Abbreviations

EGFR	epidermal growth factor receptor
miR-7	microRNA-7
UTR	untranslated region.
CRC	colorectal cancer.

including colorectal tumors, and overexpression has been found to be associated with tumor progression, resistance to chemotherapy and radiation therapy and poor prognosis (7,8). Therefore, important therapeutics used in clinical practice include antibodies targeting EGFR and its downstream signaling effectors and low-molecular-weight compounds that inhibit signal transduction (11–13). Anti-EGFR antibodies have been used to treat CRC. However, tumors can develop resistance to these agents, limiting their clinical effectiveness (14). Resistance can be caused by mutations affecting EGFR downstream signaling and can be acquired during treatment (14). New therapeutic tools are currently being sought to help overcome this resistance.

A microRNAs (miRNAs) are non-coding RNAs of 21–23 nucleotides in length. miRNAs bind to complementary sequences in the 3′-untranslated regions (UTRs) of target mRNAs and inhibit translation. miRNAs are involved in cancer growth, differentiation, proliferation and apoptosis (15). We focused on microRNA-7 (*miR-7*), which has been reported to target *EGFR in vitro* (16). Rai *et al.* determined that *miR-7* targets not only but also v-*raf*-1 murine leukemia viral oncogene homolog 1 (*RAF-1*), a gene downstream of. As such, *miR-7* regulation might help overcome the resistance of tumors to *EGFR* inhibition therapies that are currently used in clinical practice (16).

The objectives of our study were to elucidate the clinical significance of *miR-7* expression in clinical specimens of CRC and to perform functional analysis of *miR-7* by using a CRC cell line. In our study, *miR-7* expression in these clinical specimens was measured, and its relationships with clinicopathological features, prognosis and *EGFR* protein expression were examined. Binding of *miR-7* to the 3′-UTR of *EGFR* mRNA and the 3′-UTR of RAF-1 mRNA was analyzed by using a luciferase assay. Three CRC cell lines were used to determine the regulatory effects of *miR-7* on cell proliferation, expression of genes downstream of and cetuximab sensitivity.

Materials and methods

Patients and sample collection

All clinical CRC samples (n = 105) in this study were used in accordance with institutional guidelines and the Helsinki Declaration after obtaining written informed consent from all participants. All patients underwent resection of the primary tumor at the Department of General Surgical Science of Gunma University Hospital in Japan between 1999 and 2009. All patients had a clear histologic diagnosis of CRC; the diagnoses were based on the clinicopathological criteria described by the Japanese Society for Cancer of the Colon and Rectum (http://www.jscr.jp/en/index.html). All patients were closely followed and were assessed every 3 months. The follow-up periods ranged from 0.7 months to 11 years, with a mean of 6 years. All sample data, including age, gender, histology, tumor size and depth, lymphatic invasion, vascular invasion, lymph node metastasis, liver metastasis, peritoneal dissemination, distant metastasis and clinical stage, were obtained from the clinical and pathologic records and are summarized in Table I.

The resected cancer tissues and adjacent non-cancerous tissues were immediately cut, frozen in liquid nitrogen and stored at –80°C until RNA and DNA were extracted. Total RNA was extracted by using the miRNeasy Mini kit (Qiagen) in accordance with the manufacturer's instructions.

Evaluation of miR-7 expression in clinical samples

For quantitative real-time reverse transcriptase–PCR of miR-7, cDNA was synthesized from 10ng of total RNA by using the TaqMan MicroRNA Reverse Transcription Kit and specific stem-loop reverse transcription primers (Applied Bio- systems, Carlsbad, CA) according to the manufacturer's protocol. PCR was performed in a LightCycler™ 480 System (Roche, Basel, Switzerland). The 10 µl PCR reaction included 0.67 µl of reverse transcription products, 1× TaqMan Universal PCR master mix and 1 µl of primers and probe mix included in the TaqMan miRNA assay kit. The reactions were incubated in 96-well optical plates at 95°C for 10min, followed by 45 cycles at 95°C for 15 s and 60°C for 10min. The expression levels of *miR-7* were normalized to that of the small nuclear RNA RNU6B and analyzed by using the $2^{-\Delta\Delta Ct}$ method.

Cell lines

The HCT116 and SW480 human colon cancer cell lines, which contain v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog gene (KRAS) mutations, were used. The HT29 human colon cancer cell line harbors a BRAF mutation. HCT116, SW480 and HT29 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin antibiotics (Invitrogen, Carlsbad, CA) in a humidified incubator with 5% CO_2 at 37°C.

Plasmid construction

The sequences in the 3′-UTR regions of *EGFR* mRNA and *RAF-1* mRNA that are targeted by *miR-7* were predicted with TargetScan (release 5.1), and the 3′-UTRs from human EGFR mRNA and RAF-1 mRNA were amplified from the genomic DNA of normal cells. The amplified fragments were inserted into the XhoI restriction site of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) by using the In-Fusion® Dry-Down PCR Cloning Kit (Clontech, Mountain View, CA). The nucleotide sequences of the plasmids were confirmed by sequencing.

Luciferase assay

HCT116 cells were seeded in 96-well plates and then cotransfected with 0.2 µg Luc-*EGFR* and *miR-7* precursor by using Lipofectamine RNAiMAX. Forty-eight hours following transfection, the activities of firefly and Renilla luciferase in cell lysates were measured by using the Dual-Glo® Luciferase Assay System (Promega) and the Fluoroskan Ascent FL (Thermo Fischer Scientific). The firefly luciferase activities produced by each vector were normalized to that of Renilla luciferase. All transfection experiments were conducted in triplicate.

Immunohistochemistry

Immunohistochemical studies of *EGFR* were conducted on formalin-fixed, paraffin-embedded surgical sections obtained from patients with CRC. The tissue sections were deparaffinized, soaked in 0.01mol/l sodium citrate buffer and boiled in a microwave oven for 5 min at 500W to retrieve the cellular antigens. A rabbit monoclonal antibody against EGFR (Cell Signaling Technology, Danvers, MA) diluted 1:100 was used as the primary antibody. All tissue sections were immunohistochemically stained with a streptavidin–biotin peroxidase complex solution (Nichirei Company, Tokyo, Japan) and counterstained with hematoxylin.

Transfection of the miR-7 precursor and miR-7 inhibitor

The pPre-miR™ miRNA Precursor hsa-miR-7-5p (*miR-7* precursor; Applied Biosystems), Pre-miR™ miRNA Precursor Molecules Negative Control (miR-nc; Applied Biosystems), mirVana® miRNA inhibitor hsa-miR-7-5p (*miR-7* inhibitor; Applied Biosystems) and mirVana® miRNA inhibitor Negative Control (miR inhibitor-nc; Applied Biosystems) were separately transfected at 20 nmol/l into HCT116, SW480 and HT29 cells by using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions.

Protein expression analysis

Western blotting was used to confirm the expression of the *EGFR*, *RAF-1*, *ERK1/2*, *pAKT* and β-actin proteins in *miR-7* precursor- and *miR-7*

Table 1. Relationship between *miR-7* expression and clinicopathological features

Factors	*miR-7/RNU6B*		*P* value
	High expression	Low expression	
	n = 37	*n* = 68	
Age (*n*)			0.385
< 59	11	15	
≧59	26	53	
Gender (*n*)			0.724
Male	21	41	
Female	16	27	
Histology (*n*)			0.753
Well	12	20	
Others	25	48	
Tumor size (mm)			0.083
< 50	20	32	
≧50	17	36	
Depth			0.630
m, sm, mp	14	29	
ss, se, si	23	39	
Lymphatic invasion			0.822
Negative	8	12	
Positive	29	56	
Venous invasion			0.733
Negative	16	42	
Positive	21	26	
Lymph node metastasis			0.353
Negative	22	34	
Positive	15	34	
Liver metastasis			0.478
Negative	30	51	
Positive	7	17	
Peritoneal dissemination			0.133
Negative	37	64	
Positive	0	4	
Distant metastasis			0.662
Negative	36	65	
Positive	1	3	
Stage			0.627
I, II	15	31	
III, IV	22	37	

Well, well differentiated.

inhibitor-transfected cells. Total protein (40 μg) was electrophoresed and then electrotransferred at 200 mA for 180 min at 4°C. These proteins were detected by using an anti-EGFR rabbit monoclonal antibody (1:1000; Cell Signaling Technology), an anti-ERK1/2 rabbit monoclonal antibody (1:1000; Cell Signaling Technology), a rabbit monoclonal antibody against *Raf-1* (1:1000; Origene, Rockville, MD) and an anti-p*AKT* rabbit monoclonal antibody (1:1000; Cell Signaling Technology); an anti-β-actin mouse monoclonal antibody (1:1000; Sigma–Aldrich, St Louis, MO) served as a control. Bands and band intensities were detected and calculated, respectively, by using ECL Prime Western Blotting Detection Reagent and an Image Quant LAS 4000 (GE Healthcare Life Sciences).

Proliferation assay

Analysis of proliferation was performed on cells that had been transfected with either the *miR-7* precursor or the *miR-7* inhibitor. The cells were plated in 96-well plates in 100 μl of medium at ~5000 cells per well. To quantitate cell viability with the Cell Counting Kit-8 assay (CCK-8; Dojindo Laboratory, Tokyo, Japan), 10 μl of the cell counting solution was added to each well after 0, 24, 48 or 72 h, and then the plates were incubated at 37°C for 2 h. The cell proliferation rate was then determined by measuring the absorbance of the well at 450 nm with the reference wavelength set at 650 nm. The absorbances were measured with a microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Regulation of the cetuximab sensitivity of CRC cells by miR-7

HCT116, SW480 and HT29 cells, 5000 cells per well, were seeded in 96-well plates and then treated with 0, 0.01, 0.1, 1, 10, 20, 50 or 100 μg/ml cetuximab for 96 h. For each dose, cells from one plate were harvested to determine the absorbance value. Viable cells were counted 96 h posttreatment with the CCK-8 assay by measuring the absorbances of the samples at 450 nm, with the reference wavelength set at 650 nm.

