Table 4. Summary of the regulatory and approval process for clinical trials in Japan

Process	Comments
PMDA-clinical trial notification	• Early consultation recommended • Follow ICH recommendations for preclinical data • Studies must address PK, PD, and PG considerations for Japanese patients, including ADME
IRB Approval Contract and budget	• Required • Standard method for cost calculation of clinical trials, but overhead costs varies by institutions and is negotiated individually with each institution on the basis of unique and complex requirements. • In general full cost retrieval required, including the cost of unplanned investigations • Interdepartmental coordination challenging (relevant when correlative studies are included)
IDMC	• Usually required even for phase I trials • If international IDMC in place, local IDMC may not be necessary

NOTE: ADME, absorption, distribution, metabolism, and excretion; PK, pharmacokinetic; PD, pharmacodynamic; PG, pharmacogenetic; IDMC, Independent Data Monitoring Committee.

approvals for Japan have lagged a number of years behind approvals in other jurisdictions (22), owing to a number of factors, summarized in Table 2 (23–25), including the requirement for data from Japanese patients, especially in later phase studies, as well as prolonged regulatory approval times, despite Japan being one of the largest markets for pharmaceuticals. Often, these considerations have resulted in development plans for Japan being implemented only after positive signals in phase II studies conducted in other regions, further delaying the availability of new agents for cancer patients in Japan and resulting in scientifically unattractive confirmatory trials. Other factors cited in the late inclusion of Japan in early clinical trials have included prolonged timelines (26) and high costs.

A number of major initiatives have been implemented since 2004 (Fig. 1) involving the Pharmaceuticals and Medical Devices Agency (PMDA; refs. 27–29), JMACCT (an organization of the Japan Medical Association; ref. 30), and Ministry of Health, Labor and Welfare (31), including initiatives to improve the infrastructure for clinical trials in Japan by supporting clinician researchers (investigators) and medical institutions in conducting clinical trials and developing clinical trials networks. Early data suggest that these initiatives have had an impact, with an increase in the numbers of clinical trials conducted in Japan and faster accrual times.

In addition to the changes noted, increasing understanding of ethnic differences in pharmacokinetic, pharmacodynamic, and pharmacogenetics has streamlined development plans. For example, phase I data for new therapeutics metabolized by CYP 1A2, 2E1, and 3A4/5, which are independent of ethnicity (32), may thus be derived from Japanese patients, include Japanese patients, or be used to allow the early initiation of phase II studies in Japan. Higher rates of mutations in the epidermal growth factor receptor have been documented in the east Asian population. In some instances, data from other Asian countries may be used to support early trials in Japan. Nonetheless, in some instances, phase I data from studies including Japanese patients may be mandatory.

Phase I studies in Japan. The requirements for the conduct of phase I studies are summarized in Tables 3 and 4, and, since the development and adoption of ICH S9 (Nonclinical Evaluation for Anticancer Pharmaceuticals), these requirements are now congruent with those in Europe and the United States. To date, relatively few first in human studies have been conducted in Japan, for the reasons described above with the majority using data from first in human studies from other countries to support starting doses. Although there are some cultural differences in the reporting of adverse event data (for example, the reporting of any laboratory changes >grade 1 as adverse events, or the attribution of causality), the conduct and design of phase I studies mirror those in Europe and the United States. When appropriate, and included as objectives of the study, patients and researchers are comfortable with biopsies or use of archival tissue for correlative studies.

Conclusions

Both the European Union and Japan are major markets for pharmaceuticals, and as such are important participants in early clinical trials. Although each jurisdiction was traditionally considered somewhat unique in terms of early drug development, major initiatives in the last decade have aligned drug development in Japan, Europe, and the United States. Although many new agents are developed in collaboration with the

pharmaceutical industry, many academic drug discovery and development organizations exist, offering many opportunities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Association of *carboxylesterase 1A* genotypes with irinotecan pharmacokinetics in Japanese cancer patients

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Association of *UDP-glucuronosyltransferase 1A1* (*UGT1A1*) genetic polymorphisms *6 and *28 with reduced clearance of SN-38 and severe neutropenia in irinotecan therapy was demonstrated in Japanese cancer patients.
- The detailed gene structure of *CES1* has been characterized.
- Possible functional SNPs in the promoter region have been reported.

WHAT THIS STUDY ADDS

- Association of functional *CES1* gene number with AUC ratio (SN-38 + SN-38G)/irinotecan, an *in vivo* index of CES activity, was observed in patients with irinotecan monotherapy.
- No significant effects of major *CES1* SNPs on irinotecan PK were detected.

AIMS

Human carboxylesterase 1 (CES1) hydrolyzes irinotecan to produce an active metabolite SN-38 in the liver. The human *CES1* gene family consists of two functional genes, *CES1A1* (*1A1*) and *CES1A2* (*1A2*), which are located tail-to-tail on chromosome 16q13-q22.1 (*CES1A2-1A1*). The pseudogene *CES1A3* (*1A3*) and a chimeric *CES1A1* variant (*var1A1*) are also found as polymorphic isoforms of *1A2* and *1A1*, respectively. In this study, roles of *CES1* genotypes and major SNPs in irinotecan pharmacokinetics were investigated in Japanese cancer patients.

METHODS

CES1A diplotypes [combinations of haplotypes A (*1A3-1A1*), B (*1A2-1A1*), C (*1A3-var1A1*) and D (*1A2-var1A1*)] and the major SNPs (−75T>G and −30G>A in *1A1*, and −816A>C in *1A2* and *1A3*) were determined in 177 Japanese cancer patients. Associations of *CES1* genotypes, number of functional *CES1* genes (*1A1*, *1A2* and *var1A1*) and major SNPs, with the AUC ratio of (SN-38 + SN-38G)/irinotecan, a parameter of *in vivo* CES activity, were analyzed for 58 patients treated with irinotecan monotherapy.

RESULTS

The median AUC ratio of patients having three or four functional *CES1* genes (diplotypes A/B, A/D or B/C, C/D, B/B and B/D; $n = 35$) was 1.24-fold of that in patients with two functional *CES1* genes (diplotypes A/A, A/C and C/C; $n = 23$) [median (25th–75th percentiles): 0.31 (0.25–0.38) *vs.* 0.25 (0.20–0.32), $P = 0.0134$]. No significant effects of *var1A1* and the major SNPs examined were observed.

CONCLUSION

This study suggests a gene-dose effect of functional *CES1A* genes on SN-38 formation in irinotecan-treated Japanese cancer patients.

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Introduction

Human carboxylesterases (CESs) are members of the α/β-hydrolase-fold family and are localized in the endoplasmic reticulum of many different cell types. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals as well as drugs (including prodrugs) to the respective free acids. They are involved in detoxification or metabolic activation of various drugs, environmental toxicants and carcinogens. CESs also catalyze the hydrolysis of endogenous compounds such as short- and long-chain acyl-glycerols, long-chain acyl-carnitine, and long-chain acyl-CoA esters. The two major CES families CES1 and CES2 have been identified in human tissues. CES1 is abundant in the liver and lung but not in the intestine, while CES2 is highly expressed in the intestine and kidney but has low expression in the liver and lung [1].

Human CES1 and CES2 are involved in producing a topoisomerase I inhibitor SN-38, an active metabolite of irinotecan which is clinically used for colorectal, lung and other cancers [2]. SN-38 is further inactivated by UDP-glucuronosyltransferase 1As (UGT1As) to produce SN-38 glucuronide (SN-38G). Irinotecan is also converted by cytochrome P450 3A4 (CYP3A4) to an inactive compound 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin (APC) (Figure 1).

Recent pharmacogenetic studies on irinotecan have revealed significant associations of *UGT1A1* polymorphisms **28* [–54_39A(TA)$_6$TAA>A(TA)$_7$TAA or –40_39insTA] and **6* [211G>A (G71R)], the latter being specifically detected in East Asians, with reduced clearance of SN-38 resulting in severe neutropenia [3–8]. These findings have led to the clinical application of genetic testing for *UGT1A1*28* in the United States (since August 2005) and for *UGT1A1*6* and **28* in Japan (since March 2009). In addition, possible additive effects of genotypes of the transporters for irinotecan and its metabolites, such as *ABCB1, ABCC2, ABCG2* and *SLCO1B1*, have been suggested [9–12]. We previously analyzed *CES2* polymorphisms in a Japanese

