表2. リスク臓器の線量制約の1例 (JCOG 0403での線量制約)

Organ	Dose	Volume	Dose	Volume
Lung	40Gy	<= 100cc	MLD	<= 18cc
	V15	<= 25%	V20	<= 20%
Cord	25Gy	Max		
Esophagus	40Gy	<= 1cc	35Gy	<= 10cc
Pulmonary artery	40Gy	<= 1cc	35Gy	<= 10cc
Stomach	36Gy	<= 10cc	30Gy	<= 100cc
Intestine	36Gy	<= 10cc	30Gy	<= 100cc
Trachea, main bronchus	40Gy	<= 10cc		
Other organs	48Gy	<= 1cc	40Gy	<= 10cc

なお線量計算には，クラークソン法を用いている。

の臨床上の結果としてステロイドを必要とするNCI-CTC Grade 2以上の問題のある放射線性肺臓炎はわずかに4%程度であった。つまり定位放射線照射の治療適応として肺野の3～4cm以内の孤立性腫瘍を対象とする限り，照射される正常肺の体積も許容範囲内のようである。これは通常の放射線治療における合併症の頻度が20～30%であることと比較すると十分に低い。もちろん呼吸機能の不良な症例を治療する場合は，注意が必要である。特に背景に間質性肺疾患を持った患者群では，致死的放射線肺臓炎のリスクがあるので注意が必要である。日本国内における高精度放射線治療外部照射研究会の全国調査では，致死的な合併症 (Grade 5) が全症例中の0.6%で見られており，その中では放射線肺臓炎が最頻であった。また肺以外の合併症として，縦隔近傍の腫瘍には注意が必要である。現在までに国内外で致死的な喀血の報告[14]や，致死的な食道潰瘍[9]の報告がある。縦隔臓器 (心臓・大血管，気管・気管支，食道，等) の領域に照射が不可避な縦隔近傍肺癌の場合への適応は，**表2**の線量制約を満たすように慎重にならざるをえない。

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(広島大学病院放射線治療部　永田　靖)

Oligometasteses

近年注目されている再発/転移癌の概念としてOligometastasesがある[1]。全身検索の結果1個もしくは数個の遠隔転移のみの症例の場合，原発巣とともに遠隔転移部位に局所療法を施行することによりそれぞれを制御することで長期生存が可能な症例群を示す概念として，1995年にHellmannらにより提唱された。局所療法としては手術も挙げられるが，侵襲性の点から放射線療法が選択されることが多い。HellmannらによるOligometastasesの概念のうち原発部位が制御されたのち，遠隔再発として1ヵ所もしくは数ヵ所の再発のみを認め，かつ局所治療の意義のある病態はOligo-recurrence[2]と呼ばれることもあるが，適応癌種はOligometastasesとほぼ一緒である。再発・転移部位の放射線療法の適応としては，肺転移，肝転移，子宮頸癌で腹部傍大動脈リンパ節転移/再発のみを伴った場合，脳単独再発を示した場合が報告されている。

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(北里大学医学部放射線科学　新部　譲)

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SNP Communication

Genetic Variations and Haplotype Structures of the Glutathione *S*-transferase Genes, *GSTT1* and *GSTM1*, in a Japanese Patient Population

Naoko TATEWAKI[1], Keiko MAEKAWA[1,2,*], Noriko KATORI[1,3], Kouichi KUROSE[1,4], Nahoko KANIWA[1,4], Noboru YAMAMOTO[5], Hideo KUNITOH[5], Yuichiro OHE[5], Hiroshi NOKIHARA[5], Ikuo SEKINE[5], Tomohide TAMURA[5], Teruhiko YOSHIDA[6], Nagahiro SAIJO[7], Yoshiro SAITO[1,2] and Jun-ichi SAWADA[1,2]

[1]*Project team for Pharmacogenetics, National Institute of Health Sciences, Tokyo, Japan*
[2]*Division of Functional Biochemistry and Genomics, National Institute of Health Sciences, Tokyo, Japan*
[3]*Division of Drugs, National Institute of Health Sciences, Tokyo, Japan*
[4]*Division of Medicinal Safety Science, National Institute of Health Sciences, Tokyo, Japan*
[5]*Thoracic Oncology Division, National Cancer Center Hospital, National Cancer Center, Tokyo, Japan*
[6]*Genetics Division, National Cancer Center Research Institute, National Cancer Center, Tokyo, Japan*
[7]*Deputy Director, National Cancer Center Hospital East, Kashiwa, Japan*

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Summary: Glutathione *S*-transferases (GSTs) play a vital role in phase II biotransformation of many synthetic chemicals including anticancer drugs. Deletion polymorphisms in *GSTT1* and *GSTM1* are reportedly associated, albeit controversial, with an increased risk in cancer as well as with altered responses to chemotherapeutic drugs. In this study, to elucidate the haplotype structures of *GSTT1* and *GSTM1*, genetic variations were identified in 194 Japanese cancer patients who received platinum-based chemotherapy. Homozygotes for deletion of *GSTT1* (*GSTT1*0/*0* or null) and *GSTM1* (*GSTM1*0/*0* or null) were found in 47.4% and 47.9% of the patients, respectively, while 23.2% of the patients had both *GSTT1* null and *GSTM1* null genotypes. From homozygous (+/+) and heterozygous (*0/+) patients bearing *GSTT1* and *GSTM1* genes, six single nucleotide polymorphisms (SNPs) for *GSTT1* and 23 SNPs for *GSTM1* were identified. A novel SNP in *GSTT1*, 226C>A (Arg76Ser), and the known SNP in *GSTM1*, 519C>G (Asn173Lys, **B*), were found at frequencies of 0.003 and 0.077, respectively. Using the detected variations, *GSTT1* and *GSTM1* haplotypes were identified/inferred. Three and six common haplotypes (N≥10) in *GSTT1* and *GSTM1*, respectively, accounted for most (>95%) inferred haplotypes. This information would be useful in pharmacogenomic studies of xenobiotics including anticancer drugs.

Keywords: *GSTT1*; *GSTM1*; nonsynonymous SNP; haplotype; haplotype-tagging SNP

Introduction

Glutathione *S*-transferases (GSTs) (EC 2.5.1.18) are dimeric phase II metabolic enzymes that mainly catalyze conjugation of reduced glutathione (GSH) with a variety of electrophilic compounds including carcinogens, therapeutic drugs and environmental toxins as well as endogenous substances.[1)] In addition, GSTs possess selenium-independent GSH peroxidase activity to reduce organic hydroperoxides, and therefore, play significant roles in detoxification, occasionally toxification, and cellular protection against oxidative stress.[2)] Noncatalytical-

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**To whom correspondence should be addressed:* Keiko MAEKAWA, Ph.D., *Division of Functional Biochemistry and Genomics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel. +81-3-3700-9453, Fax. +81-3-5717-3832, E-mail: maekawa@nihs.go.jp*
On April 28th, 2008, the novel variations described in this paper were not found in the Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/) or SNP500Cancer Database (http://snp500cancer.nci.nih.gov/).
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ly, GSTs modulate signaling pathways by interacting with protein kinases[3] and by binding numerous ligands for nuclear hormone receptors.[4]

Human GSTs are composed of three main families: cytosolic, mitochondrial and microsomal (or membrane-bound). The cytosolic family, which is principally involved in biotransformation of toxic xenobiotics, contains at least 17 genes subdivided into seven separate classes designated alpha, mu, pi, sigma, theta, zeta, and omega.[5,6] Increasing numbers of GST genes are identified as polymorphic.

The θ-class enzyme GSTT1 and the μ-class enzyme GSTM1 exhibit gene deletion polymorphisms (*GSTT1*0* and *GSTM1*0*, respectively).[7] The null genotype of *GSTT1* (*GSTT1*0/*0*) is found in 15–40% of Caucasians and 50–60% of Asians.[7] On the other hand, about half of both Japanese and Caucasians and 30% of Africans are homozygous for the *GSTM1* deletion (*GSTM1*0/*0*).[7] In intact *GSTM1*, alleles **A* and **B* are used to discriminate the single nucleotide polymorphism (SNP) with amino acid substitution (thereafter, nonsynonymous SNP), 519C>G (Asn173Lys) in exon 7, in which both alleles encode proteins that are catalytically identical for the substrates, 1-chloro-2,4-dinitrobenzene (CDNB), *trans*-4-phenyl-3-buten-2-one (tPBO) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP).[8] In addition, a tandem duplication in *GSTM1* associated with ultrarapid enzyme activity was observed in Saudi Arabians.[9] A gene-dose effect has been clearly established: that is, homozygously deleted (**0/*0*), heterozygously (**0/+*) and homozygously intact (*+/+*) *GST* genotypes correspond to non-, intermediate, and high conjugators, respectively.[10,11]

A large number of association studies on *GSTM1* and *GSTT1* null genotypes have been performed with inter-individual differences in susceptibility to environmental toxins, cancer and other diseases, and in the outcomes of anticancer treatments. Increased risk of lung, bladder, breast and colon cancers were observed in carriers of *GSTM1* or *GSTT1* null genotypes, while other studies have reported controversial findings.[5–7] As for response to anti-cancer drugs, pharmacodynamic correlations have been investigated, but the obtained results are inconsistent.[6] It should be pointed out that despite the possible gene-dose effect, most association studies were only focused on null genotypes of *GSTM1* and/or *GSTT1*. Therefore, in addition to nonconjugators, discrimination between high and intermediate conjugators would be valuable to evaluate the clinical relevance of these GST loci. Also, certain SNPs in the intact genes might affect either the expression of the gene or the activity of the encoded enzyme.

