formed between patients 1 or 2 and each of the controls due to a coincidence in the SNP genotype. However, the RHS overlap IG-RCHH nest between the patients is likely to be longer than any of the RHS overlap IG-RCHH nests accidentally formed between a patient and a control.
doi:10.1371/journal.pone.0025059.g001

patients with Siiyama-type α1-antitrypsin deficiency; it was believed that this number was suited for demonstrating the power of the analysis and for enabling an easy interpretation of the analysis results.

Genotyping

SNP genotyping was performed using the Genome-Wide Human SNP Array 6.0 (Affymetrix).

Synthetic data

The synthetic genotyping data of a patient who shared 2 IBD fragments that contain a disease-causing gene were made as follows: (i) A subject was randomly chosen from the 261 subjects (see above) to serve as the MRCA. (ii) An SNP was randomly chosen from an autosomal region and was considered to mark the position of the disease-causing gene. (iii) The range of the chromosomal region that contained the SNP and was inherited by the patient from the MRCA was calculated according to the Haldane's Poisson process model [13]. (iv) Step (iii) was repeated for the second fragment. (v) The genotyping data of the patient corresponding to the regions that were obtained at steps (iii) and (iv) were replaced with those of the MRCA.

Variables investigated in HM on HH analysis of a population

The variables investigated were the number of subjects in the test population (20, 40, and 60), proportion of patients in the test population (0, 5, 10, 15, 20, 25, and 30%), generational distance of the MRCA (20, 40, 60, 80, and 100 generations), and the gene frequency in the general population (0.0, 0.05, and 0.1). A gene frequency of 0.0 was considered to represent a rare variant, while gene frequencies of 0.05 and 0.1 were considered to represent common variants.

Computer program

The program was written in the Ruby programming language (http://www.ruby-lang.org/en/) with an extension library written in the C programming language (http://gcc.gnu.org/). The program was executed on a MacPro computer that ran on MacOS X 10.6.

Program

HM on HH program is available at Homozygosity Haplotype Analysis Web site, http://www.hhanalysis.com

Results

HM on HH analysis in patients with Siiyama-type α1-antitrypsin deficiency

We tested the performance of HM on HH analysis by using the SNP genotypes of 6 unrelated patients with Siiyama-type α1-antitrypsin deficiency, a rare autosomal recessive disease in Japan caused by a founder mutation of the *SERPINA1* gene (MIM 107400) [12]. As controls, we employed the genotypes of 200 Japanese individuals from the general population. The results obtained after each of the 4 steps that compose HM on HH analysis are shown for a pair of patients (**Figure 3A**). After the completion of the analysis, 2 closely located regions with a total length of 1.4 centimorgans were identified, 1 of which contained *SERPINA1* (**Figure 3A**). The results of the other 14 patient-pair combinations (note that ${}_6C_2 = 15$) were similar: each combination identified candidate regions (total length: 1.2 to 21.8 centimorgans, median: 1.6 centimorgans) that contained *SERPINA1*. Using the genotyping data of only 2 patients, HM on HH analysis was able to narrow the position of the disease-causing gene to a very short chromosomal interval.

HM on HH analysis of a pair of synthetic patients

We further examined the performance of HM on HH analysis of a pair of patients using synthetic data. We investigated the MRCA at 5 different generational distances (20, 40, 60, 80 and 100 generations). For each distance, we employed 60 randomly selected subjects, so that a total of 1770 pairs (${}_{60}C_2 = 1770$) were constructed. Each pair was investigated for 100 randomly selected SNP locations, which were assumed to be the location of a disease-causing gene. The number of trials was thus 177000 (1770 combinations × 100 SNPs) for each generational distance.

Detection of the region containing the RHS overlap depended on the generational distance of the MRCA (**Figure 3B**). This was a major limitation of HM on HH analysis: at the HM step, only RHSs that were longer in length than the RHS cutoff were detected (**Figure S1A**) [10]. The detection will be improved by genotyping more SNPs at a genomewide level, which will allow the use of a smaller RHS cutoff value (**Figure S1B**). Once an RHS overlap was detected at the HM step, HM on HH analysis rarely failed to track it (**Figure 3C**): for the MRCA that occurred 20 generations earlier, the RHS overlap was falsely excluded (false negative) in only 1.5% of the cases, while the falsely included areas (false positive) were reduced from 61.7 centimorgans after the HM step to 0.47 centimorgans after the completion of the HH step, indicating that a small false positive is a prominent feature of HM on HH analysis. Data for the other generations of the MRCA are presented in **Figure S2**.

HM on HH analysis of a population

HM on HH analysis of a population targets a population containing multiple patients sharing an IBD fragment (**Figure 4A**). This simulates a situation in which the population is a collection of patients with the same disease, and some of the patients share an IBD gene. We attempt to identify (1) a patient subgroup sharing an IBD fragment and (2) the chromosomal location of the shared IBD fragment. Here, we defined the analysis level: at analysis level n, the computer program searches for a subgroup consisting of n patients, any pair of which shares an IBD fragment at the same position on the chromosome (**Figure 4B**). To achieve the aims (1) and (2) as stated above, the program identifies (a) the topmost analysis level at which any subgroup is detected, (b) the members that are contained in the subgroup, and (c) the position of the IBD fragment on the chromosome.