Statistical analysis

The differences between two groups were estimated by using the *t*-test, the chi-square test and the repeated measures analysis of variance test. Kaplan–Meier curves were generated for overall survival, and statistical significance was determined by using the log-rank test. A probability value of <0.05 was considered significant. In addition, univariate and multivariate survival analyses were performed using Cox's proportional hazards model. All statistical analyses were performed with JMP5.0 software (SAS Institute Incorporated, Cary, NC).

Results

The clinicopathological significance of miR-7 expression in CRC

The expression levels of *miR-7* in cancerous tissues (T) were higher than those in adjacent, non-cancerous tissues (N) ($P < 0.001$; Figure 1A). In this study, the receiver operating characteristic curve determined the cutoff point. Cancerous tissue below the cutoff point for *miR-7* expression of 3.21, normalized to expression of the U6 small nuclear RNA *RNU6B*, was assigned to the low-expression group ($n = 68$), whereas cancerous tissue with an expression level above the cutoff point was assigned to the high-expression group ($n = 37$). Patients in the low-*miR-7*-expression group had a significantly poorer prognosis than those in the high-*miR-7*-expression group ($P = 0.0489$; Figure 1B). In addition, the clinicopathological factors of age, gender distribution, histology, tumor depth, lymphatic or venous invasion, lymph node, liver, or distant metastasis, peritoneal dissemination, or clinical staging were not significantly different between these two groups (Table I). However, tumor size in the low-*miR-7*-expression group showed a non-significant increase ($P = 0.083$) over that seen in the high-*miR-7*-expression group (Table I). The results of univariate and multivariate Cox proportional hazards regression analyses for overall survival are shown in Supplementary Table 1, available at *Carcinogenesis* Online. Multivariate analysis indicated that low expression of *miR-7* was an independent and significant prognostic factor for survival (relative risk: 0.82; 95% confidence interval: 0.54–0.94; $P = 0.0430$; Supplementary Table 1, available at *Carcinogenesis* Online).

miR-7 regulates EGFR and RAF-1 in CRC cells

By using *in silico* miRNA target prediction tools, such as TargetScan, we identified *miR-7* binding sites in the 3′-UTRs of transcripts encoding EGFR and RAF-1 (Figure 2A and C). To investigate miRNA binding and repression, we performed a luciferase reporter assay with a vector in which the 3′-UTR sequences of *EGFR* mRNA and *RAF-1* mRNA were inserted downstream of the luciferase reporter gene (Luc-EGFR, Luc-RAF-1). The luciferase

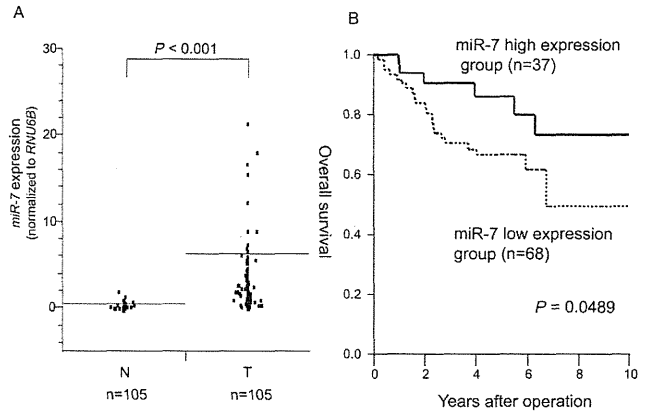

Figure 1. Clinical significance of *miR-7* expression in CRC samples. (A) *miR-7* expression in cancerous (T) (n = 105) and adjacent non-cancerous (N) (n = 105) tissues from CRC patients assessed by TaqMan reverse transcription–PCR. All data were normalized to RNU6B. Horizontal lines indicate the means ($P < 0.001$). (B) Kaplan–Meier curves according to *miR-7* expression levels in CRC patients ($P = 0.0489$).

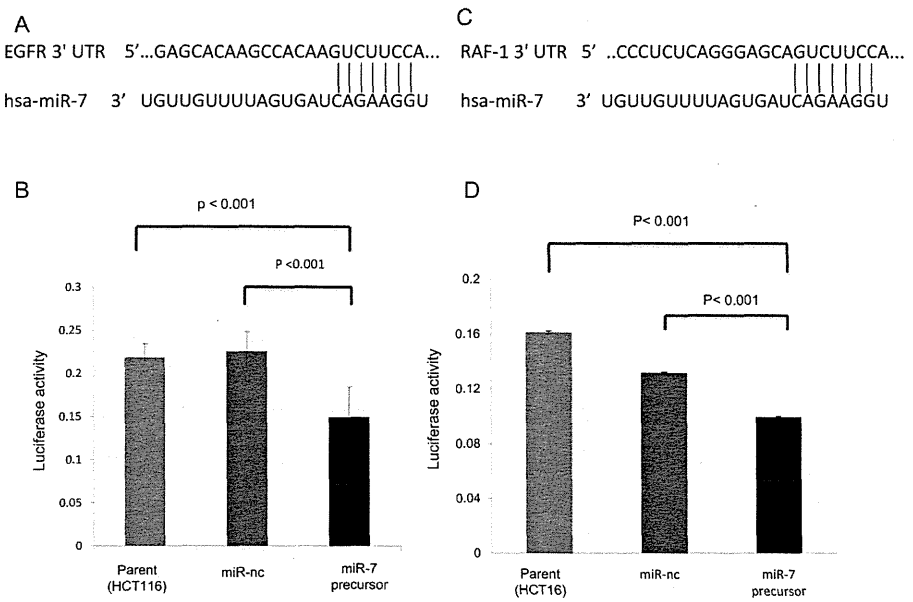

Figure 2. *EGFR* and *RAF-1* expression is directly suppressed by *miR-7* in CRC. (A) *miR-7* binding sites in the *EGFR* 3′-UTR. Putative conserved target sites in the 3′-UTR were identified using *in silico* miR target prediction tools. (B) Luciferase assays of pre-*miR-7*-transfected HCT116 cells. The error bars represent the SD from eight replicates. Left bar: *EGFR* 3′-UTR luciferase vector only. Middle bar: *EGFR* 3′-UTR luciferase vector + miR-nc. Right bar: *EGFR* 3′-UTR luciferase vector + *miR-7* precursor ($P < 0.001$). (C) *miR-7* binding sites in the *RAF-1* 3′-UTR. Putative conserved target sites in 3′-UTR were identified using *in silico* miR target prediction tools. (D) Luciferase assays of pre-*miR-7*-transfected HCT116 cells. The error bars represent the SD from eight replicates. Left bar: RAF-1 3′-UTR luciferase vector only. Middle bar: RAF-1 3′-UTR luciferase vector + miR-nc. Right bar: RAF-1 3′-UTR luciferase vector + *miR-7* precursor ($P < 0.001$).

activities of Luc-EGFR and Luc-RAF-1 were both significantly reduced when compared with that of the negative control in transient cotransfection of HCT116 cells with *miR-7* precursor ($P < 0.001$, $P < 0.001$, respectively; Figure 2B and D). These data suggest that the 3′-UTRs of both *EGFR* and *RAF-1* are direct functional targets of *miR-7*.

miR-7 and EGFR protein expression in clinical samples

For each frozen tissue sample used to measure miR-7 levels in colorectal tumors and adjacent non-cancerous tissue, representing 105 patients with CRC, there were matching, adjacent formalin-fixed, paraffin-embedded surgical sections from the same tumor the association between *miR-7* and *EGFR* protein expression in clinical samples, we used immunohistochemistry sections of the formalin-fixed, paraffin-embedded samples, which were then divided into groups based on a score of *EGFR* protein expression. Samples were further classified according to the staining patterns in the tumor cell membranes as either incomplete staining, i.e. tumor cells were stained in only part of their membrane, and complete staining, i.e. tumor cells displayed a circumferential staining of the entire tumor cell membrane (17). The following scoring system for assessing EGFR immunostaining was used: score 0 = no staining or unspecific staining of tumor cells; score 1 = weak (intensity) and incomplete staining (quality) of > 10% of tumor cells (quantity); score 2 = moderate and complete staining of > 10% of tumor cells; score 3 = strong and complete staining of > 10% of tumor cells. Representative examples for the different scores are shown in Supplementary Figure 1A, available at *Carcinogenesis* Online. The expression of *miR-7* was significantly increased in the EGFR expression-negative group (score 0) compared with the EGFR expression-positive group (score 1–3) (mean ± SEM: EGFR expression-negative group = 6.01±0.58; EGFR expression-positive group = 2.19±0.38; $P = 0.043$; Supplementary Figure 1B, available at *Carcinogenesis* Online).

