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SNP Communications

Novel Single Nucleotide Polymorphism of UGT1A7 Gene in Japanese

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Summary: We sequenced exon 1 of the UDP-glucuronosyltransferase (UGT) 1A7 gene from 52 Japanese cancer patients. Four single nucleotide polymorphisms (SNPs) were found. Three of them caused *UGT1A7*2* and *UGT1A7*3*. A novel SNP (98973G>C) causing amino acid substitution (Ser¹⁴¹Cys) was found. The sequence is as follows: SNP, 050824FujitaK002; GENE NAME, UGT1A7; ACCESSION NUMBER, AF297093; LENGTH, 25 bases; 5'-TAAAGGAGAGTTG/CTTTTGATGC-AGT-3'. One out of 52 cancer patients was heterozygous for the variant allele, resulting in the allele frequency of 0.96%. The patient did not possess *UGT1A7*2* or *UGT1A7*3*.

Key words: *UGT1A7*; novel SNP; Japanese; Irinotecan

Introduction

UDP-glucuronosyltransferase (UGT) is one of phase II drug-metabolizing enzymes that catalyzes the glucuronidation of a wide variety of endogenous and exogenous compounds, including drugs, carcinogens, and other xenobiotics. The UGT superfamily is composed of families, and two of the families (UGT1 and UGT2) have the ability to catalyze the glucuronidation of foreign chemicals.¹⁾ The *UGT1A7* gene located on chromosome 2q37 is expressed exclusively in the oropharynx, esophagus, stomach and pancreas,²⁻⁶⁾ but is absent from the liver.⁷⁾ Cloning and characterization of the *UGT1A7* gene product revealed the glucuronidation of known carcinogens, which included tobacco smoke carcinogens.^{8,9)} Chemicals with phenolic, anthraquinone, flavone, coumarin and naphthol structures are substrates for *UGT1A7*.⁷⁾ *UGT1A7* is also known to be involved in the glucuronidation of an active metabolite of an anticancer drug irinotecan (SN-38).¹⁰⁾ Various single nucleotide polymorphisms (SNPs) have been

identified in the *UGT1A7* gene so far.^{6,10-13)} *UGT1A7*3* has been known to cause the reduced capacity to metabolize SN-38, whereas the product of *UGT1A7*2* gene showed higher activity toward SN-38 than that of *UGT1A7*1*.^{10,13)} The effects of these polymorphisms on the irinotecan toxicity in humans seem somewhat contradictory. Carlini *et al.*¹¹⁾ claimed that the low *UGT1A7* activity was related to the little toxicity of irinotecan, whereas Ando *et al.*¹⁴⁾ demonstrated no relationship between the reduced *UGT1A7* activity and the irinotecan toxicity.

In the present study, the exon 1 of *UGT1A7* gene from 52 Japanese cancer patients treated with irinotecan was sequenced, and found a novel SNP causing amino acid substitution.

Materials and Methods

Patients: Fifty-two Japanese cancer patients (32 colorectal cancer, 15 gastric cancer, 5 others) who received various regimens of irinotecan-containing chemotherapy or irinotecan monotherapy from June 2003 to August 2005 were studied. All of patients gave informed consent in writing for their peripheral blood samples and medical information to be used for the research. This study was approved by the Institutional Review Board of Saitama Medical School.

Human DNA samples: Genomic DNA was extracted from 200 μ L of peripheral blood of 52 Japanese

On September 30, 2005, the variation was not found on the UDP-glucuronosyltransferase homepage (<http://som.flinders.edu.au/FUSA/ClinPharm/UGT/>), the Japanese Single Nucleotide Polymorphism (JSNP) (http://snp.ims.u-tokyo.ac.jp/index_ja.html) or dbSNP in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP/>).

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cancer patients, which had been stored at -80°C until analysis, by using QIAamp Blood Kit (QIAGEN GmbH, Hilden, Germany).

PCR conditions and DNA sequencing: Exon 1 of the *UGT1A7* gene was analyzed by direct sequencing of a PCR product by the method of Carlini *et al.*¹¹⁾, with minor modifications. Briefly, the reaction mixture for PCR consisted of 2.5 mM MgCl_2 and 1.25 unit of AmpliTaq Gold polymerase (Perkin-Elmer, Foster City, CA) in a final volume of 50 μL . The sequence of the complete *UGT1A7* gene described in the GeneBank (AF297093) was used as a reference.

Two polymorphisms of *UGT1A1* gene (*UGT1A1*6* and *UGT1A1*27*) were determined by PCR-RFLP method as described by Ando *et al.*,¹⁵⁾ with minor modifications. Briefly, the first and the second PCRs were performed with AmpliTaq Gold. The reaction mixture for the first PCR consisted of 2 mM MgCl_2 and 1.25 unit of AmpliTaq Gold polymerase in a final volume of 50 μL . The second PCR was carried out with the reaction mixture consisted of 1.5 mM MgCl_2 and 1.25 unit of AmpliTaq Gold polymerase in a final volume of 50 μL . The PCR conditions for the second PCR were: 95°C 15 min followed by 25 cycles of 94°C for 30 s, 55°C for 40 s and 72°C for 40 s. RFLP analyses for *UGT1A1*6* and *UGT1A1*27* were performed with *Msp* I (Takara, Otsu, Japan) and *Bsr* I (New England Biolabs, Ipswich, MA), respectively. The TATA box polymorphism (*UGT1A1*28*) was determined by the direct sequencing described by Ando *et al.*,¹⁵⁾ with minor modifications. Briefly, the reaction mixture for the first PCR consisted of 1 mM MgCl_2 and 1.25 unit of AmpliTaq Gold polymerase in a final volume of 50 μL . The PCR conditions for the first PCR were: 95°C 15 min followed by 30 cycles of 95°C 30 s, 58°C 40 s and 72°C 40 s.

Treatments: Irinotecan as a monotherapy was given weekly at a dose of 100 mg/m^2 for the first 3 weeks of each 4-week cycle,¹⁶⁾ or biweekly at a dose of 150 mg/m^2 ¹⁷⁾ until the disease showed progression or intolerable toxicity occurred. As combination chemotherapy, a 100 mg/m^2 irinotecan was administered with the bolus 5-fluorouracil (FU) 500 mg/m^2 and leucovorin (LV) 10 mg/m^2 (1 isomer form) weekly for the first 4 weeks of each 6-weeks cycle (IFL regimen).¹⁸⁾ The FOLFIRI regimen administered at 2-week intervals comprised irinotecan at escalating doses from 150 to 180 mg/m^2 and LV 200 mg/m^2 administered over 2 hours followed by FU 400 mg/m^2 as a bolus injection and FU 2,400 mg/m^2 as a 46-hour continuous infusion.¹⁹⁾ As the other regimen repeated every 4 week (IP regimen), irinotecan at doses from 50 to 70 mg/m^2 was followed 2 hours later by a 120-minute infusion of cisplatin 80 mg/m^2 with adequate hydration on day 1, and the same dose of irinotecan was repeated on day 15.²⁰⁾ In each regimen,

irinotecan at doses from 50 mg/m^2 to 180 mg/m^2 dissolved in 250 mL of 5% dextrose solutions was infused over 90 minutes.

Pharmacokinetic analysis: Blood sampling for pharmacokinetic analysis was typically performed at their first courses. The blood samples for the analysis were taken from the arm opposite the infusion site at the beginning of irinotecan infusion and 0, 0.25, 0.5, 1, 2, 4, 8, and 24 hours after the end of the infusion. Plasma was immediately obtained by centrifugation of the blood samples and stored at -80°C until analysis.

Total (lactone and carboxylate) plasma concentrations of irinotecan, SN-38 and SN-38 glucuronide (SN-38G) were analyzed at the institution, using a reverse-phase HPLC method. A 150 μL of plasma sample was mixed with 300 μL of methanol, 5% perchloric acid (50:50, v/v) and camptothecin as an internal standard by a vortex mixer. The 200 μL of supernatant obtained by centrifugation of the mixture at 15,000 rpm for 10 min was injected into a HPLC system (Hitachi model 7000 series, Hitachi, Tokyo, Japan) equipped with a TSK-gel ODS-120T analytical column (4.6 \times 250 mm; 4 μm ; TOSOH, Tokyo, Japan), and separated at 40°C , at a flow rate of 1.0 mL/min to quantify the total (lactone and carboxylate) plasma concentrations of irinotecan, SN-38 and SN-38G. The mobile phase consisted of 75 mM ammonium acetate (pH 4.75) for solvent A and acetonitrile for solvent B: a 20-minute run of a linear gradient of 85% to 65% of solvent A. Lower limit of quantification for irinotecan was 5 ng/mL, and those for SN-38 and SN-38G were 0.5 ng/mL. The intra- and inter-assay coefficient variations for irinotecan and the metabolites were under 10%.

Area under the time versus concentration curve (AUC, $\mu\text{mol} \cdot \text{h}/\text{l}$) from the beginning of the infusion to the last sampling was calculated by the linear trapezoidal rule, using a computer program, WinNonlin version 4.01 software (Pharsight Corporation, Mountain View, Calif).

Results and Discussion

We found a following novel SNP:
SNP, 050824Fujita002; GENE NAME, *UGT1A7*; AC-
CESSION NUMBER, AF297093; LENGTH, 25 bases;
5'-TAAAGGAGAGTTG/CTTTTGATGCAGT-3'.
The SNP was 98973G>C in exon 1 of *UGT1A7*
(Fig. 1), resulting in the amino acid substitution
(Ser¹⁴¹Cys). This novel SNP was found in one Japanese
colorectal cancer patient with a heterozygosity. Thus,
the allele frequency was calculated to be 0.96%. The
patient possessing the novel SNP did not harbor
*UGT1A7*2* or *UGT1A7*3* variant in exon 1, suggesting
the no linkage among these SNPs.