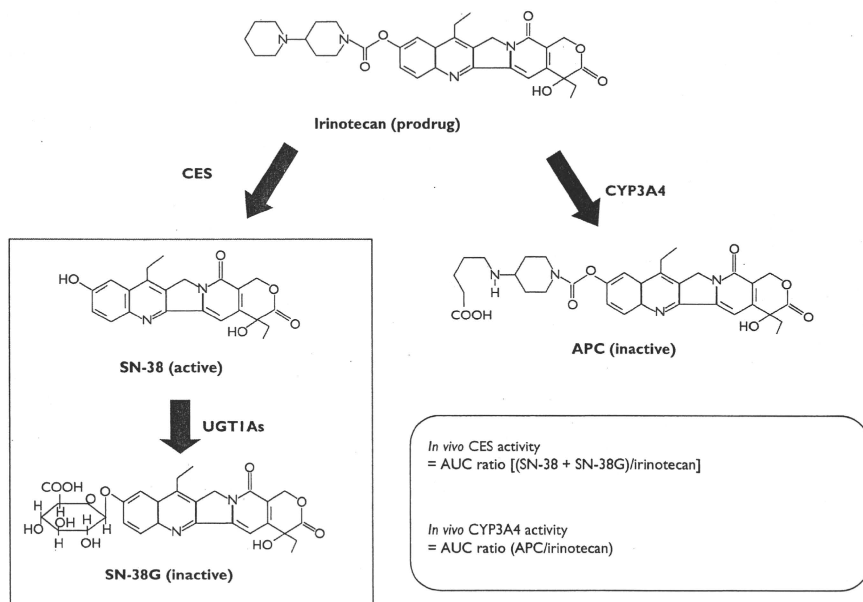

Figure 1

Metabolic pathway of irinotecan. The prodrug irinotecan is hydrolyzed by carboxylesterase (CES) to produce an active metabolite SN-38, and subsequently detoxified by UDP-glucuronosyltransferase 1As (UGT1As) to produce an inactive metabolite SN-38 glucuronide (SN-38G). Irinotecan is also metabolized by cytochrome P450 3A4 (CYP3A4) to produce another inactive metabolite APC

population and identified minor genetic variations which were associated with lower expression/function *in vitro* and *in vivo* [13, 14]. However, major *CES2* haplotypes (**1b* and **1c*) did not affect irinotecan pharmacokinetics (PK) [14]. Since CES1 is expressed at higher levels in the liver, a major organ for activating irinotecan, it is possible that *CES1* genotypes affect the plasma concentrations of irinotecan metabolites. However, their clinical relevance to irinotecan pharmacokinetics/pharmacodynamics has not yet been fully investigated.

Functional human *CES1* genes include *CES1A1* (*1A1*) and *CES1A2* (*1A2*), which are inversely located (tail-to-tail) on chromosome 16q13-q22.1 (*1A2-1A1*). Both *1A1* and *1A2* consist of 14 exons encoding 567 amino acids, and they have 98% homology with 5 nucleotide (4 amino acid) differences in exon 1, which encodes a signal peptide [1]. Recent studies also identified *CES1A1* variants (*var1A1*), in which exon 1 was replaced with exon 1 of *CES1A2*, and a pseudogene *CES1A3* (*1A3*; formerly referred to as *CES4*) replacing *CES1A2* [15, 16]. The *1A3* sequence from the promoter region to exon 1 is the same as that of *CES1A2*, but contains a stop codon in exon 3. The sequence downstream from exon 11 is highly homologous with that of *1A1* (NT_010498) [16]. Ethnic differences in these *CES1* genes (*1A1*, *var1A1*, *1A2* and *1A3*) have been reported [16].

Expression levels of CES1A2 mRNA were lower than those of CES1A1 mRNA in several tissues. This CES1A1 up-regulation could be mediated by additional Sp1 and C/EBP binding sites in the promoter region [17]. Transcript levels of CES1A2 derived from *var1A1* were reported to be higher than those from the original *1A2* [15, 16]. These findings suggest that polymorphisms in the upstream region of *CES1A1* or *var1A1* could affect their expression.

In addition to structural variations of the *CES1* gene family, several single nucleotide polymorphisms (SNPs) and small deletion/insertion variants were found. −816C in the *CES1A2* promoter region was reported to be associated with enhanced CES1A2 expression and imidapril efficacy [18]. Furthermore, −816A>C was found to be linked with several SNPs (−62T>C, −47G>C, −46G>T, −41C>G, −40A>G, −37G>C, −34del/G and −32G>T) in the proximal promoter region, leading to two additional Sp1 binding sites, and these additional sites were suggested to increase transcription of *1A2* [19].

In this context, this study investigated the clinical significance of *CES1* genotypes in irinotecan therapy. For this purpose, we analyzed the *CES1* genotypes (combinations of four *CES1A* isoforms) and major SNPs in the *CES1A1* exon 1 with its adjacent region and in the *CES1A2* and *1A3* promoter regions, which could be important for CES1 expression or function, in Japanese cancer patients treated with irinotecan, and then examined the associations of these *CES1* genotypes or SNPs with irinotecan PK.

Methods

Patients

Genetic analysis of 177 Japanese cancer patients who received irinotecan therapy at the National Cancer Center in Japan was performed. The patients were the same as those described in our previous study [7], where details on eligibility criteria for irinotecan therapy, patient profiles and irinotecan regimens were described. Since the AUC ratio [(SN-38 + SN-38G) : irinotecan], a parameter of *in vivo* CES activity, was influenced by irinotecan regimens [14], 58 patients receiving irinotecan monotherapy (100 mg m^{-2} weekly or 150 mg m^{-2} biweekly) from the 177 patients were primarily used for analysis of the association between *CES1* genotypes and irinotecan PK parameters. The patient set was the same as used in our previous study on *CES2* [14]. This study was approved by the ethics committees of the National Cancer Center and the National Institute of Health Sciences, and written informed consent was obtained from all participants.

Determination of CES1 genotypes and SNPs

For describing the *CES1* gene family, haplotypes A to D designated by Fukami *et al.* [16] were used (Figure 2): haplotype A, *CES1A3-CES1A1* (*1A3-1A1*); haplotype B, *CES1A2-CES1A1* (*1A2-1A1*); haplotype C, *CES1A3-CES1A1* variant (*1A3-var1A1*); and haplotype D, *CES1A2-CES1A1* variant (*1A2-var1A1*). To determine the diplotypes, combinations of haplotypes A to D, we sequenced *1A1/var1A1* exon 1 and its flanking region and the *1A2/1A3* promoter region of 177 patients. These regions are indicated in Figure 2, and a list of primers/probes is shown in Table 1.

For discrimination between *1A1* and *var1A1*, their exon 1s and flanking regions were sequenced (Figure 2a). Briefly, the first PCR was performed using 25 ng of genomic DNA with 0.625 units of Ex-Taq (Takara Bio. Inc., Shiga, Japan) and 0.2 μM of primers, Ces1-FP and Ces1-RP (Table 1a, first PCR). The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. Then, the second PCR was performed with the primers, Ces1_seqF and Ces1_seqR (Table 1a, second PCR) under the same reaction conditions described above. The PCR products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers listed in Table 1a (sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany), and the eluates were analyzed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems). The conditions of the PCR and sequencing procedures described in the following section were the same as described above unless otherwise noted.

1A2 and *1A3* were discriminated by the restriction fragment length polymorphism (RFLP) method for exon 5

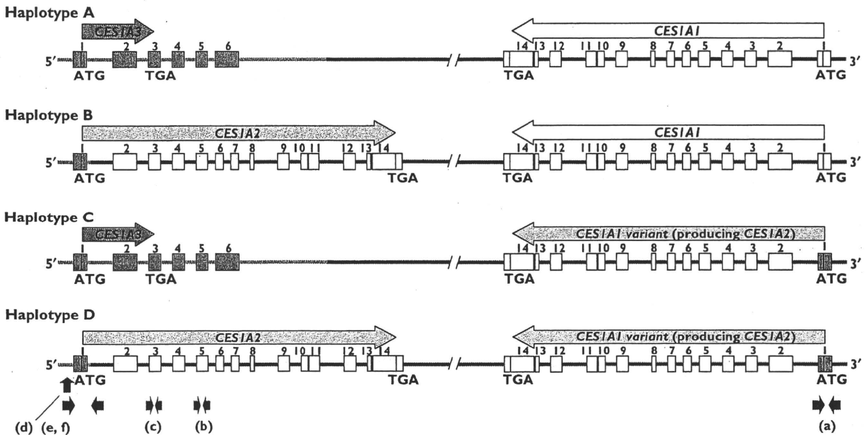

Figure 2

CES1 gene structure and haplotypes. The regions used for haplotype determination in this study are indicated with arrows (a–f)

reported by Fukami *et al.* [16] (Figure 2b). Briefly, the PCR was performed using a primer set (1A-int4F and 1A-int5AS) (Table 1b), and then the PCR products were digested with *Pvu*II to produce *CES1A3*-derived fragments (409 bp and 248 bp). UV intensity of the fragments stained with ethidium bromide was measured after electrophoresis (2% agarose gel). The number of *1A3* (0, 1 or 2) was also confirmed by direct sequencing of exon 5 using the same primer set. To verify that the *1A3* sequence is derived from the pseudogene, we confirmed the existence of a stop codon at codon 105 of *1A3* exon 3 (Figure 2c) in 11 randomly selected patients (heterozygous or homozygous) by amplification and sequencing using primers listed in Table 1c.

Genotyping for −816A>C in the *1A2* and *1A3* promoter region (Figure 2d) was conducted by the TaqMan method of Geshi *et al.* [18] (Table 1d) in all patients. We also examined attribution of −816C to *1A2* or *1A3* by specific amplifications from 5′-regions to intron 1 of the *1A2* and *1A3* (Figure 2e,f) in 23 randomly selected heterozygous patients. For specific amplifications, primers CES1A3-1A2_F1 and CES1A2 R1 for *CES1A2* (Table 1e) and primers CES1A3-1A2_F1 and CES1A3 R1 for *1A3* (Table 1f, first PCR) were used with 0.05 U μl^{-1} LA-Taq with GC buffer I (Takara Bio. Inc.); and for *1A3*, the second PCR using primers CES1A3-1A2_F2 and CES1A3 R2 (Table 1f, second PCR) was also conducted with 0.05 U μl^{-1} Ex-taq. Then, direct sequencing of the *1A2* and *1A3* PCR products was performed. Complete linkage among −816A>C and several SNPs in the proximal promoter region (between −62 to −32) [19] was confirmed for 11 randomly selected subjects.

