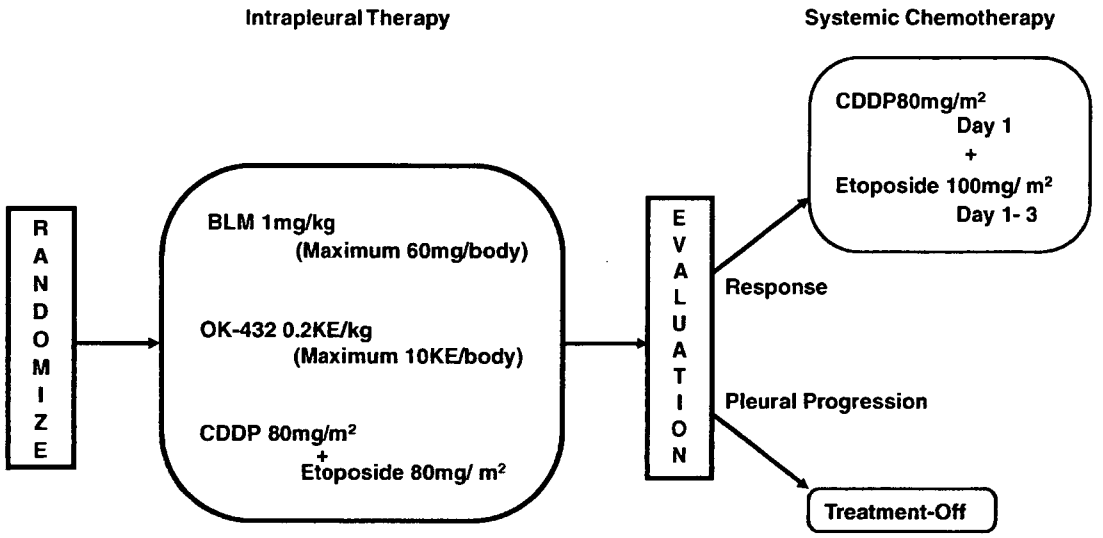

Fig. 1 Treatment schema.

above). A responder identified within 2 weeks after the first (4-week) evaluation received systemic chemotherapy consisting of cisplatin (80 mg/m^2) on day 1 and etoposide (100 mg/m^2) on days 1–3, which was repeated every 3–4 weeks for two or more courses.

2.5. Toxicity criteria and dose modification

Adverse reactions were graded according to the JCOG Toxicity Criteria [14], which are modifications of the National Cancer Institute's common toxicity criteria issued in 1991. The second or subsequent cycles of systemic chemotherapy were delayed if on day 1 the WBC count was less than 3000 μL^{-1} or the platelet count was less than 75,000 μL^{-1}. If grade 4 hematological toxicity occurred during the previous course, the dose of etoposide was reduced to 75%. Cisplatin was permanently discontinued at any time when the serum creatinine level was greater than 2.0 mg/dL. If the serum creatinine level was 1.5–2.0 mg/dL, the next cycle was delayed until it was 1.2 mg/dL or less, and the dose of cisplatin was then reduced to 75%.

2.6. Data management and statistical analysis

This study was designed as a multicenter randomized phase II trial among 21 participating centers in the Lung Cancer Study Group in the JCOG. Pleural progression-free survival (PPFS) was defined as the time from randomization to the first observation of pleural progression or death due to any cause. The primary endpoint of this study was 4-week PPFS rate. Assuming that the 4-week PPFS rate was at least 50% for these arms, the required number for each arm was 30 to select the better arm correctly with 90% probability if the better arm's 4-week PPFS rate was 70% or higher [15]. Planned accrual was set at 35 per arm. Secondary end-points were 8-, 12- and 24-week PPFS rates, overall survival (OS) and toxicity. The duration for OS was measured from the date of randomization to the date of death due to any cause or last follow-up. The mandated time to start treatment following randomization was within a week. Survival distribution was estimated by the Kaplan–Meier method, and confidence intervals were based on Greenwood's formula [16].

Patient randomization and data management were performed by the JCOG Data Center (JCOG DC). In-house interim monitoring was performed by the JCOG Data and Safety Monitoring Committee semiannually. Central review of chest X-rays for all responses in all eligible cases was performed at regular study group meetings by an extramural panel. Statistical analysis was performed by the JCOG DC with SAS software version 6.12 for Windows (SAS Institute Inc., Cary NC).

3. Results

3.1. Patients

From May 1996 to August 1999, 105 patients were enrolled onto this study from the 21 participating institutions. The clinical characteristics of the patients are listed in Table 1. Three patients were later found to be ineligible (one patient per group): one had malignant pleural effusion secondary to colon cancer; one had no reexpansion of the affected lung after tube drainage; and one had poor renal function. Thus, 102 patients were assessable for response and survival. Four patients did not receive intrapleural therapy because of one self-removal of the drain, one obstruction of the drain, and two cases of intrapleural sclerosis. These four patients were excluded from the analysis of toxicity. The three treatment arms were well balanced for age, sex, and PS.

3.2. Treatment compliance and toxicity

Table 2 outlines the compliance with treatment. Fifty-one (50.0%) of the eligible patients completed intrapleural therapy and systemic chemotherapy as defined by the protocol. Forty-one (40.1%) of the eligible patients did not receive systemic chemotherapy because of disease progression. Two

Table 1 Patient Characteristics

Characteristic	BLM	OK-432	PE
All patients	36	34	35
Eligible patients	35	33	34
Age (years)			
Median	64	60.5	61
Range	44–75	31–73	39–75
Sex			
Male	24	21	24
Female	12	13	11
PS (ECOG)[a]			
0	2	4	2
1	30	27	28
2	4	3	5
≥10% weight loss within 6 m			
No	33	27	31
Yes	3	7	4
Histology			
Adenocarcinoma	29	32	32
Squamous cell	4	1	3
Large cell	1	1	0
Other	1	0	0
TNM (N factor)			
N0	14	14	14
N1	2	0	2
N2	16	13	11
N3	3	7	8
Stage			
IIIB	23	17	25
IV	12	17	10

[a] At the time of reexpansion of the affected lung.

patients (5.7%) in the BLM arm had pneumonitis induced by BLM and one of them had treatment-related death. One patient in the PE group did not receive systemic chemotherapy due to elevation of serum creatinine. Other reasons for noncompletion of the protocol treatment were two

Table 2 Treatment compliance

Variable	BLM	OK-432	PE
Eligible patients	35	33	34
No therapy	1	2	1
End of study protocol	18	19	14
Progressive disease	14	11	16
Toxicity	1	0	1
Death	1	0	0
Patient refusal	0	1	1
Insufficient drainage	0	0	1

patient refusals in each for the OK-432 and the PE arms, and one patient in the PE arm who could not receive sufficient drainage due to self-removal of the drain 48 h after intrapleural therapy.

Toxicities for intrapleural therapy in the three arms are listed in Table 3. Hematological toxic events were well tolerated in the three arms. Grade 4 nonhematological toxicity was not found in the three arms. Grade 2–3 chest pain occurred almost equally in the three arms. Grade 2–3 fever and nausea/vomiting occurred most frequently in the OK-432 arm (59.4%) and the PE arm (50.0%), respectively.

3.3. PPFS and OS

All eligible patients in the three arms were included in the survival analysis. PPFS and OS data are shown in Figs. 2 and 3, respectively. Median PPFS for the BLM arm was 20.9 weeks (95% confidence interval (CI), 4.7–25.9 weeks); for the OK-432 arm, 27.9 weeks (95% CI, 18.6–50.0 weeks); and for the PE arm, 18.4 weeks (95% CI, 4.4–41.4 weeks). The 4-week PPFS rate, which was the primary endpoint of this study, was 68.6% for the BLM arm (95% CI, 53.2–84.0%); 75.8% for the OK-432 arm (95% CI, 61.1–90.4%); and 70.6% for the PE arm (95% CI, 55.3–85.9%). The median survival time (MST) for the BLM arm was 32.1 weeks (95% CI, 21.6–37.9 weeks); 48.1 weeks for the OK-432 arm (95% CI, 26.7–58.4 weeks); and 45.7 weeks for the PE arm (95% CI, 34.4–57.1 weeks). The 48-week survival rate for the BLM arm was 29.9% (95% CI, 14.4–45.3%); 51.1% for the OK-432 arm (95% CI,

Table 3 Toxicity (JCOG grade) for Intrapleural Therapy

	BLM ($n=35$)				OK-432 ($n=32$)				PE ($n=34$)			
	1	2	3	4	1	2	3	4	1	2	3	4
Leukocytes	3	3	0	1	1	0	1	0	8	3	2	1
Neutrophils	1	0	2	1	0	0	1	0	5	5	1	2
Hemoglobin	3	5	3	ND	3	6	1	ND	6	6	3	ND
Platelet	0	0	1	0	0	0	0	0	1	1	0	0
AST	8	0	0	0	15	2	0	0	6	0	0	0
ALT	11	0	0	0	14	7	0	0	10	2	0	0
Serum creatinine	1	0	0	0	0	0	0	0	4	1	0	0
Chest pain	10	5	4	0	15	8	1	0	13	6	1	0
Fever	12	13	0	0	6	18	1	0	9	7	2	0
Nausea/vomiting	7	3	0	ND	5	0	0	ND	10	13	4	ND

Abbreviation: ND, not defined.

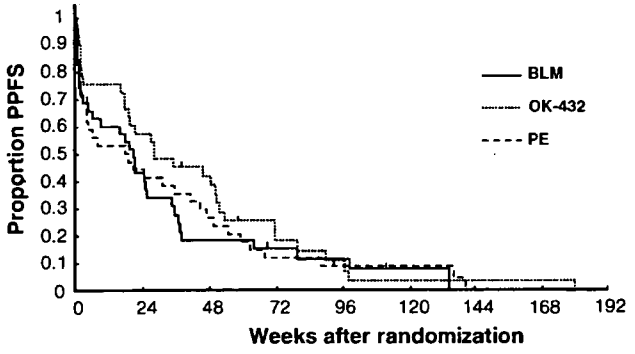

Fig. 2 Pleural progression-free survival (PPFS) in all eligible patients ($n = 102$).

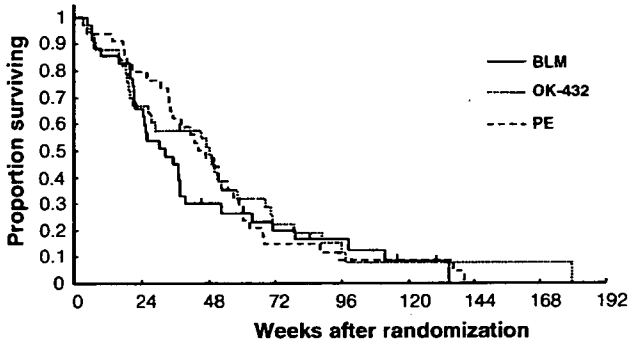

Fig. 3 Overall survival in all eligible patients ($n = 102$).