Expression of EGFR, RAF-1, ERK1/2 and pAKT is suppressed by miR-7 in vitro

We used quantitative reverse transcription–PCR to confirm that *miR-7* expression in cells transfected with *miR-7* precursor was significantly higher than that in both untreated cells ('Parent' in Figures 2–5) and cells transfected with miR-nc ($P < 0.001$, Supplementary Figure 2, available at *Carcinogenesis* Online). We also determined that *miR-7* expression in cells transfected with *miR-7* inhibitor was significantly lower than that in either untreated cells or cells transfected with the miR inhibitor-nc ($P < 0.001$, Supplementary Figure 3, available at *Carcinogenesis* Online). To determine whether *miR-7* suppresses EGFR expression and downstream signaling events in the CRC cell lines HCT116, SW480 and HT29, cell lysates of transfected cells were analyzed by Western blotting. They were then compared with untreated cells and miR-nc-treated cells. In HCT116 and SW480 cell lines (*KRAS* mutation), the expression of *EGFR*, *RAF-1*, *pAKT* and *ERK1/2* was downregulated in cells transfected with *miR-7* precursor (Figure 3A and C), while the expression of *EGFR*, *RAF-1*, *pAKT* and *ERK1/2* in cells transfected with *miR-7* inhibitor was upregulated relative to the expression in untreated cells and cells transfected with the *miR inhibitor-nc* (Figure 3B and D). However, in the HT29 cell line (*BRAF* mutation), the expression of *EGFR*, *RAF-1* and *pAKT* was downregulated in cells transfected

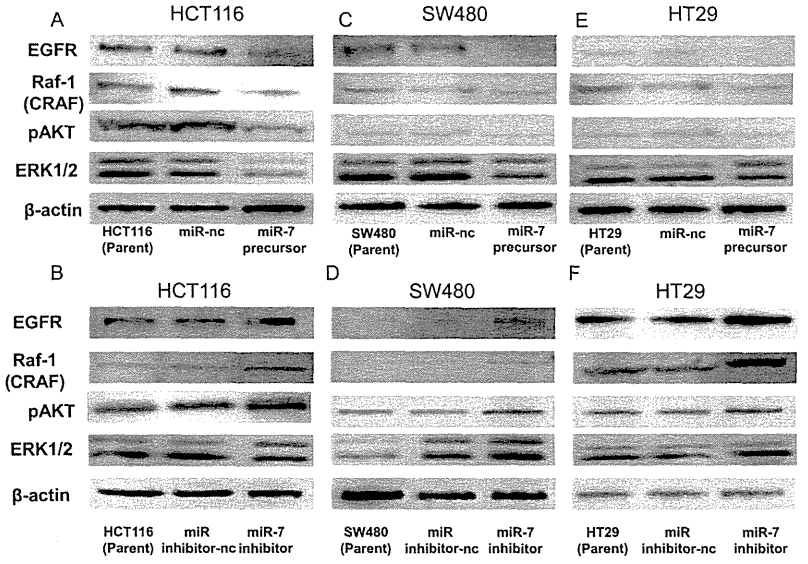

Figure 3. Expression of EGFR, *Raf-1*, *pAKT* and *ERK1/2* is suppressed by *miR-7* in CRC cells. Western blotting of *EGFR*, *Raf-1*, pAKT and ERK1/2 protein in *miR-7* precursor-transfected *HCT116* cells (A), *SW480* cells (C) and *HT29* cells (E). Western blotting of *EGFR*, *Raf-1*, *pAKT* and *ERK1/2* protein in *miR-7* inhibitor-transfected *HCT116* cells (B), SW480 cells (D) and HT29 cells (F). Protein levels were normalized with respect to beta-actin.

with the *miR-7* precursor, while *ERK1/2* expression was upregulated (Figure 3E). These cells also upregulated the expression of *EGFR*, *RAF-1*, *pAKT* and *ERK1/2* upon transfection with miR-7 inhibitor relative to the expression in untreated cells and cells transfected with miR inhibitor-nc (Figure 3F).

miR-7 regulates proliferation in HCT116, SW480 and HT29 cells

We analyzed the proliferation of HCT116, SW480 and HT29 cells that had been transfected with either miR-7 precursor or miR-7 inhibitor. In HCT116 and SW480 cells, the proliferation rate of miR-7 precursor-treated cells was significantly lower than that of untreated cells ($P < 0.001$; Figure 4A and B). In contrast, the proliferation rate of miR-7 inhibitor-treated cells was significantly higher than that of untreated cells ($P < 0.001$; Figure 4A and B). In HT29 cells, the proliferation rate of miR-7 precursor-treated cells was not significantly different from that of untreated cells ($P = 0.0636$; Figure 4C). Similarly, there was no significant difference between the proliferation rates of miR-7 inhibitor-treated cells and untreated cells ($P = 0.2151$; Figure 4C).

miR-7 regulates cetuximab sensitivity in cetuximab-resistant HCT116 and SW480 cells with a KRAS mutation and HT29 cells with a BRAF mutation

To determine if cetuximab, an EGFR-targeted antibody, enhances the antitumor efficacy of miR-7 in CRC cells, we treated HCT116, SW480 and HT29 cells with cetuximab and analyzed proliferation in cells that received only cetuximab treatment and cells that were transfected with either miR-7 precursor or miR-nc after cetuximab treatment. HCT116 and SW480 colon cancer cells carry *KRAS* mutations and, as such, are resistant to cetuximab. Whereas untreated cells and miR-nc-treated cells were highly resistant to cetuximab, the *miR-7* precursor-treated cells responded to this drug ($P < 0.001$; Figure 5A and B). However, in HT29 cells with a *BRAF* mutation, there was no change in the sensitivity to cetuximab in cells treated with the miR-7 precursor ($P = 0.8584$; Figure 5C).

Discussion

In this study, we determined that the expression of miR-7 in primary CRC is higher than in normal colorectal tissues; however, a low level of miR-7 expression is associated with cancer progression and poor prognosis. We also determined that miR-7 regulates proliferation and cetuximab sensitivity via *EGFR* suppression.

We focused on miR-7, which has been reported to target *EGFR in vitro* (16). Rai *et al.* demonstrated that miR-7 targets not only *EGFR* but also *RAF-1*, a gene downstream of *RAS*. Mir-7 targets many other genes besides those analyzed in this study. Induction of miR-7 might overcome resistance of tumors to therapies that inhibit *EGFR* (16,18). Cetuximab is one of the targeted molecular drugs that have recently been used to treat patients with CRC. However, unresolved issues with this drug persist, such as acquired treatment resistance caused by mutations in *KRAS* and *BRAF*, both of which participate in the *EGFR* signaling pathway (19). Cetuximab is known to have low effectiveness in patients with either *KRAS* or *BRAF* mutations; this feature warrants limitations on its use (19). We examined how treatment with miR-7 changes cetuximab sensitivity in cetuximab-resistant HCT116 and SW480 cells, which harbor *KRAS* mutations, and HT29 cells, which harbor a *BRAF* mutation. Cetuximab treatment did not reduce proliferation of the parent HCT116 and SW480 cells, but cetuximab sensitivity increased in miR-7 precursor-treated HCT116 and SW480 cells. Since miR-7 does not target *EGFR* alone, it would be difficult to markedly increase cetuximab sensitivity by treatment with miR-7. miR-7 has been reported to bind to the 3′-UTR in the mRNA of not only *EGFR*, but also to that of *RAF-1*, a gene downstream of *KRAS*, and inhibit translation (16). Similarly, in this study, *RAF-1*, which is downstream of *EGFR*, is also a potential target of miR-7 (Figure 2). In HT29 cells with a *BRAF* mutation, there was no change in the sensitivity to cetuximab in cells treated with the miR-7 precursor. When *KRAS* and *BRAF* are not mutated, *KRAS* signals to *ERK1/2* through *BRAF* rather than *RAF-1*. When *KRAS* is mutated, however, KRAS signaling switches from *BRAF* to *RAF-1*, and the *EGFR* pathway is hyperactivated. When *BRAF* is mutated, the *EGFR* pathway is also hyperactivated, but instead by BRAF-dependent activation of *ERK1/2* (20). In HT29 cells that do not have mutated *KRAS*, *EGFR* signaling occurs through *BRAF*, and as such the combination effect of cetuximab with *miR-7* that is targeted to *RAF-1* would not be expected to occur.

miR-7 inhibits *EGFR* signaling that may regulate the growth capacity and cetuximab sensitivity of colon cancer cells. When there is a mutation in *BRAF* in colon cancer, a combined effect of an *EGFR* inhibitor and *miR-7* does not occur, but in cancers that have both low *miR-7* expression and *KRAS* mutations, *miR-7* administration may be a strategy to overcome resistance to therapeutic agents.

We showed that expression of *miR-7* was increased in CRC specimens. In lung cancer, *EGFR* mutations have also been reported to induce *miR-7* expression (16).

When *EGFR* activation is caused by a driver mutation, the effects of changes in EGFR expression levels are small in CRC, which is in contrast to the effects of EGFR in lung cancer, suggesting that the signaling mechanisms may differ across cancer types (21). This finding suggests that activation of *EGFR* signaling and induction of *miR-7* expression might be specific to the cancer type. Our study also showed that *miR-7* expression was significantly lower in the clinical specimens of patients with CRC who had positive *EGFR* protein expression than in the clinical specimens of those with negative expression. An *in vitro* analysis revealed that *EGFR* and *RAF-1* translation were inhibited by the binding of *miR-7* to the 3′-UTR of *EGFR* mRNA and *RAF-1* mRNA in a CRC cell line. Our data suggest that *miR-7* might not be induced by *EGFR* signaling and that *miR-7* inhibits *EGFR* signaling. Thus, a low level of *miR-7* expression in CRC lesions is thought to be associated with the progression of cancer and a poor prognosis. From our *in vitro* data, it is expected that the cetuximab sensitivity in primary CRC with high *miR-7* expression is higher than in cases of low miR-7 expression, even if they have a *KRAS* mutation. *miR-7* might be a useful cetuximab sensitivity marker in CRC.

In conclusion, the low expression of *miR-7* correlated with cancer progression and poor prognosis. Low expression of *miR-7* could be a useful prognostic marker for CRC. In addition, *miR-7* regulates CRC cell proliferation and resistance to cetuximab *in vitro*. Finally, *miR-7* might be a promising candidate for targeted therapy in patients with CRC whose tumors are resistant to EGFR-directed antibodies.