All variations were confirmed by sequencing PCR products generated from new amplifications from genomic DNA. GenBank NT_010498.15 was used as the reference sequence for *CES1A1, CES1A3* and the promoter region of *CES1A2*, and AB119998.1 was used for exon 1 and its downstream region of *CES1A2*. The translational initiation site was designated as +1 to describe the polymorphism positions. Diplotype configuration was estimated with the LDSUPPORT software [20]. The diplotypes A/D and B/C could not be distinguished.

Pharmacokinetic data and association analysis

The area under the concentration–time curve (AUC) values for irinotecan and its metabolites, SN-38, SN-38G and APC, were previously obtained [4, 21]. The AUC ratio of SN-38 plus SN-38G to irinotecan [$AUC_{(SN\text{-}38\,+\,SN\text{-}38G)}/AUC_{irinotecan}$] was used as a parameter reflecting *in vivo* CES activity [14]. The AUC ratio of APC to irinotecan [$AUC_{APC}/AUC_{irinotecan}$] was used as a parameter for *in vivo* CYP3A4 activity [21].

Statistical significance (two-sided, $P < 0.05$) for associations between AUC ratios (or AUC/dose) and *CES1* genotypes or SNPs was determined by the Mann-Whitney test or the Jonckheere-Terpstra (JT) test using Prism version 4.0 (GraphPad Prism Software Inc. San Diego, CA, USA) and StatXact version 6.0 (Cytel Inc., Cambridge, MA). Correla-

Table 1

Primers and probes used in this study

Region (indicated in Figure 2)	Primer	Primer sequence	Reference
(a) *CES1A1* exon 1 and promoter region			This study
First PCR	Ces1-FP	5′-CCAGGCAAAACCTAGGAGTG-3′	
	Ces1-RP	5′-AGTACAGGGCGATCTCAGGA-3′	
Second PCR	Ces1_seqF	5′-GTATTTCCTTAGCCAGCGGTA-3′	
	Ces1_seqR	5′-CAGAGCCGGACCTGTTGT-3′	
Sequencing	Ces1_SF2	5′-AGAGCCTGGAAAGCTATGAAAA-3′	
	Ces1_SR	5′-TTTCTACGCATCTGCGCCCACC-3′	
(b) *CES1A1, 1A2* and *1A3* exon 5			[16]
PCR and sequencing	1A-int4F	5′-GCTCAGTAAATAGTTGCCAGTT-3′	
	1A-int5AS	5′-TCTCATCAGCATCACATCAAG-3′	
(c) *CES1A3* exon 3			This study
PCR and sequencing	CES1A3-15183F	5′-CAGGGAAGATCGTTGTATTGGTTT-3′	
	CES1A3-15974R	5′-TTCCTTCCACCACTAACATTATTG-3′	
Sequencing (additional primer)	CES1A3-15823R	5′-AAGATGTTCATTAAAGATGCACAG-3′	
(d) *CES1A2* and *1A3* -816A>C genotyping			[18]
PCR	F	5′-CCTTAATTTGGTGATTTCACATTGC-3′	
	R	5′-CAAGACATGGTTCAGCTTCTCAAG-3′	
TaqMan probe	FAM	5′-CATCACCCCTACTGC-3′	
	VIC	5′-CATCACACCTACTGCT-3′	
(e) *CES1A2* promoter region			This study
PCR	CES1A3-CES1A2_F1	5′-ATGATTTCCAGCTTCATCTACA-3′	
	CES1A2_R1	5′-GAGAGAACGTTCCCATGTCTTT-3′	
(f) *CES1A3* promoter region			This study
First PCR	CES1A3-CES1A2_F1	5′-ATGATTTCCAGCTTCATCTACA-3′	
	CES1A3_R1	5′-GCTTGAGTTTTCTTTACAGACA-3′	
Second PCR	CES1A3-CES1A2_F2	5′-AACAGTTTATAACCTGTTATTTTT-3′	
	CES1A3_R2	5′-TGCTTTGGATAAAGACAAGATGTT-3′	
Seqeuncing of *CES1A2/1A3* promoter region			
	CES1A3-CES1A2_F2	5′-AACAGTTTATAACCTGTTATTTTT-3′	
	CES1A3-CES1A2_R1	5′-CACACTTCCAATCTCAGGTAAA-3′	
	CES1A3-CES1A2_F3	5′-TTATGCCACAAGCAGTTGGGCG-3′	
	CES1A3-CES1A2_R2	5′-TCCAAGTCCAATTCCAAGTACGGA-3′	

NT_010498.15 was used as the reference sequence for *CES1A1, CES1A3* and the promoter region of *CES1A2*, and AB119998.1 was used for exon 1 and its downstream region of *CES1A2*.

tions between the AUC ratios [$AUC_{(SN\text{-}38\ +\ SN\text{-}38G)}/AUC_{irinotecan}$] and [$AUC_{APC}/AUC_{irinotecan}$] were analyzed by Spearman's rank correlation test. Multiplicity adjustment was not applied to bivariate analysis, and contributions of the candidate genetic markers to the AUC ratios [$AUC_{(SN\text{-}38\ +\ SN\text{-}38G)}/AUC_{irinotecan}$] were further determined by multiple regression analysis after logarithmic transformation of the AUC ratio. The variables examined were age, sex, body surface area, history of smoking or drinking, performance status, serum biochemistry (GOT, ALP, creatinine) at baseline, *CES1* genotypes and SNPs, *CES2*2* [100C>T(R34W)] or *5* [1A>T (M1L)] [13, 14], *UGT1A1*6* or **28* [7, 8], and the transporter haplotypes, *ABCB1*2* [2677G>T(A893A)], *ABCC2*1A* (−1774delG), *ABCG2#IIB* [421C>A (Q141K) and IVS12+49G>T] and *SLCO1A1*15·17* [521T>C (V174A)] [10]. The variables in the final models were selected by the forward and backward stepwise procedure at a significance level of 0.10 using JMP version 7.0.0 (SAS Institute, Inc., Cary, NC, USA). *UGT1A1*6 or *28* was grouped as '+' for stratifying patients: for example, homozygous *UGT1A1 *6 or *28* was depicted as *UGT+/+*.

Results

Genotypes and SNPs of CES1 gene family in Japanese

Frequencies of individual *CES1* genes and *CES1* diplotypes stratified according to the number of functional *CES1* genes are summarized in Table 2. The frequencies of the patients with two, three and four functional *CES1* genes were 44%, 47% and 9%, respectively, in all 177 patients.

By sequencing *1A1* and *var1A1* exon 1s and their flanking region, we detected four novel variations; three in the 5′-flanking region and one in the 5′-untranslated region (5′-UTR) (Table 3): −258C>T (allele frequency: 0.014), −233C>A (0.003), −161A>G (0.006) and −30G>A (0.042). Eleven nucleotide substitutions from the 5′-UTR to intron 1 at allele frequencies of 0.294–0.299 were closely linked with *var1A1* (Table 3). The SNP −816A>C found in the *1A2* and *1A3* promoter regions was genotyped by a TaqMan method [18], and the allele frequency of −816A>C in 177 subjects was 0.249 (Table 4). It was noted that −816C was detected only in patients with *1A3* (*1A3/1A2* and *1A3/1A3*),

Table 2

Frequency of *CES1* genes and diplotypes in Japanese cancer patients

CES1 diplotype	Number of *CES1* gene *1A1*	*var1A1*	*1A2*	*1A3*	Total*	Frequency (n = 177)†		Frequency (monotherapy: n = 58)†	
A/A	2	0	0	2	2	0.203	0.441	0.138	0.397
A/C	1	1	0	2		0.220		0.241	
C/C	0	2	0	2		0.017		0.017	
A/B	2	0	1	1	3	0.237	0.469	0.293	0.534
A/D or B/C	1	1	1	1		0.192		0.190	
C/D	0	2	1	1		0.040		0.052	
B/B	2	0	2	0	4	0.040	0.090	0.017	0.069
B/D	1	1	2	0		0.034		0.052	
D/D	0	2	2	0		0.017		0.000	
Frequency (n = 354)‡	0.703	0.297	0.325	0.675					
(monotherapy: n = 116)‡	0.690	0.310	0.336	0.664					

*Number of functional genes. †Number of subjects. ‡Number of chromosomes.

but not in the *1A2* homozygotes (*1A2/1A2*). In the *1A2/1A3* patients, 38 of the 39 patients having −816C were heterozygous for −816C (Table 4). These findings suggested a close association between −816C with *1A3*. Following specific amplifications of the regions from 5′-regions to intron 1 in *1A2* and *1A3* (Figure 2e,f) of 23 patients randomly selected from the 38 patients with −816A/C and *1A2/1A3*, we confirmed that −816C resided in the *1A3* gene (data not shown). Thus, −816A>C is the major SNP of *1A3* but very rare in *1A2*. In addition, the SNPs, −62T>C, −47G>C, −46G>T, −41C>G, −40A>G, −37G>C, −34del/G and −32G>T, in the proximal promoter region reported to be linked with −816A>C [19] were found to be completely linked with *1A3* (data not shown).