34.0–68.3%); and 47.1% for the PE arm (95% CI, 30.3–63.8%). Both the PPFS and OS for the OK-432 arm were superior to those for other two arms; however, the outcomes did not differ significantly between groups.

4. Discussion

To date, numerous chemical agents for treatment of MPE have been studied. These were antibiotics, antineoplastic agents, biological response modifiers (BRMs) and others that showed varied degrees of chemical sclerosis. Among them, BLM and talc are most frequently used for the management of MPE [5,7,17,18]. BLM is an antineoplastic antibiotic used in sclerotherapy with a success rate of 63–85% [7,8,18–21]. Talc applied as either slurry or poudrage is superior to other commonly used sclerosing agents with a success rate of 71–100% [5,7,22–24]. Because talc has not been available commercially in Japan and the use of talc was considered controversial at the beginning of this study because of severe complications, such as acute respiratory distress syndrome [25,26], we selected BLM as the sclerosing agent. A recent report demonstrated that the safety of talc pleurodesis and that acute respiratory distress syndrome can be avoided by using large-particle talc applied as thoracoscopic poudrage [27]. The thoracoscopic pleurodesis with talc is now considered to be the gold standard treatment for MPE [28,29].

OK-432 has been used as a BRM for gastric and lung cancer [9,10,30,31]. OK-432 has been reported to be effective in controlling MPE in two prospective randomized trials. One study reported a 73% success rate with OK-432 compared to 41% with mitomycin C treatment ($p = 0.03$) [11]. The other comparison found OK-432 70% effective compared to 46% in BLM subjects (statistical data not reported) [12]. OK-432 has been reported to induce various cytokines, such as tumor necrosis factor-α, interferon-γ, interleukin (IL)-1, IL-8 and IL-12 [32] and also to enhance cytotoxicity against tumor cells [33,34]. It is suggested that the main therapeutic effects of OK-432 for malignant effusion depend on increased expression of intercellular adhesion molecule-1 on tumor cells induced by interferon-γ [35].

Intrapleural combination chemotherapy is focused on achieving higher concentrations in the pleural cavity with less toxicity than systemic chemotherapy [36]. Two phase II studies with intrapleural cisplatin and cytarabine had success rates of 49% [2] and 73% [37]. Tohda et al. [13] reported that intrapleural instillation of cisplatin and etoposide for NSCLC with MPE resulted in a 46.2% overall response rate and the MST of 8 months was found to be improved, compared with previous reports for NSCLC with MPE of 3–6 months [11,18,38]. The reason for this was assumed to be that intrapleural combination chemotherapy of cisplatin and etoposide produced systemic as well as local effects. The overall response rates of intrapleural combination chemotherapy are variable and there are no prospective randomized studies compared modality of intrapleural combination chemotherapy with that of sclerotherapy.

There have been several special problems raised in the clinical trials for MPE, such as patient selection, response criteria, treatment procedures, short life expectancy, small sample sizes, and different endpoints [2–7,11,39]. To minimize the bias of patient selection, NSCLC patients with MPE who had received no prior therapy were entered into this study. Furthermore, justifiable and simplified response criteria and whether further treatment was required or not, as suggested by Ruckdeschel [18] and Rusch [40] were used and single intrapleural instillation of each agent was permitted to allow uniform estimation of responses. In many trials, successful pleurodesis was determined by assessing clinical and radiological findings. The positive response criteria have been defined generally as no pleural re-accumulation, 50% less effusion than that observed in the baseline radiograph taken immediately after the procedure, or no requirement for further thoracentesis. To determine the efficacy, we used the criterion that a decrease in effusion over one-quarter of the treated lung provides a stricter assessment of chemical pleurodesis that may relieve the symptoms of MPE. The position rotation after intrapleural instillation was recommended traditionally because it was thought to allow the agents to be distributed thoroughly throughout the entire pleural space. In contrast, studies using tetracycline and talc [41,42] demonstrated that rotation does not affect the overall intrapleural dispersion. It is unclear whether rotation is beneficial or not when applying the agents used in this study. Because a previous phase II study [13] showed that etoposide remains for a long period (β-phase half-life = 62.53 h) in intrapleural fluids, we applied the longer duration of clamping in the PE arm (72 h) than the other two arms (3 h) to provide enough exposure to the cancer cells. We found no major safety concerns such as excess pleural effusion as a result of the longer duration of clamping.

In this study, all three regimens were feasible. One treatment-related death occurred in the BLM arm 9 weeks after intrapleural instillation of BLM. Treatment compliance

rates for both intrapleural and systemic therapy was 50% (51 of the 102 eligible patients). This study lacks sufficient power to demonstrate differences between treatment arms; however, the OK-432 arm seemed to demonstrate modest benefit compared with the other two arms in terms of PPFS. It is assumed that the favorable efficacy in the OK-432 arm suggests that OK-432 has clinically meaningful activity for controlling MPE in NSCLC patients. NSCLC patients with MPE have been treated as patients with stage IV disease even when without metastasis, and systemic chemotherapy should be recommended when they have a good PS [43]. We prescribed systemic PE chemotherapy regimens, which were considered one of the standard regimens at the beginning of the study, following successful pleurodesis. However, we expect that platinum-based systemic combination chemotherapy regimens with several active new chemotherapeutic agents such as taxanes (paclitaxel and docetaxel), vinorelbine, gemcitabine and irrinotecan, which are the current standard treatment options for patients with advanced NSCLC, should enhance the survival benefit more than PE regimens.

This is the first fully reported randomized study that has evaluated the efficacy of intrapleural therapy for previously untreated patients with NSCLC and compliance with sequential systemic chemotherapy. As the results of this study demonstrate that intrapleural therapy with OK-432 shows a tendency to be more effective than BLM or PE in the management of MPE in NSCLC, in terms of PPFS, further studies are needed to compare OK-432 with talc.

Conflict of interest

None declared.

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SNP Communication

*Genetic Variations and Frequencies of Major Haplotypes in SLCO1B1 Encoding the Transporter OATP1B1 in Japanese Subjects: SLCO1B1*17 is More Prevalent Than *15*

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

Summary: A liver-specific transporter organic anion transporting polypeptide 1B1 (OATP1B1, also known as OATP-C) is encoded by *SLCO1B1* and mediates uptake of various endogenous and exogenous compounds from blood into hepatocytes. In this study, 15 *SLCO1B1* exons (including non-coding exon 1) and their flanking introns were comprehensively screened for genetic variations in 177 Japanese subjects. Sixty-two genetic variations, including 28 novel ones, were found: 7 in the 5′-flanking region, 1 in the 5′-untranslated region (UTR), 13 in the coding exons (9 nonsynonymous and 4 synonymous variations), 5 in the 3′-UTR, and 36 in the introns. Five novel nonsynonymous variations, 311T>A (Met104Lys), 509T>C (Met170Thr), 601A>G (Lys201Glu), 1553C>T (Ser518Leu), and 1738C>T (Arg580Stop), were found as heterozygotes. The allele frequencies were 0.008 for 1738C>T (Arg580Stop) and 0.003 for the four other variations. Arg580Stop having a stop codon at codon 580 results in loss of half of transmembrane domain (TMD) 11, TMD12, and a cytoplasmic tail, which might affect transport activity. In addition, novel variations, IVS12-1G>T at the splice acceptor site and -3A>C in the Kozak motif, were detected at 0.003 and 0.014 frequencies, respectively. Haplotype analysis using -11187G>A, -3A>C, IVS12-1G>T and 9 nonsynonymous variations revealed that the haplotype frequencies for **1b*, **5*, **15*, and **17* were 0.469, 0.000 (not detected), 0.037, and 0.133, respectively. These data would provide fundamental and useful information for pharmacogenetic studies on OATP1B1-transported drugs in Japanese.

Key words: ***SLCO1B1*; direct sequencing; novel genetic variation; amino acid change**

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Introduction

Organic anion transporting polypeptide 1B1 (OATP1B1, also known as OATP-C, OATP2 and LST-1) is a liver-specific transporter expressed on the sinusoidal membrane and mediates uptake of various endogenous and exogenous compounds from blood into hepatocytes.[1,2] Exogenous compounds include several 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (such as pravastatin), an active metabolite of irinotecan SN-38, methotrexate, and rifampicin; endogenous substrates include bilirubin and bilirubin glucuronide, cholate, leukotriene C_4, and estradiol-17β-glucuronide.[1,2]

OATP1B1 protein (691 amino acid residues) is encoded by *SLCO1B1*, which consists of 15 exons (including non-coding exon 1) and spans approximately 109 kb on chromosome 12p12.2-p12.1. Similar to other OATP family members, this transporter is predicted to have 12 transmembrane domains (TMDs).[1,3]

Several genetic polymorphisms and haplotypes with functional significance are already known in *SLCO1B1*. In Japanese, two haplotypes with nonsynonymous variations **1b* and **15* have been frequently reported. The *SLCO1B1*1b* haplotype with 388A>G (Asn130Asp) has been shown to have no altered transport activity from *in vitro* expression systems.[2,4-7] Recently, however, an *in vivo* study has suggested that the area under the concentration-time curve (AUC) of pravastatin is significantly lower in **1b/*1b* subjects than in **1a/*1a* subjects, suggesting increased transport activity possibly through increased protein expression.[8] Another major haplotype, *SLCO1B1*15* harboring both 388A>G (Asn130Asp) and 521T>C (Val174Ala), has been reported to show impaired plasma membrane expression[6] and reduced transport activity *in vitro*,[6,7] probably due to the Val174Ala substitution.[2,4,6] The association of the **15* haplotype with significant increases in AUC was reported for pravastatin,[2,9] and irinotecan and SN-38.[10] The haplotype frequencies of **1b*, **5* (with 521T>C, Val174Ala), and **15* were reported to be 0.46–0.54, 0.00–0.01, and 0.10–0.15, respectively, in Japanese.[5,11]

Recently, the *SLCO1B1*17* haplotype having 388A>G (Asn130Asp), 521T>C (Val174Ala), and -11187G>A was also shown to increase the AUC of pravastatin[9] and likely reduces the pravastatin efficacy on cholesterol synthesis,[12] although the effect of -11187G>A on transcriptional activity has not been clarified *in vitro*. The frequency of **17* has not, however, been reported in Japanese.

In this study, all 15 exons and their surrounding introns were resequenced for comprehensive screening of genetic variations in *SLCO1B1*. Sequence analysis detected 62 variations including 5 novel nonsynonymous ones from 177 Japanese subjects. Haplotype frequencies of **1b*, **5*, **15*, and **17* were also estimated.

Materials and Methods

Human genomic DNA samples: One hundred seventy-seven Japanese cancer patients administered irinotecan participated in this study and provided written informed consent. The ethical review boards of the National Cancer Center and the National Institute of Health Sciences approved this study. Whole blood was collected from the patients prior to the administration of irinotecan, and genomic DNA was extracted from blood leukocytes by standard methods.