In this study, we first determined the deletion genotypes (**0/0*, **0/+*, and *+/+*) of *GSTM1* and *GSTT1* by conventional PCR and TaqMan real-time quantitative PCR for 194 Japanese cancer patients treated by platinum-based chemotherapy. Then, we resequenced the homozygous and heterozygous intact *GSTM1* and *GSTT1* genes. Lastly, linkage disequilibrium (LD) and haplotype analyses were performed using the detected SNPs.

Materials and Methods

Human genomic DNA samples: All 194 patients participating in this study were administered carboplatin or nedaplatin in combination with paclitaxel for treatment of various cancers (mainly non-small cell lung cancers) at the National Cancer Center. Genomic DNA was extracted from blood leukocytes from all subjects prior to the chemotherapy. The ethical review boards of the National Cancer Center and National Institute of Health Sciences approved this study. Written informed consent was obtained from all subjects.

Conventional PCR amplification of the *GSTT1* deletion junction: We used the genotyping assay described by Sprenger *et al.*,[10] in which 1460 (for **0* allele) and 466 bp (for exon 5 of the wild-type) PCR fragments were coamplified by multiplex PCR. PCR reactions were performed according to their method with minor modification.[10] Briefly, PCR mixtures contained 100 ng of genomic DNA, 0.2 μM each of the 4 primers reported previously, 0.2 mM each of four deoxynucleotide triphospates (dNTPs), and 0.75 units of HotStarTaq polymerase (Qiagen, Tokyo, Japan) in a 50 μl volume. The PCR conditions were 95°C for 15 min, followed by 30 cycles of 94°C for 30 sec, and 65°C for 1.5 min. PCR fragments were analyzed on 1% agarose gels with ethidium bromide in TAE buffer.

Conventional PCR amplification of *GSTM1*: We used the method of McLellan *et al.* (1997),[9] in which exons 3 to 5 of *GSTM1* were coamplified with β-globin as an internal standard by multiplex PCR. The PCR reactions were carried out according to their method[9] except that 100 ng of genomic DNA and 0.75 units of HotStarTaq polymerase (Qiagen) were used in a 50 μl total volume. The PCR conditions were 94°C for 15 min, followed by 30 cycles of 94°C for 48 sec, 62°C for 48 sec, and 72°C for 1.5 min, and then a final extension for 5 min at 72°C.

Quantitative real-time PCR for *GSTM1* and *GSTT1*: Quantitative real-time PCR using the TaqMan (5′-nuclease) assay system was carried out according to the method of Covault *et al.*,[12] in which the amounts of target *GSTM1* or *GSTT1* were quantified relative to those of the reference β-2-microglobulin (*B2M*) or cannabinoid receptor 1 (*CNR1*), respectively. Briefly, triplicate reactions were performed for 5 ng of genomic DNA used as a template in 1x TaqMan Universal PCR Master Mix with Amp Erase (50 μl) (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of

95°C for 20 sec and 60°C for 1 min with the 7500 Real-Time PCR System (Applied Biosystems).

***GSTT1* DNA sequencing:** The heterozygous and homozygous samples for *GSTT1* (**0/+* and *+/+*), the 5′-flanking region (up to 801 bp upstream from the translation start site), all 5 exons with their surrounding introns and the 3′-flanking region were amplified by PCR and directly sequenced. For the 1st round PCR, the reaction mixtures contained 25 ng of genomic DNA, 1.25 units of Ex-Taq (Takara Bio. Inc. Shiga, Japan), 0.2 mM dNTPs, and 0.2 μM primers listed in **Table 1**. The 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. The regions from 5′-flanking to exon 1 and from exon 4 to 3′-flanking were amplified separately by the nested PCR with Ex-Taq (1.25 units) and the primer sets (0.2 μM) listed in "2nd round PCR" of **Table 1**. The 2nd round PCR conditions were the same as described in the 1st round PCR. The 2nd round PCR products and the 1st round PCR products for exons 2 and 3 were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and were directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with the sequencing primers listed in **Table 1** (Sequencing column). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). Eluates were analyzed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems). All novel SNPs were confirmed by repeated sequencing of the PCR products generated by new genomic DNA amplifications. The genomic and cDNA sequences of *GSTT1* obtained from GenBank (NT_011520.11 and NM_000853.1, respectively) were used as reference sequences.

***GSTM1* DNA sequencing:** For samples with **0/+* and *+/+*, genetic variations were identified by resequencing. Particular attention was paid to avoid amplification of sequences of other homologous *GSTMs* because exon 8 of *GSTM1* is 99% identical to that of *GSTM2*.[13] We confirmed that PCR fragments were not amplified from samples with *GSTM1*0/*0* genotypes to evaluate primer specificities. The entire *GSTM1* gene except for the region through exon 8 to the 3′-flanking region was amplified in the 1st round of PCR from 25 ng of genomic DNA utilizing 1.25 units of Ex-Taq with 0.2 μM of primers listed in **Table 2**. Next, three regions (from 5′-flanking to exon 3, from exon 4 to 5, and from exon 6 to 7), were separately amplified in the 2nd round PCR from the 1st round PCR product by Ex-Taq (0.625 units) with 0.2 μM primers listed in **Table 2**. The region from exon 8 to the 3′-flanking was separately amplified from 25 ng of genomic DNA using 0.625 units of Ex-Taq with 0.2 μM primers (listed in **Table 2**). All PCR conditions were the same as those described for *GSTT1*. PCR products were then directly sequenced with the primers listed in

Table 1. *GSTT1* primer sequences

Amplified and sequenced region			Forward primer		Reverse primer		PCR product (bp)
			Sequence (5′ to 3′)	Position[a]	Sequences (5′ to 3′)	Position[a]	
1st round PCR	multiplex	5′-flanking (up to −1366) to exon 1	CACTCCCGCCCCAAATTAGGTT	3776166	ATGATCCCCACCCCTTTATTCG	3774444	1723
		Exon 4 to 3′-flanking region	ATCACAAGGTCAGGAGATTG	3767902	ACTCTTGGCAAACATCAGGG	3766589	1314
		Exon 2	ACATAATCTCTTCTGCAAACTG	3773267	TGTCTCAAGGATACTCTCACCA	3772011	1257
		Exon 3	GCAAATTGTCAGAAAGGTTAAAGA	3770734	CCCACCTCCTGATTAGCTTAGAAG	3768725	2010
2nd round PCR		5′-flanking (up to −801) to exon 1	TTTCAGTGGGATTCGTTTTAGA	3775601	CCCCGTGGTCTATTCCGTGA	3774478	1124
		Exon 4 to 3′-flanking region	CATCACTAATCATTAGGGAA	3767648	CTGGGAAGGGGGTTGTCTTT	3766628	1021
Sequencing		5′-flanking (up to −801)	TTTCAGTGGGATTCGTTTTAGA	3775601	GGCTCGCTCATTTCACTTAG	3775090	
		Exon 1	GGTGGGAAATTCTGACACAC	3775162	CCCCGTGGTCTATTCCGTGA	3774478	
		Exon 2[b]	AAGGGACAAGGTAGTCAGTC	3772758	AACTGGAATAGCACGGAAGGC	3772099	
		Exon 3[b]	AAAAAAGCGACTATGTATGAAAT	3770153	AGATAAAATGGATGAACAGATGGT	3769662	
		Exon 4	CATCACTAATCATTAGGGAA	3767648	CAGACTGGGGATGGATGGTTGT	3767204	
		Exon 5 to 3′-flanking region	CATCCCCAGTCTGTACCCTTTTCC	3767216	CTGGGAAGGGGGTTGTCTTT	3766628	

[a]The nucleotide position of the 5′ end of each primer on NT_011520.11.
[b]For exons 2 and 3, the 1st round PCR product was directly sequenced.