First, we investigated the background signal that was detected in the general population (**Figure 4C**). For this purpose, we employed 260 normal subjects. Step (a): 260 normal subjects were randomly divided into a test population (60 subjects) and 200 controls. Step (b): HM on HH analysis of a population was performed. Steps (a) and (b) were repeated 500 times. The histogram of the topmost analysis level, at which any subgroup was detected (**Figure 4D**), demonstrated that a subgroup could be falsely detected (i.e., false positive) in the level 4 analysis and in an earlier analysis level. Conversely, when a positive result was

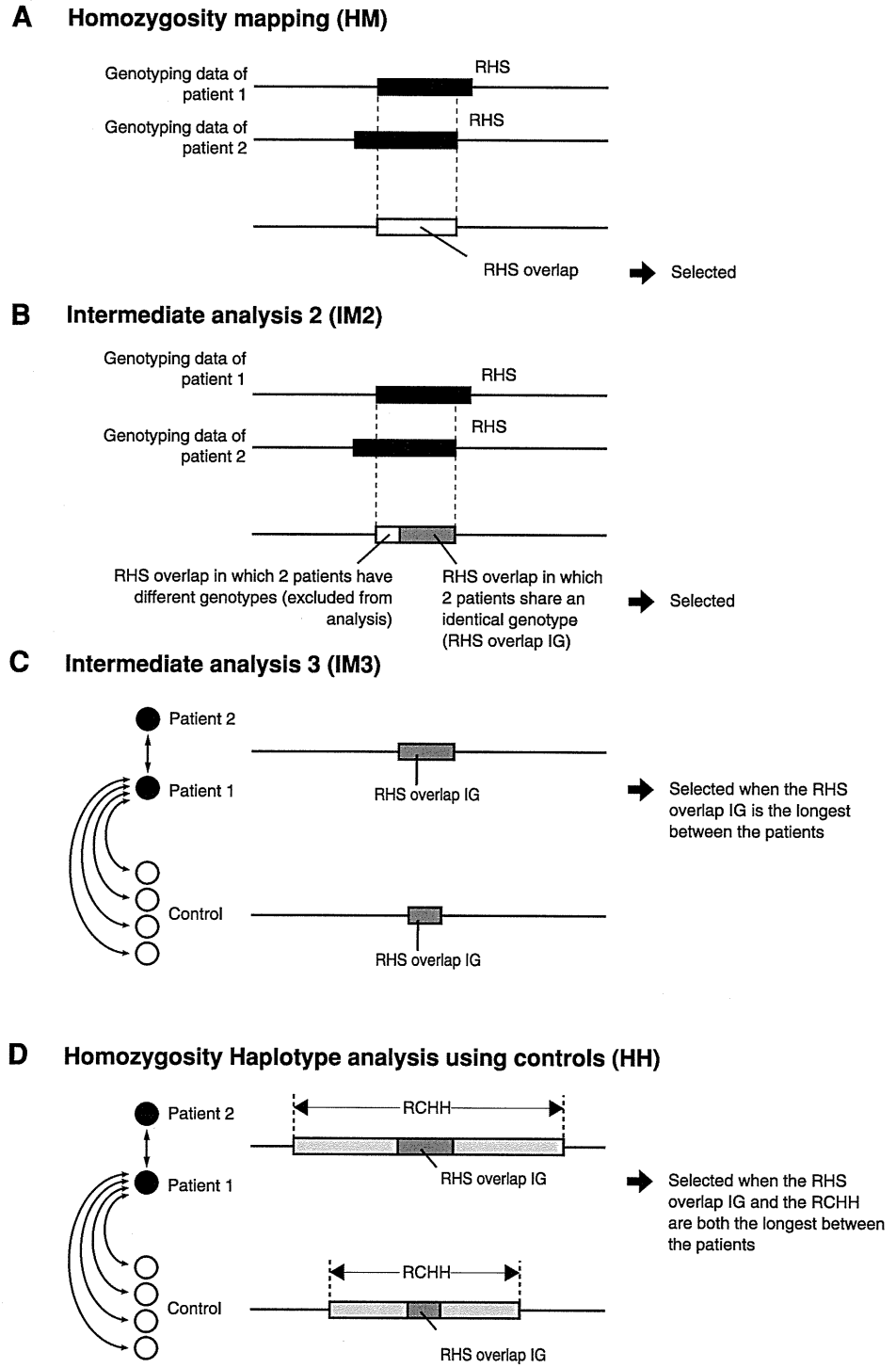

Figure 2. Steps of HM on HH analysis. HM on HH analysis is composed of 4 steps that are serially performed. (A) Homozygosity mapping (HM). The RHSs are determined for each patient, and the RHS overlaps are obtained. (B) Intermediate analysis 2 (IM2). The RHS overlap IGs are determined. (C) Intermediate analysis 3 (IM3). The RHS overlap IGs are compared. The RHS overlap IG is selected as a candidate region when the RHS overlap IG is the longest between the patients. (D) HH analysis using controls. RHS overlap IG-RCHH nests are compared. The RHS overlap IG is selected as a candidate region when the RHS overlap IG and the RCHH are both the longest between the patients.
doi:10.1371/journal.pone.0025059.g002

obtained in the level 5 analysis or in a later analysis, a subgroup sharing an IBD fragment was likely to be detected. Next, we investigated a test population comprising 6 unrelated patients with Siiyama-type α1-antitrypsin deficiency and 54 normal subjects (**Figure 4E**). A subgroup was detected at level 6 (**Figure 4F**); the members of the subgroup were the 6 patients with Siiyama-type