Although functional significance of this SNP has not been known, it may cause functional reduction of

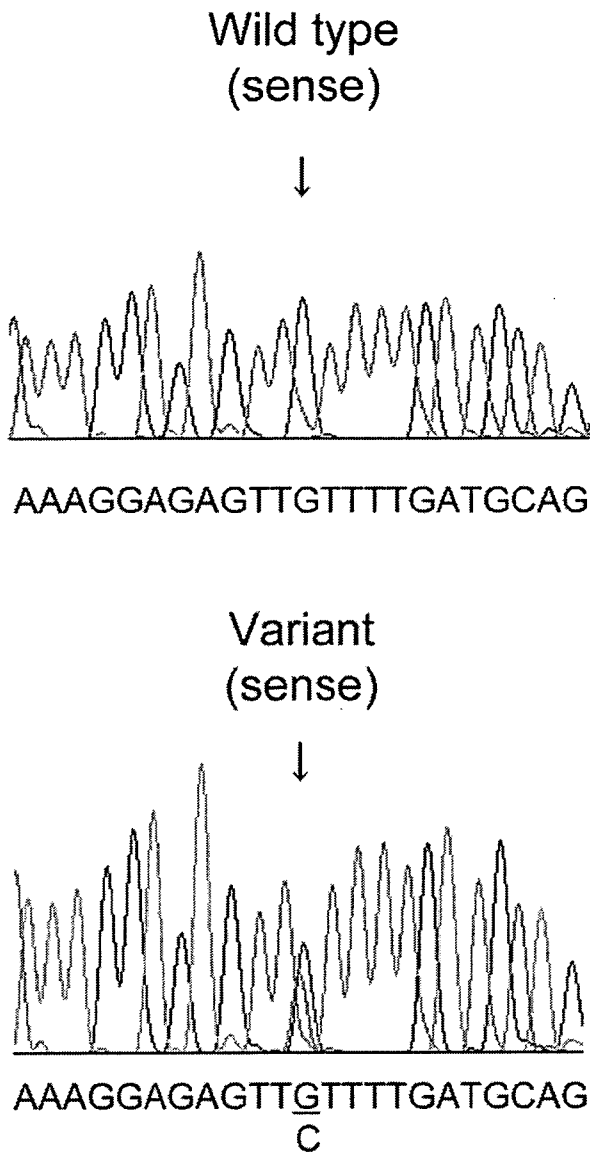


Fig. 1. Nucleotide sequences of *UGT1A7* containing novel variant 050824FujitaK002 (98973G>C) in exon 1. Arrows indicate the positions of the nucleotide change.

UGT1A7 because the amino acid of 141 is located in the putative substrate-binding domain of the enzyme (between Ser⁶¹ and Phe¹⁹⁰).²¹ The idea is considered to be supported by the fact that the decrease of the enzymatic activity of *UGT1A7* by the known amino acid substitutions in the putative substrate-binding domain (*UGT1A7.3*).

UGT1A7 plays a role in the glucuronidation of the active metabolite of irinotecan (SN-38).¹⁰ Therefore, patient with reduced activity of *UGT1A7* may have decreased ability to detoxify the SN-38. Besides *UGT1A7*, *UGT1A1* is known to be responsible for the

detoxification of SN-38,¹⁰ and the polymorphisms in the *UGT1A1* gene such as *UGT1A1*28*, *UGT1A1*6* and *UGT1A1*27* have been demonstrated to be related to the enhanced toxicity of irinotecan such as diarrhea and neutropenia.¹⁵ The analysis of the *UGT1A1* polymorphisms for the patient with the novel SNP revealed that the patient harbored the wild type of *UGT1A1* gene. Therefore, to clarify the effects of the novel SNP in *UGT1A7* gene on the glucuronidation capacity for SN-38, pharmacokinetics in SN-38 and SN-38G were compared between the patient with the novel SNP (not possessing *UGT1A7*2* or *UGT1A7*3* and *UGT1A1*28*, *UGT1A1*6* or *UGT1A1*27*) and patients without all of these SNPs in *UGT1A1* and *UGT1A7* genes. According to the genetic analysis, 16 patients possessed the wild type of *UGT1A1* and *UGT1A7*. The AUC ratio of SN-38 to SN-38G seen in the patient with the novel SNP (0.42) was not necessarily higher than those observed in the 16 patients (0.38 to 0.57; quartile range). In addition, the patient possessing the novel SNP did not show severe toxicity by the irinotecan treatment. Accordingly, heterozygosity of the novel SNP appeared not to affect the pharmacokinetics of irinotecan metabolites and the toxicity of irinotecan. The functional significance of the novel SNP in the *UGT1A7* gene is needed to be examined in the future.

In conclusion, we found a novel nonsynonymous mutation 98973G>C in exon 1 of *UGT1A7* in a DNA sample from a Japanese colorectal cancer patient. The SNP caused the amino acid substitution (Ser¹⁴¹Cys).

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SNP Communication

Novel Single Nucleotide Polymorphism of UGT1A9 Gene in Japanese

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Full text of this paper is available at <http://www.jstage.jst.go.jp/browse/dmpk>

Summary: We sequenced from 5'-franking region to intron 1 (to 337 bp downstream from exon 1) of the UDP-glucuronosyltransferase (UGT) 1A9 gene prepared from 55 Japanese cancer patients. Seven single nucleotide polymorphisms (SNPs) were found. Two of them were *UGT1A9* -118(T)_n (n=10) and *UGT1A9**5, and four were reported SNPs in intron 1 of *UGT1A9* gene (89540C>T, 89549G>A, 89616T>A and 89710A>C). A novel SNP (89587T>C) was found. The sequence is as follows: SNP, 050824FujitaK001; GENE NAME, UGT1A9; ACCESSION NUMBER, AF297093; LENGTH, 25 bases; 5'-CCTTCTTGAAGAT/CATGTATTTATAA-3'. Two patients were heterozygous for the mutant allele, resulting in the allele frequency of 1.82%.

Key words: UGT1A9; novel SNP; Japanese

Introduction

UDP-glucuronosyltransferase (UGT) is one of phase II drug-metabolizing enzymes that catalyzes the glucuronidation of a wide variety of endogenous and exogenous compounds, including drugs, carcinogens, and other xenobiotics. The UGT superfamily is composed of families, and two of the families (UGT1 and UGT2) have the ability to catalyze the glucuronidation of foreign chemicals.¹⁾ The *UGT1A9* gene located on chromosome 2q37 is expressed in the liver, kidney, small intestine, colon, and reproductive organs such as testis and ovary.²⁻⁴⁾ Cloning and characterization of the *UGT1A9* gene product demonstrated the conjugation of endogenous estrogenic and thyroid hormones,^{3,5)} as well as various xenobiotics such as phenols,⁶⁾ acetaminophen,⁷⁾ and an active metabolite of irinotecan (SN-38).⁸⁾ So far, various single nucleotide polymorphisms (SNPs) have been identified in the *UGT1A9* gene.⁹⁻¹³⁾ *UGT1A9**3 and *UGT1A9**5 have been known

to cause the reduced ability to metabolize SN-38.^{9,11)} The SNP in the 5'-franking region of the *UGT1A9* gene (-118(T)_n (n=10)) (based on assigning the 'A' in the 'ATG' translation start codon as +1) has been reported to be related to the enhanced transcriptional activity of the gene.¹²⁾

In the present study, 5'-franking region to intron 1 (to 337 bp downstream from exon 1) of the *UGT1A9* gene from 55 Japanese cancer patients treated with irinotecan was sequenced, and found a novel SNP in intron 1.

Materials and Methods

Patients: Fifty-five Japanese cancer patients (33 colorectal cancer, 15 gastric cancer, 3 ovarian cancer and 4 others) who received various regimens of irinotecan-containing chemotherapy or irinotecan monotherapy from June 2003 to August 2005 were studied. All of patients gave informed consent in writing for their peripheral blood samples and medical information to be used for the research. This study was approved by the Institutional Review Board of Saitama Medical School.

Human DNA samples: Genomic DNA was extracted from 200 μ L of peripheral blood of 55 Japanese cancer patients, which had been stored at -80°C until analysis, using QIAamp Blood Kit (QIAGEN GmbH, Hilden, Germany).

On September 30, 2005, the variation was not found on the UDP-glucuronosyltransferase homepage (<http://som.flinders.edu.au/FUSA/ClinPharm/UGT/>), the Japanese Single Nucleotide Polymorphism (JSNP) (http://snp.ims.u-tokyo.ac.jp/index_ja.html) or dbSNP in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP/>).

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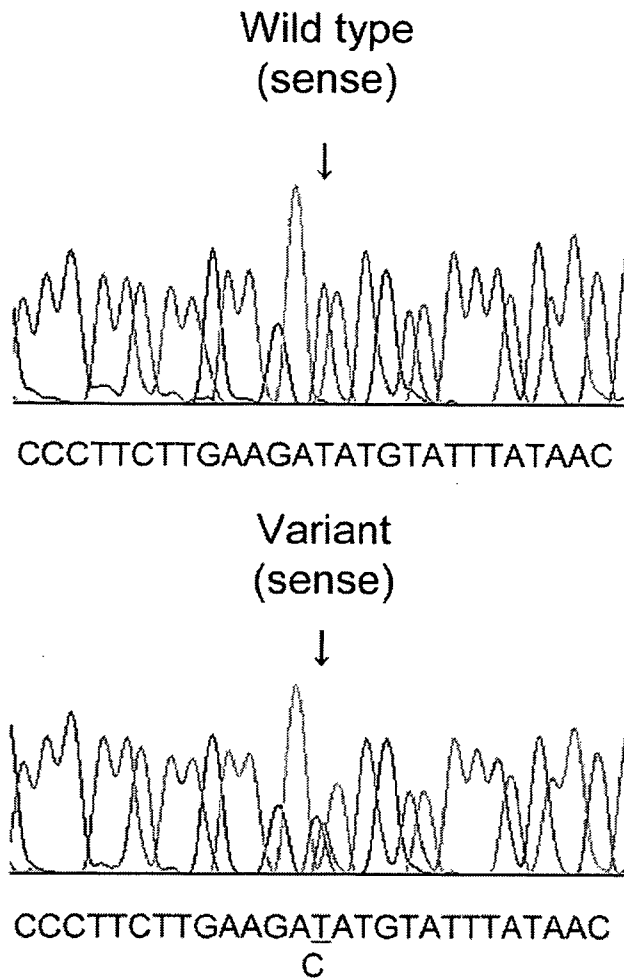


Fig. 1. Nucleotide sequences of *UGT1A9* containing novel variant 050824FujitaK001 (89587T>C) in intron 1. Arrows indicate the positions of the nucleotide change.

