15 (26)	16 (41)	74 (38)
2–3	22 (22)	16 (28)	8 (21)	46 (23)
≥4	34 (35)	27 (46)	15 (38)	76 (39)
Treatment used for recurrence				
None	26 (26)	12 (21)	14 (36)	52 (27)
Any treatment	73 (74)	46 (79)	25 (64)	144 (73)
CT	55 (56)	42 (72)	23 (59)	120 (61)
RT	34 (34)**	19 (33)	6 (15)**	59 (30)
Surgery	9 (9)	7 (12)	2 (5)	18 (9)

CT chemotherapy, *CRT* chemoradiation therapy, *RT* radiation therapy
* $P = .0453$; ** $P = .0271$

poor survival after recurrence compared with those with a lower number of recurrent tumors (Fig. 1). Patients given preoperative CRT also showed significantly poorer survival after recurrence compared with surgery-alone patients and those given preoperative chemotherapy (mean survival time; CRT 6.4 months, surgery alone 13.1 months, chemotherapy 10.9 months, Fig. 2). Univariate analysis also showed that chemotherapy, radiation therapy, and surgery for recurrent disease can all improve survival after recurrence (Fig. 3a–d).

Multivariate analysis showed that preoperative CRT, the number of recurrent tumors, and the presence of recurrence at local site and liver were independent factors affecting survival after recurrence, and it identified the number of recurrence tumors as the most important prognostic factor (Table 4, model A). In multivariate analysis, pathological tumor stage was an independent prognostic factor although clinical tumor stage was not. Multivariate analysis also showed that chemotherapy and radiotherapy performed for recurrence were associated with prolonged survival (Table 4, model B). Surgery performed for recurrence showed borderline prognostic significance in multivariate analysis.

Treatment for Recurrence According to Number of Recurrences

Finally, we examined whether the number of recurrent tumors influenced treatment for recurrence. Although chemotherapy for recurrence was performed regardless of the number of recurrent tumors, patients with multiple recurrent tumors significantly less often received radiotherapy or surgery than those with a solitary recurrence (Table 5).

DISCUSSION

In this study, we investigated factors that affect survival of patients who had recurrent disease after curative resection for esophageal squamous cell carcinoma and also examined whether preoperative therapy affects pattern of recurrence, treatment for recurrent disease, and survival after recurrence. Our study found that the number of recurrent tumors, preoperative CRT performed, pathological tumor stage, and the recurrence at local site and liver were identified as independent prognostic factors, and that any type of treatment performed for recurrence can contribute to prolonged survival. Moreover, the success of treatment for recurrence was limited by the number of recurrent tumors and the preoperative therapy.

It is well known that the number of lymph node metastases is the most important prognostic factor in patients who had undergone esophagectomy, and patients with multiple lymph node metastases are recognized as having systemic disease.[21–23] Similarly, patients with multiple recurrent tumors may be recognized as having systemic recurrence at the time of diagnosis. In fact, the proportion of patients who received systemic chemotherapy was not different according to the number of recurrent tumors, whereas the proportion of patients who received radiation therapy or surgical resection as locoregional therapy was significantly lower in patients who had multiple recurrent tumors, especially more than 3, compared with patients with a solitary recurrence.

Previous studies suggested that radiotherapy may be effective therapy for locoregional recurrence. Some previous studies that involved no more than 30 patients reported median survival times from the detection of

TABLE 3 Univariate analysis for survival after recurrence

		HR	95% CI	*P* value
Before recurrence				
Age	≥70	1.25	0.88–1.70	.2275
Gender	Male	1.28	0.81–2.02	.2857
Location	Lower third	1.16	0.87–1.56	.3104
Histology	Poorly SCC	1.22	0.88–1.68	.2309
cStage	Stage III–IV	1.49	1.05–2.13	.0263
Number of LNs	≥4	1.30	0.97–1.72	.0762
pStage	Stage III–IV	1.54	1.13–2.10	.0064
Preoperative therapy				
CRT	Performed	1.83	1.25–2.67	.0018
CT	Performed	0.96	0.70–1.33	.8242
Postoperative complications	Present	1.30	0.98–1.73	.0716
At recurrence				
Recurrence within 1 year	Present	1.84	1.32–2.56	.0004
Recurrence site				
Local	Present	1.63	1.12–2.38	.0099
Lymphatic	Present	0.99	0.74–1.32	.9299
Cervical	Present	1.24	0.86–1.80	.2509
Thoracic	Present	1.12	0.84–1.50	.4401
Abdominal	Present	1.14	0.84–1.52	.4796
Distant	Present	1.58	1.17–2.14	.0031
Lung	Present	1.12	0.80–1.58	.4989
Liver	Present	1.83	1.30–2.58	.0006
Bone	Present	1.92	1.25–2.96	.0029
Dissemination	Present	1.48	0.98–2.23	.0630
No. of recurrent tumor	≥4	2.65	1.92–3.64	<.0001
After recurrence				
CT for recurrence	Performed	0.55	0.40–0.75	.0002
RT for recurrence	Performed	0.41	0.29–0.58	<.0001
Surgery for recurrence	Performed	0.33	0.186–0.60	.0001