PCR conditions for DNA sequencing and haplotype analysis: First, two sets of multiplex PCR were performed to amplify all 15 exons of *SLCO1B1* from 100 ng of genomic DNA using 1.25 units of *Z-Taq* (Takara Bio. Inc., Shiga, Japan) with 0.2 μM each of the mixed primers (Mix 1 and Mix 2) designed in the intronic regions as listed in **Table 1** (1st PCR). Mix 1 contained primers for amplifying exons 1 and 2, and 12 to 14, and Mix 2 contained primers for exons 3 to 7, 8 to 11, and 15. The first PCR conditions consisted of 30 cycles of 98 °C for 5 sec, 55°C for 10 sec, and 72°C for 190 sec. Next, each exon was amplified separately by *Ex-Taq* (0.625 units, Takara Bio. Inc.) with appropriate primers (0.5 μM) designed in the introns (**Table 1**, 2nd PCR). The conditions for the second round PCR were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. For amplification of exons 10 and 13, PCR was carried out under the following conditions: 94°C for 5 min followed by 33 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 30 sec, and then a final extension at 72°C for 7 min. Following PCR, the 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 1** (Sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany), and the eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All variations were confirmed by sequence analysis of PCR products generated by new amplification of the original genomic DNA templates.

As of July 18, 2007, the novel variations reported here are not found in the database of Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB Database (http://www.pharmgkb.org/).

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Table 1. Primer sequences used in this study

		Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified region[a]	Length (bp)
1st PCR	Mix 1	Exons 1 and 2	ACTCTGGGGCTAAAACCTATTGGAC	CTGCTTGCCATAACATCTTGAGGGT	14041049_14055679	14,631
		Exons 12 to 14	CTAGGGCTTTTATTGATAGGCAGGT	AAACTTCCAGACTGTCTTCTACCAT	14127745_14137752	10,008
	Mix 2	Exons 3 to 7	TTTGTTGAGAAGAGACTGTTAGGCA	GGAAAATGGATGAAGAAGCACTGGA	14083429_14092020	8,592
		Exons 8 to 11	AAGGACAGCACCAAGCAATGAAGGA	CATTCAACTCAGCAATCCCACTACC	14106917_14119592	12,676
		Exon 15	CTGAGGAGAACTGTAATTTGATGTC	TTTCCAGAGGCAAGCATTTACAACT	14148708_14152868	4,161
2nd PCR		Exon 1	TAACAGGCATAATCTTTGGTCT	AAGGGCTCAGAATGTAAGCG	14041915_14043281	1,367
		Exon 2	TCCTTAGGCTAGAATTTGTGT	CAAAGTGAGTCTCAAGACATT	14053227_14053950	724
		Exon 3	TGGCTGAGTAGTAGTACCTG	ATCCTCACTATCAACATTTTCA	14084394_14084961	568
		Exon 4	TGAGTGGTCTAATGTAGGTGA	AGGTGTAAGTGTTGAGGTCTT	14086324_14086946	623
		Exon 5	ATCTTTCTTGCTGGACACTTC	TATTAAGGAATTTGTTACAGGG	14088485_14089158	674
		Exons 6 and 7	CATAAGAATGGACTAATACACC	GGGAGACATTTTACATTTGGTT	14090310_14091202	893
		Exon 8	TTCCTAGACAGTATCTGTTGC	CTTCCACTTGTTATGTGCTCA	14108658_14109289	632
		Exon 9	AGTTACAAAACAGCACTTACG	TCAGGAACTCATCTAAAATAAG	14112156_14112798	643
		Exon 10	CAGGGGTTAAAACCTAGATGA	ATCCATGTATTTCTCTAAGCC	14114169_14114904	736
		Exon 11	TGGCAAAGATGGAGAGCGTA	AGTCAAATGAGGTGCTTCTTA	14117563_14118256	694
		Exon 12	TTGTCCAAAAGAGTATGTGCT	CAGCCTTGAGAGTTCATAGT	14128668_14129375	708
		Exon 13	TTGACCCAGCAATCCAACTAT	CCTTTTTTTTTTCATCATACCTAGT[b]	14133788_14134290	503
		Exon 14	ATATTAACCAACATAACTTCCA	CCTTGAATCACAGTTTCTTCG	14136297_14136980	684
		Exon 15	GATGGCTTAACAGGGCTTGA	TGCGGCAAATGATCTAGGAA	14150619_14151844	1,226
Sequencing		Exon 1	TAACAGGCATAATCTTTGGTCT	AAGGGCTCAGAATGTAAGCG		
			TATGTGAGAGAAGGGTCTGTA	CTACAGGTTACATTGGCATTT		
			AAATGCCAATGTAACCTGTAG	CTGAAATAAAGTACAGACCCT		
		Exon 2	TCCTTAGGCTAGAATTTGTGT	CAAAGTGAGTCTCAAGACATT		
		Exon 3	TGGCTGAGTAGTAGTACCTG	ATCCTCACTATCAACATTTTCA		
		Exon 4	TGAGTGGTCTAATGTAGGTGA	AGGTGTAAGTGTTGAGGTCTT		
		Exon 5	ATCTTTCTTGCTGGACACTTC	TATTAAGGAATTTGTTACAGGG		
		Exon 6	TTAAGAGTTTACAAGTAGTTAAA	AAGCAATTTTACTAGATGCCAA		
		Exon 7	CTTCTTTGTATTTAGGTAATGTA	ATAGTATAAATAGGAGCTGGAT		
		Exon 8	TTCCTAGACAGTATCTGTTGC	CTTCCACTTGTTATGTGCTCA		
		Exon 9	AGTTACAAAACAGCACTTACG	TCAGGAACTCATCTAAAATAAG		
		Exon 10	TTGATAGGTGCAGCAAACCAC	GGAATAAAAGAATGTGTTTGAG		
		Exon 11	TCTTTTTGATATATGTCTATCAT	AGTCAAATGAGGTGCTTCTTA		
		Exon 12	TTGTCCAAAAGAGTATGTGCT	CAGCCTTGAGAGTTCATAGT		
		Exon 13	GTTCTAACCACTTCCTCATAG	CCTTTTTTTTTTCATCATACCTAGT		
		Exon 14	TCCTTTTTACCATTCAGGCTTA	ACTAAAATGAGATACGAGATTG		
		Exon 15	GATGGCTTAACAGGGCTTGA	TGCGGCAAATGATCTAGGAA		
			CACATCTTTTATGGTGGAAGT	AGGCTTATTTATACTTCCACC		

[a]The reference sequence is NT_009714.16.
[b]Mismatched nucleotides at the 5′ end are underlined.

Furthermore, rare SNPs found in single patients as heterozygotes were confirmed by sequencing the PCR fragments produced by the amplification with a high fidelity DNA polymerase KOD-Plus- (TOYOBO, Tokyo, Japan).

Hardy-Weinberg equilibrium was analyzed by SNPAlyze version 3.1 (Dynacom Co., Yokohama, Japan). Estimation of *SLCO1B1* haplotypes was performed by an expectation-maximization based program, LDSUPPORT software.[13]

Results and Discussion

Sequence analysis from 177 Japanese subjects resulted in the identification of 62 genetic variations, including 28 novel ones (**Table 2**). Of these variations, 7 were located in the 5′-flanking region, 1 in the 5′-untranslated region (UTR), 13 in the coding exons (9 nonsynonymous and 4 synonymous variations), 5 in the 3′-UTR, and 36 in the introns. All detected variations were in Hardy-Weinberg equilibrium ($p > 0.05$).

Of the 9 nonsynonymous variations, 5 variations were novel: 311T>A (Met104Lys), 509T>C (Met170Thr), 601A>G (Lys201Glu), 1553C>T (Ser518Leu), and 1738C>T (Arg580Stop). All of these variations were found as heterozygotes with frequencies of 0.008 for 1738C>T (Arg580Stop) and 0.003 for the four other variations. Arg580, residing in TMD11, is conserved among human, rat and mouse OATP families.[1] The change from arginine residue to the immature termination codon leads to loss of this conserved amino acid