Table 2. *GSTM1* primer sequences

	Amplified and sequenced region	Forward primer		Reverse primer		PCR product (bp)
		Sequence (5′ to 3′)	Position[a]	Sequences (5′ to 3′)	Position[a]	
1st round PCR	5′-flanking (up to −1309) to exon 7	CCACAAACAAGTTTATTGGGCG	6136872	GTACTAGACATCAATGTCACCGTT	6141347	4476
	Exon 8 to 3′-flanking region	ACAGTGAGATTTTGCTCAGGTATT	6142766	CTCAATTCTAGAAAAGAGCGAG	6145058	2293
2nd round PCR	5′-flanking (up to −650) to exon 3	GACCACATTTCCTTTACTCTGG	6137531	TAAGAATACTGTCACATGAACG	6139231	1701
	Exon 4 to 5	TCTGTGTCCACCTGCATTCGTTCA	6139192	CTGAACACAAACTTTACCATAC	6139883	692
	Exon 6 to 7	CTAATAAATGCTGATGTATCCAAT	6140410	CCTACTATTGCCAGCTCCATCTAT	6141315	906
Sequencing	5′-flanking (up to −650)	GTCCTTCCTATACCACTGACAC	6137567	AACCGAGCAGGGCTCAGAGTAT	6138145	
	Exon 1 to 2	CCCTGACTTCGCTCCCGGAAC	6137956	GGACACCCGTCCCAATTAGACA	6138764	
	Exon 3	TCTGCCCACTCACGCTAAGTTG	6138577	TAAGAATACTGTCACATGAACG	6139231	
	Exon 4 to 5	TCTGTGTCCACCTGCATTCGTTCA	6139192	CTGAACACAAACTTTACCATAC	6139883	
	Exon 6 to 7	CTAATAAATGCTGATGTATCCAAT	6140410	CCTACTATTGCCAGCTCCATCTAT	6141315	
	Exon 8[b]	GAACTTCTGTTTCCCACATGAG	6143164	GAGTAAAGATGGGAATAAACAG	6143735	
	3′-untranslated and flanking region[b]	TCGTTCCTTTCTCCTGTTTATT	6143701	CCTTGGGGTCCTATTCAATGAG	6144362	

[a]The nucleotide position of the 5′ end of each primer on NT_019273.18.
[b]For the region from exon 8 to 3′-flanking, the 1st round PCR product was directly sequenced.

"sequencing" of **Table 2** as described above for *GSTT1*. All novel SNPs were confirmed by repeated sequencing of PCR products that were newly generated by amplification of genomic DNA. The genomic and cDNA sequences of *GSTM1* obtained from GenBank (NT_019273.18 and NM_000561.2, respectively) were used as reference sequences.

Linkage Disequilibrium (LD) and haplotype analyses: Hardy-Weinberg equilibrium and LD analyses were performed by SNPAlyze ver 7.0 (Dynacom Co., Yokohama, Japan). Pairwise LD ($|D'|$ and r^2 values) between two variations was calculated using 102 subjects bearing one or two *GSTT1* genes and 101 subjects bearing one or two *GSTM1* genes. Some haplotypes were unambiguous from subjects with heterozygous **0* alleles. Diplotype configurations were inferred based on estimated haplotype frequencies using Expectation-Maximization algorithms by SNPAlyze software, which can handle multiallelic variations. Haplotypes containing SNPs without any amino acid change were designated as **1*, and nonsynonymous SNP-bearing haplotypes were numerically numbered. Subtypes were named in their frequency order by use of alphabetical small letters.

Results

Determination of deletion polymorphisms in *GSTM1* and *GSTT1*: Both conventional PCR[10] and TaqMan real-time PCR[12] were used to identify deletion of *GSTT1*. By conventional PCR, 92 out of 194 subjects (frequency = 0.474) were assigned as *GSTT1*0/*0*. For all 92 samples with *GSTT1*0/*0*, no significant fluorescence derived from *GSTT1* amplification was detected by TaqMan real-time PCR (mean cycle threshold, Ct, 37.6). Eighty-two (frequency = 0.423) and 20 (frequency = 0.103) subjects were identified as heterozygous (**0/+*) and homozygous (*+/+*) for intact *GSTT1* by conventional PCR, respectively. In the TaqMan real-time PCR, the mean ± SD of relative amounts of *GSTT1* was 1.0 ± 0.111, and 0.448 ± 0.058 for homozygous and heterozygous *GSTT1* carriers, respectively (the mean value for the 20 homozygotes was set as 1). Since the maximum relative amount of *GSTT1* was 1.214, no gene duplication could be inferred for *GSTT1*. The assigned genotypes were consistent between both methods, and their frequencies (**Table 3a**) were in Hardy-Weinberg equilibrium ($p = 0.785$ by Pearson's chi-square test).

As for *GSTM1*, conventional PCR[9] indicated that 93 out of 194 subjects had a homozygous deletion of *GSTM1* (**0/*0*), and that the remaining 101 subjects were either heterozygotes (**0/+*) or homozygotes (*+/+*) for intact *GSTM1*. By real-time PCR, Ct values of 93 samples with the null genotypes were greater than 36.5, which exceeded the sensitivity limits (Ct = 35) of the real-time PCR detection system, indicating that both methods gave consistent results for *GSTM1*0/*0*. As for the 101 subjects with intact *GSTM1* genes (either **0/+* or *+/+*), the distribution of relative amounts of *GSTM1* was clustered into two groups with 1.0 ± 0.083 (16 homozygotes), and 0.51 ± 0.048 (85 heterozygotes) when the mean value of the 16 homozygotes was set as 1. No individuals showed relative amounts more than 1.216, suggesting that the duplication in *GSTM1*[9] was not present in our population. Thus, the frequencies of *GSTM1*0/*0*, **0/+*, and *+/+*, were 0.479, 0.438, and 0.082, respectively (**Table 3a**), and in Hardy-Weinberg equilibrium ($p = 0.576$ by the Pearson's chi-square test).

Table 3b summarizes the results of the distribution of *GSTM1* and *GSTT1* deletions in our Japanese population.

About one-fourth (45 of 194 subjects) were null for both *GSTM1* and *GSTT1* genes.

Variations found in the intact *GSTT1* gene and their LD profiles: Six variations including three novel ones were found by sequencing the 5′-flanking regions, all 5 exons and their flanking regions in the 102 Japanese subjects with *0/+ and +/+ genotypes (**Table 4**). All detected variations were in Hardy-Weinberg equilibrium ($p \geq 0.44$ by the χ^2 test or Fisher's exact test) when assuming the presence of three alleles (wild, variant and *0 alleles) at each site. One novel nonsynonymous variation, 226C>A (Arg76Ser), was heterozygous in one subject with two intact *GSTT1* genes, and its allele frequency was 0.003 (1/388). The remaining two novel variations in the intronic regions (IVS1+71A>G and IVS2−8A>C) were also rare (allele frequency = 0.003 for both).

Three known variations (IVS1+166A>G, IVS3−36C>T and 824T>C) were found at a relatively high frequency (0.106) and were perfectly linked ($r^2 = 1.0$) with each other.

Table 3. Frequencies of *GSTT1* and *GSTM1* deletions

(a)

	Genotype	N	Frequency (%)	Allele	N	Frequency (%)
GSTT1	*0/*0	92	0.474	*0	266	0.686
	*0/+	82	0.423			
	+/+	20	0.103	+	122	0.314
GSTM1	*0/*0	93	0.479	*0	271	0.698
	*0/+	85	0.438			
	+/+	16	0.082	+	117	0.302

(b)

Genotype combination		N	Frequency (%)
GSTT1	*GSTM1*		
*0/*0	*0/*0	45	0.232
	*0/+	42	0.216
	+/+	5	0.026
*0/+	*0/*0	39	0.201
	*0/+	34	0.175
	+/+	9	0.046
+/+	*0/*0	9	0.046
	*0/+	9	0.046
	+/+	2	0.010

*0, deletion; +, intact gene

Variations found in the intact *GSTM1* gene and their LD profile: We found 23 variations, including seven novel ones, in 194 Japanese cancer patients (**Table 5**). Ten variations were located in the 5′-flanking region, 2 in the coding exons, 9 in the introns, and 2 in the 3′-flanking region. All detected variations were in Hardy-Weinberg equilibrium ($p > 0.37$ by the χ^2 test or Fisher's exact test) except for 1107+41C>T in the 3′-flanking region ($p = 0.003$ by the Fisher's exact test). Deviation from Hardy-Weinberg equilibrium for this variation was due to 2 more homozygotes than expected among 16 *GSTM1* +/+ subjects.

Seven novel variations, −416G>T and −165A>G in the 5′-flanking region, IVS1+97C>T, IVS1−79G>A, IVS1−78T>A, and IVS2+202G>A in the introns and 1107+128G>A in the 3′-flanking region, were found in single subjects (allele frequencies = 0.003). No novel nonsynonymous SNPs were detected.

Sixteen other variations were already reported or publicized in the dbSNP and/or JSNP databases. They were detected in more than 10 chromosomes (allele frequencies ≥0.026) in our population except for −423C>G and IVS2+118T>C (allele frequency = 0.003).

The pairwise |D′| values between 14 common variations (N≥10) in *GSTM1* were higher than 0.95 except for the combinations between −480A>G and other variations, which showed lower |D′| values ($0.27 < |D'| < 1.0$). As for the r^2 values, strong LDs ($r^2 > 0.87$) were observed among 10 variations,

Table 4. Summary of *GSTT1* SNPs detected in a Japanese population

SNP ID			Location	Position		Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	Allele frequency (N = 388)
This study	dbSNP (NCBI)	JSNP		NT_011520.11	From the translational initiation site or from the end of nearest exon			
MPJ6_GTT1001[a]			intron1	3774618	IVS1+71A>G	catagcttagggA/Gacttctcccagc		0.003
MPJ6_GTT1002	rs140313	ssj0002194	intron1	3774523	IVS1+166A>G	gatccaagagtcA/Ggggctcccccaaa		0.106
MPJ6_GTT1003[a]			intron2	3770088	IVS2-8A>C	catgacccccacA/Ccccacagtgtgg		0.003
MPJ6_GTT1004[a]			Exon3	3770055	226C>A	ctctacctgacgC/Agcaaatataagg	Arg76Ser	0.003
MPJ6_GTT1005	rs140308		intron3	3767603	IVS3-36C>T	ctaactccctacC/Tccagtaactccc		0.106
MPJ6_GTT1006	rs4630	ssj0002197	3′-UTR	3766891	824(*101[b])T>C	ggaatggcttgcT/Ctaagacttgccc		0.106

[a]Novel variations detected in this study.
[b]The nucleotide that follows the translation termination codon TGA is numbered and starts as *1.