Association of CES1 genotypes with in vivo CES activity

CES1 diplotypes In patients treated with irinotecan monotherapy, we found the AUC ratios of patients with haplotypes A or C (having the *1A3* pseudogene) were lower than those without A or C, indicating functional *CES1* gene number dependency. The median AUC ratio of patients having three or four functional *CES1* genes was 1.24-fold of that in patients with two functional *CES1* genes [median (25th–75th percentiles): 0.31 (0.25–0.38) *vs.* 0.25 (0.20–0.32), P = 0.0134, Mann-Whitney test)] (Figure 3a). No significant differences were observed between *1A1* and *var1A1* (among *1A1/1A1*, *var1A1/1A1* and *var1A1/var1A1*). As we previously reported, the *CES2* variations, *CES2*5* [1A>T(M1L)] and *CES2*2* [100C>T(R34W)] [13, 14] showed low CES activity as indicated in Figure 3a.

Platinum-containing regimens themselves enhance renal excretion of irinotecan and its metabolites, especially SN-38G. No significant effect of *CES1* gene number on the AUC ratio was observed. However, it was noted that the median renal excretion ratio [(SN-38 + SN-38G)/irinotecan] in patients with four functional *CES1* genes was 1.37-fold higher than that in patients with two or three functional genes (P = 0.0217, Mann-Whitney test) (data not shown).

To exclude the possibility that the higher AUC ratio observed above (Figure 3a) was biased by CYP3A4, another metabolic enzyme for irinotecan, we analyzed the association between the (SN-38 + SN-38G)/irinotecan AUC ratio and the APC/irinotecan AUC ratio, an *in vivo* parameter of CYP3A4 activity [21], in patients treated with irinotecan monotherapy. The result showed no correlation between the two parameters (Spearman r = 0.126, P = 0.345).

CES1 SNPs Next, associations of the two *1A1* SNPs, −75G>T and −30G>A (Table 3) and *1A3*-816A>C with the AUC ratio [(SN-38 + SN-38G)/irinotecan] were analyzed. The effects of the SNPs were analyzed in patients stratified by the functional *CES1* gene number and also in all the patients receiving monotherapy. A −75G>T-dependent increase in the AUC ratio was observed in the whole group of patients (P = 0.027, JT test) (Figure 3b), and this trend was remarkable in patients with three or four functional *CES1* genes. No significant effect of −30G>A was observed (Figure 3c). As for −816C in *1A3*, no association between this SNP and the AUC ratio was evident in patients with two or three functional *CES1* genes (Figure 3d). In the platinum-containing regimens, no significant effects of these SNPs on the AUC ratio or the renal recovery ratio were observed (data not shown).

Multivariate analysis The contribution of *CES1* genotypes to the AUC ratio was further analyzed by multivariate analysis, using the patient background factors and polymorphisms including the haplotypes of *CES2*, *UGT1A1* and transporters as variables [7, 8, 10, 13, 14]. The final model revealed a significant association of the functional *CES1* gene number (n = 3 or 4) with the AUC ratio. Contributions of smoking history, irinotecan dose, hepatic and renal function were also detected while that of *ABCB1*2* (+/+) was

Table 3

Summary of genetic variations of *CES1A1* and *var 1A1* exon 1s and their flanking regions detected in this study

SNP identification				Position					
This study	NCBI (dbSNP)	JSNP	Location	NT_010498.15	From the translational initiation site or the nearest exon	Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	Allele frequency (n = 354)*	*CES1A1* variant (*CES1A2* type)
MPJ6_CS1001†			5′-flank	9481424	−258	ttgggcaagtttacagctct**C/T**tgtaatctgacagtagagtc		0.014	
MPJ6_CS1002†			5′-flank	9481399	−233	atctgacagtagagtccaga**C/A**tggtttgatgaaagagggta		0.003	
MPJ6_CS1003†			5′-flank	9481327	−161	tagaagcccagggagatctg**A/G**ggaaagggagggcttttctg		0.006	
MPJ6_CS1004	rs3815583	IMS-JST175949	Exon1(5′-UTR)	9481241	−75	aactctgggcggggctgggc**G/T**ccagggctggacagcacagt		0.41	
MPJ6_CS1005	rs28429139		Exon1(5′-UTR)	9481212	−46	ggacagcacagtccctctga**A/G**ctgcacagagacctcgcagg		0.299	*var1A1*
MPJ6_CS1006	rs28494177		Exon1(5′-UTR)	9481205	−39	acagtccctctgaactgcac**A/G**gagacctcgcaggccccgag		0.299	*var1A1*
MPJ6_CS1007†			Exon1(5′-UTR)	9481196	−30	ctgaactgcacagagacctc**G/A**caggccccgagaactgtcgc		0.042	
MPJ6_CS1008	rs28520463		Exon1(5′-UTR)	9481187	−21	acagagacctcgcaggcccc**G/C**agaactgtcgcccttccacg		0.297	*var1A1*
MPJ6_CS1009	rs28499065		Exon1(5′-UTR)	9481186	−20	cagagacctcgcaggccccg**A/G**gaactgtcgcccttccacga		0.297	*var1A1*
MPJ6_CS1010	rs28515828		Exon1(5′-UTR)	9481168	−2	cgagaactgtcgcccttcca**C/G**gatgtggctccgtgccttta		0.299	*var1A1*
MPJ6_CS1011			Exon 1	9481156	11	cccttccacgatgtggctcc**G/C**tgcctttatcctggccactc	Arg4Pro	0.297	*var1A1*
MPJ6_CS1012			Exon 1	9481152	15	tccacgatgtggctccgtgc**C/T**tttatcctggccactctctc	Ala5Ala	0.297	*var1A1*
MPJ6_CS1013			Exon 1	9481151	16	ccacgatgtggctccgtgcc**T/C**ttatcctggccactctctct	Phe6Leu	0.297	*var1A1*
MPJ6_CS1014			Exon 1	9481148	19	cgatgtggctccgtgccttt**A/G**tcctggccactctctctgct	Ile7Val	0.297	*var1A1*
MPJ6_CS1015	rs28563878		Exon 1	9481133	34	tgcctttatcctggccactctc**T/G**ctgcttccgcggcttggggt	Ser12Ala	0.297	*var1A1*
MPJ6_CS1016	rs12149359		Intron 1	9481099	IVS1+16	ttggggtgagtccttctgaa**A/G**tcaaaatgcggggcactttt		0.294	*var1A1*

*Number of chromosomes. †Novel variation detected in this study.

Table 4

Frequency of *CES1A2(/1A3)* promoter SNP −816A>C in Japanese cancer patients

CES1A2 and *1A3* Genotype	-816A>C Genotype	Number of subjects	Allele frequency
1A2/1A2	A/A	16	0/32 (0%)
	A/C	0	
	C/C	0	
1A2/1A3	A/A	44	40/166 (24.1%)
	A/C	38	
	C/C	1	
1A3/1A3	A/A	41	48/156 (30.8%)
	A/C	26	
	C/C	11	
Total		177	88/354 (24.9%)

not significant (Table 5). The *CES1* genotypes explained 22.6% of variability in the final model among all the variables and 11.3% of total variability in the AUC ratio.

Effects of CES1 genotypes on SN-38 AUC and toxicity

To clarify the clinical importance of *CES1* genotyping for irinotecan therapy, the effects of *CES1* genotypes or SNPs on AUC levels of the active metabolite SN-38 and neutropenia were examined in the non-*UGT*+/+ patients. In this non-*UGT*+/+ population, significantly higher AUC ratios of (SN-38 + SN-38G)/irinotecan were also observed in the patients with three or four functional *CES1* genes (P = 0.0234, Mann-Whitney test) as observed in all the patients treated with irinotecan monotherapy (Figure 3a). With increased number of functional *CES1* genes, an increasing trend of SN-38 AUC/dose was observed in patients receiving irinotecan monotherapy (1.4-fold for four genes *vs.* two genes; P = 0.080, JT test) (Figure 4). However, multiple regression analysis revealed no statistically significant contribution of *CES1* genotypes to SN-38 AUC/dose although *UGT1A1"*6 or *28"* and *ABCB1*2/*2* showed significant contributions [10]. Regarding neutropenia, a higher incidence (though statistically insignificant) for grade 3/4 neutropenia in patients with four functional *CES1* genes was observed (50% for four genes and 16% for two or three genes, P = 0.09, Fisher's exact test). The effects of the SNPs (−75G>T, −30G>A and −816A>C) on SN-38 AUC or incidence grade 3/4 neutropenia were not significant (data not shown). In platinum-containing regimens, no significant effects of the *CES1* genotypes on SN-38 AUC/dose or incidence of grade 3/4 neutropenia were detected in the non-*UGT*+/+ patients (data not shown).

Discussion

Recent pharmacogenetic studies on irinotecan have shown the clinical significance of *UGT1A1 *6* and **28* in Japanese patients [7,8] and *UGT1A1*28* in Caucasians [5,6] for severe neutropenia. Subsequent studies have revealed additional genetic factors including transporters [10–12]. However, the clinical importance of genotypes of the irinotecan-activating enzymes *CES1* and *CES2* is still uncertain.