PCR conditions and DNA sequencing: A 1.46 kb fragment (from 88271 to 89734) including 5'-franking region, exon 1 and intron 1 (to 337 bp downstream from exon 1) of *UGT1A9* gene was amplified by PCR, according to the method of Carlini *et al.*,¹⁴⁾ with minor modifications. Briefly, the reaction mixture consisted of 2.5 mM MgCl₂ and 1.25 unit of AmpliTaq Gold polymerase (Perkin-Elmer, Foster City, CA) in a final volume of 50 μ L. Polymorphisms were detected by direct sequencing as described previously.¹⁴⁾ The sequence of the complete *UGT1A9* gene described in the GeneBank (AF297093) was used as a reference.

Results and Discussion

We found a following novel SNP: SNP, 050824Fujita001; GENE NAME, *UGT1A9*; ACCESSION NUMBER, AF297093; LENGTH, 25 bases; 5'-CCTTCTTGAAGAT/CATGTATTTATAA-3'. The SNP

Table 1. Allele frequency of polymorphisms in *UGT1A9* seen in Japanese cancer patients

		Allele number	Frequency
5'-Fraking region	<i>UGT1A9</i> -118(T) _n (n = 10)	70	63.6
Exon1	<i>UGT1A9</i> *5	1	0.91
Intron 1	89540C>T	1	0.91
	89549G>A	13	11.8
	89587T>C	2	1.82
	89616T>A	66	60.0
	89710A>C	66	60.0

was 89587T>C in intron 1 of the *UGT1A9* gene (Fig. 1). This novel SNP was heterozygously found in two Japanese cancer patients (colorectal cancer and ovarian cancer). Thus, the allele frequency was calculated to be 1.82% (Table 1). Besides the novel SNP, a known SNP was observed in the 5'-franking region (*UGT1A9* -118(T)_n (n = 10)). The allele frequency of the SNP calculated in this study was consistent with that observed in the previous study.¹²⁾ The other was seen in exon 1 (*UGT1A9**5) and four were detected in intron 1. We did not find the *UGT1A9**2, *UGT1A9**3 or *UGT1A9**4 polymorphisms, probably because the low allele frequency of these SNPs in Japanese.¹⁰⁾ In Caucasians, *UGT1A9**3 was reported to be found with the allele frequencies of 0.633 to 3.6%,^{9,15)} whereas *UGT1A9**5 was demonstrated not to be observed.¹⁵⁾ On the other hand, *UGT1A9**2 was found in Africans with the allele frequency of 2.5%.⁹⁾ These results suggest the ethnic difference in the distribution of *UGT1A9* polymorphisms. Intronic SNPs, 89616T>A and 89710A>C, were completely linked. Furthermore, these SNPs were highly related to the *UGT1A9* -118(T)_n (n = 10) polymorphism. One of patients with the novel SNP was also heterozygous for *UGT1A9* -118(T)_n (n = 10), 89616T>A and 89710A>C. No severe toxicity, diarrhea or neutropenia, was not observed in these patients. At present, the functional significance of this SNP is not known. Further studies including the analysis of linkage between the novel SNP and other SNPs are needed to clarify the importance of the novel SNP.

In conclusion, we found a novel mutation 89587T>C in intron 1 of *UGT1A9* in DNA samples from two Japanese colorectal and ovarian cancer patients.

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Pharmacogenetic impact of polymorphisms in the coding region of the *UGT1A1* gene on SN-38 glucuronidation in Japanese patients with cancer

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Pharmacogenetic testing for UDP-glucuronosyltransferase (*UGT*) 1A1*28, a promoter variant of the *UGT1A1* gene, is now carried out clinically to estimate the risk of irinotecan-associated toxicity. We studied the clinical significance of *UGT1A16 and *UGT1A1**27, two variants in exon 1 of the *UGT1A1* gene that are found mainly in Asians. The study group comprised 46 Japanese patients who received various regimens of chemotherapy including irinotecan at doses from 50 to 180 mg/m². Pharmacogenetic relationships were explored between the *UGT1A1* genotype and the ratio of the area under the plasma concentration–time curve (AUC) of the active metabolite of irinotecan (SN-38) to that of SN-38 glucuronide (SN-38G), used as a surrogate for *UGT1A1* activity (AUC_{SN-38}/AUC_{SN-38G}). No patient was homozygous for *UGT1A1**28, and none had *UGT1A1**27. Two were heterozygous for *UGT1A1**28. Two were homozygous and 15 heterozygous for *UGT1A1**6, all of whom were wild type with respect to *UGT1A1**28. Two patients were simultaneously heterozygous for *UGT1A1**28 and *UGT1A1**6, present on different chromosomes. The other 25 patients had none of the variants studied. The two patients simultaneously heterozygous for *UGT1A1**28 and *UGT1A1**6 and the two patients homozygous for *UGT1A1**6 had significantly higher AUC_{SN-38}/AUC_{SN-38G} ratios than the others ($P = 0.0039$). Concurrence of *UGT1A1**28 and *UGT1A1**6, even when heterozygous, altered the disposition of irinotecan remarkably, potentially increasing susceptibility to toxicity. Patients homozygous for *UGT1A1**6 should also be carefully monitored. *UGT1A1* polymorphisms in the coding region of the *UGT1A1* gene should be genotyped in addition to testing for *UGT1A1**28 to more accurately predict irinotecan-related toxicity, at least in Asian patients. (*Cancer Sci* 2006; 97: 1255–1259)**

Irinotecan is a camptothecin analog with potent antitumor activity resulting from the inhibition of topoisomerase. This anticancer drug is now used widely to treat colorectal, lung and other types of cancer.^(1,2) Dose-limiting toxicity of irinotecan includes severe leukopenia, neutropenia and diarrhea.^(3,4) Post-marketing studies of approximately 14 000 patients with cancer who received irinotecan in Japan have estimated that the incidence of grade 3 or higher leukopenia is 23.8% for irinotecan monotherapy and 38.3% for irinotecan combined with other drugs, whereas that of grade 3 or higher diarrhea is approximately 10%, regardless of regimen.⁽³⁾

Genetic polymorphisms of UDP-glucuronosyltransferase (*UGT*) 1A1, a key metabolizing enzyme of irinotecan, are important determinants of individual variations in susceptibility to toxicity.⁽⁵⁾ Irinotecan is a prodrug that is metabolized by carboxylesterase to its principal active metabolite, SN-38. SN-38 is subsequently conjugated mainly by *UGT1A1* to a more polar, inactive glucuronide (SN-38G). Severe toxicity is attributed, at least in part, to increased exposure to SN-38 caused by decreased *UGT1A1* activity due to genetic polymorphisms.

Pharmacogenetic studies of irinotecan toxicity have therefore focused on genetic polymorphisms of the *UGT1A1* gene, especially *UGT1A1**28, a variant sequence in the promoter region.^(6,7) Multiple studies have significantly linked this variant to irinotecan-related toxicity.⁽⁸⁾ The United States Food and Drug Administration has required that the package insert of irinotecan states that *UGT1A1**28 is associated with an increased risk of toxicity; to decrease this risk, genetic testing is encouraged. The package insert also recommends that the starting dose of irinotecan is reduced by at least one level for patients who are homozygous for *UGT1A1**28; however, whether this reduction is sufficient remains unclear. Firm recommendations for patients who are heterozygous for *UGT1A1**28 have yet to be established. Such patients are likely to have an intermediate *UGT1A1* activity leading to an increased risk of neutropenia, but clinical evidence supporting the use of lower doses of irinotecan is lacking.

The clinical consequences of *UGT1A1**6 and *UGT1A1**27, single nucleotide polymorphisms in exon 1 of the *UGT1A1* gene, also remain poorly understood. These variants are found mainly in Asians.⁽⁹⁾ Unlike *UGT1A1**28, case-control studies of Japanese patients have failed to demonstrate a significant relationship between *UGT1A1**6 alone and severe irinotecan-related toxicity.⁽¹⁰⁾ However, the study suggested that the presence of *UGT1A1**6 and *UGT1A1**27 in addition to *UGT1A1**28 might increase susceptibility to irinotecan-related toxicity considerably. To better understand the clinical significance of these variants, especially the more frequent variant of *UGT1A1**6, we examined how the coexistence of *UGT1A1**6 or *UGT1A1**27 and *UGT1A1**28 alters the pharmacokinetics of irinotecan in Japanese patients.

Materials and Methods

Patients. Forty-six Japanese patients with cancer who received irinotecan monotherapy or various regimens of irinotecan-based chemotherapy from June 2003 through April 2006 were studied. Some patients with metastatic colorectal cancer were also enrolled in a phase I study of a regimen containing irinotecan, fluorouracil (FU), and leucovorin (LV) (FOLFIRI), carried out at Saitama Medical School (Saitama, Japan). Toxicity was assessed prospectively according to the common terminology criteria for adverse events, version 3.0 (http://ctep.cancer.gov/reporting/ctc_v30.html). Tumor response was not included as a study variable because of various primary diseases and prior therapies. All subjects gave informed consent in writing for their peripheral blood samples and medical information to be used in this study. This study was approved by the Institutional Review Board of Saitama Medical School.