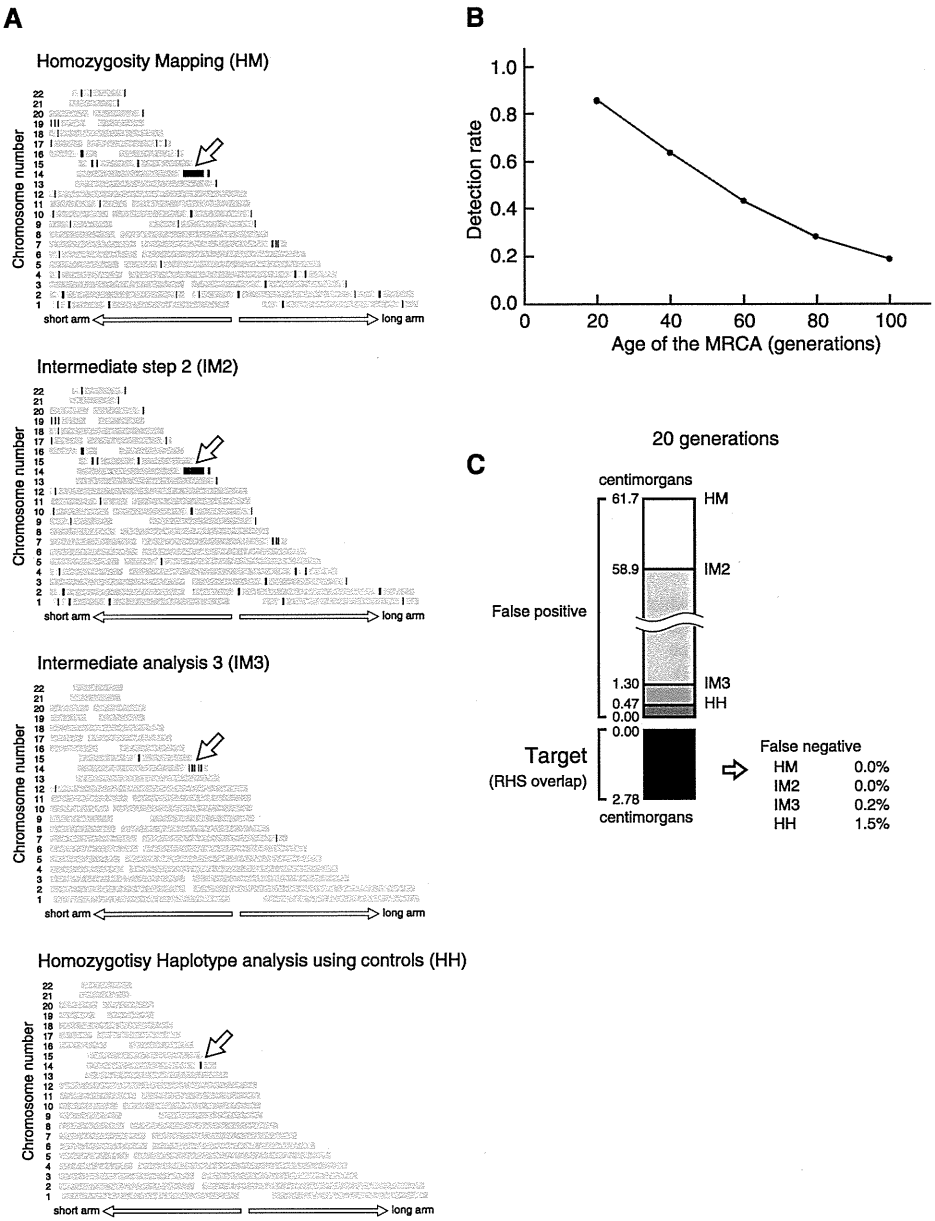

Figure 3. HM on HH analysis of a pair. (A) Analysis of patients 1 and 2 with Siiyama-type α1-antitrypsin deficiency. The position of the disease-causing gene (*SERPINA1*) is indicated by an arrow. HM on HH analysis is composed of 4 steps that are sequentially performed. The regions selected after each step are shown as black bands. The total length of the regions selected at the end was 1.36 centimorgans. (B) The rate at which the RHS overlap was detected by the HM step (i.e., the first step of the analysis) was the major determinant of HM on HH analysis. The detection rate will be improved by genotyping more SNPs genomewide. (C) False positives and false negatives for each analysis. False negatives are decreased with the progression of the analyses. False negatives are very few: 1.5% of the RHS overlap detected by the HM analysis is falsely excluded by HM on HH analysis.
doi:10.1371/journal.pone.0025059.g003

α1-antitrypsin deficiency. The candidate region, 1.2 centimorgans in width, was located on chromosome 14 and contained the *SERPINA1* gene. HM on HH accurately isolated a subpopulation that accounted for only 10% of the population and identified the position of an IBD fragment on the chromosome.

HM on HH analysis of a population containing synthetic patients

To study the performance of HM on HH analysis in more detail, we studied test populations containing synthetic patients. The synthetic patients (5, 10, 15, 20, 25, and 30% of the members of the population) were homozygous for the IBD fragment derived from MRCAs at generational distances of 20, 40, 60, 80, and 100 generations. For each combination of these parameters, the analysis was repeated 100 times by changing the disease-gene location, which was randomly selected from the SNP positions on the autosomes. The analysis was considered successful when (1) only a single candidate region was detected in the topmost level that detected any subgroup, and (2) the candidate region contained the locus of a disease-causing gene. The rates of successful trials (detection rate) were graphed for populations with 60, 40, and 20 subjects (**Figure 5A**). The results demonstrated