Since the hydrolytic activity of CES2 for irinotecan was reported to be much higher than that of CES1 [2], most studies have focused on the clinical significance of *CES2* polymorphisms in irinotecan therapy [13, 14, 22]. We previously identified minor *CES2* genetic variations in Japanese, including *CES2*2* [100C>T (R34W)] and *CES2*5* [1A>T (M1L)] which caused low *in vitro* expression/function of CES2 [13, 14] and also exhibited reduced *in vivo* CES activity in irinotecan-treated patients [14] (also see Figure 3a). However, the major *CES2* haplotypes in Japanese, **1b* (IVS10-108G>A and 1749A>G, frequency = 0.233) and **1c* (−363C>G, IVS10-108G>A and IVS10-87G>A, frequency = 0.027), did not show any significant effects on irinotecan PK [14]. No clinical significance of *CES2* polymorphisms has been reported in Caucasians [22]. Neither *CES1* nor *CES2* SNPs affecting their mRNA expression in normal colonic mucosa were found in European and African populations [23]. Since precise structures of the *CES1* genes and their promoter regions had not been elucidated, evaluation of the roles of the *CES1* genotypes in irinotecan therapy has been rather difficult.

In the present study, the frequencies of individual *CES1* genes (*1A1, var1A1, 1A2* and *1A3*) (Table 2) were almost comparable with the previous report in the Japanese population (0.748, 0.252, 0.313 and 0.687, respectively) [16]. To our knowledge, the present study is the first report suggesting a possible effect of *CES1* genotypes on irinotecan PK. This study showed that the AUC ratio [(SN-38 + SN-38G)/irinotecan], and probably *in vivo* CES activity, was elevated depending on the number of functional *CES1* genes (*1A1, var1A1* and *1A2*) in patients treated by irinotecan monotherapy (100 or 150 mg m^{-2} irinotecan) (Figure 3a). This gene-dose effect was not clearly shown in the platinum-containing combination therapy (60–70 mg m^{-2} irinotecan), where renal excretion of irinotecan and its metabolites (especially SN-38G) is highly enhanced by a large volume of infusion fluid. However, the median renal excretion ratio [(SN-38 + SN-38G)/irinotecan] in patients with four functional genes was 1.37-fold higher than that in patients with two or three functional genes in the platinum-containing therapy (data not shown), supporting a partial but significant contribution of the CES1s to activate irinotecan. The present study showed no significant differences in the AUC ratios between *1A1* and *var1A1* (Figure 3a), indicating a common upstream region may be involved in regulation of gene expression of *1A1* and *var1A1*. The previous reports showed the expression levels of CES1A2 were lower than those of CES1A1 [17] and suggested that CES1A2 mRNA was derived mainly from transcription of *var1A1* rather than the original *1A2* [15, 16]. The present study, on the other hand, has suggested that the

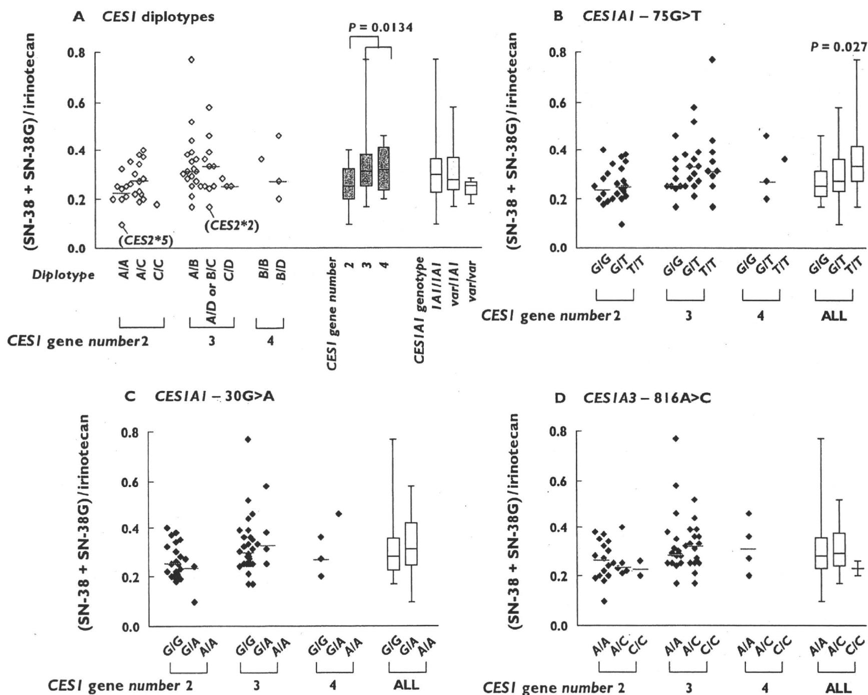

Figure 3

Association of *CES1* diplotypes (A) or SNPs (B–D) with AUC ratio [(SN-38 + SN-38G)/irinotecan], an *in vivo* index of CES activity, in Japanese cancer patients treated with irinotecan monotherapy ($n = 58$). '*CES1* gene number' means the number of functional genes (*1A1*, *var1A1* and *1A2*). Higher AUC ratios were observed in patients with three or four functional *CES1* genes than with two functional genes ($P = 0.0134$, Mann-Whitney test) in (A). Patients with *CES2*5* [*CES2* 1A>T (M1L)] (*CES2*5*) and *CES2*2* [*CES2* 100C>T (R34W)] (*CES2*2*) were found to have reduced CES activity in our previous study [13, 14]

Table 5

Multiple regression analysis of AUC ratio [(SN-38 + SN-38G)/irinotecan]* in Japanese cancer patients treated with irinotecan monotherapy

Variable	Coefficient	SE	*P* value
Smoking	0.073	0.034	0.0375
Initial dose of irinotecan (mg m^{-2})	−0.002	0.001	0.0005
Serum GOT and ALP†	0.082	0.027	0.0038
Serum creatinine (mg dl^{-1})	0.130	0.062	0.0399
*ABCB1*2*‡ (+/+)	0.042	0.024	0.0831
CES1 functional gene (n = 3 or 4)	0.038	0.016	0.0215

$r^2 = 0.500$, Intercept = −0.248, $n = 58$. * Values after logarithmic conversion were used. † Grade 1 or greater for both GOT and ALP. ‡ *ABCB1*2* [2677G>T (A893S)].

1A2 transcript could contribute to the total CES activity because the [(SN-38 + SN-38G)/irinotecan] AUC ratios of patients without *1A2* (with two functional *CES1* genes) were lower than those with *1A2* (with three or four functional genes) (Figure 3a). However, it must be noted that the increase in the AUC ratio by three or four functional *CES1* genes was only 20% compared with two functional genes (Figure 3a), and that such alterations might be masked by other non-genetic factors. In fact, hepatic and renal function, irinotecan dosage and smoking history were found to be potent contributors to this parameter (Table 5).

−816A>C SNP in *1A2* was reported to be associated with imidapril efficacy and a higher promoter activity for

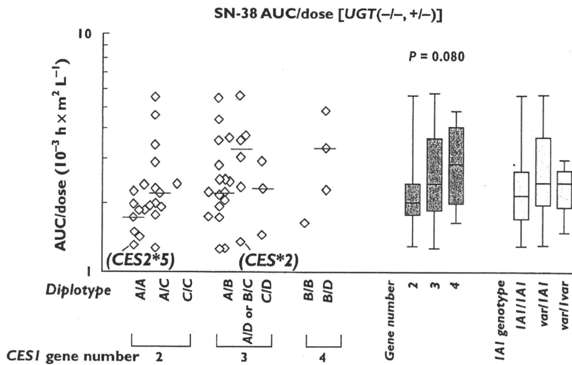

Figure 4

Association of *CES1* genotypes with SN-38 AUC/dose in *UGT*(–/– and +/–) patients treated with irinotecan monotherapy ($n = 51$).'*CES1* gene number' means the number of functional genes (*1A1*, *var1A1* and *1A2*). One patient with an outlying value who had *ABCB1**2* [2677G>T (A893S)] and **14* [2677G>T (A893S) and 1345G>A 230 (E448K)] was excluded from this analysis [10]. A slightly increasing trend in SN-38 AUC(/dose) was observed depending on functional *CES1* gene number. ($P = 0.080$, Jonckheere-Terpstra test). The patients with *CES2*5* [*CES2* 1A>T (M1L)] (*CES2*5*) and *CES2*2* [*CES2* 100C>T (R34W)] (*CES2*2*) [13, 14] are marked

CES1A2 [18] and had strong linkage with SNPs in the proximal promoter region (between –62 to –32) which resulted in additional Sp1 binding sites in the *1A2* promoter region [19]. However, our current study showed no significant effect of –816A>C on the AUC ratio. This can be explained by our finding that –816C and several linked SNPs were mostly located on the *CES1A3* psuedogene but not the functional *1A2* gene.

We newly detected three SNPs (–258C>T, –233C>A and –161A>G) in the 5′-flanking region and one SNP (–30 G>A) in the 5′-UTR of *CES1A1* (Table 3). The effect of –30 G>A on the AUC ratio was not significant (Figure 3c). The frequencies of three other SNPs in the 5′-flanking region were very low (0.003–0.014) which made statistical analysis difficult. These SNPs are not located in the putative transcriptional regulatory regions of *CES1A1*, the binding sites of transcription factors Sp1 and C/EBP [17]. The AUC ratios of the patients with these SNPs were within the 25th–75th percentiles except that slightly higher values were shown in the two -258T patients who received platinum-combination therapy (data not shown). Thus, clinical impact of these SNPs would be small.