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Treatments. Irinotecan as a monotherapy was given weekly at doses of 50, 75 or 100 mg/m² for the first 3 weeks of a 4-week cycle or every 2 weeks at a dose of 150 mg/m² until the onset of disease progression or intolerable toxicity. For combination chemotherapy, a 100-mg/m² dose of irinotecan was administered with bolus FU 500 mg/m² and LV 10 mg/m² (*l* isomer form) weekly for the first 4 weeks of a 6-week cycle (IFL). The FOLFIRI regimen, administered at 2-week intervals, comprised irinotecan starting at 150 or 180 mg/m² and LV 200 mg/m² administered over the course of 2 h, followed by FU 400 mg/m² as a bolus injection and FU 2400 mg/m² as a 46-h continuous infusion. Regimens containing irinotecan plus cisplatin (IP) were repeated every 4 weeks. Irinotecan was given at a dose of 50–70 mg/m², followed 2 h later by a 120-min infusion of cisplatin 80 mg/m² with adequate hydration on day 1. The same dose of irinotecan was given again on day 15. For all regimens, 50–180 mg/m² of irinotecan was dissolved in 250 mL of 5% dextrose solution and infused over the course of 90 min.

Pharmacokinetic analysis. Blood samples for pharmacokinetic analysis were usually obtained during the first course of treatment with irinotecan. The samples were taken from the arm opposite the infusion site, at the beginning of irinotecan infusion and 0, 0.25, 0.5, 1, 2, 4, 8 and 24 h after the end of the infusion. The blood samples were centrifuged immediately, and the plasma was stored at –80°C until analysis. Total (lactone and carboxylate) plasma concentrations of irinotecan, SN-38 and SN-38G were analyzed by a modified reverse-phase high-performance liquid chromatographic (HPLC) method.⁽¹¹⁾ A 150- μ L plasma sample was mixed with 300 μ L of methanol, 5% perchloric acid (50:50, v/v) and camptothecin, serving as an internal standard, in a vortex mixer. The mixture was centrifuged at 20 600 *g* for 10 min, and 200 μ L of the supernatant was injected into an HPLC system (Hitachi model 7000 series; Hitachi, Tokyo, Japan), equipped with a TSK-gel ODS-120T analytical column (4.6 \times 250 mm, 4 μ m; TOSOH, Tokyo, Japan). HPLC was carried out at 40°C at a flow rate of 1.0 mL/min. Irinotecan, SN-38 and SN-38G were determined fluorometrically (excitation 355 nm; emission 515 nm). The total (lactone and carboxylate) concentrations of irinotecan, SN-38 and SN-38G in plasma were quantified. The mobile phase consisted of 75 mM ammonium acetate (pH 4.75) for solvent A, and acetonitrile for solvent B; a 20-min run was carried out with a linear gradient of 85–65% for solvent A.

The lower limit of quantification was 5 ng/mL (7.4 nM) for irinotecan and 0.5 ng/mL (1.2 nM and 0.88 nM, respectively) for SN-38 and SN-38G. The intra-assay and interassay coefficients of variation were less than 10% for irinotecan and the metabolites.

The area under the plasma time–concentration curve (AUC; μ mol·h/L) from the beginning of the infusion of irinotecan to the time of obtaining the last blood sample was calculated by a linear trapezoidal rule. The ratio of the AUC of SN-38 to that of SN-38G (AUC_{SN-38}/AUC_{SN-38G}) was used as a surrogate for UGT1A1 enzyme activity *in vivo*.

Genotyping. Genomic DNA was extracted from peripheral blood, which had been stored at –80°C until analysis, with the use of a QIAamp Blood Kit (QIAGEN, Hilden, Germany).

To analyze exon 1, first-step PCR amplification of a 923-bp fragment containing exon 1 was carried out as reported previously.⁽¹⁰⁾

For analysis of UGT1A1*6, the second set of polymerase chain reaction (PCR) amplifications was carried out with nested primers designed to amplify a 235-bp segment. The mismatched forward primer and the reverse primer were 5'-CTAGCACCT-GACGCCTCGTTGTACATCAGAGCC-3' (+178 to +210; underlining indicates mismatched site) and 5'-CCATGAGCTC-C TTGTTGTGC-3' (+393 to +412), respectively. The forward primer was designed to introduce an *Msp*I (Takara Shuzo Co., Otsu, Japan) restriction site into the wild-type sequence from

+209 to +212. A 1000-fold diluted product of the first PCR was subjected to nested PCR in a volume of 50 μ L containing 0.2 mM of each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.5 μ M of each primer and 1.3 units of *Taq* polymerase (AmpliTaQ Gold; Perkin-Elmer, Foster City, CA, USA). The PCR conditions were 95°C for 15 min followed by 25 cycles of 94°C for 30 s, 55°C for 40 s and 72°C for 40 s. A 1- μ L PCR product was digested with 4 units of *Msp*I for 1 h at 37°C. DNA from the wild-type sequence was digested into 203- and 32-bp fragments, whereas DNA from UGT1A1*6 gave an undigested 235-bp fragment.

For the sequence of UGT1A1*27, another set of the second PCR amplifications was carried out using hemi-nested primers 5'-AGTACCTGTCTCTGCCAC-3' (+485 to +503) and 5'-GTCCCACTCCAATACACAC-3' (+865 to +867 and intron 1), designed to amplify a 399-bp segment. Two *Bsr*I (New England Biolabs, Ipswich, MA, USA) restriction sites exist in UGT1A1*27 (+552 to +556 and +684 to +688), but only one site (+552 to +556) exists in wild type. The method of PCR amplification was identical to that for the *Msp*I restriction fragment length polymorphism, as described above. Digestion of the PCR products with 2.5 units of *Bsr*I for 1 h at 65°C gave 199-, 132- and 68-bp fragments from UGT1A1*27, or 331- and 68-bp fragments from wild type.

UGT1A1*28 was distinguished from wild type by direct sequencing (–147 to +106) of the 253–255 bp produced by PCR.

A set of PCR amplifications of a 1030-bp fragment containing UGT1A1*28 and UGT1A1*6 was used to determine the location of these variant sequences on the respective alleles. The reaction mixture was similar to that described previously,⁽⁷⁾ except that the concentration of MgCl₂ was 2.0 mM, and the amount of *Taq* polymerase was 1.3 units. The PCR primers were 5'-AAGTGAAGTCCCTGCTACCTT-3' (–147 to –127) and 5'-GTCCCACTCCAATACACAC-3' (+865 to +867 and intron 1). The PCR conditions were 95°C for 15 min, followed by 30 cycles of 94°C for 30 s, 58°C for 40 s and 72°C for 40 s. The PCR fragments obtained were subcloned into pT7 Blue T-Vector (Novagen, Darmstadt, Germany), and sequence analysis was used to examine the gene arrangement of the variants on homologous chromosomes.

Statistical analysis. The statistical significance of differences in the AUC_{SN-38}/AUC_{SN-38G} was assessed using the Mann–Whitney *U* test. This and other statistical analyses were carried out with SPSS for Windows, version 12.0 J (SPSS Japan, Tokyo Japan). Differences were considered statistically significant when the two-tailed *P*-value was less than 0.05.

Results

A total of 46 patients were studied (Table 1). Performance status was generally good; most patients had gastrointestinal tract cancers. Fourteen patients received IP, 20 FOLFIRI, three IFL and nine irinotecan monotherapy. Two of the 14 patients treated with IP, which is one of the experimental arms of an ongoing randomized phase III trial against unresectable gastric cancer in Japan (JCOG9912), were enrolled into JCOG9912, and the other 12 patients treated with IP also fulfilled the recruitment criteria of JCOG9912 except for having no prior chemotherapy.

Genomic DNA from all patients was genotyped to examine UGT1A1 polymorphisms. No patient was homozygous for UGT1A1*28 or had UGT1A1*27 (Table 2). Two patients were heterozygous for UGT1A1*28. Two patients were homozygous and 15 were heterozygous for UGT1A1*6, all of whom were wild type with respect to UGT1A1*28. Two patients were simultaneously heterozygous for UGT1A1*6 and UGT1A1*28. Sequencing of the subcloned products obtained from the two

Table 1. Patient characteristics (n = 46)

Characteristic	n
Median age (years)	62 (range: 42–85)
Sex	
Female	20
Male	26
Performance status	
0	28
1	16
2	2
Primary organ	
Gastric	14
Colorectal	25
Other	7
Previous treatments	
Surgery	31
Radiotherapy	3
Systemic chemotherapy	38
1 regimen	30
2 regimens	8
None	6

Table 2. UGT1A1 genetic profiles and plasma concentration–time curve (AUC) ratios

UGT1A1*6	UGT1A1*28	n†	AUC _{SN-38} /AUC _{SN-38G}
–/–	–/–	24	0.43 (0.08–1.39)‡
+/-	–/–	15	0.61 (0.26–1.42)‡
+/+	–/–	2	1.40, 1.10
–/–	+/-	2	0.48, 0.44
+/-	+/+	2	1.04, 2.16

†Total n = 45. One patient was excluded because of an incomplete set of blood samples; ‡median (range).

patients who were heterozygous for both *UGT1A1*6* and *UGT1A1*28* revealed that the variants existed on different chromosomes (*UGT1A1*6/UGT1A1*28* diplotype), which was consistent with the results of Saeki *et al.*⁽¹¹⁾ The other 25 patients had none of the variants studied.

Complete sets of blood samples were obtained in 45 of the 46 patients. In the remaining one patient who received FOLFIRI, a blood sample could not be obtained for technical reasons at the end of the infusion. Pharmacokinetic profiling showed that SN-38G levels were higher than SN-38 levels; AUC_{SN-38}/AUC_{SN-38G} was therefore usually under 1 (Table 2; Fig. 1A). There was no apparent relationship between AUC_{SN-38}/AUC_{SN-38G} and the dose of irinotecan (data not shown). In the patient who was excluded from AUC analysis because of the missing blood sample, SN-38G levels were higher than SN-38 levels at all other time points. This patient had none of the variants studied.