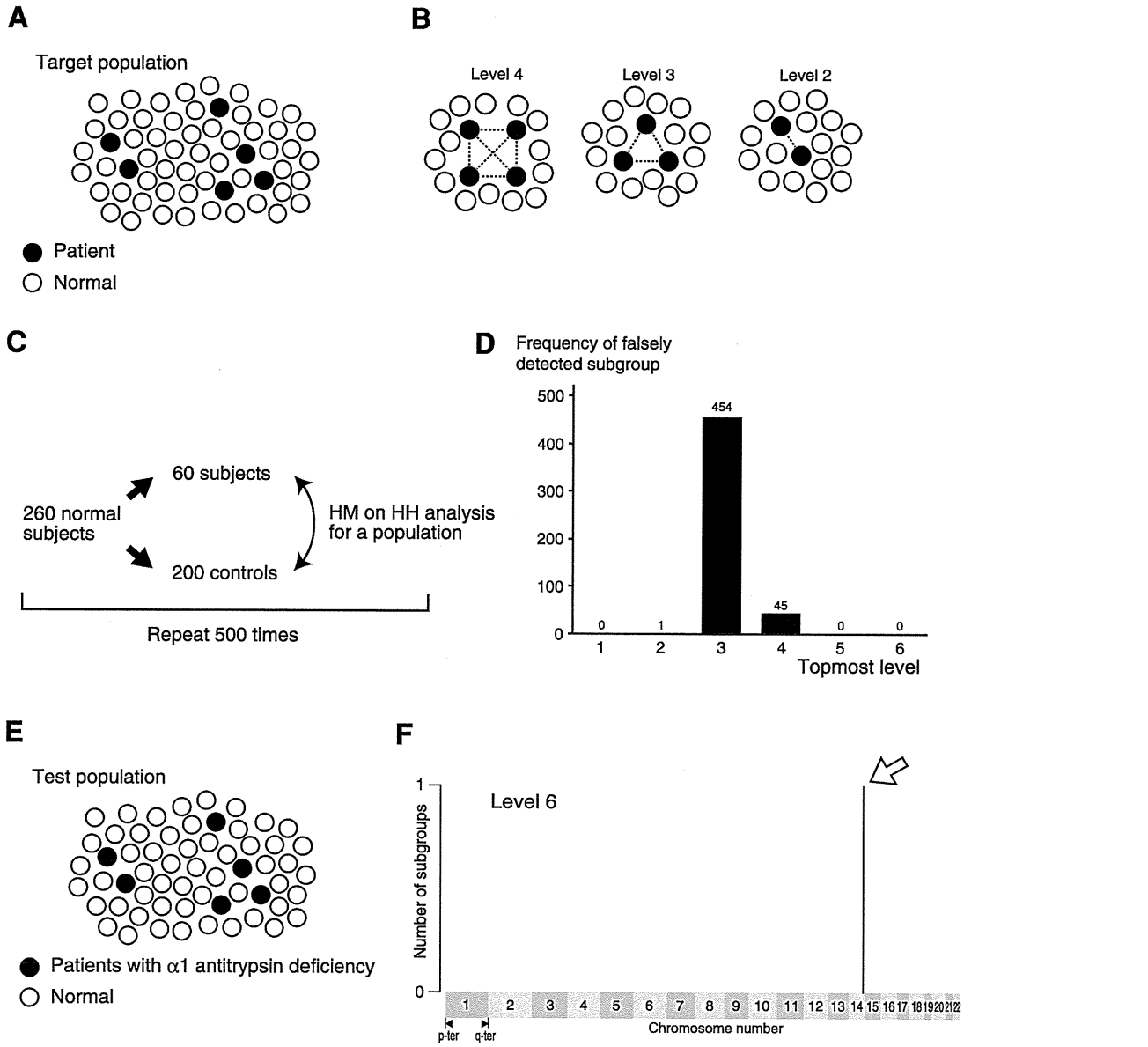

Figure 4. HM on HH analysis of a population. (A) Targets. Targets of HM on HH analysis of a population are populations containing multiple subjects sharing an IBD fragment. (B) Analysis level. At the level *n* analysis, a subgroup of *n* members, each pair of which shares an IBD fragment at the same position on the chromosome, are sought. (C) Scheme of the background determination. (D) Background. A subgroup(s) was falsely detected at level 3, 4, and 5 analyses. (E) A test population. The population is composed of 6 patients with Siiyama-type α1-antitrypsin deficiency (black circles) and 54 normal subjects (white circles). (F) Result. The horizontal position indicates the location on the autosomes, each of which is aligned from the p terminal (left side) to the q terminal (right side). A single subgroup was identified at the level 6 analysis, and the candidate region contained the *SERPINA1* gene. The members of the subgroup, which was the output on the computer console and thus is not shown here, were the 6 patients with Siiyama-type α1-antitrypsin deficiency.
doi:10.1371/journal.pone.0025059.g004

that HM on HH could identify a subpopulation sharing an IBD fragment that accounted for only a small fraction of the population.

The analysis described above assumed that the frequency of the allele containing the disease-causing gene was 0.0 in the general population. However, the disease-causing gene may be a common variant. We investigated the performance of HM on HH analysis when the frequency of the disease-causing gene in the general population was 0.05 or 0.1 (**Figure 5B**). The results indicated that the performance was severely degraded for a frequency of 0.1. HM on HH analysis was considered to work well for a frequency <0.1. Therefore, the HM on HH analysis targets a recessive gene that is the cause of a disease, in which less than 1% of the people in the general population are homozygous for the gene and thus may suffer from the disease somewhere in their lifetime.

Analysis without utilizing HH information

Analyses similar to HM on HH analysis may be performed by stopping the analysis after the HM, IM2, or IM3 steps (**Figure 3A**). When stopping after either the HM or the IM2,