Supplementary material

Supplementary Table 1 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

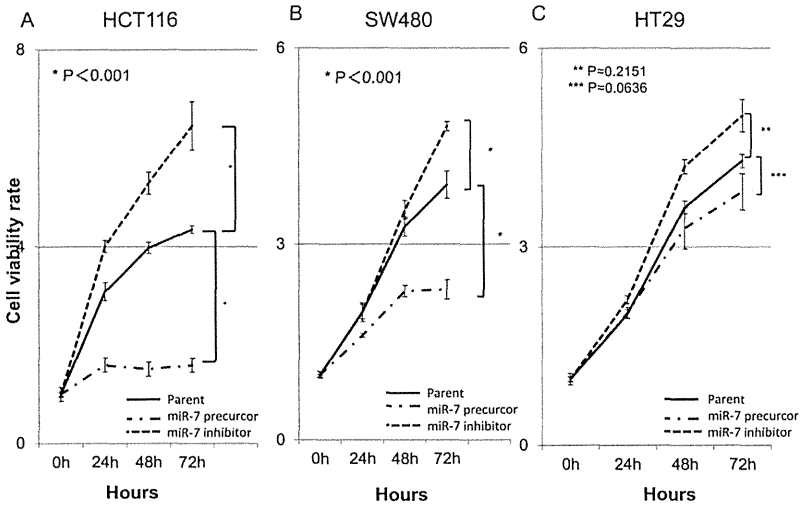

Figure 4. Proliferation potency is suppressed by *miR-7* in HCT116, SW480 and HT29 cells. (A and B) In HCT116 and SW480 cells, the proliferation rate of *miR-7* precursor-treated cells was suppressed in comparison with that of untreated cells. In contrast, the proliferation rate of *miR-7* inhibitor-treated cells was significantly higher than that of untreated cells. (C) In HT29 cells, the proliferation rate of *miR-7* precursor-treated cells was not significantly different from that of untreated cells. Similarly, there was no difference between the proliferation rates of *miR-7* inhibitor-treated and untreated cells. The data represent the means ± SD of five replicates.

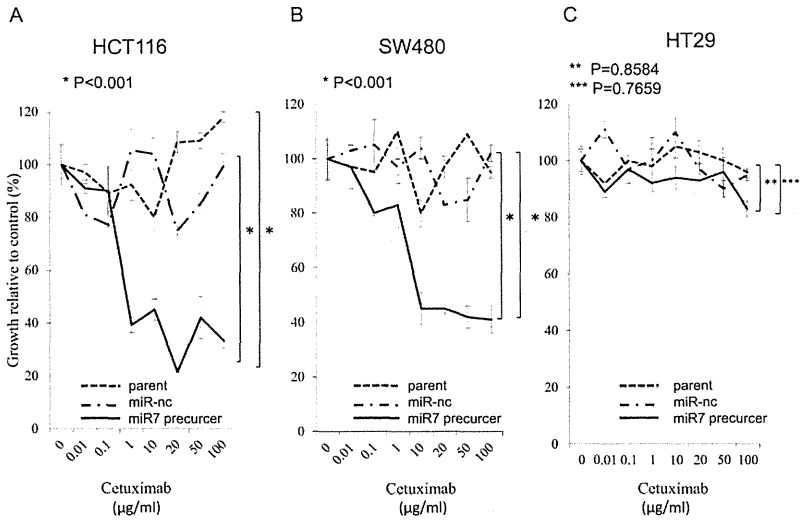

Figure 5. *miR-7* inhibits the proliferation of the CRC cell line EGFR in combination with cetuximab. To determine the cetuximab sensitivity of the cetuximab-resistant (A) HCT116 and (B) SW480 cells with KRAS mutations and (C) HT29 cells with a *BRAF* mutation, 5000 cells per well were seeded in 96-well plates and then treated with 0, 0.01, 0.1, 1, 10, 20, 50 or 100 μg/ml cetuximab for 96 h. Viable cells were counted 96 h posttreatment with the CCK-8 assay. The results are presented as means ±SD of three replicates.

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ORIGINAL ARTICLE

Phase I/II study of docetaxel, cisplatin, and 5-fluorouracil combination chemoradiotherapy in patients with advanced esophageal cancer

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Abstract
Purpose This phase I/II study was aimed to determine the recommended dose (RD) of docetaxel, cisplatin, and 5-fluorouracil as combination chemoradiotherapy (DCF-RT) for patients with esophageal cancer and to evaluate the efficacy and safety of this protocol.
Methods Fourteen patients with esophageal cancer enrolled in this dose escalation study to determine the RD for a phase III trial. Efficacy and toxicity in DCF-RT of RD were evaluated in 37 patients with esophageal cancer.
Results The RD for DCF-RT for esophageal cancer in the present study was 50 mg/m^2 docetaxel plus 60 mg/m^2 cisplatin on day 1 and day 29 plus 600 mg/m^2 5-FU on days 1–4 and days 29–32 and concurrent radiation of 60 Gy/30 fractions/6 weeks. The main toxicities were myelotoxicity and radiation esophagitis. In this phase I/II study, we could have safety and feasibility by RD, because there was low mortality and most toxicities were manageable level. The complete response (CR) rate and response rate were 54.1 and 83.8 %, respectively, in the phase II study. In patients with a classification of clinical T4, the CR rate and response rate were 47.6 and 85.7 %, respectively. The 2-year overall survival rate, 2-year progression-free survival rate, and median survival time (MST) were 52.9, 50.0 %, and 24.7 months, respectively. In patients with clinical T4 classification, the 2-year overall survival rate, 2-year progression-free survival rate, and MST were 43.5, 44.9 %, and 21.6 months respectively.
Conclusions DCF-RT keeps safety and feasibility by management of myelotoxicity adequately in RD. This protocol might produce a high CR rate and favorable prognosis compared with standard chemoradiotherapy for advanced esophageal cancer.

Keywords Docetaxel · Cisplatin · 5-Fluorouracil · Chemoradiotherapy · Esophageal cancer

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Introduction

Esophageal cancer is a malignant tumor that is the sixth most common cause of cancer death worldwide, with occurrence rates varying greatly by geographic location [1]. Surgery is the standard therapy for patients with resectable esophageal cancer. However, survival after surgery alone is still not satisfactory although new surgical techniques and perioperative multimodality treatments have been developed. Neoadjuvant chemotherapy and chemoradiotherapy (CRT) increase local control and improve survival according to a recent meta-analysis of randomized trials [2].

CRT is standard therapy for patients with unresectable esophageal cancer and will-less of surgery. The prognosis of esophageal cancer with other organ invasion (T4) is unfavorable. The treatment strategy for T4 esophageal cancer is to control invasiveness to other organs using chemotherapy and radiotherapy or to resect the lesion after downstaging by CRT. It is controversial whether which is better: definitive CRT or surgery after CRT. Cisplatin and 5-FU therapy (CF therapy) is a standard protocol for patients

with unrespectable esophageal carcinoma [3–5]. In phase II studies of CRT with PF therapy plus 60 Gy of radiotherapy in T4 tumors and/or M1 lymph node metastasis, the rate of complete response (CR) was 15–33 % with a 3-year survival rate of 23 % [6, 7]. This performance was not satisfactory in these studies. It is a priority to establish a more effective chemoradiotherapeutic regimen for patients with unrespectable esophageal cancer or recurrent esophageal cancer.

Response rates (RR) of 20–30 % were obtained in phase II studies of docetaxel (70–100 mg/m^2) for advanced and recurrent esophageal squamous cell carcinoma [8–10]. Docetaxel is a radiosensitizer just like cisplatin and 5-FU [11, 12]. There are some reports describing the use of a combination of docetaxel, cisplatin, and 5-FU with concurrent radiotherapy (DCF-RT) for esophageal carcinoma [13–16]. However, there are very few reports about definitive CRT using DCF-RT [13]. Therefore, we conducted a phase I/II clinical trial of a DCF regimen with concurrent radiotherapy in patients with advanced esophageal carcinoma.

The objective of this study was to determine the recommended dose (RD) of DCF combination CRT for patients with esophageal cancer and to evaluate the efficacy and safety of this protocol to determine whether this regimen merited further investigation by a phase III trial.

Patients and methods

This open-label, prospective, phase I/II study was conducted at Gunma University Hospital, Gunma, Japan. This study was approved by the institutional review board of Gunma University Hospital, and all patients gave written informed consent before enrollment.

Eligibility criteria

Patients fulfilling the following criteria were eligible for the study: histopathologic or cytologic diagnosis of esophageal carcinoma; unresectable tumor, rejection of surgery, or recurrent esophageal carcinoma; no prior chemotherapy and/or CRT was allowed. Eligibility required that subjects be 20–75 years of age and an Eastern Cooperative Oncology Group performance status of 0–2. We did not restrict patients according to whether there was an evaluable lesion or not by RECIST. Patients also had to fulfill the following criteria in the 2 weeks before registration in the study: white blood cell count 4,000–12,000/mm^3; mature granulocyte count ≥2,000/mm^3; blood platelet count ≥100,000/mm^3; hemoglobin ≥9.5 g/dL; AST and ALT ≤1.5 times the upper limit of normal at our hospital; total bilirubin ≤1.5 mg/dL; A1-P ≤2.5 times the upper limit of normal at our hospital; creatinine ≤1.2 mg/dL; PaO_2 ≥60 torr (in room air); and an expected prognosis ≥3 months.

Patients were excluded for the following reasons: known sensitivity to docetaxel, cisplatin, 5-FU, or polysorbate 80; presence of other severe diseases, including malignant hypertension, severe heart failure, liver failure, liver cirrhosis, inadequately controlled diabetes mellitus, or bleeding disorders; current infectious disease with fever; presence of motor paralysis, peripheral neuropathy, or severe edema; pleural effusion or cardiac effusion needing treatment; the presence of multiple primary cancers; pregnancy or breast-feeding; presence of interstitial pneumonia on chest X-rays or CT, or pulmonary fibrosis; known psychosis or neurologic manifestations, or patients considered unlikely to fully cooperate in the study; and patients deemed inappropriate for the study by the investigator for any other reason.