In the two patients who had the heterozygous *UGT1A1*6/UGT1A1*28* diplotype, SN-38G levels were lower than or approximated SN-38 levels. Consequently, their AUC_{SN-38}/AUC_{SN-38G} ratios were higher than those of the patients who had no variants or who were heterozygous for either *UGT1A1*6* or *UGT1A1*28* alone (Table 2; Figs 1B,C, 2). One of these patients who received the FOLFIRI regimen at an irinotecan dose of 150 mg/m² was studied again, after obtaining additional informed consent, to confirm reproducibility of the pharmacokinetic profile. The dose of irinotecan was reduced from 150 mg/m² in the first course to 100 mg/m² in the second course because of grade 4 neutropenia during the first course. The patient had

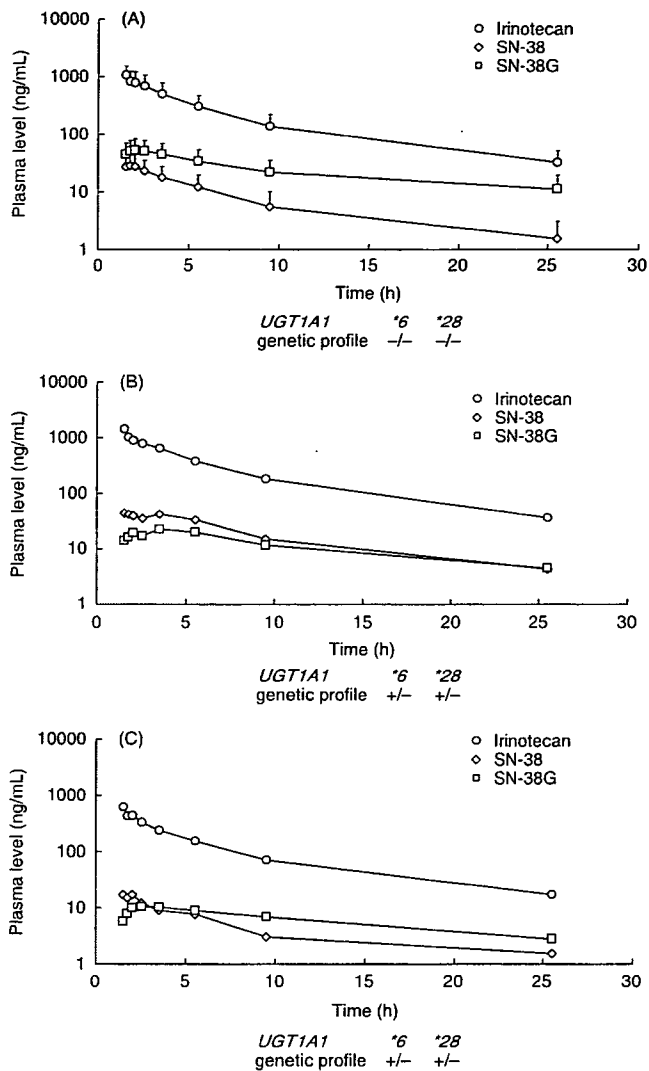


Fig. 1. Pharmacokinetic profiles of patients who received irinotecan. (A) The profile of 24 patients without *UGT1A1*28*, *UGT1A1*6* and *UGT1A1*27* showed that SN-38 glucuronide (SN-38G) levels were higher than the SN-38 levels reported in previous studies. Each data point indicates mean \pm SD. (B) The patient with colon cancer received irinotecan at a dose of 150 mg/m² in FOLFIRI. (C) The patient with gastric cancer received irinotecan at a dose of 70 mg/m² in IP. SN-38G levels were lower than SN-38 levels in these two patients who were heterozygous for *UGT1A1*6* and *UGT1A1*28*.

grade 2 neutropenia in the second course. The pharmacokinetic profiles were similar for the first and second courses of treatment; the AUC_{SN-38}/AUC_{SN-38G} was 2.16 in the first course and 1.56 in the second. The other patient who had the heterozygous *UGT1A1*6/UGT1A1*28* diplotype received IP safely, with no apparent toxicity.

Two patients were homozygous for *UGT1A1*6*. Their AUC_{SN-38}/AUC_{SN-38G} ratios were relatively higher than those of the other patients (Table 2; Fig. 2). One received irinotecan at a dose of 180 mg/m² in the FOLFIRI regimen and had grade 2 neutropenia in the first course of treatment. Pharmacokinetic studies were repeated in this patient with the same dose of irinotecan after obtaining additional informed consent. The patient had grade 3 neutropenia in the second course. Similar pharmacokinetic profiles were obtained; the AUC_{SN-38}/AUC_{SN-38G} ratio was 1.40

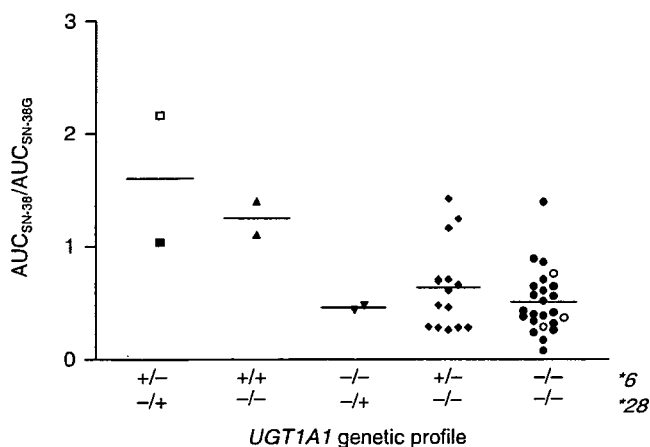


Fig. 2. Ratio of the area under the plasma concentration–time curve (AUC) for SN-38 to the AUC for SN-38 glucuronide (SN-38G), used as a surrogate for UGT1A1 activity (AUC_{SN-38}/AUC_{SN-38G}). Results are shown according to UGT1A1 genetic profile for 45 Japanese patients who received various regimens of irinotecan chemotherapy. Open symbols in the figure represent the patients suffering from grade 4 neutropenia. Lines indicate median values. One patient was excluded because of an incomplete set of blood samples.

in the first course and 2.73 in the second. The other patient received irinotecan monotherapy at a dose of 75 mg/m² and had grade 3 nausea and grade 1 neutropenia. AUC_{SN-38}/AUC_{SN-38G} in this patient was 1.10. The AUC_{SN-38}/AUC_{SN-38G} ratios in the four patients who were heterozygous for UGT1A1*28/UGT1A1*6 or homozygous for UGT1A1*6 were significantly higher than those in the other 41 patients ($P = 0.0039$).

Among the 45 patients, four patients suffered from grade 4 neutropenia. Two of them received FOLFIRI regimen. One of the patients with UGT1A1*28/UGT1A1*6 received a FOLFIRI regimen including irinotecan 150 mg/m², and the absolute AUC for SN-38 was the highest (1.06 μmol·h/L) among all patients who received FOLFIRI (median 0.56 μmol·h/L; range 0.49–1.06 μmol·h/L; irinotecan dose 150 or 180 mg/m²; $n = 19$). One who had wild-type UGT1A1 alleles received FOLFIRI with irinotecan 180 mg/m², and the absolute AUC for SN-38 was not high (0.66 μmol·h/L). Another two who harbored wild-type UGT1A1 alleles received IP regimens with irinotecan 60 or 70 mg/m². The AUCs for SN-38 were, respectively, 0.39 μmol·h/L and 0.26 μmol·h/L, similar to the values in the other patients given IP (median 0.30 μmol·h/L; range 0.20–0.68 μmol·h/L; $n = 12$). No patient had grade 3 or higher diarrhea in this study.

Discussion

The present study demonstrated that patients who were heterozygous for both UGT1A1*6 and UGT1A1*28 (UGT1A1*6/UGT1A1*28 diplotype) had markedly lower SN-38 glucuronidation activity than those who were heterozygous for either of these variants, let alone patients who had neither variant. Previous pharmacokinetic studies of irinotecan have reported that UGT1A1*6 decreases SN-38 glucuronidation, despite the effects being somewhat lower than UGT1A1*28 does.^(12,13) Heterozygous UGT1A1*28 status is considered to result in UGT activity intermediate between that of wild type and that of homozygous UGT1A1*28. Thus, the presence of both UGT1A1*6 and UGT1A1*28, even when heterozygous, apparently lowers SN-38 glucuronidation activity additively, resulting in a phenotypic effect similar to that associated with homozygous UGT1A1*28. The mechanisms responsible for this add-on effect are unclear. UGT1A1*6 and UGT1A1*28 decrease

UGT1A1 activity via different mechanisms; the former variant lessens protein function directly, whereas the latter reduces transcriptional activity of the promoter. Genetic effects on UGT activity compensate for each other when these variants exist separately. In contrast, compensation might be hindered by the coexistence of UGT1A1*6 and UGT1A1*28, resulting in remarkably lower UGT activity.

Our results suggest that patients who have the UGT1A1*6/UGT1A1*28 diplotype are at elevated risk for severe irinotecan-related toxicity due to increased exposure to SN-38. This notion is supported by the fact that one of the four patients with severe toxicity had the UGT1A1*6/UGT1A1*28 diplotype. The AUC for SN-38 in this patient was higher than that of any other patients who received the same FOLFIRI regimen. We therefore believe that patients who have the UGT1A1*6/UGT1A1*28 diplotype should be treated carefully, similar to those who are homozygous for UGT1A1*28. Furthermore, 2.5–4.6% of the Japanese population is estimated to have both UGT1A1*6 and UGT1A1*28,^(13–15) which is similar to, or slightly higher than, the percentage homozygous for UGT1A1*28. These findings suggest that pharmacogenetic testing for irinotecan toxicity should include not only UGT1A1*28, but also the coding region variant UGT1A1*6, at least in Japanese patients.