Table 2. Summary of *SLCO1B1* variations detected in this study

SNP ID					Position				
This Study	dbSNP (NCBI)	JSNP	Reference	Location	NT_009714.16	From the translational initiation site or from the end of the nearest exon	Nucleotide change	Amino acid change	Allele frequency (n = 354)
MPJ6_SB1_001[a]				5′-flanking	14042128	-11355 (-1078)[b]	ccaatactctcaA/Gtaattaaccaag		0.003
MPJ6_SB1_002	rs4149015	ssj0003132	9, 15	5′-flanking	14042296	-11187 (-910)[b]	tatgtgtatacaG/Agtaaaagtgtgt		0.153
MPJ6_SB1_003[a]				5′-flanking	14042494	-10989 (-712)[b]	atctctactcaaG/Aaaaacttttaac		0.076
MPJ6_SB1_004[a]				5′-flanking	14042530	-10953 (-676)[b]	cttcctttctccA/Tcaagtcaagtca		0.003
MPJ6_SB1_005	rs11835045			5′-flanking	14042793	-10690 (-413)[b]	atttgtcttaaaT/Ctatttcctattt		0.076
MPJ6_SB1_006[a]				5′-flanking	14042860	-10623 (-346)[b]	ttaaagaaaaaaA/-tcttatgccacc		0.003
MPJ6_SB1_007[a]				5′-flanking	14043018	-10465 (-188)[b]	aactaggttttaT/Catgtttgactag		0.003
MPJ6_SB1_008[a]				Intron 1	14043209	IVS1 + 65	ttcacggaagaaG/Catttgatggtc		0.014
MPJ6_SB1_009	rs2010668	ssj0003141		Intron 1	14053267	IVS1-155	tcctacttttgtG/Ttccagcattgac		0.113
MPJ6_SB1_010[a]				5′-UTR	14053480	-3	atctatatttcaA/Ctcatggaccaaa		0.014
MPJ6_SB1_011[a]				Intron 2	14053635	IVS2 + 69	tagaaaagcaagT/Ctgttaaaaagaa		0.003
MPJ6_SB1_012[a]				Intron 2	14053648	IVS2 + 82	tgttaaaaagaaC/Tattatgtttcaa		0.003
MPJ6_SB1_013[a]				Intron 2	14053734	IVS2 + 168	aaaccagtctttT/Caatctgattaag		0.008
MPJ6_SB1_014	rs4149021	ssj0003142	9	Intron 2	14053759	IVS2 + 193	tatttctttggcG/Aaaatttttgatg		0.153
MPJ6_SB1_015	rs12812795		9	Intron 2	14053769	IVS2 + 203	gcgaaatttttgA/Ttgcttaatagtt		0.003
MPJ6_SB1_016[a]				Intron 2	14053807	IVS2 + 241	aatttagaaaataT/Ctttgatagcttc		0.006
MPJ6_SB1_017	rs12303784			Intron 2	14053814	IVS2 + 248	aaaatattttgatA/Ggcttctctttgg		0.003
MPJ6_SB1_018[a]				Intron 2	14084429	IVS2-129	aaagggaaaactA/Gagtatggttttt		0.003
MPJ6_SB1_019[a]				Intron 2	14084478	IVS2-80	aaagaagaaagcT/Cattataattcca		0.008
MPJ6_SB1_020	rs2291073	JST-043317		Intron 3	14084788	IVS3 + 89	actggggtaaatT/Gtatctctcacag		0.271
MPJ6_SB1_021	rs2291074	JST-043318		Intron 3	14084923	IVS3 + 224	attctataatgcA/Gcaaagaatgatg		0.243
MPJ6_SB1_022[a]				Exon 4	14086569	311	gttgtttcattaT/Agggaattggagg	Met104Lys	0.003
MPJ6_SB1_023	rs4149036	ssj0003160		Intron 4	14086714	IVS4 + 97	ataggcagttacC/Attttgagaagat		0.427
MPJ6_SB1_024[a]				Intron 4	14088523	IVS4-161	cacttttacccaT/Ccacatctcttaa		0.017
MPJ6_SB1_025	rs2306283	JST-063865	4, 5, 9, 11, 15	Exon 5	14088712	388	gaaactaatatcA/Gattcatcagaaa	Asn130Asp	0.667
MPJ6_SB1_026	rs2306282	JST-063864	11	Exon 5	14088776	452	ttttatcactcaA/Gtagagcatcacc	Asn151Ser	0.034
MPJ6_SB1_027	rs4149044	ssj0003170	9	Intron 5	14088970	IVS5 + 165	cacagttcgcccA/Tttaacaacacag		0.427
MPJ6_SB1_028	rs4149045	ssj0003171	9	Intron 5	14088994	IVS5 + 189	ggttttaaactacG/Acgttttcacttc		0.429
MPJ6_SB1_029	rs4149046	ssj0003172	9	Intron 5	14088996	IVS5 + 191	tttaaactacgcG/Attttcacttcta		0.331
MPJ6_SB1_030	rs4149096	ssj0003230	9	Intron 5	14090372_14090377	IVS5-107_112	aaattacttgtaCTTGTA/-aattaaaaaaaa		0.427
MPJ6_SB1_031[a]				Intron 5	14090469	IVS5-15	aaatgaaacactC/Gtcttatctacat		0.003
MPJ6_SB1_032[a]				Exon 6	14090511	509	ctgggtcatacaT/Cgtggatatatgt	Met170Thr	0.003
MPJ6_SB1_033	rs4149056	ssj0003182	4, 5, 9, 11, 15	Exon 6	14090523	521	tgtggatatatgT/Cgttcatgggtaa	Val174Ala	0.175
MPJ6_SB1_034	rs4149057	ssj0003183	9, 11, 15	Exon 6	14090573	571	cccatagtaccaT/Ctggggctttctt	Leu191Leu	0.333
MPJ6_SB1_035[a]				Exon 6	14090578	576	agtaccattgggG/Actttcttacatt	Gly192Gly	0.003
MPJ6_SB1_036	rs2291075	JST-043319	9, 11, 15	Exon 6	14090599	597	cattgatgatttC/Tgctaaagaagga	Phe199Phe	0.427
MPJ6_SB1_037[a]				Exon 6	14090603	601	gatgatttcgctA/Gaagaaggacatt	Lys201Glu	0.003
MPJ6_SB1_038	rs2291076	JST-043320	9	Intron 7	14090961	IVS7 + 33	gtaccatgataaC/Tgtctttctaagc		0.336
MPJ6_SB1_039			11	Exon 9	14112452	1007	tccttactaatcC/Gcctgtatgttat	Pro336Arg	0.006
MPJ6_SB1_040[a]				Intron 9	14114331	IVS9-68	ttgacatacattG/Ctgtttcatctat		0.003
MPJ6_SB1_041	rs4149099	JST-080069	9	Intron 10	14117669_14117670	IVS10-106_-107	tttatctacttt-/CTTttttccctcttt		0.647
MPJ6_SB1_042			11	Intron 10	14117728_14117730	IVS10-46_-48	cttctttgtcttTTT/-cttctctctctc		0.003
MPJ6_SB1_043	rs4149070	ssj0003204	9	Intron 11	14128857	IVS11-170	gaaagaaatccaC/Gaaaactatttta		0.280
MPJ6_SB1_044	rs4149071	ssj0003205	9	Intron 11	14128938	IVS11-89	agtttgaacaagT/Cgagacttcacta		0.280
MPJ6_SB1_045	rs4149100	ssj0003234	9	Intron 11	14128952	IVS11-75	agacttcactaaA/-tataatgcaatg		0.395
MPJ6_SB1_046	rs4149072	ssj0003206	9	Intron 11	14128959	IVS11-68	actaaatataatG/Acaatgtatttgc		0.280
MPJ6_SB1_047			11	Intron 11	14129015	IVS11-12	catattttatacA/Gcaacgcttaagg		0.014
MPJ6_SB1_048[a]				Exon 12	14129082	1553	acagaaattactC/Tagcccatttggg	Ser518Leu	0.003
MPJ6_SB1_049	rs987839			Intron 12	14133812	IVS12-396	tccaactattggG/Atatctaccaaaa		0.316
MPJ6_SB1_050[a]				Intron 12	14134097	IVS12-111	ggggccattcaaC/Ttgtgagcttaat		0.020
MPJ6_SB1_051[a]				Intron 12	14134207	IVS12-1	tgtcttgtttcaG/Taattgttcaacc		0.003
MPJ6_SB1_052[a]				Exon 13	14134263	1738	tcaatggttataC/Tgagcactaggta	Arg580Stop	0.008
MPJ6_SB1_053	rs4149080	ssj0003214	9	Intron 13	14136533	IVS13-97	ctccaaatttttG/Caacttttattta		0.395
MPJ6_SB1_054	rs11045875		11	Intron 14	14136797	IVS14 + 50	gactatattaatT/Gcctaaaaaatat		0.011
MPJ6_SB1_055[a]				Intron 14	14150655	IVS14-232	tatatttttctcG/Attttatgaagaa		0.006
MPJ6_SB1_056[a]				Intron 14	14150656	IVS14-231	atatttttctcgT/Ctttatgaagaag		0.251
MPJ6_SB1_057[a]				Exon 15	14151004	1983	tgcatcagaaaaT/Cggaagtgtcatg	Asn661Asn	0.006
MPJ6_SB1_058[a]				3′-UTR	14151137	2116 (*40)[c]	tgtgtttccaaaC/Gagcattgcattg		0.011
MPJ6_SB1_059	rs4149085	ssj0003219		3′-UTR	14151264	2243 (*167)[c]	acaaactgtaggT/Cagaaaaaatgag		0.251
MPJ6_SB1_060	rs4149086	ssj0003220		3′-UTR	14151425	2404 (*328)[c]	aaacaaatgagtA/Gtcatacaggtag		0.025
MPJ6_SB1_061	rs4149087	ssj0003221	15	3′-UTR	14151536	2515 (*439)[c]	gaactataatacG/Taaggcctgaagt		0.333
MPJ6_SB1_062	rs4149088	ssj0003222		3′-UTR	14151560	2539 (*463)[c]	tctagcttggatG/Atatgctacaata		0.333

[a]Novel variations detected in this study.
[b]Intron 1 is skipped for counting.
[c]Positions are shown as * and bases from the translational termination codon TAA.

along with the subsequent half of TMD11, TMD12 and the cytoplasmic tail,[1] which very likely affects transport activity. Other variations 311T>A (Met104Lys), 509T>C (Met170Thr), 601A>G (Lys201Glu), and 1553C>T (Ser518Leu) are located in TMD3, TMD4, the short cytoplasmic loop between TMD4 and TMD5, and the large extracellular loop between TMD9 and TMD10, respectively.[1] Using the PolyPhen program (http://genetics.bwh.harvard.edu/pph/) to predict the functional effects of the four amino acid substitutions, three substitutions, Met104Lys, Met170Thr and Ser518Leu, were expected to alter the protein function based on the PSIC (position-specific independent count) score differences derived from multiple alignments. The functional significance of these 5 novel nonsynonymous variations should be clarified in the future. In addition, a novel variation at the splice acceptor site, IVS12-1 G>T, was detected at a 0.003 frequency. This variation might cause aberrant splicing of *SLCO1B1* pre-mRNA and thus influence the expression level of active protein. Furthermore, -3A>C might reduce translational efficiency since this purine-to-pyrimidine alteration results in a deviation from the Kozak motif, where the purine nucleotide at position -3 from the translational initiation codon is important.[14]

Four known variations, 388A>G (Asn130Asp), 452A>G (Asn151Ser), 521T>C (Val174Ala), and 1007C>G (Pro336Arg), were detected at 0.667, 0.034, 0.175, and 0.006 frequencies, respectively, which are similar to the Japanese data reported previously.[5,11] The allele frequencies of 521T>C (Val174Ala) in Japanese (0.11–0.18) are comparable to those in other Asian populations (0.04–0.25) and Caucasians (0.14–0.22), but higher than that in African-Americans (0.02).[10,15,16] The frequencies of 388A>G (Asn130Asp) in Japanese (0.63–0.67) are also similar to those in other Asians (0.57–0.88) and African-Americans (0.75), but higher than those in Caucasians (0.30–0.51).[10,15,16] Variations 452A>G (Asn151Ser) and 1007C>G (Pro336Arg) have not been reported in other ethnic populations. Analysis of these four known variations with PolyPhen program showed that only Val174Ala was expected to alter protein function, which is consistent with the previous functional analysis.[2,4,6] Variations 1454G>T (Cys485Phe) and 1628T>G (Leu543Trp) previously reported in Japanese were not detected in this study.[11,17] Hepatocyte nuclear factor 1α is known to transactivate *SLCO1B1* through binding to the promoter region (from -10432 to -10420 from the translational start codon);[18] however, no variation was found in this region.

Using -11187G>A, -3A>C, IVS12-1G>T and 9 nonsynonymous variations, diplotype configuration was estimated for each subject. The configuration was estimated with >0.99 probabilities for all but four subjects. The predicted haplotype frequencies for **1b* [harboring 388A>G (Asn130Asp)], **5* [harboring 521T>C (Val174Ala)], **15* [harboring 388A>G (Asn130Asp) and 521T>C (Val174Ala)] and **17* [harboring -11187G>A, 388A>G (Asn130Asp), and 521T>C (Val174Ala)] were 0.469, 0.000 (not detected), 0.037 and 0.133, respectively. The haplotype frequencies for **1b* and **5* are similar to those in the previous studies in Japanese.[5,11] The **17* frequency is higher than those in Chinese (0.085), Finnish Caucasians (0.069), Malay (0.029) and Indians (0.009).[15,16] It should be noted that 76% (n=47 alleles) of 521T>C (Val174Ala)-bearing haplotypes were assigned as **17*, and 21% (n=13) of them as **15*. The remaining two (3%) was estimated to exist with 1007C>G (Pro336Arg) and **17* variations [-11187G>A, 388A>G (Asn130Asp), and 521T>C (Val174Ala)] on the same chromosomes. The **17* ratio in 521T>C (Val174Ala)-bearing haplotypes is similar to that in Chinese (65%), but higher than those in Finnish Caucasians (34%), Malay (26%) and Indians (14%).[15,16] Variation 452A>G (Asn151Ser, n=12 alleles) or 1738C>T (Arg580Stop, n=3) were predicted to be on the **1a* background (no other variation).