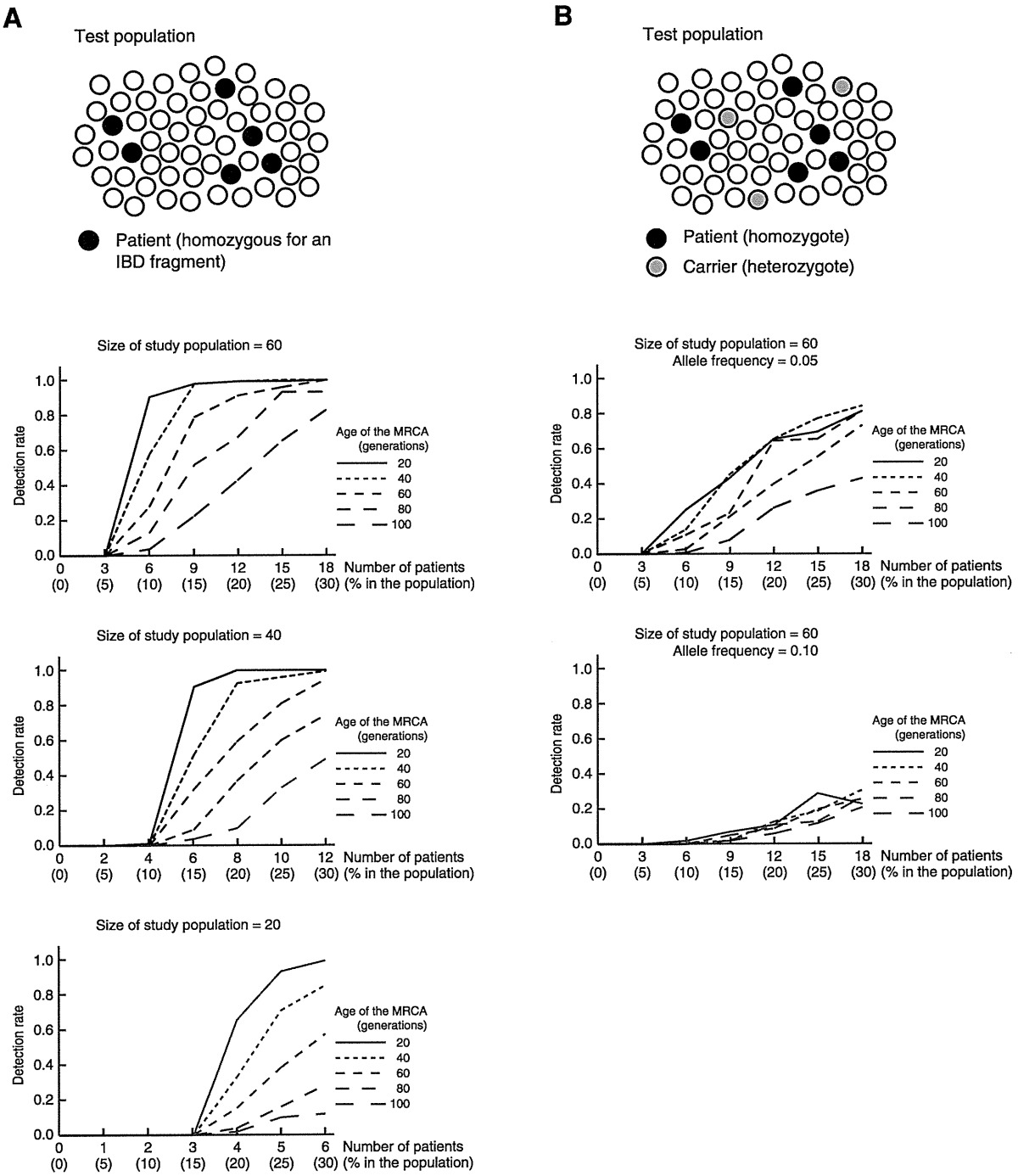

Figure 5. HM on HH analysis of a population performed on populations containing synthetic patients. (A) Scheme of the analysis and Result. The test population is composed of patients homozygous for a gene identical by descent (black circles) and subjects who do not have the gene (white circles). The horizontal line of each graph indicates the number (percentage) of patients homozygous for the gene in the test population. (B) Scheme of the analysis and Result for a gene that is widely shared in the general population. The test population is composed of patients homozygous for a gene identical by descent (black circles), subjects heterozygous for the gene (gray circles), and subjects who do not have the gene (white circles). In the case that the gene was widely shared in the general population (the frequency of the allele of the disease-causing gene was 0.05 and 0.1), the detection rate was decreased.
doi:10.1371/journal.pone.0025059.g005

the program ran out of memory from the explosion in the numbers of subgroups that resulted from a large amount of false positives. When stopping after the IM3 step, the detection rate was much lower than that after the HH analysis, because of a greater amount of false positives (**Figure s3**). These results demonstrated that the small amount of false positives attained by the HH step is important for the performance of HM on HH analysis of a population.

Discussion

In the current report, we demonstrated that HM on HH is able to narrow the candidate region for a disease-causing gene to a very small chromosomal interval either by employing 2 outbred patients sharing an IBD fragment, or by using a small population in which 10%≤ of the patients share an IBD fragment. Haplotype information obtained from the region that flanks the RHSs was the component of the HM on HH analysis that enabled them. By using the HM on HH analysis, genes with a recessive trait are exploited in the very early stage of a project attempting to identify a disease-causing gene.

It has been reported that HM is able to identify a candidate region from as few as 3 inbred patients [5,8]. Although this number is small, the clinical characteristics often do not provide information sufficient for selecting 3 patients who may have an IBD gene. Furthermore, the total length of the candidate regions detected by HM is usually large [8,11], which necessitate an enormous effort for an in-depth search of the regions. HM on HH analysis offers the advantage of being capable of using only 2 patients to obtain a relatively narrow candidate region, typically it is a few centimorgans in length. This may enable novel strategies for identifying disease-causing genes. One such strategy is to collect several patients who are likely to share a fragment IBD, identify the candidate regions by a pairwise comparison, and scrutinize all of these regions by high-throughput sequencing [14].

A small number of founder mutations often largely accounts for the occurrence of a recessive disease or its predisposition. Examples are α1-antitrypsin deficiency and cystic fibrosis in Europeans [15,16], and Gaucher disease and Tay-Sachs disease in Ashkenazi Jews [17]. The cause of the prevalence may be heterozygote advantage, a founder effect, or genetic drift [17,18]. Whatever is the cause, this suggests that the predominance of a limited number of founder mutations is worth taking into consideration in an attempt to search for disease-causing genes with a recessive trait. HM on HH analysis is suitable for pursuing the possibility.

The generational distance of the MRCA has a major effect on the performance of HM on HH analysis. In the analysis of pair of patients, the effect was large (**Figure 3B**). In the analysis of population, the effect was moderate (**Figure 5A**). Use of arrays with a greater number of SNPs will accomplish a better performance (**Figure S1B**). Data obtained using SNP Array 6.0 were investigated in the current study; they were considered suitable for the MRCA with a generational distance ≤60 generations (**Figures 3B and 5A**). Founder populations that settled in recent centuries are amenable to the analysis. These include the French-Canadian population that settled in Quebec in the 17th century [19,20], or the Icelandic population that was founded in the 10th century [21], because the generational distance of the MRCA may be less than 20–60 generations in many diseases.

Isolated populations may also be suitable for this analysis; in such population, a single IBD gene from an MRCA existed in a recent generation may predominate among patients with a specific disease. In many countries, there may be many geographical areas in which MRCAs for a disease-causing gene have a generational distance of 20–60 generations. A small number of patients that HM on HH analysis requires will make the analysis easily performable in small populations from such areas.