Although the patients who were homozygous for UGT1A1*6 had lower SN-38 glucuronidation activity than the others in this study, UGT1A1*6 alone appears to have only a limited effect on irinotecan-related toxicity. Indeed, UGT1A1*6 was not associated with severe toxicity, and even patients who were homozygous for UGT1A1*6 could be treated safely in a previous study.⁽¹⁰⁾ Thus, patients who are heterozygous or even homozygous for UGT1A1*6 alone can most likely tolerate conventional starting doses of irinotecan, unless they have other conditions associated with an increased risk of toxicity. In *in vitro* expression studies, however, homozygosity and heterozygosity for UGT1A1*6 were associated with approximately 30 and 60% reductions in UGT activity, respectively.⁽¹⁶⁾ Otherwise, UGT1A1*6 could be partially linked to other genetic polymorphisms that decrease overall UGT activity. We have recently identified genetic linkages of UGT1A7 and UGT1A9 polymorphisms to UGT1A1*6, including those related to lower catalytic or transcriptional activities of UGT enzymes (K. Fujita *et al.*, unpublished data, 2006). In addition, a previous pharmacokinetic study showed that the patients who had UGT1A1*6 alone showed slightly but not significantly decreased SN-38 glucuronidation activity,⁽¹³⁾ consistent with the results of our study. As for hyperbilirubinemia, UGT1A1*6 is a significant contributory factor to unconjugated hyperbilirubinemia, including Gilbert's syndrome, especially among Japanese neonates.^(14,15) These findings suggest that patients who have UGT1A1*6, especially when homozygous, are at increased risk for irinotecan-related toxicity. Although definitive evidence of this risk is lacking, we believe that toxicity should be monitored rigorously during irinotecan chemotherapy in patients who have UGT1A1*6 alone.

The genetic basis for irinotecan-related toxicity apparently differs among distinct ethnic populations. That is to say, the same genetic variants affect toxicity differently. UGT1A1 genetic polymorphisms differ considerably among genetically distinct populations; the allele frequency of UGT1A1*28 is several times higher in whites (0.3–0.4) than in Asians (around 0.15).^(17,18) UGT1A1*6 and UGT1A1*27 have been identified only in Asians (0.11–0.23 for UGT1A1*6 and 0.01–0.03 for UGT1A1*27).^(6,9,12,14) As for hyperbilirubinemia, bilirubin levels are generally higher in Asians than in whites, at least among infants, suggesting that the genetic basis for hyperbilirubinemia differs between these two ethnic groups.^(19–21) The neonatal hyperbilirubinemia found in Japanese is significantly related to UGT1A1*6, not UGT1A1*28,^(14,15) whereas that occurring in whites during the first 2 days of life is associated with homozygosity

for *UGT1A1*28*.⁽²²⁾ In the present study, the total bilirubin levels observed in the patients who were homozygous for *UGT1A1*6* and heterozygous for both of *UGT1A1*6* and *UGT1A1*28* were not significantly higher than those observed in the other patients. The complex genetic basis for *UGT1A1* activity makes it difficult to compare irinotecan-related toxicity among different ethnic groups. In addition, other factors potentially related to irinotecan-associated toxicity include patients' age, organ functions, prior treatments, dosing schedule and concurrently administered drugs. However, because genotype-based individualized chemotherapy with irinotecan is now practiced widely, studies comparing irinotecan toxicity among the different ethnic populations appear to be warranted.

In conclusion, our study showed that the coexistence of two *UGT1A1* variants, the promoter variant *UGT1A1*28* and the coding region variant *UGT1A1*6*, remarkably altered the disposition of irinotecan, potentially increasing susceptibility to

toxicity, even when these variants were heterozygous. Patients who had the *UGT1A1*6/UGT1A1*28* diplotype should therefore be treated similarly to those who are homozygous for *UGT1A1*28*. Genotyping for *UGT1A1*6* in addition to *UGT1A1*28* is necessary to predict the risk of irinotecan-related toxicity, at least in Asian patients. Genomic information is hereafter expected to play an increasingly important role in optimizing the use of irinotecan therapy.

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Late toxicity in complete response cases after definitive chemoradiotherapy for esophageal squamous cell carcinoma

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Background. We retrospectively investigated long-term toxicity after concurrent chemoradiotherapy (CRT) for patients with esophageal squamous cell carcinoma (ESCC). **Methods.** Concurrent chemoradiotherapy was performed in 110 patients with T1 to T4 disease containing M1 lymph node (LYM) disease. Chemotherapy consisted of protracted infusion of 5-fluorouracil 400 mg/m² per 24 h on days 1 to 5 and 8 to 12, combined with 2-h infusion of cisplatin 40 mg/m² on days 1 and 8. Radiation treatment of the mediastinum at a dose of 30 Gy in 15 fractions was administered concomitantly with chemotherapy. A course schedule with a 3-week treatment and a 2-week break was applied twice, with a total radiation dose of 60 Gy. For the assessment of toxicity, the Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer late radiation morbidity scoring schema was adopted. **Results.** A total of 81 patients were recruited in patients with stage I to IVA. Of 34 patients with complete response, 1 patient died as a result of acute myocardial infarction. Grade 2, 3, and 4 late toxicities occurred with the following incidences: pericarditis in 3 patients, 1 patient, and 2 patients, respectively; heart failure in 0, 0, and 3 patients; pleural effusion in 2, 3, and 0 patients; and radiation pneumonitis in 0, 0, and 1 patient, respectively. **Conclusions.** Definitive chemoradiotherapy for ESCC is effective with substantial toxicities. Further investigation is warranted to minimize the normal tissue toxicities.

Key words: esophageal carcinoma, chemoradiotherapy, late toxicity, prognosis

Introduction

In Western countries, the number of patients with esophageal adenocarcinoma has been increasing, whereas most patients in Japan have squamous cell carcinoma of the esophagus. Previous studies indicated that 95% of esophageal cancers in Japanese patients are squamous cell carcinomas.¹ In recent years, most patients are still diagnosed with advanced-stage ESCC, although the number of patients found with early-stage ESCC has been increasing. The standard therapy in Japan for patients with resectable disease has been surgery. The 5-year survival rate for T1, T2, and T3 disease was 52%, 37%, and 28%, respectively.²

On the other hand, the effects of chemotherapy combined with radiotherapy on esophageal carcinoma have been investigated since the 1980s. Several investigators have reported successful results with these modalities, either with or without surgery, against locoregional carcinoma.^{3–8} The combination of 5-fluorouracil (5-FU) and cisplatin (CDDP) has become a standard regimen, not only because of the clinical outcome, but also because of the synergism between the two agents and their radiosensitizing effects.^{9–11} Recently published results on chemoradiotherapy (CRT) indicated that it offers various advantages for the treatment of carcinoma of the esophagus.^{7,12} In a prospective randomized trial by the Radiation Therapy Oncology Group, which compared chemoradiotherapy with radiotherapy alone, the combined-modality arm demonstrated a significant improvement of survival;¹³ with a 5-year survival rate of 27%, compared with 0% for radiotherapy alone.¹⁴ With regard to the indications for CRT as a curative treatment for patients with locally advanced diseases, our multicenter study suggested that concurrent CRT was potentially curative even in cases with unresectable carcinoma of the esophagus (i.e., T4 and/or M1 LYM disease).^{15,16}

Esophageal cancer deaths often occur in non-CR cases or in recurrent cases. However, recent data indi-

cate that the risk of early death from esophageal carcinoma is not quite as daunting for patients who achieve complete response (CR) after CRT. We have already reported that concurrent CRT was effective for inoperable patients. Therefore, a significant proportion of CR patients may have a sufficiently long survival time to allow for the adequate assessment of treatment-related late toxicity. We retrospectively investigated the long-term toxicity after definitive CRT for patients with squamous cell carcinoma of the thoracic esophagus.

Methods

Patient population

From May 1996 through March 2002, 110 consecutive patients were diagnosed at Showa University School of Medicine as having esophageal carcinoma. Patients were recruited from our database on the basis of the following criteria: age ≤ 75 years, performance status (Eastern Cooperative Oncology group) 0 to 2, clinical stage I to IVA (International Union Against Cancer tumor-node-metastasis system, 1997), adequate organ function, and no other site of carcinoma except for early stage. None of the patients had surgery or chemotherapy for previous diseases. In agreement with that observation, none of the patients enrolled in this study had esophageal adenocarcinoma; thus, all patients had squamous cell carcinoma of the esophagus.

Eligibility criteria

Patients who were eligible for this trial had previously untreated, histologically confirmed squamous cell carcinoma of the thoracic esophagus. The tumors had to show evidence of T1–T4 disease, containing M1 LYM disease, based on the staging criteria of the UICC. The prestudy clinical evaluation included air-contrast barium esophagography, esophagoscopy, neck computed tomography (CT), chest CT, abdominal CT, endoscopic ultrasonography (EUS), bronchoscopy, and bone scan. However, EUS was optional because the endoscope could not be passed through stenotic lesions in most cases (68%). Bronchoscopy was performed in some cases when tracheobronchial involvement was suspected. Because the prognosis of patients with T4 disease differed significantly from that of patients with T3 disease,¹⁶ we defined T3 and T4 disease in clinical staging. Adjacent organs were considered to be involved (T4 disease) if the tumors extended into the esophageal lumen, or caused deformity of the tracheobronchial tree, or if the tumors appeared to be attached to the organs at $>90^\circ$ angle to the thoracic aorta as

observed on the CT scan.^{17,18} T3 or lesser extent of the disease was determined by EUS. In those patients who could not undergo this procedure, T3 was defined based on the lack of any associated abnormal bronchoscopic findings; i.e., no deformity of the airway and tracheobronchial tree on the CT scan. Furthermore, we modified the criteria described previously for the definition of T3 disease¹⁷ to include tumors attached to the organs at $\leq 90^\circ$ angle to the thoracic aorta as observed on the CT scan. The patients were considered to have lymph node metastasis if the tumor was ≥ 1 cm in diameter.¹⁹ Radiologic evaluations for staging were reviewed by radiologists and medical oncologists at Showa University School of Medicine, as was reported in previous studies.^{17–19} However, although the UICC staging criteria were adopted in these previous studies, we used a nonstandard staging technique in this study, especially when evaluating the depth of tumor infiltration.

The following criteria were used for enrollment for chemoradiotherapy: (1) an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 2 or less, (2) satisfactory hematological function (leukocyte count $\geq 3000/\text{mm}^3$ and platelet count $\geq 100\,000/\text{mm}^3$), (3) satisfactory hepatic function [aspartate aminotransferase (AST) or alanine transferase (ALT) levels within three times the normal upper limit and a serum bilirubin level $< 2.0\text{ mg/dl}$], (4) good renal function (creatinine level $\leq 1.5\text{ mg/dl}$ and creatinine clearance $\geq 50\text{ ml/min}$), (5) satisfactory pulmonary function ($\text{PaO}_2 \geq 70\text{ mm Hg}$), (6) normal electrocardiogram, and (7) life expectancy ≥ 8 weeks. Patients with serious complications, such as history of ischemic heart disease, pulmonary fibrosis, or active carcinoma at another site, were excluded from the study. After explaining the true disease status and predicted complications of the treatment, including the possibility of treatment-related death, each patient gave informed consent for the study. The study protocol was approved by the Human Ethics Review Committee of Showa University School of Medicine.