With respect to the clinical importance of *CES1* genotyping for irinotecan therapy, the effects of *CES1* genotypes on the AUC level of the active metabolite SN-38 and incidence of grade 3/4 neutropenia should be considered. Since the patients homozygous for *UGT1A1*6 or *28* (*UGT+/+*: **6/*6, *6/*28* and **28/*28*) showed higher SN-38 AUC/dose levels and severe neutropenia [7], we examined the effects of *CES1* genotypes and SNPs in the non-*UGT+/+* patients. Increasing trends of SN-38 AUC/dose (Figure 4) and incidence of grade 3/4 neutropenia were observed depending on the functional *CES1* gene number in patients with irinotecan monotherapy although statistical significance was not obtained. For the platinum-containing regimens, no significant effects of *CES1* genotypes were shown. Thus, although possible effects of the *CES1* genotypes on neutropenia could not be excluded in irinotecan monotherapy, this study was still insufficient to establish the clinical importance of *CES1* genotyping in irinotecan therapy. Since the sample size will be twice that of the present study to detect a statistically significant decrease of absolute neutrophil counts in the patients with four functional *CES1* genes, future clinical data obtained in a larger number of patients could clarify this point.

In conclusion, this study suggests that the total number of functional *CES1A* genes could influence the formation of the active metabolite of irinotecan in Japanese cancer patients.

Competing interests

HK has received lecture honorarium from Yakult Honsha, the manufacturer of irinotecan. HM has been paid by Yakult Honsha, the manufacturer of irinotecan, for speaking and research.

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Clinical Studies

A phase II trial of dose-dense chemotherapy, followed by surgical resection and/or thoracic radiotherapy, in locally advanced thymoma: report of a Japan Clinical Oncology Group trial (JCOG 9606)

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BACKGROUND: This study aimed to evaluate the safety and efficacy of dose-dense weekly chemotherapy, followed by resection and/or thoracic radiotherapy.

METHODS: Patients with histologically documented thymoma with unresectable stage III disease received 9 weeks of chemotherapy: cisplatin 25 mg m^{-2} on weeks 1–9; vincristine 1 mg m^{-2} on weeks 1, 2, 4, 6 and 8; and doxorubicin 40 mg m^{-2} and etoposide 80 mg m^{-2} on days 1–3 of weeks 1, 3, 5, 7 and 9. Patients went on to surgery and post-operative radiotherapy of 48 Gy; those with unresectable disease received 60 Gy radiotherapy.

RESULTS: A total of 23 patients were entered. The main toxicities of the chemotherapy regimen were neutropenia and anaemia, and 57% of patients completed the planned 9 weeks of therapy. There were no toxic deaths. Of the 21 eligible patients, 13 (62%) achieved a partial response (95% confidence interval: 38–82%). Thirteen patients underwent a thoracotomy and nine (39%) underwent complete resection. Progression-free survival at 2 and 5 years was 80 and 43%, respectively. Overall survival at 5 and 8 years was 85 and 69%, respectively. Survival did not seem to be affected by resection.

CONCLUSION: In thymoma patients, weekly dose-dense chemotherapy has activity similar to that of conventional regimens. Although some patients could achieve complete resection, the role of surgery remains unclear.

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Thymoma is one of the most common tumours to originate in the mediastinum (Giaccone, 2005; Girard *et al*, 2009). Although its clinical behaviour tends to be indolent, it eventually disseminates into the pleural space or sometimes leads to distant metastases. Masaoka's classification has been widely used for clinical staging (Masaoka *et al*, 1981; Girard *et al*, 2009).

The majority of thymomas are discovered at a limited stage, Masaoka's stage I or II, and surgical resection is the treatment of choice for such cases (Giaccone, 2005; Girard *et al*, 2009). Even when the tumour invades neighbouring organs, namely, stage III disease, surgical resection with post-operative radiotherapy is the preferred treatment when the tumour can be completely resected (Curran *et al*, 1988; Urgesi *et al*, 1990; Ogawa *et al*, 2002; Strobel *et al*, 2004).

However, for stage III, unresectable tumours, a combination of chemotherapy and radiotherapy with or without surgical resection is frequently used, but optimal management remains controversial (Ciernik *et al*, 1994; Loehrer *et al*, 1997; Kim *et al*, 2004; Mangi *et al*, 2005; Lucchi *et al*, 2006). There are very few prospective trials with limited numbers of cases, some including stage IV cases (Loehrer *et al*, 1997; Kim *et al*, 2004; Girard *et al*, 2009).

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On the other hand, thymomas are generally reported to be chemotherapy-sensitive tumours, with a response rate of 50–70% to combination chemotherapy (Loehrer *et al*, 1994, 1997, 2001; Giaccone *et al*, 1996; Berruti *et al*, 1999; Kim *et al*, 2004; Lucchi *et al*, 2006; Yokoi *et al*, 2007). Active agents include cisplatin (CDDP), vincristine (VCR), doxorubicin (ADM), etoposide (ETP), cyclophosphamide (CPM) and ifosfamide (IFX).

Dose-dense chemotherapy with the CODE combination (CDDP–VCR–ADM–ETP), combined with granulocyte colony-stimulating factor (G-CSF), has been shown to be safe when administered to patients with advanced lung cancer (Murray *et al*, 1991; Fukuoka *et al*, 1997). Theoretically, it might be suitable for chemo-sensitive tumours such as small-cell lung cancers and thymomas, especially in cases with limited tumour burden (Goldie and Coldman, 1983; Levin and Hryniuk, 1987; Murray, 1987). Because of the pilot data in Japan that had suggested that administration of 12 weeks of CODE chemotherapy was barely feasible, subsequent Japanese trials used a modified schedule that was shortened to 9 weeks (Fukuoka *et al*, 1997; Furuse *et al*, 1998).

In 1996, we, the Japan Clinical Oncology Group (JCOG), initiated two clinical trials for advanced thymoma: one aimed to evaluate the safety and efficacy of the CODE regimen in stage IV, disseminated thymoma (JCOG 9605), and the other aimed to evaluate the safety and efficacy of CODE combination chemotherapy, followed by surgical resection and post-operative radiotherapy, in initially unresectable stage III thymoma (JCOG 9606). The primary end point in each study was progression-free survival (PFS). The results of JCOG 9606 are reported herein.

PATIENTS AND METHODS

Eligibility criteria

Patients with previously untreated, histologically documented thymomas with Masaoka's stage III disease that was judged to be unresectable by the surgeons, radiologists and medical oncologists at each institute were eligible for entry. Thymoma had to be confirmed histologically, and thymic tumours with other histology, such as thymic carcinoma, carcinoid or lymphoma, were excluded. Each patient was required to fulfil the following criteria: 15–70 years of age; Eastern Cooperative Oncology Group (ECOG) performance status, 0–2; and adequate organ function, that is, leukocyte count $\geqslant 4000/\mu l$, platelet count $\geqslant 10^5/\mu l$, haemoglobin $\geqslant 10.0$ g per 100 ml, serum creatinine <1.5 mg per 100 ml, creatinine clearance $\geqslant 60$ ml min^{-1}, serum bilirubin <1.5 mg per 100 ml, serum alanine aminotransferase and aspartate aminotransferase less than double the upper limit of the institutional normal range, PaO_2 $\geqslant 70$ mm Hg and predicted post-operative forced expiratory volume in 1 s to be 50% or more of the age-, sex- and height-predicted vital capacity. The exclusion criteria included patients with uncontrolled heart disease, uncontrolled diabetes or hypertension, pulmonary fibrosis or active pneumonitis as evident on chest X-ray, infections necessitating systemic use of antibiotics, disease necessitating emergency radiotherapy, such as superior vena cava obstruction syndrome, active concomitant malignancy, as well as pregnant or lactating women. Also excluded were those with grave complications of thymoma, such as pure red cell aplasia or hypogammaglobulinaemia; myasthenia gravis was allowed and these patients were not excluded *per se*.

Patient eligibility was confirmed by the JCOG Data Center before patient registration. This study protocol was confirmed by the JCOG protocol committee, and then approved by the institutional review boards at each participating centre. Written informed consent was obtained from all patients.

Treatment plan

Chemotherapy Patients received the 9-week CODE combination chemotherapy described below. Each chemotherapeutic agent was administered intravenously.

Week 1: CDDP 25 mg m^{-2} on day 1 with antiemetics and ample hydration; VCR 1 mg m^{-2} on day 1; ADM 40 mg m^{-2} on day 1; and ETP 80 mg m^{-2} on days 1–3.
Weeks 2, 4, 6 and 8: CDDP 25 mg m^{-2} on day 1 with antiemetics and ample hydration and VCR 1 mg m^{-2} on day 1.
Weeks 3, 5, 7 and 9: CDDP 25 mg m^{-2} on day 1 with antiemetics and ample hydration, ADM 40 mg m^{-2} on day 1 and ETP 80 mg m^{-2} on days 1–3.

Each week, G-CSF (filgrastim 50 μg m^{-2} per day or lenograstim 2 μg kg^{-1} per day) was administered by subcutaneous injection, except on days when chemotherapy was administered or when the leukocyte count was $\geqslant 10\,000/\mu l$. Corticosteroid was used only as part of the antiemetic regimen, and the specific drug and dosage were not regulated by the protocol.

Dose and schedule modifications were carried out as previously reported (Kunitoh *et al*, 2009).