Table 5. Summary of *GSTM1* SNPs detected in a Japanese population

SNP ID			Location	Position		Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	Reported alleles	Allele frequency (N = 388)
This study	dbSNP (NCBI)	JSNP		NT_019273.18	From the translational initiation site or from the end of nearest exon				
MPJ6_GTM1001	rs412543	ssj0002146	5′-flanking	6137629	−552C>G	agactaagcccct**C/G**ggagtagctttc			0.044
MPJ6_GTM1002	rs3815029	ssj0002147	5′-flanking	6137641	−540C>G	gggagtagctttt**C/G**ggatcagaggaa			0.026
MPJ6_GTM1003	rs412302	ssj0002148	5′-flanking	6137701	−480A>G	tcccaggttggg**A/G**ccaccactttt			0.064
MPJ6_GTM1004	rs3815026		5′-flanking	6137758	−423C>G	ccctgggaact**C/G**ggcagcggagag			0.003
MPJ6_GTM1005*			5′-flanking	6137765	−416G>T	gaactcggcagc**G/T**gagagaaggctg			0.003
MPJ6_GTM1006	rs4147561	ssj0002149	5′-flanking	6137783	−398C>T	aaggctgagggac**C/T**accgcgggcagg			0.077
MPJ6_GTM1007	rs4147562	ssj0002150	5′-flanking	6137784	−397A>T	aggctgagggac**A/T**ccgcgggcaggg			0.077
MPJ6_GTM1008	rs4147563	ssj0002151	5′-flanking	6137788	−393T>C	tgagggacaccg**T/C**ggcagggagga			0.080
MPJ6_GTM1009	rs28549287	ssj0002152	5′-flanking	6137823	−358G>A	gagctttgctcc**G/A**ttaggatctggc			0.075
MPJ6_GTM1010*			5′-flanking	6138016	−165A>G	cttactgagtgc**A/G**ccccaggcgcc			0.003
MPJ6_GTM1011*			intron1	6138313	IVS1+97C>T	tcctcttcaggg**C/T**gcccgcctcag			0.003
MPJ6_GTM1012*			intron1	6138398	IVS1−79G>A	ggtacgtgcagt**G/A**taaactgggggc			0.003
MPJ6_GTM1013*			intron1	6138399	IVS1−78T>A	gtacgtgcagtg**T/A**aaactgggggct			0.003
MPJ6_GTM1014	rs4147564	ssj0002153	intron2	6138670	IVS2+118T>C	ctgcaggctgtc**T/C**cttccctgagcc			0.003
MPJ6_GTM1015*			intron2	6138754	IVS2+202G>A	ctgtctaattgg**G/A**acgggtgtccct			0.003
MPJ6_GTM1016	rs737497	ssj0002154	intron3	6139277	IVS3−78C>T	cccggtctcctc**C/T**ctgctcttgctt			0.077
MPJ6_GTM1017	rs4147565	ssj0002155	intron4	6139462	IVS4+26A>G	gctgcaatgtgt**A/G**ggggaaggtgg			0.080
MPJ6_GTM1018	rs4147566	ssj0002156	intron5	6139772	IVS5+140C>T	cagttattctca**C/T**gactccaatgtc			0.077
MPJ6_GTM1019	rs1065411	ssj0002159	Exon7	6140823	519C>G	atttgagcccaa**C/G**tgcttggacgcc	Asn173Lys	*B	0.077
MPJ6_GTM1020	rs1056806	ssj0002160	Exon7	6140832	528C>T	caagtgcttgga**C/T**gccttcccaaat	Asp176Asp		0.077
MPJ6_GTM1021	rs4147569	ssj0002161	intron7	6143292	IVS7−221G>A	tgtagaatcttc**G/A**taagtgttagct			0.080
MPJ6_GTM1022	rs4147570	ssj0002162	3′-flanking	6144093	1107(*450)+41C>T[b]	ctggccatctac**C/T**cagactgtctgt			0.026
MPJ6_GTM1023*			3′-flanking	6144180	1107(*450)+128G>A[b]	ggattctgctgg**G/A**catagtaaggcg			0.003

*Novel variations detected in this study.
[b]The position of the 3′ end of exon 8 (1107 or *450)+the position in the 3′-flanking region. (*450 indicates the position from the termination codon TAG.)

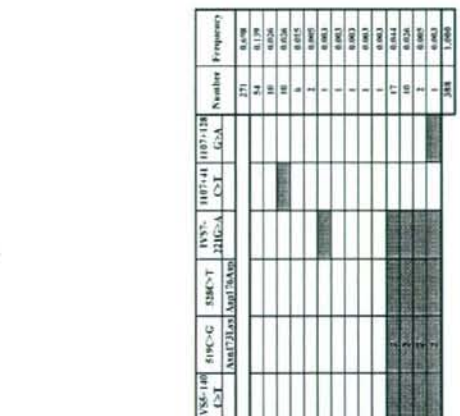

Fig. 1. *GSTT1* (a) and *GSTM1* (b) haplotypes in a Japanese population
Each haplotype is shown in the row, and the alleles are in the columns with the white cell being the major allele and gray cell the minor (nucleotide alteration). [a]Haplotypes were inferred in only one patient and were ambiguous except for the marker SNPs.

−398C>T, −397A>T, −393T>C, −358G>A, IVS3−78C>T, IVS4+26A>G, IVS5+140C>T, 519C>G (Asn173Lys), 528C>T (Asp176Asp), and IVS7−221G>A. Of these variations, two (−398C>T and −397A>T) and four (IVS3−78C>T, IVS5+140C>T, 519C>G, and 528C>T) pairs of SNPs were in perfect LD ($r^2 = 1.0$).

Haplotype estimation and selection of haplotype-tagging SNPs (htSNPs): Based on results of the LD profiles, haplotypes of *GSTT1* and *GSTM1* were analyzed as one LD block that spans at least 7.7 kb and 6.5 kb, respectively. Using the six variations and null alleles in *GSTT1*, three common haplotypes (*GSTT1*0*, **1a* and **1b*) and three rare haplotypes (**1c*, **1d* and **2a*) were identified or inferred (**Fig. 1a**). Frequencies of the common haplotypes, **0*, **1a*, and **1b*, were 0.686, 0.201, and 0.106, respectively. Thus, the htSNPs are either one of IVS1+166A>G, IVS3−36C>T, and 824T>C for **1b* and 226C>A for **2*.

For the *GSTM1* gene, three groups of haplotypes (*GSTM1*0*, **1* and **2*), each containing 1, 10 and 4 subtypes, were identified or inferred using the 23 variations and the null allele (**Fig. 1b**). The **2* group (**2a* to **2d*) was defined as the haplotypes harboring the known nonsynonymous SNP, 519C>G (Asn173Lys), which was previously assigned **B*.[8] The most dominant haplotype was **0* (0.698 frequency), followed by **1a* (0.139), **2a* (0.044), **1b* (0.026), **1c* (0.026), and **2b* (0.026). These six haplotypes accounted for 95% of all haplotypes. The htSNPs that were able to resolve the 5 common haplotypes of the intact genes were −552C>G (**1b* and **1d*), −540C>G (**2b*), −480A>G (**1b* and **2b*), 519C>G (Asn173Lys) (**2*), and 1107+41C>T (**1c*).

Discussion

The present study provides the first comprehensive data on genetic variations of *GSTT1* and *GSTM1* in Japanese, the genes encoding the phase II metabolic enzymes important for cellular defense systems. Moreover, SNPs in intact genes were identified, and haplotype structures and tagging SNPs were shown.

It is well recognized that **0* alleles in *GSTT1* and *GSTM1* distribute with different frequencies in several ethnicities. We have shown that 47.4% and 47.9% of our Japanese population homozygously lack *GSTT1* (*GSTT1*0/*0*) and *GSTM1* (*GSTM1*0/*0*), respectively. The *GSTT1*0/*0* frequency is comparable to that reported previously in Japanese (54.0%)[14] and east Asians such as Koreans (46–62%)[7,15] and Chinese (49–58%),[16,17] but was higher than Malay (38%),[17] Indians (16%),[17] Caucasians (15–24%),[7,18] African Americans (22–24%),[7,18] Mexican Americans (10%),[7] and Scandinavians (15%).[7] On the other hand, no marked differences are found in the frequencies of *GSTM1*0/*0* between Caucasians (42–60%)[7,18] and East Asians including Japanese, Koreans

and Chinese (44–63%),[7,14–16] although these frequencies were higher than that of Africans (16–36%).[7,18] The subjects bearing neither *GSTT1* nor *GSTM1* were observed at 23.2%, the frequency of which is similar to Koreans (29.1%)[15] and Shanghai Chinese (24%),[16] but higher than Caucasians (7.5–10.4%)[7,18] and Africans (3.9%).[18]

A number of association studies of the *GSTM1* and *GSTT1* genotypes with cancer susceptibility and cancer therapy outcome have been reported; however, the results are sometimes conflicting.[5–7] In our 194 patients with mainly non-small cell lung cancers, the frequency of *GSTT1*0/*0* and *GSTM1*0/*0* was similar to those in healthy Japanese. This result is in good agreement with a body of literature where the effects of *GSTT1* and *GSTM1* null genotypes on lung cancer development were not clear unless other genetic traits affecting carcinogen metabolism such as *CYP1A1*2A* and *GSTP1*B* (Ile105Val) were combined.[7]

One novel *GSTT1* nonsynonymous variation (226C>A, Arg76Ser) was found in one subject. Arg76 is located in the α3 helix of N-terminal domain I, which forms glutathione binding sites.[19,20] In the crystal structure of human GSTT1-1, this residue closely (2.7 Å) contacts Tyr85 of another subunit (Protein Data Bank, 2C3T).[21] Arg76 is conserved among human, bovine and chicken, whereas this residue is a histidine in mouse and rat. Interestingly, rat and mouse GSTT2 have Ser at position 76.