Treatment schedule

Chemotherapy consisted of protracted infusion of 5-FU at a dose of 400 mg/m^2 per day on days 1–5 and 8–12, combined with 2-h infusion of CDDP at 40 mg/m^2 on days 1 and 8 (Fig. 1). A 10-MV radiation treatment was administered for 3 weeks (5 days/week) at 2 Gy/day , concomitantly with chemotherapy. The targeted area for carcinoma of the upper and middle third of the esophagus included the primary tumors with a 3-cm margin craniocaudally and any metastatic nodes with 1- to 1.5-cm margin, in the supraclavicular fossa and mediastinum. For carcinoma of the lower third of the esophagus, the field was extended to include the

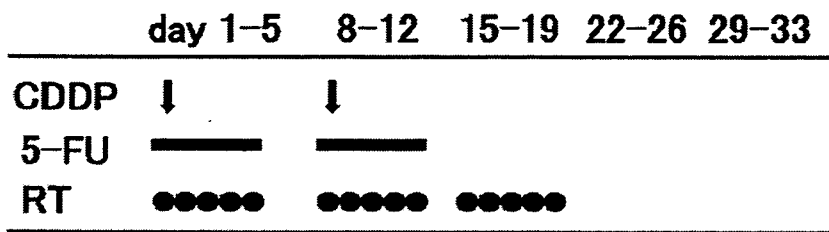


Fig. 1. The schema of treatment schedule for chemoradiotherapy. The treatment course included 3 weeks of radiotherapy followed by a 2-week break, and the course was repeated twice, with a total radiation dose of 60 Gy. RT, radiation treatment

perigastric nodes, while the supraclavicular fossa was excluded if the cervical nodes were found to be negative. The daily fractional dose of radiotherapy was 2 Gy administered 5 days a week. When the planned volume included both the supraclavicular fossa and upper abdominal nodes, the daily dose of 1.8 Gy was allowed. After a dose of 30 Gy, we allowed a 2-week treatment-free period. Radiotherapy was restarted on day 36, along with the same schedule of chemotherapy as described above. The treatment course included 3 weeks of radiotherapy followed by a 2-week break, and the course was repeated twice, with a total radiation dose of 60 Gy. The irradiation techniques were initially applied in anterior and posterior opposed fields. At 40 Gy, the radiation portals were reduced to shield the spinal cord and to encompass the primary tumor craniocaudally with a 2- to 3-cm margin, usually by using an oblique opposed field. Metastatic nodes were encompassed with a 1- to 1.5-cm margin. The total radiation dose to the spinal cord was kept at a maximum of 40 Gy. The homogeneity of the dose within the planning volume was within $\pm 10\%$ of the prescribed dose.

Patients who were evaluated for an objective response to this treatment received additional chemotherapy consisting of continuous infusion of 5-FU at a dose of 800 mg/m² on days 1-5 and CDDP at a dose of 80 mg/m² on day 1. This treatment schedule of 1-week treatment followed by a 3-week break was only repeated once in some patients, and no further treatment was applied if no disease progression was observed. When a single course consisted of treatment followed by >5-week break, we defined the latter as interruption. All patients receiving CRT were monitored by neck CT, chest CT, abdominal CT, endoscopy, and air-contrast esophagography every 4 weeks.

Evaluation of response and toxicity of chemoradiotherapy

For measurable lesions, response was assessed using the World Health Organization criteria. Briefly, a complete response (CR) was defined as the complete disappearance of all measurable and assessable disease for at least 4 weeks. A partial response (PR) was defined as more

than 50% reduction in the sum of the products of the longest perpendicular diameter of measurable disease for a period of at least 4 weeks. Stable disease (SD) was defined as the failure to observe CR, PR, or progressive disease for at least 4 weeks. Progressive disease (PD) was defined as >25% increase in the sum of the products of the longest perpendicular diameter of measurable disease or the appearance of new lesions.

For primary tumors, CR was defined as when all visible tumors, including ulceration, disappeared for at least 4 weeks, confirmed by normal endoscopic biopsy specimens. Uncertain CR was defined as the persistence of small nodes (≤ 1 cm) with no evidence of progression for ≥ 3 months after completion of treatment, and patients with uncertain CR were included in the analysis of those with CR. The response was evaluated by esophagography, esophagoscopy, and neck, chest, and abdominal CT scans during each course.

Toxicity was evaluated using the criteria defined by the National Cancer Institute Common Toxicity Criteria (NCI-CTC, version 2.0). Toxicity was assessed on a weekly basis during chemoradiotherapy and then biweekly during the subsequent chemotherapy. Late toxicity assessment for cardiac ischemia, pericardial effusion, heart failure, and pleural effusion was performed according to the NCI-CTC. Radiation Therapy Oncology Group (RTOG)/European Organization for Research and Treatment of Cancer (EORTC) late radiation morbidity scoring schema was used for assessment of radiation pneumonitis. Late toxicity was defined as that occurring more than 90 days after the treatment initiation.

Statistical analysis

Follow-up evaluations after CRT were performed every 3 months for the first 2 years and every 6 months thereafter by endoscopy and CT scan. Differences between the two groups were calculated by the chi-square test. Survival was calculated from the data at the initiation of treatment by the actuarial Kaplan-Meier method.²⁰ Survival differences between the two groups were assessed by the log-rank test. P values less than 0.05 were considered significant.

Results

Patient characteristics

There were 110 patients who received definitive or palliative CRT during the periods: 110 patients matched the recruitment criteria, and 29 patients were excluded from the analysis. The reasons for exclusion were stage IVB (5 patients), double cancer (3 patients), age > 75 years (10 patients), inadequate organ function (5 patients), fistula (2 patients), small cell carcinoma (1 patient), and carcinoma of the cervical esophagus (3 patients). The characteristics of the participating 81 patients are listed in Table 1. Seventy-four patients were men and 7 were women, and the median age was 63 years. Most patients had a good performance status. According to our criteria, the clinical staging was classified as follows: stage I in 8 patients, stage II in 14 patients, stage III in 25, and stage IV in 34. There were 22 patients (27%) with T3 disease, and 44 (54%) with T4 disease. Of 81 patients, 33 (41%) had M1 LYM disease. Clinically involved sites in the 44 cases with T4 disease were thoracic aorta (28 patients), tracheobronchial tree (13 patients), and both sites (3 patients). Five patients had cervical node metastasis, 25 had abdominal nodes, and 3 had metastases in both nodes. All 81 patients had histopathologically confirmed squamous cell carcinoma. Seventy-five patients (93%) completed at least the CRT

Table 1. Patient characteristics

No. of patients	81
Sex (male/female)	74/7
Age, years (range)	63 (45–75)
Performance status	
0/1/2	64/13/4
Location ^a	
Upper	8
Middle	43
Lower	30
Histopathology	
Well differentiated	9
Moderately differentiated	58
Poorly differentiated	14
Stage (UICC)	
Stage I	8
Stage II	14
Stage III	25
Stage IV	34
Tumor ^b	
1	10
2	5
3	22
4	44
Metastasis ^b	
0	48
1a	33

^aLocation of the tumor according to the TNM classification (UICC, International Union Against Cancer)

^bNumbers correspond to the tumor-node-metastasis system of classification

segment with a total radiation dose of 60 Gy. The remaining 6 patients did not complete CRT; 4 experienced disease progression, and 2 died of treatment-related esophagoaortic fistula. Of 63 patients who responded to CRT, 55 (87%) received the additional two or more courses of chemotherapy.

Clinical response to chemoradiotherapy

Of the 81 eligible patients, 34 (42%) achieved CR. The CR rates in patients with stage I, II, III, and IV were 100% (8 of 8 patients), 57% (8 of 14), 36% (9 of 25), and 26% (9 of 34), respectively. Although the CR rate in patients with T4 tumors was 18% (8 of 44 patients), the CR rate in patients with non-T4 tumors was 70% (26 of 37). A significant difference was seen between T4 and non-T4 ($P < 0.0001$). Furthermore, the CR rate in patients with M1 LYM (8 of 33; 24%) disease was significantly different from that in patients with M0 LYM (26 of 48; 54%, $P = 0.0073$). Of 25 patients with T4M1 disease, 4 (16%) achieved a CR and 2 (8%) had more than 3-year survival.