In conclusion, 62 genetic variations were identified, including 28 novel ones, in *SLCO1B1*. One novel nonsynonymous variation results in a truncated protein and four novel nonsynonymous variations result in amino acids substitutions. In addition, novel variations IVS12-1 G>T at the splice acceptor site and -3A>C in the Kozak motif were detected. Approximately 76% of 521T>C (Val174Ala)-bearing haplotypes were assigned as **17* and the majority of the remaining haplotypes were **15*. This information would be useful for pharmacogenetic studies to investigate the associations of *SLCO1B1* variations with interindividual differences in drug disposition.

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SNP Communication

Genetic Variations of VDR/NR1I1 Encoding Vitamin D Receptor in a Japanese Population

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Summary: The vitamin D receptor (VDR) is a transcriptional factor responsive to 1α,25-dihydroxyvitamin D_3 and lithocholic acid, and induces expression of drug metabolizing enzymes CYP3A4, CYP2B6 and CYP2C9. In this study, the promoter regions, 14 exons (including 6 exon 1's) and their flanking introns of *VDR* were comprehensively screened for genetic variations in 107 Japanese subjects. Sixty-one genetic variations including 25 novel ones were found: 9 in the 5′-flanking region, 2 in the 5′-untranslated region (UTR), 7 in the coding exons (5 synonymous and 2 nonsynonymous variations), 12 in the 3′-UTR, 19 in the introns between the exon 1's, and 12 in introns 2 to 8. Of these, one novel nonsynonymous variation, 154A>G (Met52Val), was detected with an allele frequency of 0.005. The single nucleotide polymorphisms (SNPs) that increase VDR expression or activity, -29649G>A, 2T>C and 1592(*308)C>A tagging linked variations in the 3′-UTR, were detected at 0.430, 0.636, and 0.318 allele frequencies, respectively. Another SNP, -26930A>G, with reduced *VDR* transcription was found at a 0.028 frequency. These findings would be useful for association studies on *VDR* variations in Japanese.

Key words: *VDR*; SNPs; nonsynonymous variation; Japanese

Introduction

The vitamin D receptor (VDR) is a nuclear receptor, which acts as a transcriptional factor upon binding of the active form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], and lithocholic acid.[1-3] Ligand-activated VDR forms a heterodimer with retinoid X receptor, binds to the vitamin D responsive-element and induces expression of its target genes, resulting in partial arrest in G_0/G_1 of the cell cycle, induction of differentiation, or control of calcium homeostatis and maintenance of bone. In addition, VDR has been shown to be involved in induction of drug metabolizing enzymes CYP3A4, CYP2B6 and CYP2C9 in human primary hepatocytes,[2,4] and CYP3A4 in intestinal cell lines.[2,5] VDR and pregnane X receptor share 63% amino acid sequence identity in their DNA binding domains.[2] Like pregnane X receptor and constitutive androstane receptor, VDR transactivates *CYP3A4* through binding to its distal DR3 and proximal ER6 elements.[4,5] Recently, CYP3A4 has been shown to catalyze hydroxylation of

As of August 16, 2007, the novel variations reported here are not found in the database of Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB Database (http://www.pharmgkb.org/).

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$1,25(OH)_2D_3$ in human liver and intestine, suggesting negative feedback control of $1,25(OH)_2D_3$ action.[6]

The *VDR* gene consists of 6 exon 1's (exons 1f, 1e, 1a, 1d, 1b, and 1c in this order) and 8 other exons (exons 2 to 9), and spans approximately 100 kb at chromosome 12q13.11.[7] The VDR protein is expressed in many tissues including kidney, skin and liver.[1] Alternative usage of exon 1's yields 14 transcripts including 3 major types: exon 1a-1c, exon 1d-1c or exon 1f-1c combinations, followed by common exons 2–9.[7] A translational initiation codon in exon 2 is used in transcripts starting from exon 1a or 1f. In the transcript from exon 1d (1d transcript), translation could initiate from the ATG codon in exon 1d, generating an active protein 50 amino acids longer than that from the exon 1a- or 1f-containing transcript (1a or 1f transcript).[7,8] However, the 1d and 1f transcripts are assumed to be minor (expression levels were less than 10 and 20%, respectively, of the 1a transcript).[7]

Genetic polymorphisms of transcriptional factors involved in induction of drug metabolizing enzymes could influence their expression levels, and as a result drug pharmacokinetics/pharmacodynamics. As for *VDR*, several polymorphisms with functional significance have been reported.[9] For example, the single nucleotide polymorphism (SNP) 2T>C (*Fok*I polymorphism) in the first ATG codon of 1a and 1f transcripts results in generation of a three amino acid shorter protein, which has a significantly higher transcriptional activity than the longer transcript.[9,10] Another SNP -29649G>A upstream of exon 1e (thus exons 1a and 1d) is located in the Cdx2 (an intestinal transcriptional factor)-binding element. This nucleotide change facilitates Cdx2 binding, resulting in increased *VDR* transcription.[9,11]

Recently, Nejentsev *et al.* resequenced *VDR* and found 245 genetic variations in Caucasians (Britons).[12] However, no comprehensive screening of *VDR* polymorphisms has been reported for Asian populations including Japanese. In this study, we searched for *VDR* variations by resequencing the promoter regions, all 14 exons and their surrounding introns from 107 Japanese subjects.

Materials and Methods

Human genomic DNA samples: One hundred and seven Japanese cancer patients administered paclitaxel were analyzed. Written informed consent was obtained from all subjects. The ethical review boards of the National Cancer Center and the National Institute of Health Sciences approved this study. DNA was extracted from whole blood, which was collected from the patients prior to paclitaxel administration.

DNA sequencing

Amplification of exon 1's, and exons 2 to 6: First, three sets of PCRs were separately performed to amplify exons 1f to 1b (Mix 1 primer set), the Cdx2 region (Mix 2), and exons 1c to 6 (Mix 3) from 50 ng of genomic DNA using 1.25 units of Z-Taq (Takara Bio Inc., Shiga, Japan) with 2 μM of each primer designed in the intronic regions (**Table 1**, 1st PCR). The first PCR conditions were 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 190 sec. Next, exon 1's and exon 2 with high GC content were amplified separately from the 1st PCR product as a template with 2.5 units of LA-Taq (Takara Bio Inc.) in GC buffer I and 1 μM of each primer (2nd PCR in **Table 1**). For the Cdx2 region and exons 3 to 6, PCR reactions were performed using Ex-Taq (1 unit, Takara Bio Inc.) and 0.4 μM of primers in **Table 1**. The second PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension for 7 min at 72°C.

Amplification of exons 7 to 9: These exons were directly amplified from 50 ng DNA. Amplification was performed with Ex-Taq for exons 7 to 8, 9-1 and 9-3, and with LA-Taq in GC buffer I for exon 9-2. Primer concentrations, polymerase units and PCR conditions were the same as described above for the second round PCR.

Sequencing: 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 ver. 3.1 (Applied Biosystems, Foster City, CA, USA) with sequencing primers listed in **Table 1** (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). All novel SNPs were verified by sequencing PCR products obtained by a new genomic DNA amplification. Under the conditions used, up to 1,640 bases upstream of exon 1f, up to 4,090 (4,460) bases upstream of exon 1a (1d), all exons and their flanking introns were successfully sequenced for all subjects. *VDR* genomic sequence (NT_029419.11) and cDNA sequence (exon 1a-1c transcript, NM_000376.2) obtained from GenBank were used as reference sequences. Nucleotide positions based on cDNA sequence were numbered from the adenine of the translational start site (10416202 in NT_029419.11 and 161 in NM_000376.2) or the nearest exons (for introns 2 to 8).

Other analysis: Hardy-Weinberg equilibrium and linkage disequilibrium (LD) analyses were performed with SNPAlyze version 3.1 (Dynacom Co., Yokohama, Japan), and pairwise LDs between variations were obtained for rho square (r^2) values. P-values for Hardy-Weinberg equilibrium were corrected by false-discovery