Dose-limiting toxicity and recommended dose

Toxicity was graded using the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 4.0. Complete blood count was determined every week in all treatment cycles. The patient's full medical history and biochemistry profiles were assessed before starting each treatment cycle. If grade 4 neutropenia occurred, the complete blood count was repeated daily during the treatment cycle to determine its duration.

Dose-limiting toxicity (DLT) during CRT was defined as follows: (1) grade 4 leukopenia or neutropenia lasting for ≥5 days, or grade 3/4 febrile neutropenia; (2) grade 4 thrombocytopenia; (3) any other grade 3 non-hematologic toxicity (excluding alopecia, nausea, vomiting, appetite loss, esophagitis, general fatigue); or (4) planned treatment delayed by ≥2 weeks.

A minimum of three patients were enrolled at each dose level (Table 1). We used the typical phase I procedure of enrolling further patients if no DLT was observed in more than 1/3 patients. The RD was defined as the dose level below the maximum tolerated dose (MTD) in which DLTs were observed in ≥3 patients from a cohort of 3–6 patients. The phase II study used the recommended dosages determined in the phase I study.

Table 1 Dose levels applied in the study

Dose level	Docetaxel (mg/m^2)	Cisplatin (mg/m^2)	5-Fluorouracil (mg/m^2)
1	40	50	500
2	50	50	500
3	50	60	600
4	60	60	600
5	60	70	700

Dose modification and delay

In the event of toxicity, the administration of all three drugs was delayed until adequate hematologic recovery was achieved (defined as a white blood count 2,500–12,000/mm^3, neutrophil count $\geq$1,000/mm^3, and platelet count $\geq$75,000/mm^3). Non-hematologic toxicities, excluding nausea and alopecia, were grade 2 or less before starting each treatment cycle. If the event did not resolve within 2 weeks after the planned treatment date, the patient was withdrawn from the study. If the event of a DLT and/or grade 4 myelotoxicity at first administration occured, the dose of three drugs was reduced by 20 % for the second cycle.

Treatment plan

Patients received docetaxel diluted in 250 mL of normal saline at the assigned dose intravenously over 2 h. Then cisplatin was prepared in normal saline at the assigned dose and administered over 2 h on day 1. 5-FU was prepared in normal saline at the assigned dose and administered continuously on days 1–4. Docetaxel and cisplatin were given on days 1 and 29, and 5-FU was given on days 1–4 and 29–32.

External radiotherapy was delivered by a 2-field technique using 10 MV X-rays at 2 Gy daily and five fractions per week. The initial 40 Gy was delivered using anterior–posterior opposed fields. The field of radiation included the 5-cm margin of the primary tumor craniocaudally, and 2-cm beyond the radial margins. The boost dose of 20 Gy with a shrinking field technique was delivered using oblique parallel–opposed fields to avoid the spinal cord. Prophylactic irradiation to the regional lymph nodes was not performed.

Assessment of side effects, tumor stage, and response

Side effects were assessed at least every week during treatment using the NCI-CTC (version 4.0). Side effects were managed using standard supportive measures, and granulocyte colony-stimulating factor (G-CSF) was administered if medically necessary.

Tumor stage was classified according to the 7th edition of the Tumor–Node–Metastasis (TNM) classification system developed by the International Union against Cancer (UICC). Standard clinical measurements and radiological examinations were used to assess tumor response according to RECIST. Treatment response of the primary lesion (non-target lesion) was evaluated according to the Japanese classification of esophageal cancer 10th edition [17]. One month after completion of treatment, the clinical response of each primary tumor and metastatic nest was assessed.

Follow-up

Patients were assessed at 1 month after completion of treatment, every 3 months for the first 2 years, and every 6 months thereafter. CR was established by esophageal endoscopy, biopsy specimens, and CT. The date of the first progression and death was recorded. With a median follow-up period of 20 months (range, 4–85 months), the median survival time (MST) of the 37 patients was 24 months in the phase II study.

Results

Patients

Phase I

Fourteen patients were consecutively enrolled from April 2005 to January 2008. The cohort included 14 men, aged 52–72 years (mean age, 63.7 years). Their demographic and clinical characteristics are summarized in Table 2. The performance status was good in all patients (ECOG 0–1).

Phase II

A Phase II study to assess the precise antitumor effect and safety of this protocol using the dosages determined in the phase I study included three patients who received treatment as a level 3 dose in the phase I study, and 37 patients were consecutively enrolled from January 2008 to November 2012 and evaluated in phase II. Their demographic and clinical characteristics are summarized in Table 2.

Toxicity

Phases I and II

In the phase I study, the side effects are summarized in Table 3. Dose level 4 was defined as the MTD, and dose level 3 was adopted as the RD. The toxicity observed in the phase II study is shown in Table 4. There were no treatment-related deaths in this study. Leukopenia and neutropenia were frequent hematologic toxicities, but there was no serious infection. All patients with a high degree of neutropenia improved relatively quickly by administration of G-CSF. Nausea and radiation esophagitis were frequent in non-hematologic toxicity. The hematologic and non-hematologic toxicity was as expected and manageable. Two patients had an allergic reaction of grade 2 when docetaxel was given by continuous intravenous infusion. Then this medication was stopped and docetaxel was not been administered in second chemotherapy. Two patients

Table 2 Patient characteristics

Characteristic	Phase I	Phase II
Total	14	37
Age (years)		
Mean (range)	63.7 (52–72)	66.2 (54–74)
Sex		
Male	14	30
Female	0	7
Location		
Ce	3	7
Ut	7	5
Mt	3	20
Lt	1	2
Lymph node	0	3
Pathology		
SCC	13	36
AC	1	1
TNM clinical classification (7th)		
T		
T0	0	2
T1	1	5
T2	0	4
T3	1	5
T4	12	21
N		
N0	2	7
N1	5	12
N2	6	13
N3	1	5
M		
M0	10	23
M1	4	14
Stage		
I	1	3
II	0	6
III	9	13
IV	4	15

SCC squamous cell carcinoma, *AC* adenocarcinoma

had esophageal fistula to the bronchi and lung, respectively, after CRT (4 days after, 56 days after). They have been treated after CRT and fed using gastrostomy.

All patients received full-dose radiation therapy. Of the 37 patients undergoing CRT, 23 (60.5 %) received full-dose radiation therapy together with full doses of all medication. In 10 patients, the dose of docetaxel, cisplatin, and 5-FU had to be reduced by 20 % for the second cycle, mainly because of first-term severe myelosuppression and late recovery myelosuppression. In two patients, a second medication was not administrated for patient rejection and myelosuppression.

Treatment response and prognosis

Phase I

At dose level 1, two patients had a partial response (PR) and one patient had a CR. At dose level 2, two patients had PR and one patient had CR. At dose level 3, two patients had PR and one patient had CR. At dose level 4, four patients had CR and one patient had PR. Therefore, the treatment RR was 100 % and the CR rate was 50 %. There was no stable disease (SD) or progressive disease (PD) patients in phase I.

Phase II

There were 19 CR (51.4 %), 12 PR (32.4 %), 4 SD (10.8 %), and 2 PD (5.4 %) at one month after completion of treatment. There were 18 CR (52.9 %), 16 PR (47.1 %) in the primary lesion and 9 CR (31.0 %), 13 PR (44.8 %), 7 SD (24.1 %) in lymph node metastases at one month after completion of treatment. The final CR rate and RR were 54.1 and 83.8 %, respectively. In patients with cT4, the CR rate and RR were 47.6 and 85.7 %, respectively. The 2-year overall survival (OS) rate and progression-free survival (PFS) rate were 52.9, 50.0 %, respectively (Fig. 1). The MST was 24.7 months in all patients. In clinical T4 cases ($n = 21$), the 2-year OS rate and PFS rate were 43.5 and 44.9 %, respectively (Fig. 2). The MST was 21.6 months in patients with T4.

In terms of patient status at the end of the study, 28 patients had received chemotherapy, three patients underwent salvage surgery, and one patient underwent salvage endoscopic treatment. Five patients had not received adjuvant therapy (two patients were stage I, and 3 patients did not have adaptation). One patient with a primary tumor that invaded the thoracic aorta was lost due to major bleeding after 5 months. He died during the resting period of additional chemotherapy.

Discussion

In the present phase I study, we sought to determine the RD for DCF combination CRT that did not cause severe side effects in patients with esophageal cancer. Based on the dose levels tested, the RD for docetaxel, cisplatin, and 5-FU were 50 mg/m^2 (day 1, 29), 60 mg/m^2 (day 1, 29), and 600 mg/m^2 (days 1–4, 29–32), respectively. The DLT was febrile neutropenia for all agents. If we could control this toxicity, a higher dose of anti-cancer agents might be administered.