Of the six SNPs detected in *GSTT1*, three were perfectly linked, resulting in a simple haplotype structure. One of the linked SNPs, 824T>C, was analyzed for various ethnicities in the SNP500Cancer Database (http://snp500cancer.nci.nih.gov/). Its frequency in Japanese (0.106) was comparable to that in Caucasians (0.121), but lower than that in Africans and African-Americans (0.70).

In the *GSTM1* 5′-flanking region (up to −650), eight known SNPs in the NCBI dbSNP database were also detected in this study. This was in contrast to *GSTT1*, in which no SNPs were detected in the 5′-flanking region (up to −801 bp). Murine GSTM1 is transcriptionally upregulated by the Myb proto-oncogene protein through the Myb-binding site (−58 to −63) in the *GSTM1* promoter,[22] whereas no studies on the mechanisms of transcriptional regulation have been performed with human GSTM1. The four common SNPs, −398C>T, −397A>T, −393T>C, and −358G>A (0.075–0.080 in frequencies), were almost perfectly linked with the known SNP, 519C>G (Asn173Lys, *GSTM1*B*) in Japanese. The GSTM1a-1a isozyme (Asn173) and GSTM1b-1b isozyme (Lys173) were reported to have similar catalytic activities *in vitro*.[8] Nevertheless the association of the *GSTM1*A* alleles has been shown with a reduced risk for bladder cancer.[23] Therefore, the functional significance of promoter SNPs on *GSTM1* expression should be further elucidated.

In conclusion, deletions of *GSTT1* and *GSTM1* in Japanese were analyzed by conventional PCR and Taq-Man real-time PCR. About one-fourth (0.232 in frequency) of subjects had double *GSTM1* and *GSTT1* null genotypes. In the intact *GSTT1* and *GSTM1* genes, six and 23 SNPs were identified, respectively, and three (*GSTT1*0*, **1a*, **1b*) and six (*GSTM1*0*, **1a*, **2a*, **1b*, **1c* and **2b*) common haplotypes were inferred. Only one rare nonsynonymous SNP (226C>A, Arg76Ser) was found in *GSTT1*, suggesting that this gene is highly conserved. These findings would be useful for pharmacogenetic studies that investigate the relationship between the efficacy of anticancer drugs and *GST* haplotypes.

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Weekly Administration of Epoetin Beta for Chemotherapy-induced Anemia in Cancer Patients: Results of a Multicenter, Phase III, Randomized, Double-blind, Placebo-controlled Study

Masahiro Tsuboi[1], Kohji Ezaki[2], Kensei Tobinai[3], Yasuo Ohashi[4] and Nagahiro Saijo[5]

[1]Department of General Thoracic and Thyroid Surgery, Tokyo Medical University Hospital, Tokyo, [2]Department of Internal Medicine, Fujita Health University School of Medicine, Aichi, [3]Hematology and Stem Cell Transplantation Division, National Cancer Center Hospital, Tokyo, [4]Department of Biostatistics, School of Public Health, University of Tokyo, Tokyo and [5]National Cancer Center Hospital East, Chiba, Japan

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Objective: The efficacy and safety of weekly administration of epoetin beta (EPO) for chemotherapy-induced anemia (CIA) patients was evaluated.
Methods: One hundred and twenty-two patients with lung cancer or malignant lymphoma undergoing chemotherapy were randomized to the EPO 36 000 IU group or the placebo group. Hematological response and red blood cell (RBC) transfusion requirement were assessed. Quality of life (QOL) was assessed using the Functional Assessment of Cancer Therapy-Anemia (FACT-An) questionnaire.
Results: Mean change in hemoglobin level with EPO increased significantly over placebo (1.4 ± 1.9 g/dl versus -0.8 ± 1.5 g/dl; $P < 0.001$). The proportion of patients with change in hemoglobin level ≥ 2.0 g/dl was higher for EPO than those for placebo ($P < 0.001$). After 4 weeks of administration, the proportion of RBC transfusion or hemoglobin level < 8.0 g/dl was significantly lower for EPO than those for placebo ($P = 0.046$). The changes in the FACT-An total Fatigue Subscale Score (FSS) were less deteriorated with EPO than those with placebo. Progressive disease (PD) did not influence the change in hemoglobin level but there was less decrease in FSS in non-PD patients. No significant differences in adverse events were observed. Thrombovascular events and pure red cell aplasia related to EPO were not observed. Retrospective analysis of survival showing the hazard ratio of EPO to placebo was 0.94.
Conclusion: Weekly administration of EPO 36 000 IU significantly increased hemoglobin level and ameliorated the decline of QOL in CIA patients over the 8-week administration period.

Key words: anemia – erythropoietin – cancer – chemotherapy-induced anemia – quality of life – survival

INTRODUCTION

One of the causes of anemia in cancer patients is myelosuppression due to chemotherapy or radiation therapy (1). Anemia occurs at a high frequency when using platinum agents, taxanes or anthracyclines often used in cancer patients, especially in patients with lung cancer and malignant lymphomas. Clinical symptoms associated with anemia such as tachycardia, palpitations, fatigue, vertigo and dyspnea are observed in patients with hemoglobin level < 10.0 g/dl, and quality of life (QOL) patients is markedly reduced.

In Japan, only red blood cell (RBC) transfusions have been approved for the treatment of chemotherapy-induced anemia (CIA). However, although the safety of RBC transfusions has improved, there are still concerns about viral infections and graft-versus-host disease, as well as adverse effects on survival prognosis. Erythropoiesis-stimulating agents (ESAs) were approved for the treatment of CIA in the 1990s in the United States and in Europe, but they have still not

For reprints and all correspondence: Masahiro Tsuboi, Department of Thoracic Surgery and Oncology, Tokyo Medical University and Hospital 6-7-1, Nishi-shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan.
E-mail: mtsuboi@za2.so-net.ne.jp

been approved for this indication in Japan. It has been reported that the requirement for RBC transfusion can be reduced and QOL improved by increasing the hemoglobin level by ESA administration (2–7). In the United States, 'Use of epoetin in patients with cancer: evidence-based clinical practice guidelines of the American Society of Clinical Oncology and the American Society of Hematology' (8) (the ASH/ASCO guidelines) was published in 2002. The present placebo-controlled, double-blind, comparative study was planned in 2003 based on the ESAs guidelines and applications for ESAs in the United States and Europe for reference. Since 2003, however, several clinical studies have reported that ESAs worsened prognosis in cancer patients (9–16), and the risks of ESAs were investigated by three meetings of the Oncologic Drugs Advisory Committee (ODAC) (May 2004, May 2007 and March 2008). Since 2007, a safety alert (17) including a change in the upper hemoglobin limit has been issued, and the package inserts have been revised. The ASH/ASCO guidelines were also revised in 2007 (18). The effects of ESAs on cancer patient prognosis are not clear at present, and the US Food and Drug Administration (FDA) revised the labeling for ESAs following the 13 March 2008 ODAC's recommendations to impose additional restrictions.

As a result of a previous dose-finding study, once a week epoetin beta (EPO) 36 000 IU was recommended for Japanese cancer patients (19). In this prospective, placebo-controlled, double-blind comparative study, the efficacy and safety of weekly administration of EPO 36 000 IU was evaluated. Efficacy was assessed based on the hematological response and QOL. In addition, considering the recent regulatory conditions in the United States and in Europe, a survival survey was retrospectively performed, and survival in the EPO group and in the placebo group was compared.

PATIENTS AND METHODS

Patient population

The study protocol was approved by the institutional review board at each study site, and written informed consent was obtained before study-related procedures were begun. Patients eligible for this study were required to be patients of age $\geq$20 to $<$80 years, who had lung cancer or malignant lymphoma, were receiving a platinum-, taxane- or anthracycline-containing chemotherapy regimen with at least two cycles of chemotherapy scheduled after the first study drug administration and had CIA (8.0 g/dl $\leq$ hemoglobin level $\leq$ 11.0 g/dl), an Eastern Cooperative Oncology Group performance status (PS) $\leq$2, life expectancy $\geq$3 months as well as adequate renal and liver function. Exclusion criteria included iron-deficiency anemia (serum iron saturation $<$15% or mean corpuscular volume (MCV) $<$80 μm^3), history of myocardial, pulmonary or cerebral infarction, severe hypertension beyond control by drug therapy, pregnancy, obvious hemorrhagic lesions or other severe complications, myeloid malignancy or ESA/RBC transfusion within 4 weeks before the first study drug administration.