Survival

After a median follow-up period of 57 months (range, 34–114 months), the median survival time of the 81 patients was 14 months. One- and 3-year overall survival rates were 62% (50 of 81) and 22% (18 of 81), respectively. The median survival times of 37 patients with T1–T3 disease and 44 patients with T4 disease were 35 and 11 months, respectively; the survival rate of patients with T1–T3 disease was significantly longer than that of patients with T4 disease ($P = 0.001$, log-rank test). Figure 2 shows the survival curves of 81 patients based on the CR rates (CR or non-CR). There was a

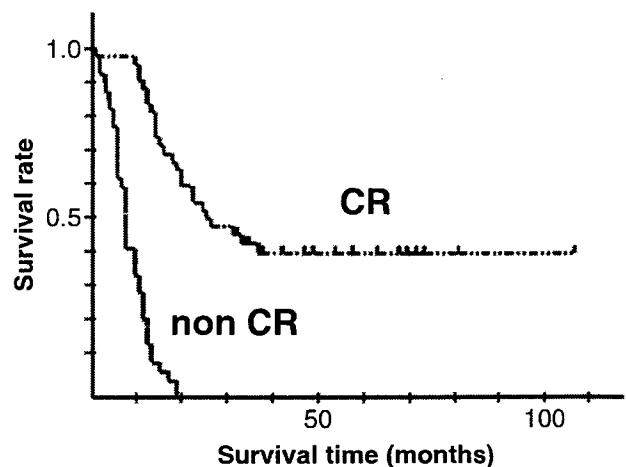


Fig. 2. Survival curves for 34 patients with complete response (CR) and for 47 patients without complete response (non CR)

Table 2. Major complications appearing during chemoradiotherapy

	Grade 1		Grade 2		Grade 3		Grade 4	
	n	%	n	%	n	%	n	%
Leukocytopenia	15	19	32	40	20	25	4	5
Anemia	4	5	31	39	26	33	0	0
Thrombocytopenia	7	9	9	11	7	9	4	5
Nausea/vomiting	24	30	7	9	13	16	0	0
Diarrhea	7	9	15	19	7	9	3	4
Mucositis	9	11	9	11	7	9	0	0
Esophagitis	32	40	15	19	13	16	7	9
Renal	7	9	15	19	0	0	0	0

Table 3. Patients with grade 2 or greater late cardiopulmonary toxicities

Case No.	Cardiac Ischemia (grade)	Pericardial Effusion (grade)	Heart Failure (grade)	Pleural Effusion (grade)	Radiation Pneumonitis (grade)	D/A	Survival (months)
1	3					Alive	37
2	4					Alive	114
3	4		4			Died	39
4		3		2		Alive	50
5		4	4			Died	14
6		4	4			Died	20
7				2		Alive	67
8		2		3		Alive	72
9		2		3		Alive	37
10		2		3	4	Died	45

D, died; A, alive; NCI-CTC, National Cancer Institute Common Toxicity Criteria; RTOG, Radiation Therapy Oncology Group; EORTC, European Organization for Research and Treatment of Cancer
 Source: NCI-CTC version 2.0, RTOG / EORTC late radiation morbidity scoring scheme

significant difference between CR and non-CR cases ($P < 0.0001$). In the CR cases, the patterns of first treatment failure were local recurrence in 3 patients, pleuritis carcinomatosa in 1 patient, liver metastasis in 2 patients, distant lymph node metastasis in 4 patients, bone metastasis in 1 patient, and brain metastasis in 1 patient, respectively. Of 34 CR cases, 12 patients (35%) died of distant or local recurrence of ESCC, while the frequency of local recurrence was low (25%). In contrast, 4 patients died as a result of late toxicities without carcinoma.

Acute toxicity

The major side effects of CRT encountered in our patients during treatment (Table 2) included myelosuppression and esophagitis. Grade 3 and higher leukocytopenia, anaemia, thrombocytopenia, and esophagitis occurred in 30%, 33%, 14%, and 25% of the patients, respectively. Two patients (2%) developed sepsis associated with leukocytopenia; however, these patients recovered from sepsis with use of filgrastim and antibiotics. Of 44 patients with T4 disease, 3 (7%) developed treatment-related perforation of the esoph-

ageal wall: 1 developed a mediastinal fistula whereas each of the other 2 developed an aortic fistula. These 3 patients had T4 disease before treatment, and these events occurred during chemoradiation. One patient showed spontaneous healing of the mediastinal fistula after the disappearance of inflammatory findings, despite continuation of treatment, and achieved a PR. The remaining 2 patients with esophagoaortic fistulae died of massive bleeding during two and four courses of chemoradiation, respectively.

Late toxicity

Grade 3 and higher cardiopulmonary toxicities are summarized in Table 3. The median time to the onset of pericarditis from the initiation of treatment was 19 months, ranging from 10 to 36 months for grade 2 and higher pericarditis. Of 3 patients with grade 3 and higher pericarditis, 2 patients suffered grade 4 heart failure at 12 and 20 months after initiation of treatment, respectively, and died as a result of heart failure without cancer recurrence (cases 5 and 6). Two patients with grade 2 pericarditis were manageable with diuretics (cases 8 and 9). Three patients suffered ischemic heart

disease at 28, 30, and 54 months, respectively, after the initiation of treatment. One patient suffered grade 3 ischemic change at 28 months, and is alive taking a medicine without cancer recurrence 9 months later (case 1). Another patient suffered grade 4 acute myocardial infarction at 54 months and is alive after receiving percutaneous transluminal coronary angioplasty (PTCA) without cancer recurrence 40 months later (case 2). The third patient suffered grade 4 acute myocardial infarction at 36 months and died as a result of heart failure without cancer recurrence 3 months later (case 3).

The median time to the onset of pleural effusion from the initiation of treatment was 16 months, ranging from 5 to 39 months for grade 2 and higher pleural effusion. Of three patients who required pleurocentesis, all patients suffered grade 2 pericarditis, and one of three patients suffered grade 4 radiation pneumonitis simultaneously at 30 months (case 10). This patient without cancer recurrence died as a result of pneumonia 6 months later, although this patient received corticosteroid therapy for radiation pneumonitis. Two patients with grade 2 pleural effusion were manageable with only diuretics (cases 4 and 7). Two patients suffered benign esophageal stricture and required frequent repeated esophageal dilatation.

In total, four patients died without cancer recurrence, and these causes of death may have been related to cardiopulmonary toxicity.

Discussion

The value of CRT for the treatment of unresectable esophageal carcinoma remains controversial, and only a few clinical studies have been published since the 1980s.^{3-8,13} Although some reports included patients with T4 disease, the proportion of such patients was usually low and the clinical outcomes were not clearly described. The results of these studies, including CR rates and survival rates, were confusing, because the clinical and pathological backgrounds varied, especially with regard to the stage of the disease. Therefore, stratification by clinical stage should be applied when evaluating the impact of treatment on survival and response. Zeone et al. reported the results of curative nonsurgical treatment that consisted of 5-FU, CDDP, and 64Gy radiotherapy combined with neodymium:yttrium-aluminium garnet (Nd:YAG) laser therapy in appropriate patients.²¹ They treated 65 patients who had predominantly T1-T3 disease, but their study included 5 patients with T4 disease. Although the 3-year survival rate of the 65 eligible patients was 37%, all 5 patients with T4 disease died within 18 months. However, clinical stages based on the TNM clas-

sification were not described; therefore, the stage at which patients survived longer is unknown. Although there are some "gray zones" with respect to determining T3 or T4 disease by imaging, several studies have reported the successful use of CT scans and/or magnetic resonance imaging with an accuracy rate $\geq 80\%$.^{17,18} We adopted their reported criterion to define T3 or T4 disease. Diagnostic radiologists, together with medical oncologists, were responsible for the final staging. A literature search produced no other studies that specifically investigated CRT for locally advanced disease, such as T4 and/or M1 LYM. In our previous study, a CR rate of 33% and a 3-year survival rate of 23% were achieved in patients with unresectable T4 tumors and/or M1 LYM disease,¹⁵ suggesting that concurrent CRT was potentially curative for locally advanced carcinoma. Although our study, including 40 patients with T4 and/or M1 LYM, was retrospective and may be biased, the results with a 3-year survival of 30% were comparable with the reported trials of CRT, including RTOG studies.^{22,23} The pattern of failure in all eligible patients of our study showed that local failure was still dominant, and the RTOG study also showed a similar pattern of failure for CRT. Improvement of local control may affect survival. In contrast, the pattern of failure in CR patients showed that distant metastasis was dominant and local recurrence was not dominant. Furthermore, fatal complication due to radiation-induced late toxicity was found in patients who achieved CR. Therefore, the dose-escalation strategy of radiotherapy was not thought to be effective in patients with ESCC.

Radiation-induced heart disease is one of the complications in patients who undergo thoracic radiotherapy.^{24,25} Pericardial disease is the most common manifestation of radiation-induced heart disease. There have been many reports of pericardial disease after thoracic radiotherapy in patients with malignant lymphoma.²⁶⁻²⁸ According to recent observation, ischemic heart disease after thoracic radiotherapy is not negligible, and it should be considered in the radiotherapy treatment planning.²⁹⁻³² We observed 2 patients with myocardial infarction and 1 patient with angina pectoris, although we were not sure whether these were related to the CRT. Because daily consumption of alcohol and cigarettes are high risk factors for coronary disease, ischemic heart disease may occur in our subjects with esophageal carcinoma. All 3 patients with ischemic heart disease smoked cigarettes daily. The patient with angina pectoris was accompanied by hyperlipemia. One of 2 patients with myocardial infarction had hypertension, hyperlipemia, and diabetes mellitus. However, the possibility that ischemic heart disease is related to late toxicity for CRT is not denied because there have been few reports on pericardial disease in patients with esophageal carcinoma. There have been many reports

with regard to definitive CRT for ESCC, and survival time was improved as a result of wide-range radiotherapy combined with use of chemotherapy. In our study, myocardial infarction was found in 2 patients, while 1 patient accompanied by pericardial effusion (grade 3 pericarditis) died and another patient without effusion is still alive more than 3 years after PTCA. Further, 1 patient with heart failure accompanied by pericardial effusion (grade 3 pericarditis) died. Treatment of pericardial effusion should be actively performed, and medication, pericardiocentesis, or pericardial window placement are manageable.^{24,33} Radiation-induced late toxicity, such as acute myocardial infarction and pericarditis, can cause the risk of treatment-related death even in CR cases. Examination to evaluate the function of the heart should be performed constantly. In contrast, the incidence of grade 2 and more benign pleural effusion in our study was 15% in 34 CR cases, and we believe that benign pleural effusion is common in patients after definitive CRT. The cause of the pleural effusion may be related to heart disease, such as heart failure and pericardial effusion. Treatment of pleural effusion, such as medication, pleurocentesis, and pleurodesis, should be actively performed.

Severe late toxicities, such as myocardial infarction, pericardial effusion, and pleural effusion, were found in patients who achieved CR after definitive CRT. The definitive CRT is potentially curative and is improved survival time, while treatment-related death of radiation-induced late toxicities was found in 11% of CR cases. More than half of the entire heart volume received at least 40Gy radiation in most patients. Three-dimensional conformal radiotherapy and intensity-modulated radiotherapy have potential advantages over traditional radiotherapy in reducing doses to the heart. Because a precise analysis using a dose-volume histogram is performed, a decreasing radiation volume to the heart will be required due to reducing radiation-induced late toxicities.

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