Surgery and radiotherapy

When the tumour was clinically judged to be resectable by the surgeons, radiologists and medical oncologists in each institution, surgical resection of the tumour and a total thymectomy were performed within 6 months (preferably within 3 months) after completion of chemotherapy. For completely resected tumours, post-operative thoracic radiotherapy up to 48 Gy/24 fractions was administered to the surgical margin and the mediastinum. For incompletely resected or unresected tumours, thoracic radiotherapy of up to 60 Gy/30 fractions was administered to the mediastinum and the residual tumour with 1.5-cm margins. The radiation dose per fraction, 2 Gy, and the total doses were determined by the study group in view of previous reports (Girard *et al*, 2009). The actual treatment delivery method was determined at each institution.

Thoracic radiotherapy was started with a linear accelerator ($\geqslant$4 MeV) within 6 months of surgery or, for those who did not undergo surgery, on completion of chemotherapy.

Patient evaluation and follow-up Before enrolment into the study, each patient underwent a complete medical history and physical examination (including neurological examination for signs of myasthenia gravis), blood cell count determinations, serum biochemistry testing, arterial blood gas analysis, pulmonary function test, electrocardiogram, chest X-ray, computed tomography (CT) scan of the chest, CT scan or ultrasound of the upper abdomen, whole-brain CT or magnetic resonance imaging and an isotope bone scan. Blood cell counts were determined, serum biochemistry testing was carried out and chest X-rays were taken weekly during each course of chemotherapy.

The toxicity of the chemotherapy was evaluated according to the Japan Clinical Oncology Group Toxicity Criteria (Tobinai *et al*, 1993), modified from the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 1. Tumour responses were assessed radiographically according to the standard, two-dimensional WHO criteria (Miller *et al*, 1981) and classified into complete response (CR), partial response (PR), no change (NC), progressive disease (PD) and non-evaluable. Response confirmation at 4 weeks or longer intervals was not required in the protocol. After completion of the protocol therapy, the patients were followed up with periodic re-evaluation, including chest CT every 6 months for the first 2 years and yearly thereafter.

Clinical Studies

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Central review Radiographic reviews for eligibility of the enrolled patients and their clinical responses were carried out at the time of the study group meetings. The study coordinator (HK) and a few selected investigators reviewed the radiographic films. The clinical data presented below were all confirmed by this central review. Reviews of pathological specimens were not carried out, because the logistics of the study group were insufficient at the time of study activation in 1997.

End points and statistical considerations

Because of the rarity of the tumour and the accrual to the US trials (Loehrer *et al*, 1994, 1997), we presumed that we would be capable of accruing 30 patients in the target accrual period of 4 years. The sample size was, therefore, not based on statistical calculations. The expected 5-year PFS rate was 60%, which would give a 95% confidence interval of 40-77% with 30 cases.

Hence, the initial study design envisioned enrolment of 30 fully eligible cases over 4 years, with a follow-up period of 5 years.

The secondary end points included toxicity and safety, objective tumour response to chemotherapy, pattern of relapse, overall survival (OS) and complete resection rate.

The PFS and OS were calculated from the date of enrolment and estimated by the Kaplan-Meier method. Progression-free survival was censored at the date verifiable to be progression free, and OS was censored at the date of last follow-up. During the accrual period, an interim analysis for futility was planned after half of the patients were registered and followed up for at least 6 months. All analyses were performed using SAS software version 8.2/9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics

A total of 23 patients from eight institutions were enrolled from July 1997 to April 2005, when the study was terminated because of slow accrual. Two patients were ineligible because of wrong histology; one had thymic carcinoma, and the other had lymphoma. These mistakes occurred because of technical problems in the patient registry. As the ineligible cases did receive the protocol therapy, all 23 patients were analysed for characteristics and toxicity. In all, 21 eligible patients were analysed for clinical response, survival (PFS and OS) and surgical results. The patients' characteristics are shown in Table 1. Diagnostic procedure was CT-guided needle biopsy in most of the cases.

Reasons for surgical unresectability (one patient could have more than one reason) included invasion into the following: the pulmonary artery trunk in 10 cases, superior vena cava in 8, aorta in 6, extensive pericardium or myocardium in 4, and sternum in 1.

Chemotherapy delivery and toxicity

Thirteen patients (57%) received the planned 9 weeks of chemotherapy. The other 10 patients included 2 who received 8 weeks, 5 who received 7 weeks, 2 who received 6 weeks and 1 who received 1 week of therapy. Reasons for ceasing chemotherapy were patient refusal (six cases), attending doctors' decision for earlier local therapy (two cases), disease progression (one case) and ineligibility (one case). The median duration of chemotherapy for the 13 patients who underwent the planned 9 cycles was 9 weeks (range: 9-12 weeks). Among the nine patients who received 6-8 cycles, six received chemotherapy without delay and the remaining three received chemotherapy with a delay of 1-4 weeks.

Table 2 summarises the major toxicities of the chemotherapy. They were mainly haematological, and although about half of the patients experienced grade 4 neutropenia, it was generally transient and complicated by infection in only 1 case. Substantial anaemia was frequently observed, consistent with other reports of dose-dense CODE therapy (Fukuoka *et al*, 1997; Furuse *et al*, 1998). Overall, the toxicities were well tolerated. There were no deaths related to toxicity.

Table 1 Patients' characteristics

Item	Number
Sex (male/female)	17/6
Age, years (median/range)	56 (28-70)
ECOG performance status	
PS0/PS1/PS2	9/14/0
Smoking history	
No	13
Yes (median pack-years)	10 (28)
Myasthenia gravis (no/yes)	21/2
Histology: thymoma and eligible	21
Lymphocyte predominance	10
Mixed cell	4
Epithelioid cell	6
Spindle cell	1
Histology: not thymoma (ineligible)	2
Carcinoma	1
Lymphoma	1

Abbreviations: ECOG = Eastern Cooperative Oncology Group; PS = performance status.

Table 2 Toxicity of the chemotherapy ($N = 23$)

Toxicity	Grades 1/2	Grade 3	Grade 4	%Grade 3/4
Leukopenia	4/5	8	5	57
Neutropenia	1/6	3	11	61
Anaemia	0/3	19	ND	83
Thrombocytopenia	6/4	4	2	26
ALT	10/1	1	0	4
Creatinine	2/0	0	0	0
PaO_2	5/6	0	0	0
Emesis	10/8	3	ND	13
Diarrhea	3/3	1	0	4
Stomatitis	5/2	0	0	0
Constipation	2/1	0	0	0
Neuropathy	7/2	0	ND	0
Infection	5/2	3	0	13

Abbreviations: ALT = alanine aminotransaminase; ND = not defined (the Japan Clinical Oncology Group toxicity criteria did not define grade 4 in these toxicities).

Clinical response to induction therapy

The clinical responses of the 21 eligible patients to the chemotherapy were judged radiologically and confirmed by central review. The responses were as follows: CR, 0; PR, 13; NC, 7; and PD, 1. The overall response rate was 62% (95% confidence interval: 38-82%).

Surgical and pathological results

Of the 21 eligible patients, a thoracotomy was performed in 13 (62%). Thoracotomy was performed 26-73 days (median: 47 days) after completion of chemotherapy. The results of the surgery were as follows: probe thoracotomy, two cases; gross residual tumour (R2 resection), one case; microscopically residual tumour on pathological review (R1 resection), one case; and complete surgical and pathological resection (R0 resection), nine cases (43% of all eligible cases). A combined resection of the adjunct organs included pericardium in eight, lung parenchyma in eight, pleura

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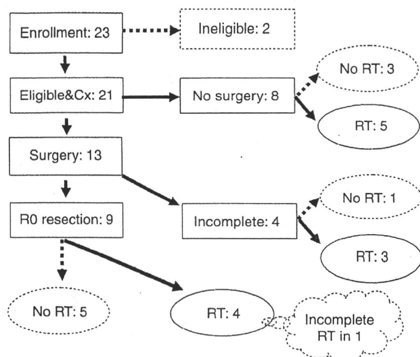

Figure 1 Study schema of the Japan Clinical Oncology Group (JCOG) 9606 trial, with the number of patients who actually received each of the protocol therapies. Cx, chemotherapy; RT, radiotherapy.

in seven, superior vena cava in two, brachiocephalic vein in two and others in five cases. Pathological CR (pCR), with no residual viable tumour cells in resected specimens, was achieved in three patients (14% of the 21 eligible patients).

The major post-operative morbidities included one case of pulmonary infarction, which subsequently recovered.

Boost radiotherapy

Post-operative radiotherapy was administered to 7 of the 13 patients who underwent thoracotomy: four of the nine patients with R0 resection received radiotherapy of 48, 48, 48 and 8 Gy, respectively; one of the two patients with incomplete resection received radiotherapy of 50 Gy; and each of the two patients with probe thoracotomy received radiotherapy of 60 Gy. Reasons for not carrying out radiotherapy included surgery-related complication or incomplete recovery (three cases), disease progression (two cases) and patient refusal (one case). Of the eight patients without thoracotomy, five received radiotherapy, with a dose of 60 Gy for each case. The other three patients refused radiotherapy.

The study schema with the actual numbers of patients receiving the protocol therapy is shown in Figure 1.