Study design

Patients were randomized 1:1 to receive EPO 36 000 IU or placebo subcutaneously once a week for 8 weeks. The planned number of patients was 120 (60 in each group). Randomization was conducted by central registration system and a dynamic balancing method using tumor type, PS, age and institution as the adjusting factors. Administration was terminated if the hemoglobin level reached 14 g/dl or more. Oral iron-supplementing drugs were administered if serum iron saturation fell below 15% or MCV fell $<$80 μm^3. Hemoglobin level and clinical laboratory tests were monitored weekly until 1 week after last study drug administration. RBC transfusion was allowed at the discretion of the investigator during the study.

Study endpoints

The primary endpoint was change in hemoglobin level from baseline, and the last evaluation was performed 8 weeks after the first study drug administration or at study discontinuation. The last observation carried forward method was used for evaluation of the change in hemoglobin level. The secondary endpoints were change in the Functional Assessment of Cancer Therapy Anemia total Fatigue Subscale Score (FSS) (0–52, where a higher score means less fatigue) from baseline to last evaluation, RBC transfusion requirement, nadir hemoglobin level, proportion of patients who achieved a hemoglobin level increase $\geq$2.0 g/dl from baseline, proportion of the patients with hemoglobin level $<$8.0 g/dl during the study and incidence of either RBC transfusion or hemoglobin level $<$8.0 g/dl. Safety was assessed by National Cancer Institute – Common Toxicity Criteria, ver. 2, translated by the Japan Clinical Oncology Group. Anti-erythropoietin antibodies were measured by enzyme-linked immunosorbent assay and radioimmunoprecipitation assay, and compared with the data of the first study drug administration with the data of the last observation. Detection by either method was judged as positive. A retrospective analysis of survival was performed.

Statistics

Efficacy analyses were performed using the full-analysis-set (FAS) population, comprising all eligible patients who received a study drug. In both EPO and placebo groups, changes in hemoglobin level and changes in FSS at the last evaluation were compared using Student's *t*-test. Stratified analyses in the groups with baseline FSS $>$36 and $\leq$36, respectively, were also performed.

RESULTS

Patient disposition

One hundred and twenty-two patients were recruited from February 2004 to March 2005 at 11 sites in Japan. Sixty-five patients had lung cancer and 57 had malignant lymphoma. The patients were randomly assigned to the EPO group ($n = 63$) or the placebo group ($n = 59$). One patient in each group never received a study drug, one patient in each group never received chemotherapy and one patient in the placebo group did not have laboratory data after administration. Thus, the FAS population was 117 patients (61 patients in the EPO group, 56 patients in the placebo group).

Demographics, clinical and baseline characteristics

Patient demographics were well balanced between the two groups, except for baseline hemoglobin levels and serum erythropoietin concentrations (Table 1). The mean hemoglobin level in the EPO group was slightly lower than in the placebo group (10.0 versus 10.4 g/dl). The baseline hemoglobin level did not influence the evaluation of the primary endpoint by analysis of covariance.

Hematological evaluations

Mean change in hemoglobin level at the last evaluation significantly increased in the EPO group (1.4 ± 1.9 g/dl) than in the placebo group (-0.8 ± 1.5 g/dl) ($P < 0.001$). The hemoglobin level started to elevate in the EPO group at 3 weeks after the first administration (Figs 1 and 2). After 4–8 weeks of administration, the proportion of patients who achieved changes in hemoglobin level ≥ 2.0 g/dl from baseline was 42.6% (26/61) for the EPO group and 1.8% (1/56) for the placebo group ($P < 0.001$).

During the study, the proportion of patients with the hemoglobin level increased 12.0 g/dl or more was evaluated in the patients with hemoglobin level below 12.0 g/dl at baseline, the proportion was higher in the EPO group than in the placebo group [49.2% (29/59) versus 9.6% (5/52), $P < 0.001$]. The nadir hemoglobin level was 9.4 ± 1.5 g/dl in the EPO group and 8.6 ± 1.3 g/dl in the placebo group ($P = 0.002$). The proportion of patients with hemoglobin level decreased <8.0 g/dl was evaluated in the patients with hemoglobin level >8.0 g/dl at baseline, the proportion was 18.6% (11/59) in the EPO group and 32.1% (18/56) in the placebo group ($P = 0.096$).

RBC transfusion

The incidence of RBC transfusion was not different between the EPO group and the placebo group throughout the study [11.5% (7/61) versus 12.5% (7/56), $P = 0.865$] or from Week 5 to Week 8 [8.2% (5/61) versus 12.5% (7/56), $P = 0.443$]. However, the incidence of RBC transfusion or hemoglobin level <8.0 g/dL from Week 5 to Week 8 was significantly lower in the EPO group than those in the placebo group [16.4% (10/61) vs. 32.1% (18/56), $P = 0.046$], and fewer RBC transfusion units were required in the EPO group (10 units, $n = 5$) than in the placebo group (26 units, $n = 7$).

Quality of life

At the last observation, the FSS data for two patients were missing because of progressive disease (PD). The missing scores were substituted by the maximum decrease in score

Table 1. Patient demographics of full-analysis-set population

	Placebo group ($n = 56$)	EPO group ($n = 61$)
Sex		
Male	33	34
Female	23	27
Age (years), mean ± SD	62.1 ± 9.6	61.8 ± 11.9
Tumor		
Lung cancer	30	32
Small cell lung cancer	7	8
Non-small cell lung cancer	23	24
Malignant lymphoma	26	29
Hodgkin lymphoma	0	3
Non-Hodgkin lymphoma	26	26
Chemotherapy		
1st line	38	41
2nd line	6	8
3rd line	1	1
Relapse/recurrence	11	11
ECOG performance status		
0	38	33
1	17	26
2	1	2
Weight (kg), mean ± SD	54.5 ± 8.8	55.2 ± 10.0
Hemoglobin (g/dl), mean ± SD	10.4 ± 1.0	10.0 ± 1.0
Serum endogenous erythropoetin (mU/ml), mean ± SD	49.1 ± 33.4	67.3 ± 72.0
MCV (fl), mean ± SD	93.5 ± 6.0	91.9 ± 5.5
Transferrin saturation (%), mean ± SD	29.4 ± 19.8	32.4 ± 22.0
Baseline QOL: FACT-An		
Fatigue subscale (0–52), mean ± SD	33.9 ± 10.0	35.5 ± 9.7
≤36	29	29
>36	26	32
Data missing	1	0

SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; QOL, quality of life; FACT-An, Functional Assessment of Cancer Therapy-Anemia; MCV, mean corpuscular volume; EPO, epoetin beta.

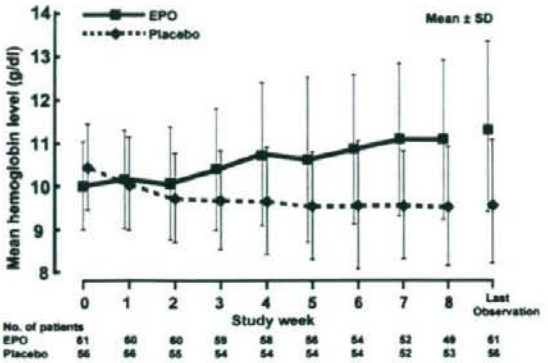

Figure 1. Hemoglobin level during the treatment period. A colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org. SD, standard deviation; EPO, epoetin beta.

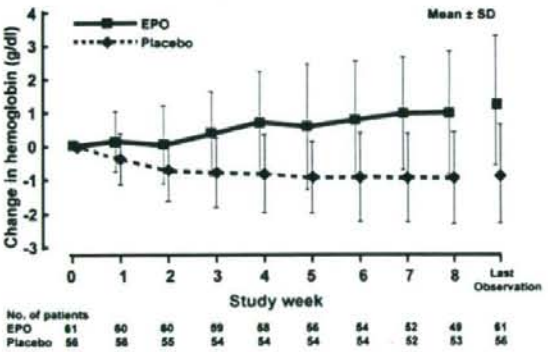

Figure 2. Change in hemoglobin level during the treatment period. A colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org.

for all patients. This substitution was decided before blinded data review. The changes in FSS from baseline were less in the EPO group than those in the placebo group (Mean ± SD: -0.5 ± 9.4 versus -4.5 ± 10.0, $P = 0.031$). But excluding these two patients with missing data at the last observation, the change in FSS from baseline was not significant in the EPO group and in the placebo group (-0.5 ± 9.4 versus -3.6 ± 9.0, $P = 0.082$). The factors that influenced the change in FSS were baseline FSS, change in hemoglobin level, treatment group and PS at the last observation (analysis of variance). It has been suggested that if the baseline FSS is higher than 36, the change in FSS will decrease after administration of ESA because of the high baseline and the lack of symptoms (ceiling effect and regression to the mean) (20,21). Thus, we also analyzed patients whose baseline FSS was ≤36. In the baseline FSS ≤ 36 patients, change in FSS was 2.1 ± 11.7 in the EPO group and -1.3 ± 9.6 in the placebo group, so the EPO group showed improvement in FSS ($P = 0.225$). However, in the baseline FSS > 36 patients, the change in FSS was -2.9 ± 5.9 in the EPO group and -7.9 ± 9.4 in the placebo group ($P = 0.016$), so the EPO group showed suppression of the decline in FSS (Fig. 3). In subset analysis of the EPO group, the mean change in hemoglobin level did not differ in PD and non-PD patients (1.3 ± 1.8 versus 1.4 ± 2.0 g/dl), but PD patients showed a more marked decrease in FSS than non-PD patients (-6.8 ± 9.4 versus 0.2 ± 9.2).