SCC squamous cell carcinoma, *CT* chemotherapy, *CRT* chemoradiation therapy, *RT* radiation therapy, *HR* hazard ratio, *95% CI* 95% confidence interval

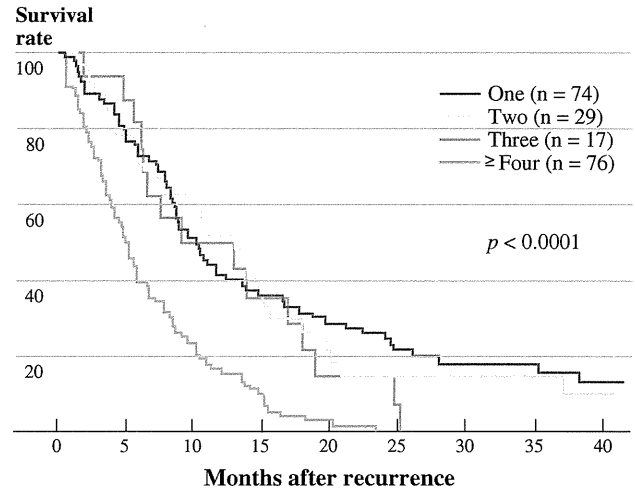

FIG. 1 Overall survival rate in 196 patients with recurrent disease after curative resection of esophageal cancer, according to the number of recurrent tumors. Patients who had more than three recurrent tumors showed significantly poor survival compared with those who had 1–3 recurrent tumors

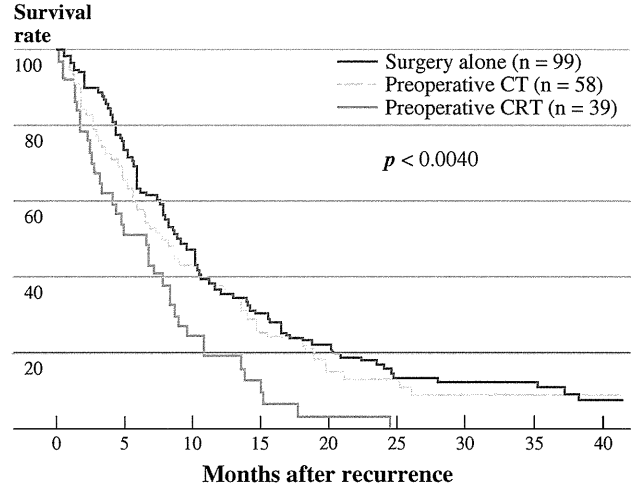

FIG. 2 Overall survival rate in 196 patients with recurrent disease after curative resection of esophageal cancer, according to type of preoperative therapy. Patients who underwent preoperative CRT showed significantly poor survival compared with those who underwent preoperative chemotherapy and surgery alone. *CT* chemotherapy, *CRT* chemoradiation therapy

recurrence of only 7–16 months.[24–27] However, the same studies found that patients with locoregional recurrence without distant organ metastasis and those with small recurrent tumors tended to show longer-term survival.[24,27] In our study, although median survival time after recurrence in 61 patients who received radiotherapy for recurrence was 13.8 months, 16 (26.2%) survived longer than 2 years after recurrence. Thus, it is clear that radiotherapy for recurrence will be effective for some patients. However, the majority of patients who had locoregional recurrence (local and/or lymphatic) after preoperative CRT followed by surgery was excluded from indication for radiation therapy, although proportion of those patients is not negligible. This could contribute partly to current result that patients who received preoperative CRT showed significantly shorter survival after recurrence than those who underwent surgery alone or preoperative chemotherapy.

In general, patients with recurrent disease after esophagectomy showed a poor prognosis, with previously reported survival times after recurrence less than 1 year. Dresner and Griffin reported a median survival time after recurrence of only 2.7 months in patients who developed