Other and late complications

Thirteen patients received thoracic radiotherapy. The toxicities were generally mild and manageable. There were four patients with grade 2 oesophagitis, one patient with a grade 3 skin reaction and another with a grade 2 skin reaction. All other adverse events were grade 0 or 1.

One patient was reported to have pure red cell aplasia, which occurred while receiving post-operative radiotherapy. Radiotherapy was terminated, and the patient recovered with immunosuppressant therapy.

Progression-free and overall survival

Survival data were last updated in May 2009, 4 years after accrual of the last patient. Figure 2 shows the PFS and OS curves for the 21 eligible cases. The median PFS was 4.5 years (95% confidence interval: 2.3 not calculable years), and the PFS at 2, 5 and 8 years was 80, 43 (95% confidence interval: 21–63%) and 32%,

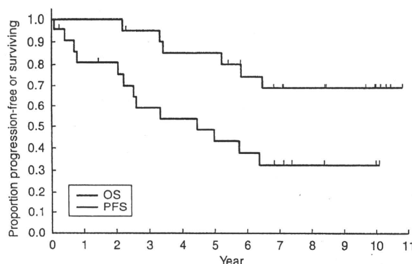

Figure 2 Progression-free survival (PFS) and overall survival (OS) of the 21 eligible patients.

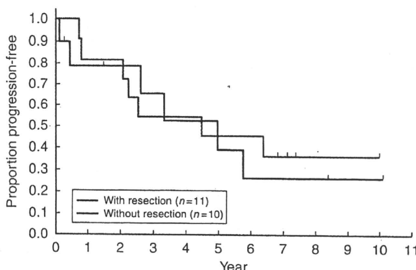

Figure 3 Progression-free survival of the 21 eligible patients, according to the surgery undergone. Resection was performed in 11 patients (complete resection in nine), and 10 patients did not undergo resection (including two with probe thoracotomy). There was no significant difference (log rank $P = 0.75$).

respectively. The median OS was not reached, and the OS at 2, 5 and 8 years was 100, 85 (95% confidence interval: 61–95%) and 69%, respectively.

Of the 21 eligible patients, 11 underwent surgical resection (nine complete resection and two incomplete resection), whereas 10 did not (including two who underwent a probe thoracotomy). The PFS and OS were quite similar for those with or without surgical resection. The 5- and 8-year PFS rates for those who underwent resection were 46 and 36% for those with surgical resection and 39 and 26% for those without, respectively (Figure 3). The 5- and 8-year OS rates were 91 and 73% for those with surgical resection and 79 and 63% for those without, respectively (Figure 4).

For the nine patients who underwent R0 resection, the outcomes were marginally better, with 5- and 8-year PFS rates of 56 and 44%, respectively, and 5- and 8-year OS rates of 89 and 78%, respectively. The case with R1 resection had relapse at 2.3 years, and the case with R2 resection had relapse at 0.7 year.

All three patients who achieved pCR were alive and disease free at 6.3–7.4 years of follow-up.

Pattern of relapse

So far, 13 of the 21 eligible patients have had tumour relapse. All of the 13 relapsed patients initially demonstrated regrowth of the primary and/or pleural or pericardial dissemination: primary only

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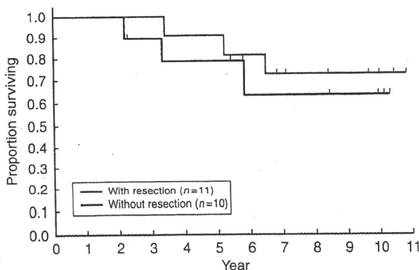

Figure 4 Overall survival of the 21 eligible patients, according to the surgery undergone. Resection was performed in 11 patients (complete resection in nine), and 10 patients did not undergo resection (including two with probe thoracotomy). There was no significant difference (log rank $P = 0.59$).

in five; pleura or pericardium only in six, and both in two2. None had initial relapse involving distant organs. There was no report of needle biopsy-track recurrence.

DISCUSSION

The optimal management of unresectable stage III thymoma remains unclear. There are some reports of combined modality approaches including chemotherapy and surgery, but many reports included stage IV disease and/or thymic carcinoma histology (Berruti *et al*, 1999; Kim *et al*, 2004; Lucchi *et al*, 2006; Yokoi *et al*, 2007). Reports of multicentre prospective trials are very few (Table 3).

In the current trial, we prospectively accrued patients with unresectable stage III thymoma, and excluded thymic carcinoma; it is now evident that thymoma and thymic carcinoma differ in clinical presentation and in prognosis, and trials on them should be reported separately (Eng *et al*, 2004; Giaccone, 2005).

We previously reported the results of another trial, JCOG 9605 (Kunitoh *et al*, 2009), in which we treated patients with stage IV thymoma with CODE chemotherapy. The results were similar to conventional chemotherapy, and we concluded that intensive chemotherapy does not seem to be promising enough in disseminated thymoma. However, dose-dense chemotherapy might still have a role in patients with limited tumour burden, in combination with definitive local therapy.

Although our results showed that CODE chemotherapy in combination with local therapy could be safely administered to thymoma patients, the efficacy was not remarkable. Compliance to chemotherapy was poorer; only 57% of patients completed the planned 9-week schedule, as compared with the 87% rate in the JCOG 9605 study for stage IV disease (Kunitoh *et al*, 2009). Doctors' and patients' decisions were the main reasons for ceasing chemotherapy and early local therapy. Therefore, although chemotherapy itself was well tolerated, toxicities such as malaise or fatigue, which the old JCOG toxicity criteria did not define, might have compromised the completion of chemotherapy before surgery.

Moreover, although the sample size was smaller than expected because of poor accrual, the 5-year PFS rate was 43% (95% confidence interval: 21–63%), which fell short of the expected 60%. Although the OS rate was favourable, it would be difficult to make a valid conclusion because of the small sample size (Table 3).

Table 3 Reports of prospective trials of combined modality therapy for locally advanced thymoma

Treatment	Stage	Patients[a]	ORR	5-yr OS
PAC, R (Loehrer *et al*, 1997)	III	23	70%	52.5%[b]
PAC, S, R (Kim *et al*, 2004)	III/IV	22	77%	95%[c]
CODE, S, R (current study)	III	21	62%	85%[d]

Abbreviations: CODE = combination chemotherapy with cisplatin/vincristine/doxorubicin/etoposide; ORR: overall response rate; PAC = combination chemotherapy with cisplatin/doxorubicin/cyclophosphamide; R = thoracic radiotherapy; S = surgical resection; 5-yr OS = overall survival rate at 5 years. [a]Number of assessable patients. [b]Including patients with thymic carcinoma. [c]7-year OS rate was 79%. [d]8-year OS rate was 69%.

In this study, we did show that about half of the patients with an initially unresectable thymoma were able to undergo complete resection after induction CODE chemotherapy. However, both PFS and OS were surprisingly similar for patients with and without complete resection.

Those who underwent complete resection got numerically better PFS and OS rates, but the difference with unresected cases was marginal, especially considering the selection bias. Only those who received pathological CR enjoyed clearly favourable outcomes. Low compliance to radiotherapy in patients with surgery could partly account for the unexpected results.

Complete resection has been reported to be associated with good prognosis in patients with stage III thymoma (Regnard *et al*, 1996; Girard *et al*, 2009). On the other hand, the role of 'debulking' surgery, in patients in whom complete resection is not feasible, remains unclear. Although some have suggested it to be beneficial (Liu *et al*, 2006), others reported that it could not affect the outcome as compared with biopsy only, followed by radiotherapy (Ciernik *et al*, 1994).

Taken together with our results, we believe that the role of surgery in locally advanced thymoma, as compared with definitive radiotherapy, still remains to be established, especially in combination with systemic chemotherapy. More studies are warranted.

One major limitation of the study is that we did not perform a central review of the histology, and thus could not provide WHO classifications of histology (Okumura *et al*, 2002; Travis *et al*, 2004). This makes comparisons with results from other reports difficult. Central pathology review and, preferably, tissue collection would be very important in future trials.

Now JCOG is discussing our next study on thymoma. As intensification of the current chemotherapy does not seem to be promising enough, our next approach would be trials with new agents, cytotoxic (such as amrubicin or irinotecan) or target based. More translational research of the tumour would be necessary, as well as international cooperation, given the rarity of the disease.

In conclusion, we found that weekly dose-dense chemotherapy could be administered safely to patients with thymoma, even when combined with local therapy in localised disease. However, the efficacy seemed to be no better than that of conventional chemotherapy. More research on the optimal systemic therapy, as well as on the role of surgery in locally advanced disease, seems to be necessary.

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Appendix 1

Study participants

The following institutions and investigators participated in the trial:

National Cancer Center Hospital East (Yutaka Nishiwaki, Kaoru Kubota, Nagahiro Saijo), National Cancer Center Hospital (Tomohide Tamura, Noboru Yamamoto, Hideo Kunitoh), Kanagawa Cancer Center (Kazumasa Noda, Fumihiro Oshita), Yokohama Municipal Citizen's Hospital (Koshiro Watanabe, Hiroaki Okamoto), Niigata Cancer Center Hospital (Akira Yokoyama, Yuko Tsukada), Kinki University Hospital (Kazuhiko Nakagawa, Isamu Okamoto), Osaka City General Hospital (Koji Takeda, Haruko Daga), and Kobe City Medical Center General Hospital (Nobuyuki Katakami, Hisashi Nishimura).

Clinical Studies

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