Inclusion of the subjects who share the IBD fragment degrades the performance of the analysis. The frequency of the gene in the control should be less than 0.1, i.e., less than 20% of the control subjects may be heterozygous for the gene and less than 1% of the control subjects may be homozygous for the gene. The analysis is not suitable for the common variants for the common diseases that are often the targets of the genome-wide association studies.

The calculation time of the HH on HH analysis is short. The analysis of a pair of patients is completed in a fraction of a second; the analysis for a test population of 60 subjects is completed in 15 seconds. Theoretically, an analysis of a study population of 60 subjects requires an investigation of 1.15×10^{18} subgroups. However, many of the comparison of 2 patients generate a result without any candidate region, and thus eliminate the need for investigating any subgroups containing a given pair. A small amount of false positive of HM on HH analysis enables an exhaustive search for the subgroups.

We used 200 controls in the current study, but it is possible to decrease this number with minimal loss in performance. When the analysis was performed with 100 controls, we found that the performance was only mildly decreased. Moreover, the International HapMap3 project (see International HapMap project Web page) has genotyped and released about 100 or more subjects for each of the 10 ethnic groups, and these data may be used for controls.

The RHS overlap IG-RCHH nest was selected when both the length of the RHS overlap IG and the length of the RCHH between the patients were both at the top. The criteria may be weakened to "top 1%," "top 10%," etc. However, we found that the condition of "at the top" worked best for almost all cases (data not shown). The current criterion is thus considered good for HM on HH analysis.

The RHS cutoff for the Genome-Wide Human SNP Array 6.0 was selected so that the total length of the false-positive RHS was acceptable (1.5 centimorgans per a patient). The equivalent RHS cutoff values for other high-density SNP arrays are 0.75 centimorgans for the Human Omni2.5 BeadChip (Illumina), 1.1 centimorgans for the Human1M-Duo BeadChip (Illumina), and 1.9 centimorgans for the GeneChip Human Mapping 500K Array Set (Affymetrix) [10].

In conclusion, HM on HH analysis used genetic information on both the RHS and the flanking regions, and thus detected the locus for a recessive, disease-causing genes with a very low background from a small number of patients. HM on HH analysis will accelerate the elucidation of the genetic causes of many diseases.

Supporting Information

Figure S1 Errors in the HM. (A) The false positive rate is the ratio of the total length of RHSs that are falsely detected along the entire length of the autosomes. The false negative rate is the ratio of the total length of the autozygous segments (i.e., chromosomal regions in which both chromosomal fragments are IBD) that fail to be detected as RHSs along the total length of the autozygous segments. The false positive rate is dependent on the kind of high-density array and thus is shown for each array. 2.5 M, Human Omni2.5 BeadChip (Illumina); 1 M, Human1M-duo BeadChip (Illuina); SNP6.0: Genome-Wide Human SNP Array 6.0 (Affymetrix); 500K, 500K GeneChips Mapping Array Set (Affymetrix). m: the age of the MRCA. (B) Detection rates for each array. The figure corresponds to **Figure 3B**; this figure summarizes the theoretical calculation, while the result in **Figure 3B** is the result using the actual genotyping data.
(EPS)

Figure S2 Errors in HM on HH for a pair of patients. The data corresponding to those of **Figure 3C** for the MRCAs with a generational distance of 40, 60, 80, and 100 generations.
(EPS)

Figure S3 Result of the analysis stopped after the IM3 step. (A) Background. The background was observed to a higher analysis level than that for the HH analysis (compare with **Figure 4D**). (B) Detection rate. Positive results obtained at a level 7 analysis or higher were considered successful. (C) Detection rate. Positive results obtained at a level 9 analysis or higher were considered successful. Figures (B) and (C) correspond with those shown in **Figure 5A**. In both conditions, the detection rate was lower than those shown in **Figure 5A**.
(EPS)

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Acknowledgments

We thank Prof. Tokunaga and Dr. Nao Nishida, Department of Human Genetics, Graduate School of Medicine, University of Tokyo, for providing SNP data for 198 Japanese individuals.

Author Contributions

Conceived and designed the experiments: KH H. Morino HK. Performed the experiments: JS TT H. Miyazawa TS. Analyzed the data: MK YO. Contributed reagents/materials/analysis tools: KS. Wrote the paper: KH MK HK.

VOLUME 29 · NUMBER 12 · APRIL 20 2011

JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Clinical Significance of Circulating Tumor Cells, Including Cancer Stem-Like Cells, in Peripheral Blood for Recurrence and Prognosis in Patients With Dukes' Stage B and C Colorectal Cancer

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See accompanying editorial on page 1508 and article on page 1556

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Submitted May 15, 2010; accepted December 8, 2010; published online ahead of print at www.jco.org on March 21, 2011.

Supported by a Grant-in-Aid for Cancer Research, No. 13-21, 17-15, and a Grant-in-Aid for Scientific Research, No. C 21591734, from the Ministry of Health, Labor and Welfare of Japan.

Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/11/2912-1547/$20.00

DOI: 10.1200/JCO.2010.30.5151

ABSTRACT

Purpose
Using multiple genetic markers, including cancer stem-like cells, we evaluated the clinical significance of circulating tumor cells (CTCs) as a prognostic factor for overall survival (OS) and disease-free survival (DFS) in the peripheral blood (PB) of patients with colorectal cancer (CRC) who had undergone curative surgery.

Patients and Methods
In a multi-institutional study, 735 patients with CRC were assigned to a retrospective training set (n = 420) or prospective validation set (n = 315). CTCs that expressed carcinoembryonic antigen (CEA), cytokeratin (CK) 19, CK20, and/or CD133 (CEA/CK/CD133) mRNA in PB were detected using real-time reverse transcription polymerase chain reaction assay.