In the phase II study, the myelotoxicity was severe with febrile neutropenia seen in 8 (21.6 %) patients. It was adequately controlled by G-CSF, a fourth-generation antimicrobial agent, and an isolator system with HEPA

Table 3 Incidence of hematologic and non-hematologic toxicities in phase I study

Grade	Dose level 1 ($n = 3$)			Dose level 2 ($n = 3$)			Dose level 3 ($n = 3$)			Dose level 4 ($n = 5$)		
	1–2	3	4	1–2	3	4	1–2	3	4	1–2	3	4
Anemia	1	0	0	2	1	0	1	0	0	2	0	0
Leukopenia	2	1	0	1	2	0	0	2	1	1	1	3
Neutropenia	2	1	0	3	0	0	2	0	1	1	1	3
Thrombocytopenia	0	0	0	0	0	0	1	0	0	0	0	0
Febrile neutropenia	0	0	0	0	0	0	0	0	0	0	3[a]	0
Nausa	2	0	0	1	0	0	1	1	0	1	3	0
Diarrhea	0	0	0	0	0	0	0	0	0	0	0	0
Esophagitis	3	0	0	3	0	0	3	0	0	3	2	0

NCI-CTC National Cancer Institute Common Toxicity Criteria

[a] Dose-limiting toxicity

Table 4 Incidence of hematologic and non-hematologic toxicities in phase II study (dose level 3)

Grade	1–2	3	4	Grades 3 and 4 (%)
Anemia	33	1	0	1 (2.7)
Leukopenia	2	24	10	34 (91.9)
Neutropenia	8	15	13	28 (75.7)
Thrombocytopenia	3	1	0	1 (2.7)
Febrile neutropenia	–	8	0	8 (21.6)
Nausea	13	12	0	12 (32.4)
Diarrhea	1	2	0	2 (5.4)
Esophagitis	20	9	0	9 (24.3)
Dermatitis radiation	9	1	0	1 (2.7)
Allergic reaction	2	0	0	0 (0)
Esophageal fistula	0	2	0	2 (5.4)

NCI-CTC National Cancer Institute Common Toxicity Criteria

filter. There were no serious infections. Sixty percentage of patients received full-dose radiation therapy together with full doses of all medication. In 10 patients, the dose of docetaxel, CDDP, and 5-FU had to be reduced by 20 % for the second cycle, mainly because of first-term severe myelosuppression or late recovery myelosuppression. In those cases, we considered prophylactic antimicrobial therapy and administration of G-CSF. In this phase I/II study, we could keep safety by adequate blood cell count and medication in treatment of RD. We think that feasibility of this protocol is acceptable in hospital which performs cancer chemotherapy routinely, because there was low mortality and most toxicities were manageable level and improved quickly in our study.

Nausea and radiation esophagitis were frequent non-hematologic toxicities. Temporary failures of oral intake were corrected with appropriate intravenous infusion. Dermatitis and esophagitis might have been due to radiation therapy.

Esophageal fistula is serious problem when patients with T4 esophageal cancer are treated. In our study, two patients (5.4 %) had esophageal fistula to the bronchi and lung, respectively, after CRT (4 days after, 56 days after). They have been treated after CRT and fed using gastrostomy. Ohtsu et al. [6] reported that 10 % of patients developed treatment-related perforation of the esophageal wall in patients with T4 and/or M1 lymph node squamous cell carcinoma. This frequency was same our results. Whether the fatal case where the primary tumor invaded the thoracic aorta was caused by major bleeding after 5 months or was due to the impact of tumor progression was uncertain.

The dose of 60 Gy set in this protocol seems excessive, considering the result of the INT 0123 study that a dose of 64.8 Gy is not superior to 50.4 Gy [18]. We adopted total 60 Gy of radiation because of the Japanese standard dose of definitive CRT according to guidelines [19, 20].

In the phase II study, the RR and CR rate were 83.8 and 54.1 %, respectively. These effects were satisfactory for advanced esophageal cancer, including 21 with T4 of 37 cases. In clinical T4 cases ($n = 21$), the 2-year OS rate and PFS rate were 43.5 and 48.6 %, respectively. Ohtsu et al. [6] have reported CR and OR rates of 33 and 87 %, respectively, and a MST in 54 patients with T4/M1 lymph esophageal cancer of 9 months and one- and 3-year survival rates of 41 and 23 %, respectively. The discrepancy between the CR and PR rates may have been caused by different evaluation methods. Ishida et al. [7] have reported that the CR rate and RR were 15 and 68.3 %, respectively. The MST was 10 months, and the 2-year survival rate was 31.5 %. There was limitation of our study to compare with large scaled studies, because our study consisted of relatively smaller number of patients with shorter follow-up time. However, definitive CRT with DCF might be superior to PF therapy in cT4 esophageal cancer patients. We think that superiority of DCF-RT will be proved by large scaled randomized controlled phase III study.

Higuchi et al. [13] have reported a phase I trial of definitive CRT with docetaxel, cisplatin, and 5-FU for esophageal squamous cell carcinoma with T4 tumor and/or M1 lymph node metastasis. They planned dose escalation with

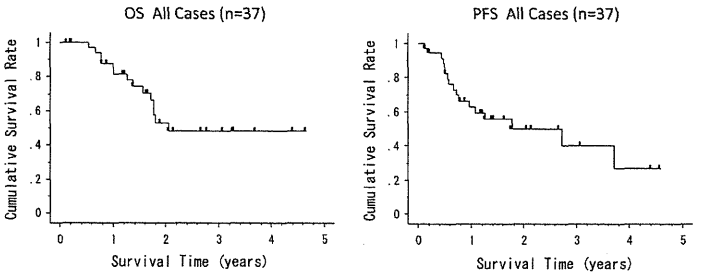

Fig. 1 Overall survival and progression-free survival in all cases in phase II ($n = 37$)

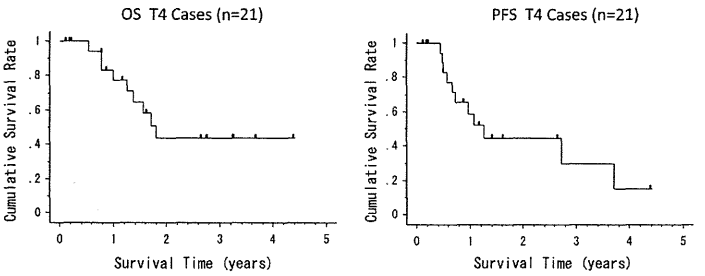

Fig. 2 Overall survival and progression-free survival in T4 patients ($n = 21$)

docetaxel and cisplatin (40 mg/m2) on days 1, 15, 29, and 43 plus a continuous infusion of 5-FU (400 mg/m^2/day) on days 1–5, 15–19, 29–33, and 43–47 with 61.2 Gy/34 fractions/7 weeks. The RR was 89.5 %, including a CR rate of 42.1 % in their study. Because this outcome was almost the same as our results, it might be considered even for T4 esophageal cancer; definitive CRT with DCF was one of promising protocols. In their study, the main toxicities were also myelosuppression and radiation esophagitis. DLT was grade 3 febrile neutropenia and grade 4 leukopenia lasting 3 days. The main toxicity is myelosuppression when we give the DCF-RT protocol to Japanese patients.

Pasini et al. [15] reported a phase II study of weekly DCF chemotherapy with concurrent radiotherapy in untreated stage II–III adenocarcinoma and squamous cell carcinoma of the mid-distal thoracic esophagus. Their schedule consisted of a first phase of chemotherapy alone and of a second phase of concurrent CRT (50 Gy). In their study, the rate of pathological CR was 47 % and the overall major pathological RR was 62 %. They attributed the high percentage of pCR to intense treatment, with a full dose of radiation concurrent with doses of chemotherapy approximately corresponding to two cycles of the classical DCF. The total dose of their protocol (docetaxel 280 mg/m^2, cisplatin 200 mg/m^2, 5-FU 9030 mg/m^2) was larger than that of our protocol (docetaxel 100 mg/m^2, cisplatin 120 mg/m^2, 5-FU 4800 mg/m^2). It is uncertain whether to use high doses or smaller divided doses as a weekly treatment schedule. In our study, we investigated the additional effect of plus taxane of CF therapy. We reduced the total dose of cisplatin and 5-FU and escalated doses of the triple-drug combination. The rather small weekly administration may have reduced side effects. The treatment effect was high with their protocol.

Zanoni et al. [16] have reported a neoadjuvant concurrent CRT using DCF for locally advanced esophageal cancer. The pathological CR rate was 43 % in patients with esophageal squamous cell carcinoma. Five-year OS and disease-related survival were 43 and 49 %, respectively. Although most patients were cT2 (13 %) and cT3 (70 %), this treatment had a favorable outcome. These results suggested that this protocol was effective for esophageal squamous cell carcinoma and adenocarcinoma. Combination CRT including a taxane was effective for treatment of patients with locally advanced esophageal cancer.

Although most patients were stage cT4 in our study, treatment effects were favorable. Because there is no means of treating the other for other organs invasive esophageal cancer, more effective protocol such as this protocol may be chosen for radical cure at first treatment. Even though there is high frequency of side effects, it may be possible to obtain more favorable survival by the strict monitoring

and precautions, such as blood cancer. The prognosis was relatively favorable in our study probably due to additional therapy after definitive CRT [21, 22]. In T4 cases, one case underwent salvage surgery and 19 case (90.5 %) including patients with salvage operation underwent additional chemotherapy.

In summary, the RD for DCF combination CRT for esophageal cancer in the present study was 50 mg/m^2 docetaxel plus 60 mg/m^2 cisplatin on days 1 and 29 plus 600 mg/m^2 5-FU on days 1–4, 29–32 and concurrent radiation. This protocol produced high CR rate and favorable prognosis, especially for T4 esophageal cancer.

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ORIGINAL ARTICLE – GASTROINTESTINAL ONCOLOGY

Phase II Study of Docetaxel, Nedaplatin, and 5-Fluorouracil Combined Chemotherapy for Advanced Esophageal Cancer

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ABSTRACT

Background. We performed a prospective, multi-institutional, phase-II, clinical trial of a docetaxel, nedaplatin, and 5-fluorouracil (DNF) regimen in patients with unresectable esophageal cancer. Our goal was to determine the efficacy and feasibility of this DNF protocol.