Table 1. Primers used for sequencing *VDR*

		Amplified or sequensed region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified length (bp)
1st PCR	Mix 1	Exon 1f	TTGTTGACTCTCCTGGCTTTATCAG	CCAGTCACTTTGAAGAGAAACCTGC	3,823
		Exon 1e, 1a, 1d, 1b	TGGTCCCTTCTGTCTTTCTAACTCC	TTGGGAGGATGTAGAACCGTGGGAT	10,421
	Mix 2	Cdx2	ACTGGTGGTGCCCTACTCTTTCT	TTGGGAGGATGTAGAACCGTGGGAT	11,835
	Mix 3	Exon 1c to Exon 2	GCTAAAAGTGGCAGAAAACATCCTG	TTGTGTTGACAGAGAGACCCCAAGT	5,476
		Exon 3	TAGCATCCTTACTTCTCATAGCGGC	CCTGTTCTGTGACTTATCCTCTGTC	2,556
		Exons 4 to 6	TGGTGTGGCTGGCAGAAACAGGTCT	TCTCTCAAAGTGTTGGGATAGGCAT	4,847
		Exons 7 to 8	AACACTCTTGTCCCTTCCAG	TCTGTGTCTCCTTTTGCTAC	753
		Exon 9-1	GTCAGCAGTCATAGAGGGGT	CAGATGGAGAAGATGCGGCT	1,327
		Exon 9-2	AGAGGGTCTGGAGAAGCAGT	AATGAGGGGATTGACTCGTT	1,012
		Exon 9-3	TCTTCAGTGGGAGAAAACAC	CTGTCAAATGGGGGTAATAA	1,417
2nd PCT		Exon 1f	GAACCCTTTTCTCTGCCCTCACCTT	AGACAGAGGACTGGAGAAGGAGATA	606
			ACTCTGCTCCTTCACCACCTCTACA	AAACTGAGTGCGTGTGAGTGAGAGA	628
			GCTGGAGGTGTTGAACTGGTTGCTT	CGCATACCCGACACTTGTTCACCTC	851
		Cdx 2	GTAATCCTTCCACCTCAACTTCCTA	CCACAGAGTCCAAAGAAAGGCAGGG	1,148
		Exon 1e	AGAGGAGAGGGTCTGGAGAG	TTCCAACCACCAATACCTTG	640
		Exon 1e to 1a	GCAGAGAATGTCCCAAGGTA	GACCGTCGTCCATAGGGCAA	1,411
		Exon 1a to 1d	AGAAGCGTGCCTTGCCCTAT	CTGAACATCTATTGACAGGC	871
		Exon 1b	CTGGAAGGCAAATAGGAAAC	GCCGTGTAAAGCAGTGGTTA	368
		Exon 1c	GCTGTGAGAGGAGAAGGAGT	GCAAAATCCTGGGTGGTATC	517
		Exon 2	GTGAACCACTAAACCCAAAT	GAAGGAGATGTGAAAAATGC	528
		Exon 3	TCTGTTGGAGAAATGGAGAC	GCCTCTGACACCAACACACA	478
		Exons 4 to 5	TGCCTTCCTTTTCACCATAG	AGAGGGGCTGTTGTGAAGAC	912
		Exon 6	TGAAAGAGGCAGAGAGAGTC	GGTTATAGTGAGCCAAGATAGTG	719
Sequencing		Exon 1f	GAACCCTTTTCTCTGCCCTCACCTT	AGACAGAGGACTGGAGAAGGAGATA	
			ACTCTGCTCCTTCACCACCTCTACA	AAACTGAGTGCGTGTGAGTGAGAGA	
			GCTGGAGGTGTTGAACTGGTTGCTT	CAGGACAGCAGGACCTTCAGGGAAC	
			CACTGACTCTCACCTTCCTTTCCTC	CGCATACCCGACACTTGTTCACCTC	
			GGTGGCTCCCTCCTGCTGTGTGG		
		Cdx2	AGGAAGGAAGGAAAAGAGGAT	TGGAGTTAGAAAGACAGAAGGGACC	
		Exon 1e	TGTTTTTTAGAGGCAGCAT	TTCCAACCACCAATACCTTG	
			TAGAGGCAGCATGAAACAGT	GATGGATGGATTCTCTACCT	
		Exon 1e to 1a	GCAGAGAATGTCCCAAGGTA	CTCACAAAATCATCCAGCAG	
			CTGGCTAAAGGAGGTCATCG	CGCTCGCCAACCTGTTACTG	
			CAGTAACAGGTTGGCGAGCG	GACCGTCGTCCATAGGGCAA	
			CGACTGCTGGATGATTTTGT	CGATGATTATAGGTGCGGAT	
		Exon 1a	AGCGTGCCTTGCCCTATGGA	TTTACCGCTGAGACTTAGAC	
		ERxon 1d	AAACTTGGCTACTGAGGTCC	TAAAAGACCCAACTCCACC	
		Exon 1b	CTGGAAGGCAAATAGGAAAC	GCCGTGTAAAGCAGTGGTTA	
		Exon 1c	CTTCCCACTGCTCCTGCTAC	GGTGGTATCCCTTCCTTCCC	
		Exon 2	GTGAACCACTAAACCCAAAT	GAAGGAGATGTGAAAAATGC	
		Exon 3	TCTGTTGGAGAAATGGAGAC	GCCTCTGACACCAACACACA	
		Exon 4	TGCCTTCCTTTTCACCATAG	CGTCCCTACCCCAGTTCTGT	
		Exon 5	ACAGAACTGGGGTAGGGACG	AGAGGGGCTGTTGTGAAGAC	
		Exon 6	TGAAAGAGGCAGAGAGAGTC	GAAGTTTCTTACCTGAATCCTGG	
		Exon 6	CACTCATCCACCACTTCTTT	TGGTGTCTGGTGCCTGTAATCCC	
		Exons 7 to 8	AACACTCTTGTCCCTTCCAG	TCTGTGTCTCCTTTTGCTAC	
		Exon 9-1	GGTGCTGCCGTTGAGTGTCT	ACATCAGTCAGCAGCCACTT	
			AAGTGGCTGCTGACTGATGT	GAAGATGCGGCTCACTGCTT	
		Exon 9-2	AGCCGCATCTTCTCCATCTG	GCCGCATTCCCCAAACTCAA	
			TTGAGTTTGGGGAATGCGGC	AATGAGGGGATTGACTCGTT	
		Exon 9-3	TCTTCAGTGGGAGAAAACAC	ATGTTGGTCAGGTTGGTCTC	
			GGAGGCTGAGGCAGAAGAAT	CCAGGGCTGAGTAACTGATA	
			CCTGCCTTCTTCGGGGAACT	CTGTCAAATGGGGGTAATAA	

Table 2. Summary of *VDR* variations detected in this study

SNP ID		Location	Position		Nucleotide change	Amino acid change	Allele frequency (n = 214)
This Study	dbSNP (NCBI)		NT_029419.11	From the translational start site[d] or from the end of the nearest exon			
MPJ6_VDR_001	rs4547172	5′-flanking	10481728	-65526	tccatatttatcT/Cttttatacttct		0.238
MPJ6_VDR_002	rs4583039	5′-flanking	10481720	-65518	tatccttttataC/Tttctttcaagt		0.252
MPJ6_VDR_003	rs7970376	5′-flanking	10481502	-65300	cattaaacagatG/Atatcatcatctg		0.037
MPJ6_VDR_004	rs4237856	5′-flanking	10481356	-65154	cccaggctcagcT/Ggccctttgacat		0.196
MPJ6_VDR_005[a]		5′-flanking	10480937	-64735	tgtgccaggtagG/Ccgtggtgccccc		0.028
MPJ6_VDR_006	rs3923693	5′-flanking	10480935	-64733	tgccaggtaggcG/Atggtgcccccgc		0.327
MPJ6_VDR_007	rs4073726	5′-flanking	10480623	-64421	cgtcctccctggG/Ccaagccatctcc		0.037
MPJ6_VDR_008[a]		5′-flanking	10480382	-64180	cttctggtgcccC/Gcctgctccctca		0.061
MPJ6_VDR_009	rs4073729	5′-flanking	10480375	-64173	tgccccccctgctC/Tctcatggccag		0.229
MPJ6_VDR_010	rs11168297	inton 1f-1e[b]	10446202	-30000	gtactgggattaC/Taggcctgagcca		0.028
MPJ6_VDR_011	rs17880972	inton 1f-1e[b]	10446166	-29964	aggatttttaatA/Gtgtattttggg		0.028
MPJ6_VDR_012	rs11568820	inton 1f-1e[b]	10445851	-29649 (Cdx2)	aactaggtcacaG/Ataaaaacttatt		0.430
MPJ6_VDR_013[a]		inton 1f-1e[b]	10445734	-29532	cattctatttttA/Gccatctggaaca		0.005
MPJ6_VDR_014[a]		inton 1f-1e[b]	10445338	-29136	catggggacaggG/Atctctgggagac		0.005
MPJ6_VDR_015	rs11614332	inton 1f-1e[b]	10445204	-29002	gatgaggacaggC/Ttgcagtcctgtg		0.028
MPJ6_VDR_016[a]		inton 1e-1a[b]	10443873	-27671	aacaaaggctgtG/Aaaaaaagactaa		0.028
MPJ6_VDR_017[a]		inton 1e-1a[b]	10443771	-27569	gtcaagcaccacA/Taaagtgacttct		0.028
MPJ6_VDR_018	rs7139166	inton 1e-1a[b]	10443640	-27438	tagctttcccacG/Catgctttgggca		0.028
MPJ6_VDR_019[a]		inton 1e-1a[b]	10443419	-27217	cagtgcttaacaA/Gacttcactgttg		0.005
MPJ6_VDR_020	rs4516035	inton 1e-1a[b]	10443132	-26930 (GATA)	gcgaatagcaatA/Gtcttccctggct		0.028
MPJ6_VDR_021	rs11574006	inton 1e-1a[b]	10442825	-26623	tgccacggggcgG/Tgggggaaggcg		0.023
MPJ6_VDR_022	rs11574007	inton 1e-1a[b]	10442819_10442818	-26617_-26616	gggcgggggggg/insG/aaggcggaactc		0.014
MPJ6_VDR_023	rs11574007	inton 1e-1a[b]	10442819_10442818	-26617_-26616	gggcgggggggg/insGG/aaggcggaactc		0.023
MPJ6_VDR_024[a]		inton 1e-1a[b]	10442795	-26593	cgggaccagggaC/Tcagggaagctga		0.028
MPJ6_VDR_025[a]		inton 1e-1a[b]	10442587	-26385	tggcgagcggagC/Tccgggatttccc		0.028
MPJ6_VDR_026[a]		inton 1e-1a[b]	10442542	-26340	cggtgccagtcgG/Tcaggegccccc		0.023
MPJ6_VDR_027	rs11574012	inton 1d-1b[b]	10441601	-25399	ggtcacctgtgaT/Cggtggagttggg		0.093
MPJ6_VDR_028	rs11168293	exon 1b (5′-UTR)	10437022	-20820	agcccagctggaC/Aggagaaatggac		0.028
MPJ6_VDR_029[a]		exon 1b (5′-UTR)	10436972	-20770	ccaggccccgtgC/Tacattgctttgc		0.014
MPJ6_VDR_030	rs11168292	inton 1b-1c[b]	10436911	-20709	aagtacagttatG/Cttctctagcagg		0.028
MPJ6_VDR_031	rs10735810	exon 2	10416201	2 (*FokI*)	ttcttacagggaT/Cggaggcaatggc	Translational start site change	0.636
MPJ6_VDR_032	rs10783218	intron 2	10416049	IVS2+8	ttcaggtgagccC/Ttcctcccaggct		0.065
MPJ6_VDR_033[a]		intron 2	10415986	IVS2+71	tccatgaagggaG/Acccttgcatttt		0.005
MPJ6_VDR_034[a]		exon 3	10402259	154	ggcaggcgaagcA/Gtgaagcggaagg	Met52Val	0.005
MPJ6_VDR_035	rs11168267	intron 3	10394848	IVS3-71	ggacctttacccC/Tcaaccgcaggag		0.151
MPJ6_VDR_036	rs11168266	intron 3	10394839	IVS3-62	cccccaaccgcaG/Agaggaaggtttc		0.360
MPJ6_VDR_037[a]		intron 3	10394802	IVS3-25	ctggccagccctC/Tctgactcccctt		0.005
MPJ6_VDR_038[a]		intron 4	10394565	IVS4+28	gggaggatgagcC/Tggtccagaggag		0.014
MPJ6_VDR_039[a]		exon 5	10394225	576	tcactgtatcacC/Ttcttcaggtaag	Thr192Thr	0.005
MPJ6_VDR_040[a]		intron 6	10392586	IVS6+133	cttgctctgtcaC/Tgcaggctggagt		0.014
MPJ6_VDR_041	rs11168265	intron 6	10392565	IVS6+154	gagtgcagtggcG/Acgatctcggctc		0.150
MPJ6_VDR_042	rs11574093	intron 6	10392429_10392428	IVS6+290_291	atttatttattt/insATTT/ttatttttttc		0.187
MPJ6_VDR_043[a]		exon 7	10383807	846	cttcaccatggaC/Tgacatgtcctgg	Asp282Asp	0.005
MPJ6_VDR_044	rs11574113	intron 8	10382206	IVS8-112	catagaggggtgG/Ccctaggggtgc		0.154
MPJ6_VDR_045[a]		intron 8	10382176	IVS8-82	ttgagtgtctgtG/Atgggtgggggt		0.047
MPJ6_VDR_046	rs7975232	intron 8	10382143	IVS8-49 (*ApaI*)	tgagcagtgaggG/Tgcccagctgaga		0.318
MPJ6_VDR_047	rs731236	exon 9	10382063	1056 (*TaqI*)	cgccgcgctgatT/Cgaggccatccag	Ile352Ile	0.164
MPJ6_VDR_048[a]		exon 9	10381988	1131	cccgggcagccaC/Tctgctctatgcc	His377His	0.005
MPJ6_VDR_049	rs2229829	exon 9	10381913	1206	caagcagtaccgC/Atgcctctccttc	Arg402Arg	0.014
MPJ6_VDR_050	rs739837	3′-UTR	10381527	1592 (*308)[c]	ctccaccgctgcC/Ataagtggctgct		0.318
MPJ6_VDR_051	rs3847987	3′-UTR	10381374	1745(*461)[c]	gataaaataatcgG/Tcccacagctccc		0.154
MPJ6_VDR_052[a]		3′-UTR	10381348	1771 (*487)[c]	ccccaccccttC/Tagtgcccaccaa		0.005
MPJ6_VDR_053[a]		3′-UTR	10381047	2072 (*788)[c]	cgacctcgtcctC/Acccctgccagtg		0.005
MPJ6_VDR_054	rs11574125	3′-UTR	10381042	2077 (*793)[c]	tcgtcctccccc/delT/gccagtgcctta		0.318
MPJ6_VDR_055[a]		3′-UTR	10380870	2249 (*965)[c]	gaagaattttcaG/Caccccagcggct		0.005
MPJ6_VDR_056	rs11574129	3′-UTR	10380609	2510 (*1226)[c]	tcaagtgcatgcT/Cctctgcagccag		0.126
MPJ6_VDR_057	rs11574131	3′-UTR	10380516	2603 (*1319)[c]	aggtgtgcgggaC/Tcggtacagaaag		0.042
MPJ6_VDR_058	rs9729	3′-UTR	10379929	3190 (*1906)[c]	aatcccctcattC/Aaggaaaactgac		0.322
MPJ6_VDR_059[a]		3′-UTR	10379840	3279 (*1995)[c]	gcggtggctcacG/Acctgtaatccca		0.005
MPJ6_VDR_060	rs2853562	3′-UTR	10379692	3427 (*2143)[c]	catggtggcgcaA/Tgcctgtaatccc		0.322
MPJ6_VDR_061[a]		3′-UTR	10379608	3511 (*2227)[c]	tgagatcgtgccG/Attactctccaac		0.037