Results
In the training sets, OS and DFS of patients who were positive for CEA/CK/CD133 were significantly worse than those of patients who were negative for these markers ($P < .001$). At each staging analysis, OS and DFS of patients with Dukes' stage B or C cancer who were positive for CEA/CK/CD133 were significantly worse than those of patients who were negative for these markers ($P < .003$ and $P < .001$ in Dukes' stage B; $P < .001$ in Dukes' stage C). In contrast, in patients with Dukes' stage A, no significant differences were seen between patients who were positive for these markers and those who were negative. Cox multivariate analysis demonstrated that CEA/CK/CD133 was a significant prognostic factor for OS (hazard ratio [HR], 3.84; 95% CI, 2.41 to 6.22; $P < .001$) and DFS (HR, 3.02; 95% CI, 1.83 to 5.00; $P < .001$). In particular, in patients with Dukes' stage B and C cancer, CEA/CK/CD133 demonstrated significant prognostic value. In validation sets, similar results were confirmed in patients with Dukes' stage B and C cancer.

Conclusion
In patients with Dukes' stage B and C CRC who require adjuvant chemotherapy, detection of CEA/CK/CD133 mRNA in PB is a useful tool for determining which patients are at high risk for recurrence and poor prognosis.

J Clin Oncol 29:1547-1555. © 2011 by American Society of Clinical Oncology

INTRODUCTION

Despite advances in therapeutic approaches for patients with colorectal cancer (CRC), approximately 20% to 45% of those who undergo curative resection subsequently develop local tumor recurrence or metastasis at distant sites.[1] It is known that approximately 30% of the metastasis in CRC is liver metastasis.[2] To improve the prognosis, adjuvant chemotherapy such as fluorouracil (FU) plus leucovorin (LV; FU/LV) and uracil/tegafur plus LV have been established as generalized regimens, and FU/LV plus irinotecan and FU/LV plus oxaliplatin combined with molecular-targeted compounds such as bevacizumab and cetuximab have been approved for the treatment of patients with advanced-stage CRC.[3] Although these combination therapies involving chemotherapy and molecular-targeted agents have demonstrated survival benefits, the substantial financial cost of these treatments remains a serious problem.[4] Furthermore, chemotherapy without certain selection tends to lead to

overtreatment of patients with toxic agents that exert severe adverse effects. To facilitate individually tailored treatment for CRC, useful biomarkers for the determination of patients who are at high risk for recurrence and/or poor prognosis are required. In particular, markers for the determination of patients with Dukes' stage B CRC who are at high risk are desirable because the role of adjuvant chemotherapy in these patients remains controversial.[5]

Many investigators[6-9] have shown the utility of circulating tumor cells (CTCs) in predicting the clinical outcome of patients with various solid cancers. The usefulness of CTCs as a predictive tool for patients with metastatic breast cancer and those with CRC has been reported using the CellSearch system (Veridex, Raritan, NJ).[10,11] However, the prognostic relevance of CTCs in patients with early-stage disease without metastasis remains unclear.

Recently, a new concept relating to cancer stem cells has arisen and is expected to lead to the development of novel diagnosis and therapeutic procedures.[12] Stem cell markers are frequently overexpressed in the CTCs of patients with metastatic breast cancer, which suggests that metastasis may be evoked by the subpopulation of CTCs that have the cancer stem cells' marker.[13] Two interesting studies have demonstrated that CD133-positive cells in CRC have high tumorigenic ability in nude mice, and it is thought that CD133 is one of the key markers of cancer stem cells in CRC.[14,15] Furthermore, it was reported that cancer stem cells are often characterized by downregulation of epithelial markers, including the epithelial cell adhesion molecule (EpCAM) and cytokeratin.[16] In this study, we hypothesized that multimarkers, including the phenotypes of cancer stem-like cells (CD133), may be useful for detecting aggressive CTCs. However, large-scale multicenter studies that use these markers are still required.

Our study was a multicenter validation study that focused on clarifying the prognostic value of CTCs in the PB of patients with CRC who were treated with curative surgery, on the basis of real-time reverse transcription polymerase chain reaction (RT-PCR) using multimarkers that included cancer stem-like cells.

PATIENTS AND METHODS

Study Design

This study was undertaken at four clinical institutions in Japan (The National Cancer Center, Tokyo; Kyushu University, Oita; Kumamoto University, Kumamoto; and Teikyo University, Tokyo). The study protocol conformed to the guidelines of the ethics committee of each institution and was approved by each institution's review board; all patients provided written informed consent. A total of 735 patients with CRC who were undergoing curative surgery at these hospitals between January 2000 and December 2008 were enrolled. The diagnosis of CRC was confirmed in all patients by endoscopic biopsy, and the primary tumor stage was confirmed by histologic examination of the resected primary tumor. Overall survival (OS) was defined as the time between the date of diagnosis and the date of death. Disease-free survival (DFS) was defined as the length of time after surgical treatment for the cancer during which the patient survived with no sign of the cancer. The study consisted of a sequential retrospective training set and a prospective validation set. The first 420 patients were enrolled as a retrospective training set between January 2000 and December 2004. The cutoff values of carcinoembryonic antigen (CEA), cytokeratin 19 (CK19), cytokeratin 20 (CK20), and CD133 mRNA were determined from the training set by analyzing receiver-operating characteristic curves on the basis of mRNA levels of these markers and clinical follow-up data. The ability of these genetic markers to predict recurrence and prognosis on the basis of the predetermined cutoff values was then examined in the prospective validation set. Between January 2005 and December 2008, 315 patients were enrolled in this set. Disease status was assessed without knowledge of the CTC levels. Patients with Dukes' stage A and B cancer were not treated with chemotherapy. Patients with Dukes' stage C cancer were treated with a standard Roswell Park Memorial Institute 1640 regimen that involved administration of FU/LV for 6 months. As a follow-up, development of local recurrence, distant metastases, and death were re-evaluated in all patients at 3-month intervals during the first year and at 6-month intervals thereafter.