Methods. Thirty-four patients with unresectable esophageal cancer were enrolled and received DNF therapy. The DNF regimen was repeated every 4 weeks for up to 8 weeks, based on the following recommended doses: docetaxel, 60 mg/m^2 (day 1); nedaplatin, 70 mg/m^2 (day 1); and 5-fluorouracil, 700 mg/m^2 (days 1–5). The primary endpoint was the response rate. The secondary endpoints were overall survival and chemotherapy toxicities.

Results. The complete response rate and response rate were 5.9 and 47.1 %, respectively. The 2-year overall survival rate and progression-free survival rate were 44.3 and 27.3 %, respectively. The median survival time was 594 days. The median progression-free time was 277 days. No treatment-related deaths occurred. Thirty patients (30/34) with grade 3, 4 neutropenia improved relatively quickly with administration of granulocyte colony-stimulating factor.

Conclusions. DNF combination chemotherapy is a useful regimen with relatively minor adverse events and may serve as an effective protocol in patients with unresectable esophageal cancer.

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Chemotherapy plays important roles in adjuvant surgical therapy and amplifies the effect of radiation therapy in patients with esophageal cancer.[1,2] The use of chemotherapy that is not combined with other modalities is limited to patients with distant metastasis or postoperative distant recurrence. However, although 15–44 % of patients have been estimated to respond to monotherapy, cases of complete response (CR) are rare, and no monotherapy has been shown to have a survival-prolonging effect.[3]

Cisplatin and 5-fluorouracil (5-FU) therapy is a standard protocol for patients with unresectable esophageal cancer.[4–6] The response rate (RR) is reportedly approximately 35 %, and the mean response duration is approximately 3.5–5.8 months in patients with a partial response (PR).[6,7] In a previous study, the mean survival time after the first dose was 9.5 months for patients who responded to the treatment versus 5.6 months for patients who showed no response.[6] Therefore, this regimen is not sufficiently effective.

In recent years, a new combination chemotherapeutic regimen comprising docetaxel, cisplatin, and 5-FU (DCF) has received much attention in the treatment of esophageal cancer.[8,9] The DCF regimen exploits the strong clinical effects of each component. However, cisplatin has some problems associated with renal toxicity, myelosuppression, and nausea. It is important to reduce these side effects to allow for continuous treatment and maintain the patient's quality of life.

Nedaplatin is a second-generation platinum complex that was shown to have pronounced activity against solid tumors but less nephrotoxicity than cisplatin in preclinical and clinical studies.[10–14] Very few reports have described the use of a combination of docetaxel, nedaplatin, and 5-FU (DNF) for esophageal cancer.[15] Therefore, we performed the first phase-I trial of DNF therapy for advanced esophageal cancer in which we determined the recommended dose of DNF therapy.[16] We then conducted a phase-II trial of a DNF regimen. Our goal was to determine the efficacy and feasibility of DNF combination chemotherapy for patients with esophageal cancer.

MATERIALS AND METHODS

This study was conducted as a prospective, multi-institutional, phase-II trial from August 2008 to December 2012 among four institutions in Gunma Prefecture, Japan (Gunma University Hospital, Gunma; Gunma Prefectural Cancer Center, Ohta; Gunma Chuo Hospital, Maebashi; and Isesaki Municipal Hospital, Isesaki). This study was approved by the institutional review board of each hospital, and all patients provided written, informed consent before enrollment. The study is registered with UMIN-CTR, Number 000005081.

Eligibility Criteria

Patients who fulfilled the following criteria were eligible for the study, as in the above-mentioned, phase-I study: histopathologic or cytologic diagnosis of esophageal carcinoma, unresectable esophageal cancer or recurrent esophageal carcinoma, an interval of ≥4 weeks between the last treatment and entrance into the present study, and an Eastern Cooperative Oncology Group performance status of 0–2.[16] We defined unresectable esophageal cancer as tumor invasion of another organ, distant metastases to another organ, or distant multiple lymph node metastases (no regional lymph nodes except supraclavicular nodes). Indications for conversion surgery were not noted in the protocol. These were cases that became resectable after chemotherapy or had a single or a few distant metastases that did not arise anew after chemotherapy. We did not restrict patients on the basis of the presence of a measurable lesion. When we evaluated the tumor response in patients without the target lesion, we used the tumor response in nonmeasureable lesions (primary lesions). Patients also were required to fulfill the following criteria during the 2 weeks before registration in the study: white blood cell count of 4000–12,000/mm^3, mature granulocyte count of ≥2000/mm^3, blood platelet count of ≥100,000/mm^3, hemoglobin level of ≥9.5 g/dL, aspartate transaminase and alanine transaminase levels of ≤1.5 times the upper limit of the reference range at our hospital, total bilirubin level of ≤1.5 mg/dL, alkaline phosphatase level of ≤2.5 times the upper limit of the reference range at our hospital, creatinine level of ≤1.2 mg/dL, partial pressure of oxygen in arterial blood of ≥60 torr (in room air), and expected prognosis of ≥3 months. Finally, written agreement to participate in the final examination was required from each patient.

Treatment and Study Protocols

All patients received adequate antiemetic medications during chemotherapy. A total of six intravenous doses of dexamethasone (8 mg or equivalent) were given. Prophylactic antibiotics were not given. The DNF regimen comprised 60 mg/m^2 of docetaxel, which was infused over 1 h on day 1, followed by 70 mg/m^2 of nedaplatin, which was infused over 1 h on day 1, and 700 mg/m^2 of 5-FU, which was administered by continuous infusion on days 1–5. This regimen was established in the above-mentioned phase-I study.[16] The DNF regimen was repeated every 4 weeks (defined as 1 cycle) for up to 2 cycles unless progressive disease (PD) or unacceptable toxic effects occurred. The tumor size and development of new lesions were assessed by helical CT, esophagogastroduodenoscopy, and ^{18}F-fluorodeoxyglucose positron emission tomography CT (FDG-PET CT) after 2 cycles. We confirmed the best clinical response at 4-week intervals.

Assessment of Side Effects and Tumor Stage

Side effects were assessed at least every week during the first two treatment cycles using the National Cancer Institute Common Toxicity Criteria version 4.0. Side effects were managed using standard supportive measures, and granulocyte colony-stimulating factor (G-CSF) was administered if medically necessary. The tumor stage was classified according to the sixth edition of the Tumor-Node-Metastasis classification system developed by the International Union Against Cancer. Standard clinical measurements and radiological examinations were performed to assess tumor response according to the Response Evaluation Criteria In Solid Tumors version 1.0. Pretreatment evaluations included barium esophagography, esophagogastroduodenoscopy, endoscopic ultrasonography, helical CT scanning of the neck, chest, and abdomen, and FDG-PET CT scanning of the whole body. The results of all staging evaluations were reviewed by both radiologists and medical oncologists.

Dose Modification

The patient's full medical history, biochemistry profile, and chest X-rays were assessed before starting each

treatment cycle. A complete blood count and biochemistry parameters were measured every week in all treatment cycles. If grade 4 neutropenia occurred, the complete blood count was repeated daily during the treatment cycle to determine the duration of the neutropenia.

If any of the following occurred after administration, the next dose administration was reduced by 80 %: (1) if platelet transfusion was performed for patients with thrombocytopenia of $\leq$25,000/μL; (2) if the patient had grade 4 leukopenia or neutropenia for $\geq$4 days; (3) if the patent had grade 3 neutropenia or leukopenia with a fever of $\geq$38.0 °C; and (4) if the patient had acute kidney injury at grade 2 or higher (administration was stopped in patients with grade 3 acute kidney injury). If hepatic failure was at grade 3 or higher, the study was stopped.

Study Design and Statistical Analysis

The primary endpoint was the response rate (CR and PR). The secondary endpoints were overall survival (OS) and chemotherapy toxicities. The sample size of this study was set at 25 patients based on an expected DNF response rate of 60 % compared with a minimal, clinically meaningful response rate of 35 % with α error of 0.05 and β error of 0.2. We decided to enroll 30 cases for a thorough analysis and inadequate case. However, we had enrolled more than 30 cases by the end of study and analyzed 34 cases for a more robust analysis. The Kaplan–Meier method with the log-rank test was used for survival analysis. OS and progression-free survival (PFS) were measured from the date of chemotherapy initiation to death of any cause and PD, respectively.

Follow-Up

Patients were assessed 1 month after completion of treatment, every 3 months for the first 2 years and every 6 months thereafter. CR was determined by esophageal endoscopy, biopsy specimens, and CT. The dates of first progression and death were recorded. The minimum follow-up period in surviving patients was 7.3 months. The mean follow-up period was 16 (range 3.4–42) months.

RESULTS

Patients

Thirty-four patients were enrolled in the study. The cohort included 31 male and 3 female patients aged 48–74 years (median age 62 years). Thirty patients had squamous cell carcinoma and four had adenocarcinoma. Their demographic and clinical characteristics are summarized in Table 1. Three cases of recurrence comprised 2 cases of lung metastasis and 1 case of adrenal gland metastasis.

TABLE 1 Patient characteristics

Characteristic	
Total	34
Age (years)	
Median (range)	62 (48–74)
Sex	
Male	31
Female	3
Location	
Ce	1
Ut	4
Mt	13
Lt	13
TNM clinical classification (initial treatment cases)	
T	
T1	2
T2	0
T3	17
T4	12
N	
N0	2
N1	29
M	
M0	7
M1	24
Stage	
III	7
IV	24
Initial treatment or not	
Initial treatment	31
Recurrence	3
Reason for unresectability	
Other organ metastasis	20
Multiple lymph nodes metastasis	6
Other organ invasion	5
Recurrence	3

Treatment Response

There were 2 patients with CR (5.9 %), 14 with PR (41.2 %), 14 with stable disease (SD) (41.2 %), and 4 with PD (11.8 %). There were 5 patients with CR (16.1 %) and 26 with incomplete response/stable disease (IR/SD) (83.9 %) in the primary lesion, and 2 patients with CR (6.5 %), 10 with PR (32.3 %), 18 with SD (58.1 %), and 1 with PD (3.2 %) in lymph node metastases. Among patients with distant organ metastasis, there were 2 patients

with CR (15.4 %), 5 with PR (38.5 %), 4 with SD (30.8 %), and 2 with PD (15.4 %). The CR rate and RR were 5.9 and 47.1 %, respectively.