[a]Novel variations detected in this study.
[b]The intronic region between the two exon 1's indicated.
[c]The position with an asterisk in parenthesis is numbered from the translational termination codon TGA.
[d]The translational start site in exon 2 was used for numbering according to NM_000376.2.

rate methods.[13]

Results and Discussion

We found 61 genetic variations, including 25 novel ones, from 107 Japanese subjects (**Table 2**). Of them, 9 were located in the 5′-flanking region of exon 1f, 19 in the introns between the exon 1's, 2 in the 5′-untranslated region (UTR), 7 in the coding exons (5 synonymous and 2 nonsynonymous variations), 12 in the 3′-UTR, and 12 in introns 2 to 8. All observed allele frequencies were in Hardy-Weinberg equilibrium ($p>0.05$) after correction for multiple comparison, except for 3 linked variations -27671G>A, -26593C>T and -26385C>T. Deviations from equilibrium were derived from an unexpected occurrence of one minor allele homozygote in these low frequency variations. However, the occurrence at these positions was confirmed by repeated sequencing.

One novel nonsynonymous variation, 154A>G (Met52Val), was found heterozygously with an allele frequency of 0.005. The Met52Val is found in the nuclear localization signal between two zinc finger regions.[14] Furthermore, the precedent amino acid Ser51 is phosphorylated by protein kinase C, which could modulate the VDR binding to the vitamin D responsive-element and transactivation activity.[15] Thus, functional significance of this Met52Val variation should be clarified in future studies.

One known nonsynonymous SNP, 2T>C (the *Fok*I polymorphism) that results in increased transcriptional activity, was also detected at a 0.636 allele frequency, which is similar to frequencies reported for Japanese women (0.59)[10] and Caucasians (0.62),[16] but slightly higher than that in Chinese (0.53)[17] and lower than that in African-Americans (0.79).[16]

Other functionally relevant SNPs are -29649G>A and -26930A>G. The SNP -29649G>A, leading to higher affinity for Cdx2 and increased *VDR* transcription, was detected at a 0.430 frequency, which is higher and lower than those in Caucasians (0.17) and Africans (0.75), respectively.[18] By luciferase reporter and electrophoretic mobility shift assays, the other SNP -26930A>G was shown to two-fold reduce *VDR* transcription with decreased binding affinity to a GATA protein.[18] The allele frequency of this variation was 0.028, which is comparable to that in Africans (0.06) but considerably lower than that in Caucasians (0.43).[18] Since GATA family proteins are expressed in many tissues,[19] this variation might be important for interindividual differences in VDR expression levels.

Fang *et al.* (2005) reported that the 3'-UTR SNPs 1592(*308)C>A, 2077(*793)delT, 3190(*1906)C>A, 3387(*2103)_3406(*2122)$A_{20}>A_{13\text{-}24}$ and 3427(*2143)A>T were in high LD in Caucasians, and that the major haplotype 1592(*308)C-2077(*793)T-3190(*1906)C-3387(*2103)_3406(*2122)A_{22}-3427(*2143)A showed a 15% lower level and 30% faster decay of VDR mRNA than the minor reciprocal haplotype.[18] However, the variation responsible for these functional alterations has not been identified. In our hands, the polymorphism 3387(*2103)_3406(*2122)$A_{20}>A_{13\text{-}24}$ could not be precisely genotyped by direct sequencing and thus is not listed in **Table 2**. However, the four other SNPs were in very high LD ($r^2\geq 0.979$) also in our Japanese population. Note that IVS8-49G>T (known as the *Apa*I polymorphism)[9] also linked to these four variations ($r^2\geq 0.979$). Thus, VDR mRNA stability is probably influenced by this haplotype also in Japanese. The tagging SNP 1592(*308)C>A (or IVS8-49G>T) was detected at a 0.318 frequency, which is lower than that in Caucasians (0.55).[18] Moreover, it was shown that this SNP was also closely linked to IVS8+283G>A (the *Bsm*I polymorphism).[18]

In conclusion, we identified 61 genetic variations, including 25 novel ones, from 107 Japanese subjects in *VDR*. One novel variation results in an amino acid substitution. Close associations of 5 SNPs [(IVS8-49G>T, 1592 (*308) C>A, 2077 (*793) delT, 3190 (*1906)C>A, and 3427(*2143)A>T] were also shown in Japanese. This information would be useful for pharmacogenetic studies to investigate the associations of *VDR* variations with interindividual differences in drug metabolism catalyzed by VDR-regulated drug metabolizing enzymes in Japanese.

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ORIGINAL REPORT

Phase II Trial of Preoperative Chemoradiotherapy Followed by Surgical Resection in Patients With Superior Sulcus Non–Small-Cell Lung Cancers: Report of Japan Clinical Oncology Group Trial 9806

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ABSTRACT

Purpose
To evaluate the safety and efficacy of preoperative chemoradiotherapy followed by surgical resection for superior sulcus tumors (SSTs).

Patients and Methods
Patients with pathologically documented non–small-cell lung cancer with invasion of the first rib or more superior chest wall were enrolled as eligible; those with distant metastasis, pleural dissemination, and/or mediastinal node involvement were excluded. Patients received two cycles of chemotherapy every 4 weeks as follows; mitomycin 8 mg/m^2 on day 1, vindesine 3 mg/m^2 on days 1 and 8, and cisplatin 80 mg/m^2 on day 1. Radiotherapy directed at the tumor and the ipsilateral supraclavicular nodes was started on day 2 of each course, at the total dose of 45 Gy in 25 fractions, with a 1-week split. Thoracotomy was undertaken 2 to 4 weeks after completion of the chemoradiotherapy. Those with unresectable disease received boost radiotherapy.

Results
From May 1999 to November 2002, 76 patients were enrolled, of whom 20 had T4 disease; 75 patients were fully assessable. Chemoradiotherapy was generally well tolerated. Fifty-seven patients (76%) underwent surgical resection, and pathologic complete resection was achieved in 51 patients (68%). There were 12 patients with pathologic complete response. Major postoperative morbidity, including chylothorax, empyema, pneumonitis, adult respiratory distress syndrome, and bleeding, was observed in eight patients. There were three treatment-related deaths, including two deaths owing to postsurgical complications and one death owing to sepsis during chemoradiotherapy. The disease-free and overall survival rates at 3 years were 49% and 61%, respectively; at 5 years, they were 45% and 56%, respectively.

Conclusion
This trimodality approach is safe and effective for the treatment of patients with SSTs.

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INTRODUCTION

Superior sulcus tumors (SSTs), involving structures at the thoracic inlet, represent a small subtype of non–small-cell lung carcinoma (NSCLC). These SSTs, first described by Henry Pancoast[1,2] and thus also called Pancoast tumors, have posed a challenging problem for surgeons, radiation oncologists, and medical oncologists alike, ever since they were first described.[3]

Preoperative radiotherapy has long been the community standard in the management of SSTs.[4-17] However, both the complete resection rate (approximately 50%) and long-term survival rate (approximately 30%) have remained poor and unchanged over the last 40 years, since the first treatment strategy was reported in the 1960s. Local control has remained the main problem,[15,17,18] adversely affecting quality of life as well as survival of patients. Presence of mediastinal lymph node metastasis (N2 status) has been reported to be associated with a particularly poor prognosis.[9,18]

However, a series of clinical trials over the last two decades have shown concurrent chemoradiotherapy to be beneficial in the treatment of unresectable stage III NSCLC.[19-21] The addition of chemotherapy to thoracic radiotherapy seems to suppress distant micrometastases,[22,23] and giving

concurrent chemotherapy with radiotherapy has been shown to yield improved local control[19,24] with survival benefit.