Safety

The incidence of adverse events was evaluated for the 120 patients who receive a study drug. Adverse events were observed in 62 patients (100%) in the EPO group and 57 patients (98.3%) in the placebo group, and no significant differences were found between the two groups ($P = 0.299$). The adverse events related to the study drug were 24 events in the EPO group (17 of 62 patients, 27.4%) and 19 events in the placebo group (11 of 58 patients, 19.0%) ($P = 0.274$). Adverse drug reactions observed in at least 3% of the patients in the EPO group were increased blood pressure (6.5%), increased lactate dehydrogenase (3.2%) and increased urinary glucose (3.2%). In the placebo group, rash (3.4%), increased blood pressure (3.4%) and decreased activated partial thromboplastin time (3.4%) were reported. Grade 3 abdominal pain and Grade 3 liver dysfunction were both observed in the same patients in the EPO group. Five patients (5 events) in the EPO group and five patients (12 events) in the placebo group experienced serious adverse events. Of these, only Grade 3 liver dysfunction was considered related to EPO treatment (Table 2). One thrombovascular event (TVE), a lacunar infarction, was reported in the EPO group. No other TVEs were reported in either group. No anti-erythropoietin antibodies were reported.

Survival

A retrospective analysis of survival was performed. The median follow-up duration was 670 days for the EPO group

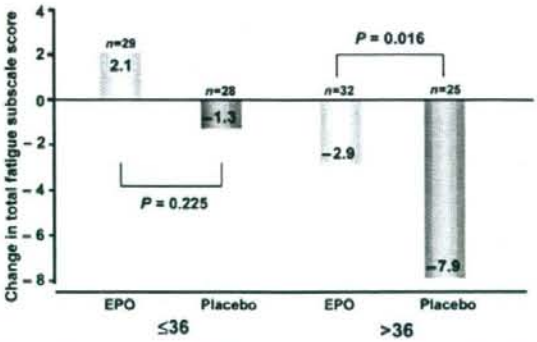

Figure 3. Mean change in FACT-An total fatigue subscale score stratified by baseline total fatigue subscale score (≤36, >36). A colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org. FACT-An, Functional Assessment of Cancer: Therapy-Anemia.

Table 2. Incidence of the most common adverse events

	Placebo group ($n = 58$)		EPO group ($n = 62$)	
	No. of patients	%	No. of patients	%
Adverse events	57	98.3	62	100
Adverse events with incidence ≥20% in the EPO group				
Neutropenia	47	81.0	47	75.8
Leukopenia	46	79.3	47	75.8
Thrombocytopenia	28	48.3	31	50.0
Nausea	28	48.3	27	43.5
Fatigue	26	44.8	28	45.2
Anorexia	24	41.4	27	43.5
Lymphopenia	24	41.4	32	51.6
Alopecia	17	29.3	22	35.5
Increased LDH	15	25.9	16	25.8
Constipation	10	17.2	14	22.6
Adverse drug reactions	11	19.0	17	27.4
Adverse drug reactions with incidence ≥3% in either group				
Increased blood pressure	2	3.4	4	6.5
Increased LDH	1	1.7	2	3.2
increased urinary glucose	0	0.0	2	3.2
Rash	2	3.4	0	0.0
Decreased APTT	2	3.4	0	0.0
Adverse drug reactions with severity ≥Grade 3				
Abdominal pain	0	0.0	1	1.6
Liver dysfunction	0	0.0	1	1.6

LDH, lactate dehydrogenase; APTT, activated partial thromboplastin time.

and 641 days for the placebo group. The 1-year survival population based on Kaplan–Meier estimates was 64.9% in the EPO group and 65.9% in the placebo group. The hazard ratio was 0.94 for the EPO group relative to the placebo group (95% CI: 0.57–1.53).

DISCUSSION

Improvements in hemoglobin level were observed in Japanese patients with CIA on administration of EPO 36 000 IU once a week for 8 weeks. In the evaluation of QOL, it is necessary to consider the effects of scores at baseline, such as the ceiling effect and regression to the mean (20). It has been reported that in patients with less symptoms as baseline FSS is more than 36, the change in FSS became negative (21). The results of a stratified analysis of groups with baseline FSS ≤36 and >36 (performed for reference) showed that in patients with baseline FSS ≤36 (severe anemia symptoms), the symptoms of anemia improved in the EPO group, but worsened in the placebo group. In patients with baseline FSS >36 (mild anemia symptoms), worsening occurred in both groups, but the worsening was significantly inhibited in the EPO group compared with the placebo group. In the United States, at present, the FDA has not approved the use of ESAs to improve QOL, but the results of this study suggest that EPO may be useful in the prevention of worsening of symptoms of anemia.

In the United States, it has been stressed that the purpose of using ESAs is to treat CIA in order to avoid RBC transfusions. In the present study, the incidence of RBC transfusion during administration was low and the hemoglobin level when RBC was transfused was 5.5–8.8 g/dl. In Japan, most physicians and patients are reluctant to use RBC transfusions, but in the United States and in Europe, RBC transfusions are often started when the hemoglobin level is 8.0–10.0 g/dl (22). In this study, the proportion of patients with either severe anemia requiring a RBC transfusion or hemoglobin level of <8.0 g/dl (NCI-CTC Grades 3 and 4) was examined. Evaluation of this proportion from 4 weeks after the start of administration, when ESAs exhibited hematopoietic effects (23–25), indicated that this proportion was significantly lower in the EPO group (16.4%, 10 of 61 patients) than in the placebo group (32.1%, 18 of 56 patients) ($P = 0.046$).

One TVE was observed in this study, a lacunar infarction (Grade 1) in one patient (69-year-old male with lung cancer) in the EPO group. The investigator judged without causal relationship to the study drug but by aging, because the event was observed 1 day after the first study drug administration. No other TVEs were reported. Increased blood pressure and hypertension occurred in 10 patients (six in the EPO group, four in the placebo group). Marked differences from the placebo group were not observed for other adverse events.

The FDA has issued several safety alerts regarding data that demonstrated adverse survival outcomes in ESA-treated cancer patients. In this study, however, based on the results of a survey of overall survival, the 1-year survival proportion showed no significant difference between the groups. The effects of ESAs on survival of cancer patients have been examined by the ODAC and other groups since 2007, based on new clinical trial reports. So far, the reported safety data have been insufficient to rule out the risk of mortality in chemotherapy-treated patients, but ESAs are considered a therapeutic option for the management of CIA. Clinical studies based on the doses and hemoglobin levels recommended on the labels will continue to accumulate evidence on the effects of ESAs on survival.

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Conflict of interest statement

The author, Yasuo Ohashi, receives consultation fee from Chugai Pharmaceutical Co., Ltd.: the author advises on design and data analysis of clinical trials.

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Clinical Studies

A randomised trial of intrapericardial bleomycin for malignant pericardial effusion with lung cancer (JCOG9811)

H Kunitoh*[,1], T Tamura[1], T Shibata[2], M Imai[2], Y Nishiwaki[3], M Nishio[4], A Yokoyama[5], K Watanabe[6], K Noda[7] and N Saijo[8], JCOG Lung Cancer Study Group, Tokyo, Japan

[1]Department of Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; [2]JCOG Data Center, Center for Cancer Control and Information Services, National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; [3]Department of Thoracic Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanohara, Kashiwashi, Chiba 277-8577, Japan; [4]Department of Medical Oncology, Cancer Institute Hospital, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan; [5]Department of Medical Oncology, Niigata Cancer Center, 2-15-3, Kawagishi-cho, Niigata-shi, Niigata 951-8566, Japan; [6]Department of Respiratory Medicine, Yokohama Municipal Citizen's Hospital, 56 Okazawa-cho, Hodogaya-ku, Yokohama, Kanagawa 240-8555, Japan; [7]Division of Thoracic Oncology, Kanagawa Cancer Center, 1-1-2 Nakao, Asahi-ku, Yokohama, Kanagawa 241-0815, Japan; [8]National Cancer Center Hospital East, 6-5-1 Kashiwanohara, Kashiwashi, Chiba 277-8577, Japan

Safety and efficacy of intrapericardial (ipc) instillation of bleomycin (BLM) following pericardial drainage in patients with malignant pericardial effusion (MPE) remain unclear. Patients with pathologically documented lung cancer, who had undergone pericardial drainage for MPE within 72 h of enrolment, were randomised to either arm A (observation alone after drainage) or arm B (ipc BLM at 15 mg, followed by additional ipc BLM 10 mg every 48 h). The drainage tube was removed when daily drainage was 20 ml or less. The primary end point was survival with MPE control (effusion failure-free survival, EFFS) at 2 months. Eighty patients were enrolled, and 79 were eligible. Effusion failure-free survival at 2 months was 29% in arm A and 46% in arm B (one-sided $P = 0.086$ by Fisher's exact test). Arm B tended to favour EFFS, with a hazard ratio of 0.64 (95% confidence interval: 0.40–1.03, one-sided $P = 0.030$ by log-rank test). No significant differences in the acute toxicities or complications were observed. The median survival was 79 days and 119 days in arm A and arm B, respectively. This medium-sized trial failed to show statistical significance in the primary end point. Although ipc BLM appeared safe and effective in the management of MPE, the therapeutic advantage seems modest.
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Keywords: malignant pericardial effusion; lung cancer; drainage; sclerosis; intrapericardial instillation; bleomycin

Malignant pericardial effusion (MPE) is a grave complication of malignant tumours. The frequency of pericardial involvement by malignancy has been estimated to be 10–21% at autopsy (Theologides, 1978; Klatt and Heitz, 1990).