Additional Treatment After Study

After this study, 19 patients (55.9 %) continued to receive additional DNF therapy (mean 4 sessions; range 3–6 sessions). In terms of patient status at the end of the study, 5 patients had received definitive chemoradiotherapy, 11 had undergone surgery, 12 had undergone another chemotherapy protocol, and 6 had undergone best supportive care. Nine patients (26.5 %) underwent radical esophagectomy and two underwent partial resection of lung metastasis. Eleven patients underwent conversion surgery and had distant metastases that were unresectable (2 cases of lung metastasis, 1 case of liver metastasis, 6 cases of lymph node metastasis): two cases of direct invasion to other organs, and recurrence of lung metastasis. Treatment effects were CR: one case, PR: six cases, SD: four cases. Five cases with unresectable tumors improved unresectable causes (2 cases of direct invasion, 3 cases of distant metastasis). Distant metastatic lesions were controlled and new lesions had not appeared in six cases (5 cases of distant metastasis and 1 recurrence). Three of nine patients showed postoperative complications requiring subtotal esophagectomy with lymphadenectomy. The complications were anastomotic insufficiency in two cases, recurrent nerve paralysis in one case, and pericardial effusion and pleural effusion (duplicated anastomotic insufficiency) in one case. None of the cases had serious complications. Two cases underwent partial resections of lung metastases without complications. Recurrences after surgery appeared in seven (63.6 %) patients. The patterns of recurrence were lung metastasis in two cases, liver metastasis in one case, carcinomatous pleuritis in three cases, and distant lymph node metastasis in three cases.

Prognosis

The 2-year OS and PFS rates were 44.3 and 27.3 %, respectively (Figs. 1, 2). The median survival time (MST) was 594 days. The median PFS time was 277 days.

Toxicity

The side effects are summarized in Table 2. There were no treatment-related deaths in this study. Leukopenia and neutropenia were frequent hematologic toxicities, but there was no serious infection. All patients with grade 3, 4 neutropenia improved relatively quickly by administration of G-CSF. No patients developed acute kidney injury or nervous system disorders.

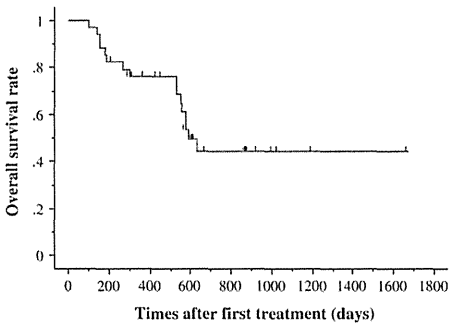

FIG. 1 Overall survival. The 2-year overall survival rate was 44.3 %, and the median survival time was 594 days

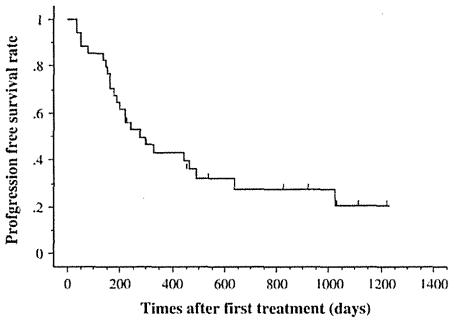

FIG. 2 Progression-free survival. The 2-year progression-free survival rate was 27.3 %, and the median progression-free time was 277 days

TABLE 2 Adverse events of DNF therapy

Grade	1–2	3	4
Anemia	21	0	0
Leukopenia	7	21	6
Neutropenia	2	17	13
Thrombocytopenia	9	1	0
Febrile neutropenia	–	8	0
Nausea	14	4	0
Diarrhea	7	3	0
Mucositis oral	6	0	0
Gastric ulcer	0	1	0
Fever	1	0	0
Nervous system disorders	0	0	0
Acute kidney injury	0	0	0

NCI-CTC National Cancer Institute Common Toxicity Criteria

DISCUSSION

The CR rate, RR, and prognosis in this protocol were better than those in reports of cisplatin + 5-FU therapy, which is recognized as a standard protocol.[6,7] Yamasaki et al. reported the results of DCF combination chemotherapy in patients with advanced or recurrent squamous cell carcinoma of the esophagus.[17] Their protocol was almost identical to ours. Like us, they administered 60 mg/m^2 of docetaxel on day 1, 70 mg/m^2 of cisplatin on day 1, and 700 mg/m^2 of 5-FU on days 1–5. However, the type of platinum agent (nedaplatin vs. cisplatin) and duration of therapy (4 vs. 3 weeks) differed from our study. In their study, the CR rate, RR, and median PFS (10.0 %, 72.5 %, and 14 months, respectively) were better than those in our study. These differences might have been caused by the patients' backgrounds; the rate of distant organ metastasis in their study was 12.5 %, whereas ours was 35.3 %. The reason for this result is considered to be the fact that many patients could undergo additional treatment after this chemotherapeutic protocol according to the treatment effect obtained. One important factor common to both studies is that many patients underwent surgery after the chemotherapy (our study 32.4 %; Yamasaki et al. 43.5 %). In our study, 11 patients underwent surgery, 9 underwent radical esophagectomy, and 2 underwent resection of lung metastases. Ten patients underwent R0 operations. Docetaxel, platinum, and 5-FU combination chemotherapy might be a powerful treatment for patients with a high probability of conversion to surgery.

Guo et al. conducted a phase II study of DNF. In their study, the CR rate was 4.65 % and the overall RR was 62.8 %.[15] The MST was 310 days, and the median time to progression was 201 days. Their RR was superior to that in our study, but their survival times were shorter. The reason for this difference is that many of our patients were able to undergo conversion surgery as a result of tumor downstaging.

The reasons for unresectability before treatment included 20 cases of metastasis to other organs, 6 cases of multiple lymph node metastases, 5 cases of invasion to other organs, and 3 cases of recurrence in other organs after the operation. Five patients with metastasis to other organs underwent conversion surgery after chemotherapy because their lesions were controlled. Three patients with multiple lymph node metastases, two with invasion to other organs, and one with distant-organ recurrence underwent conversion surgery after chemotherapy. After 2–6 courses (mean 2.8 courses) of DNF chemotherapy, these patients were able to undergo surgery. Ten patients underwent definitive or palliative chemoradiotherapy after this protocol; however, their prognosis was poor. Seventeen patients were treated with DNF, cisplatin + 5-FU, docetaxel, paclitaxel, S-1 (tegafur, gimeracil, and oteracil potassium), and tegafur + uracil chemotherapy.

Hematological toxicities were the main side effects associated with this protocol. The most serious side effect was febrile neutropenia, which was controlled by the administration of G-CSF, a fourth-generation antimicrobial agent, and an isolator system with HEPA filter. Yamasaki et al. reported that 90 % of patients developed grade 3 or 4 neutropenia and that 72.5 % of patients developed grade 3 or 4 leukopenia. These results are similar to ours. In one study, neutropenia rapidly improved after G-CSF administration following completion of the DCF protocol.[18] DNF resulted in the same degree of rapid recovery of neutropenia as did G-CSF in our study. Guo et al. reported that grade 3–4 events included neutropenia (20.93 %), febrile neutropenia (4.65 %), thrombocytopenia (6.98 %), and vomiting (9.3 %).[15] Thrombocytopenia is one of most important adverse events associated with nedaplatin. In our study, grade 1, 2, and 3 thrombocytopenia was observed in 8, 1, and 1 patients, respectively. Guo et al. reported that the incidence of grade 3–4 thrombocytopenia was 6.98 % and that one patient died of intracranial hemorrhage secondary to grade 4 thrombocytopenia.[15]

There were no grade 2–4 creatinine elevations, but two patients (4.65 %) developed grade 1 creatinine elevations in the study by Guo. Their protocol was as follows: docetaxel (75 mg/m^2, day 1), nedaplatin (100 mg/m^2, day 1), leucovorin (200 mg/m^2, day 1), and 5-FU (375 mg/m^2, day 1) followed by a 46-h infusion of 5-FU (2600 mg/m^2). The treatment cycle was repeated every 3 weeks in their protocol. Although their doses were higher than the recommended doses in our study, their side effects were generally tolerable. The reasons for this are unclear, but there were some differences between their study and ours, such as patients' race and the timing of drug administration.

None of the patients in the present study developed nephrotoxicity. This may be attributed to the use of nedaplatin, which is less nephrotoxic than cisplatin. Minamide et al. reported that renal dysfunction occurred in 17 % of their patients; grade 3 or higher events occurred in 1.5 % of patients treated with docetaxel (70 mg/m^2 on day 1), CDDP (80 mg/m^2 on day 1), and 5-FU (800 mg/m^2 on days 1–5) combination chemotherapy.[19] The high incidence of renal dysfunction might have been caused by the higher doses used in their protocol than in our protocol, and the renal toxicity of cisplatin may be stronger than that of nedaplatin. Although Yamazaki et al. reported no side effects associated with renal function, we should evaluate the kidney function of patients undergoing combination chemotherapy involving three drugs, one of which is a platinum drug.[17] We should give particular attention to kidney function when we administer platinum drugs to patients with low renal function, elderly patients, and patients with comorbid disorders. DNF may be suitable in these patients.

In conclusion, DNF combination chemotherapy is an effective regimen for patients with unresectable esophageal cancer. This protocol may be suitable for treatment of patients with esophageal cancer with low renal function.

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