Encouraged by the promising data of concurrent chemoradiotherapy for N2 NSCLC, the Southwest Oncology Group (SWOG) applied this modality as preoperative therapy for patients with SSTs (SWOG 9416, Intergroup Trial 0160), and reported favorable results.[25]

The Japan Clinical Oncology Group (JCOG) launched another trial of this preoperative concurrent chemoradiotherapy, or the trimodality approach, for the treatment of SSTs in 1999, before the first report of SWOG 9416 was published. Our study was initiated to evaluate the safety and efficacy of this treatment strategy in this rare subset of patients with NSCLC. As the induction treatment, we used mitomycin, vindesine, and cisplatin (MVP) combination chemotherapy, which has been demonstrated to be safe and effective for concurrent chemotherapy with thoracic radiotherapy in Japanese trials.[19]

PATIENTS AND METHODS

Eligibility Criteria

Patients with untreated histologically or cytologically documented NSCLC involving the superior sulcus with clinical stage T3 or T4 disease were eligible for entry onto this study. T4 diseases included tumor invasion to the spine (including to a transverse process of vertebra), aorta, or superior vena cava; invasion to the chest wall or subclavian vessels was included in T3 disease. Involvement of the superior sulcus was confirmed by computed tomographic (CT) or magnetic resonance imaging (MRI) evidence of tumor invasion of the first rib or more superior chest wall. Patients with pleural or pericardial dissemination, malignant effusion, and/or distant metastasis (M1) were excluded. Those with clinical N2 disease (mediastinal node involvement) were also excluded; all mediastinal nodes measuring $\geq$ 1.0 cm in size on CT images were required to be biopsied and documented to be negative for metastasis before patient enrollment. However, those with ipsilateral supraclavicular node involvement (N3) were eligible, unless it was accompanied by mediastinal node metastasis. Each patient was required to fulfill the following criteria: 15 to 74 years of age, Eastern Cooperative Oncology Group performance status of 0 to 1; adequate organ function (ie, leukocyte count $\geq$ 4,000/μL, platelet count $\geq$ $10^5/\mu$L, hemoglobin $\geq$ 11.0 g/dL, serum creatinine less than 1.5 mg/dL, creatinine clearance $\geq$ 60 mL/min, serum bilirubin less than 1.5 mg/dL, serum ALT and AST less than double the upper limit of the institutional normal range, arterial partial pressure of oxygen $\geq$ 70 mmHg, and predicted postoperative forced expiratory volume in 1 second $\geq$ 0.8 L. From July 2001, when the protocol was revised after the death of a patient from septic shock during chemoradiotherapy, those patients with systemic use of corticosteroids were excluded.

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

Treatment Plan

Induction chemotherapy. Patients received two courses of MVP combination chemotherapy with a 4-week interval in between. Mitomycin was administered at 8 mg/m^2 on chemotherapy day 1, and vindesine was administered at 3 mg/m^2 on days 1 and 8; both were administered as bolus injections. Cisplatin was administered at 80 mg/m^2 as a 2-hour infusion on day 1, with ample hydration and antiemetic administration.

The second cycle of chemotherapy was postponed until all the severe toxicities recovered to grade 1 or 0. If the second cycle could not be started within 2 weeks of the due date, it was canceled, and the patient received only preoperative radiotherapy, if possible.

Induction radiotherapy. Thoracic radiotherapy was started with a linear accelerator ($\geq$ 4 MeV) on chemotherapy day 2. The first session was scheduled to be given with the first chemotherapy cycle at 27 Gy in 15 fractions over 3 weeks. Then the second session was started after a week's interval until day 2 of the second course of chemotherapy. The second session, given with the second cycle of MVP, was administered at 18 Gy in 10 fractions over 2 weeks. The total radiation dose was thus 45 Gy in 25 fractions administered over 6 weeks, including the 1-week split, or interval, between the two sessions; this schedule, including the split, basically followed that of the original method reported by Furuse et al.[19] The radiation field included the primary tumor and the ipsilateral supraclavicular nodes. The mediastinal and hilar nodes were not irradiated, even in cases with hilar node involvement (clinical N1 cases).

Surgery. After the induction chemoradiotherapy, each case was re-evaluated to determine the clinical response and resectability. The resectability of the tumor was determined by the multimodality team of each institution, irrespective of the clinical response (tumor shrinkage). Surgical resection of the tumor was performed 2 to 4 weeks after the completion of the induction therapy. The surgical procedures undertaken included lobectomy or pneumonectomy, with systematic node dissection. Standard systematic node dissection, ND2, includes complete removal of the hilar and mediastinal nodes. Less complete dissection includes ND0 (ie, no systematic dissection with or without lymph node sampling) or ND1 (ie, hilar node dissection with or without mediastinal lymph node sampling).

Boost therapy. For unresected or incompletely resected cases, boost radiotherapy of 21.6 Gy in 12 fractions was given. Those who were judged to have undergone complete resection were followed up without additional therapy until clinical evidence of recurrence.

Patient Evaluation and Follow-Up

Before enrollment onto the study, each patient underwent complete medical history taking and physical examination, blood cell count determinations, serum biochemistry testing, arterial blood gas analysis, chest x-ray, ECG, CT scan of the chest, bronchoscopy, CT scan or ultrasound of the upper abdomen, whole-brain CT or MRI, and an isotope bone scan. Chest MRI was recommended for evaluation of the local tumor status but was not mandatory. Blood cell counts, serum biochemistry testing, and chest x-ray were performed weekly during each course of chemotherapy. Chest CT was performed every 3 to 4 weeks during the induction therapy.

Chemotherapy toxicity was evaluated according to the JCOG Toxicity Criteria,[26] modified from the National Cancer Institute Common Toxicity Criteria version 1. Tumor responses were assessed radiographically according to the standard, two-dimensional WHO criteria[27] and were classified into complete response (CR), partial response, no change, progressive disease (PD), and not assessable. Response confirmation at 4 weeks or longer intervals was not necessitated. After curative resection and/or definitive boost radiotherapy, the patients were followed up with periodic re-evaluation, including with chest CT, as well as a systemic survey every 6 months for the first 3 years.

Central Review

Radiographic reviews for eligibility of the enrolled patients and the clinical responses were performed at the time of the JCOG Lung Cancer Surgical Study Group meeting, held every 3 to 4 months. The study coordinator (H.K., a medical oncologist), the group coordinator (M.T., a surgical oncologist), and a few selected investigators of the group reviewed the radiographic films. The clinical response data presented below were all confirmed by this central review.

Statistical Considerations

The primary end point of the study was the survival rate at 3 years. The sample size calculation was performed, as described in Appendix 1 (online only).

Secondary end points included the objective tumor response to chemotherapy, complete resection rate, and postsurgical morbidity/mortality. Both overall survival (OS) and progression-free survival (PFS) were calculated from the date of enrollment by the Kaplan-Meier method. For exploratory analysis to identify prognostic factors, the OS or PFS of subgroups was compared by two-sided log-rank tests. All analyses were performed with the SAS software version 8.2 (SAS Institute, Cary, NC).

RESULTS

Patient Characteristics

From May 1999 to November 2002, 76 patients from 19 institutions were enrolled onto the study. Three patients were ineligible. One patient was found to have concomitant anemia and did not receive the protocol treatment. Two others were found ineligible by the central review, after completion of the protocol therapy; the tumor was judged not to involve the first rib in one case, and in the other, a mediastinal node was judged to be enlarged on chest CT, without confirmation by mediastinoscopy. These two cases were included in the analysis. Therefore, 75 patients were analyzed to determine the toxicities, response rates, surgical and pathologic results, PFS, and OS. All 76 patients were included in the analysis of the patient characteristics, as shown in Table 1. In each of the T4 cases, the tumor was judged to have involved the spine. Nodal status was clinically determined and was pathologically confirmed in only a few cases.

Induction Therapy Delivery and Toxicity

The study schema with the actual numbers of patients receiving the protocol therapy is shown in Appendix Figure A1 (online only).

Table 1. Patient Characteristics (n = 76)

Characteristic	No. of Patients	%
Sex		
Male	67	88
Female	9	12
Age, years		
Median	57.5	
Range	34-74	
ECOG performance status		
0	30	39
1	46	61
Clinical T stage		
T3	56	74
T4	20	26
Clinical N stage		
N0	59	78
N1	9	12
N2*	1	1
N3	7	9
Smoking history		
No	4	5
Yes	72	95
Median smoking history	1.5 packs for 37 years	
Body weight loss within 6 months		
≤ 5%	61	80
5-10%	7	9
> 10%	5	7
Missing	3	4
Histology		
Adenocarcinoma	34	45
Squamous cell carcinoma	27	36
Others/unclassified	15	20
Primary site		
Right	39	51
Left	37	49

Abbreviation: ECOG, Eastern Cooperative Oncology Group.
*Found ineligible by central review but included in the subsequent analyses.

The induction therapy could be completed in 71 (95%) of the 75 patients. The treatment was terminated in the remaining four patients after only one course of chemotherapy (owing to the development of adverse events in two cases, patient refusal in one case, and early toxicity-related death in one case).

Table 2 lists the major toxicities of the protocol therapy. They were mainly hematologic, and although more than 80% of the patients experienced neutropenia/leukopenia, they were generally transient and not complicated by infection/fever. Overall, toxicities were well tolerated. There was one toxic death on chemoradiotherapy day 6 as a result of severe myelosuppression and subsequent development of septic shock.

Clinical Response to the Induction Therapy

The clinical responses of the 75 eligible patients to induction therapy were judged radiologically and confirmed by the central review. The responses were as follows: CR, 0 patients; partial response, 46 patients; no change, 22 patients; PD, five patients; not assessable, two patients. The overall response rate was 61% (95% CI, 49% to 72%).

Surgical and Pathologic Results

Thoracotomy was performed in 57 (76%) of the 75 patients who received the induction therapy. The surgical procedures undertaken

Table 2. Major Toxicities of Induction Therapy (N = 75) and Postsurgical Complications

Toxicity or Complication	No. of Patients			
	Grade 1/2	Grade 3	Grade 4	% Grade 3/4
Acute toxicity*				
Leukopenia	1/11	37	26†	84
Neutropenia	3/9	26	36†	83
Anemia	19/47	5	0	7
Thrombocytopenia	14/12	9	2†	15
ALT	27/5	2	0	3
Creatinine	18/2	0	0	0
PaO_2	37/6	0	0	0
Emesis	32/25	2	— (not defined)	3
Diarrhea	7/5	1	0	1
Constipation	22/3	1	0	1
Esophagitis	22/9	0	0	0
Infection	10/9	6	1†	9
Neuropathy	8/0	0	— (not defined)	0
Skin toxicity	16/2	1	0	1
Fever	25/19	1	1	3
Postsurgical complications‡				
ARDS	0	1	1 (grade 5)	
Empyema	0	2	0	
Cylothorax	1	1	0	
Pneumonitis	0	1	0	
Late complications‡				
Pneumonitis	0	1	0	
Bleeding	0	0	1 (grade 5)	

Abbreviations: PaO_2, alveolar-arterial difference in partial pressure of oxygen; ARDS, adult respiratory distress syndrome.
*During induction therapy.
†Includes one patient with toxic death owing to septic shock.
‡Report of each complication was evaluated by National Cancer Institute Common Toxicity Criteria version 3.0.