Malignant pericardial effusions are often asymptomatic and detected incidentally by echocardiography or computed tomography. Symptomatic cases, however, often manifest cardiac tamponade, which can rapidly lead to cardiovascular collapse and death, unless promptly treated (Press and Livingston, 1987).

Lung cancer is the most frequent cause of MPE, and other common primary sites include breast cancer, oesophageal cancer, lymphoma and leukaemia (Abraham *et al*, 1990; Wilkes *et al*, 1995; Yonemori *et al*, 2007). The prognosis of MPE in lung cancer patients is particularly poor, with a reported median survival of 3 months or less (Okamoto *et al*, 1993; Gornik *et al*, 2005).

Although prompt diagnosis and pericardial drainage result in good palliation of symptoms, drainage alone is often inadequate to prevent re-accumulation of the fluid after the drainage tube is removed (Shepherd, 1997). There are numerous reports of pericardial sclerosis for MPE by the instillation of various agents, such as tetracycline/doxycycline (Shepherd *et al*, 1987; Maher *et al*, 1996), a streptococcal preparation (Imamura *et al*, 1991), bleomycin (BLM) (Vaitkus *et al*, 1994; Liu *et al*, 1996; Maruyama *et al*, 2007), thiotepa (Colleoni *et al*, 1998; Martinoni *et al*, 2004), cisplatin/carboplatin (Moriya *et al*, 2000; Tomkowski *et al*, 2004), 5-fluorouracil (Lerner-Tung *et al*, 1997), anthracyclines (Kawashima *et al*, 1999), vinblastine (Primrose *et al*, 1983), mitoxyantrone (Norum *et al*, 1998), mitomycin C (Kaira *et al*, 2005) and ^{32}P-colloid (Dempke and Firusian, 1999), after drainage. Platinum agents are actually not 'classic' sclerosants to induce inflammatory adhesion of the pericardial sac; they were apparently used as local chemotherapy. Whereas each study reports favourable outcomes in terms of MPE control and prevention of re-accumulation, almost all were performed as phase II trials, and no definite conclusions could be drawn (Press and Livingston, 1987; Vaitkus *et al*, 1994).

In one of the very few randomised trials conducted to date, Liu *et al* (1996) reported that BLM is the preferred agent for sclerosis, because of the lower morbidity associated with it. However, to the best of our knowledge, the efficacy and safety of pericardial sclerosis itself has never been evaluated by a prospective randomised trial.

This trial was aimed at evaluating the safety and efficacy of pericardial sclerosis induced by intrapericardial (ipc) BLM

*Correspondence: Dr H Kunitoh; E-mail: hkkunito@ncc.go.jp
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instillation, as compared with pericardial drainage alone, in lung cancer patients with MPE.

PATIENTS AND METHODS

Patient eligibility criteria

Patients with pathologically documented lung cancer, who had undergone pericardial drainage for clinical MPE (moderate to large accumulation of fluid), were eligible for study entry. Indications for the drainage were clinically determined; cases after emergent drainage and those after elective one were both included. Patient registration should be done within 72 h of drainage. The eligibility criteria were as follows: 75 years of age or less, expected life prognosis of 6 weeks or more with control of the MPE and minimum organ functions (leukocyte count $\geqslant 3000$ per mm^3, platelet count $\geqslant 75\,000$ per mm^3, haemoglobin $\geqslant 9.0\,g\,dl^{-1}$ and no renal or hepatic failure; however, laboratory abnormalities related to cardiac tamponade were allowed). Patients with chemotherapy-naive small cell cancer were excluded. Other exclusion criteria included apparently non-malignant effusion (e.g., purulent effusion), recurrent MPE, myocardial infarction or unstable angina within the previous 3 months, constrictive pericarditis, active interstitial pneumonia, severe infection and disseminated intravascular coagulation. Those with an unstable clinical condition attributable to other severe complications, such as superior vena cava syndrome, central airway obstruction or uncontrollable massive pleural effusion, were also excluded.

Patient eligibility was confirmed by the Japan Clinical Oncology Group Data Center before patient registration. The study protocol was approved by the institutional review boards at each participating centre and all the patients provided written informed consent.

Treatment plan

The study protocol did not limit the method used for the pericardial drainage. Both percutaneous tube pericardiostomy (non-surgical method), in which a drainage catheter is inserted using the Seldinger technique, and subxiphoid pericardiostomy (surgical method), in which a drainage tube is placed surgically, were allowed; each participating institution, however, basically adhered to one method, which they used in routine practice. The drainage method used was recorded on the case report form.

After registration with telephone or facsimile, the patients were randomly assigned to one of the two treatment arms with block randomisation stratified by the institution. In arm A, no additional intervention was performed and the patient was observed clinically after the pericardial drainage. In arm B, 15 mg of BLM dissolved in 20 ml of normal saline was instilled through the drainage catheter into the pericardial space immediately after the patient registration. The catheter was then clamped and reopened after 2 h, allowing resumption of the drainage. Additional doses of BLM at 10 mg were instilled similarly every 48 h, unless the criteria for tube removal, as described below, were met.

The drainage tube was removed, in both arm A and arm B, when the drainage volume per 24 h was 20 ml or less. If the criterion was met during the 24 h preceding randomisation in a patient allocated to arm A, the tube was immediately removed.

Patient evaluation and follow-up

Primary control of the MPE was considered to be achieved when the drainage tube could be successfully removed within 7 days of randomisation. When the criterion for tube removal, that is 20 ml per 24 h, could not be met by 7 days, the case was judged to show primary failure of the protocol therapy: treatment after off-protocol was not limited by the study protocol. When the drainage tube had to be removed because of obstruction, but re-drainage was clinically unnecessary, it was judged to have been successfully removed with primary control of MPE.

Monitoring for recurrence of the MPE in those who showed primary control was conducted by echocardiography at 1, 2, 4, 6 and 12 months. When the estimated fluid volume in the recurrent effusion exceeded 100 ml, the case was labelled as showing MPE re-accumulation and recurrence. Re-drainage was performed as clinically indicated.

The adverse effects of the therapy were evaluated according to the Japan Clinical Oncology Group Toxicity Criteria (Tobinai *et al*, 1993), modified from the National Cancer Institute Common Toxicity Criteria version 1.

The primary end point of the study was effusion failure-free survival (EFFS) rate at 2 months; EFFS was patient survival without MPE recurrence as defined above, in patients showing primary control. It was calculated as the period from the date of pericardial drainage to the date of MPE recurrence or the patient's death. For those patients with primary failure, MPE recurrence was considered to have occurred at the date of drainage, with an EFFS of zero. Effusion failure-free survival was judged regardless of the other disease status.

The secondary end points included the primary MPE control rate, time to drainage tube removal, EFFS, treatment-related morbidity, proportion of late pericardial or cardiac complication, overall survival (OS) and symptom scores.

Study-specific four-item symptom scores were completed by patients at the time of randomisation (i.e., after pericardial drainage) and at 1 month after the enrolment. The scores were to be interviewed by the health professionals other than the attending physicians. The items consisted of cough, pain, anorexia and shortness of breath. The scoring was conducted as follows: as not at all present (0), a little (1), moderate (2) and very much (3). The score for each item and the sum of the total score for all the four items were compared between the baseline and the follow-up assessments, and judged to be improved (lower scores in the follow-up assessments), stable (no change of scores) or worsened (higher scores, or the patient could not fill out the questionnaire, in the follow-up assessments).

Statistical considerations

From the historical data, the EFFS rate at 2 months in arm A was assumed to be 30% and that in arm B was presumed to be 60%. The study was designed to provide 80% power with 5% one-sided α. The required sample size was calculated as 80 patients, 40 in each arm, for comparing independent proportions.

The OS, time to tube removal and EFFS of both arms were calculated by the Kaplan–Meier method and compared by log-rank tests. The primary MPE control rate, symptom scores, complication rates and EFFS at each of the follow-up points were compared using Fisher's exact test. All analyses were performed with the SAS software version 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics and treatment delivery

From August 1999 to January 2006, 80 patients from 14 institutions were enrolled and randomised, 42 to arm A and 38 to arm B. One patient in arm B was found to be ineligible because of late registry, 2 weeks after the pericardial drainage. All 80 patients were analysed for their characteristics and chemotherapy morbidity, and the 79 eligible patients were analysed for efficacy and survival.

Table 1 lists the characteristics of the patients, which were generally well balanced between the arms, except for the effusion cytology: there were numerically more patients with