Blood Sampling and Quantitative Real-Time RT-PCR

PB samples were obtained at the presurgery stage before chemotherapy was begun. Ten-milliliter blood samples were collected and stored at −80°C. Extraction of total RNA and cDNA synthesis was performed according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The expression of CEA, CK19, CK20, and CD133 mRNA of blood samples was examined; target genes of CTCs and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) mRNA were used as internal control genes. The expression levels of these mRNA were measured by real-time quantitative RT-PCR using the LightCycler instrument (Roche Diagnostics, Mannheim, Germany), as described previously.[9,17] All samples were measured in duplicate. The levels of CEA, CK19, CK20, and CD133 mRNA were normalized by GAPDH mRNA.

Statistical Analysis

The sample size was calculated by SAS statistical software (V 9; SAS Institute, Cary, NC) on the basis of the results of a small-scale test ($n = 50$) under which the one-sided P value of .025 would have 80% power to detect a difference between the DFS and OS curves of patients with and without CTC.

The cutoff values of markers were determined by receiver-operating characteristic curves that were constructed by plotting all possible sensitivity/1 − specificity pairs in the training sets. Sensitivities of genetic markers were calculated as a ratio of the number of patients with PCR and hematogenous metastasis relative to the number of patients who developed hematogenous metastasis. Specificities were calculated as the ratio of the number of patients without PCR and hematogenous metastasis relative to the number of all patients without hematogenous metastasis. The relationships between OS and DFS and genetic makers were analyzed by Kaplan-Meier survival curves and the log-rank test. Cox proportional hazards regression was used to determine univariate and multivariate hazard ratios for OS and DFS. The comparison of clinicopathologic factors in the training and validation sets were analyzed using the t test, χ^2 tests, and analysis of variance. All P values were two sided, and P values of less than .05 were considered statistically significant. Data were analyzed using JMP software (V 7; SAS Institute).

RESULTS

Clinicopathologic Characteristics and Genetic Markers

In the comparison of clinicopathologic characteristics of the patients in the training and validation sets, there were no significant differences found in tumor size, depth of invasion, lymphatic invasion, venous invasion, lymph node metastasis, histologic type, and tumor stage (Table 1). In the training set, cutoff values for each marker were predetermined (5.2×10^{-6} as CEA/GAPDH; 8.7×10^{-5} as CK19/GAPDH; 1.9×10^{-5} as CK20/GAPDH; and 3.4×10^{-4} as CD133/GAPDH), and the positive rates, sensitivities, and specificities of various combinations of genetic markers were examined (data not shown). In the single-marker groups, CEA showed the highest positive rates and highest sensitivity for hematogenous metastasis. In contrast, CD133 showed the highest specificity for hematogenous metastasis. In the multimarker groups, CEA^+, $CK19^+$, $CK20^+$, and/or $CD133^+$ (CEA/CK/CD133) showed the highest positivity, sensitivity, and specificity. Therefore, the CEA/CK/CD133 group was selected as representative of PCR positivity and used for the prognostic analysis. Between the training and validation sets, there were no significant differences in positive rates, sensitivity, and specificity of CEA/CK/CD133 (Table 1).

Table 1. Patient Characteristics in Training and Validation Sets

Characteristic	No. of Patients	Training Set		Validation Set		*P*
		No.	%	No.	%	
Total No. of patients	735	420		315		
Age (mean ± SD)		66.0 ± 12.4		67.5 ± 11.8		.524
Sex						
Women	336	196	46.67	140	44.44	.550
Men	399	224	53.33	175	55.56	
Tumor size						
< 5	440	252	60.00	188	59.68	.940
≥ 5	295	168	40.00	127	40.32	
Depth of invasion						
≤ pT2	188	107	25.48	81	25.71	.942
≥ pT3	547	313	74.52	234	74.29	
Lymphatic invasion						
Negative	511	295	70.24	216	68.57	.628
Positive	224	125	29.76	99	31.43	
Venous invasion						
Negative	387	217	51.67	170	53.97	.536
Positive	348	203	48.33	145	46.03	
Lymph node metastasis						
Negative	488	270	64.29	218	69.21	.162
Positive	247	150	35.71	97	30.79	
Histologic type						
Well	590	328	78.10	262	83.17	.086
Nonwell	145	92	21.90	53	16.83	
Dukes' stage						
A	169	94	22.01	75	24.35	.101
B	319	176	41.22	143	46.43	
C	247	150	35.13	97	31.49	
CEA/CK/CD133						
Positive rates*		106 of 427	25.24	75 of 308	23.81	.143
Sensitivity		55 of 67	82.09	34 of 46	73.91	.352
Specificity		302 of 353	85.55	228 of 269	84.76	.147

Abbreviations: SD, standard deviation; CEA, carcinoembryonic antigen; CK, cytokeratin.
*Ratio of number of CEA/CK19/CK20/CD133–positive patients (according to polymerase chain reaction) to number of all patients.

Kaplan-Meier OS and DFS Curve Analysis

In the training set, the average follow-up time for OS was 36.9 ± 19.3 months (95% CI, 35.0 to 38.8 months) and that of DFS was 32.7 ± 20.2 months (95% CI, 30.7 to 34.6 months). In the validation set, the average follow-up time was 37.1 ± 18.1 months (95% CI, 35.0 to 39.2 months) for OS and 33.4 ± 19.1 months (95% CI, 31.2 to 35.4 months) for DFS. In the training set, all patients with CEA/CK/CD133 positivity showed significantly worse OS and DFS than those with PCR negativity. The same results were found in the validation set (data not shown).

Figure 1 shows the OS and DFS at each tumor stage according to the PCR status in the training set. In patients with Dukes' stage A cancer, there were no significant differences in OS and DFS in the patients with CEA/CK/CD133 positivity as compared with those with negativity (data not shown). In all patients with Dukes' stage B cancer, the OS and DFS of those with CEA/CK/CD133 positivity were significantly worse than those of patients who were negative for these markers (Figs 1A and 1B). Furthermore, when patients with Dukes' stage B and CEA/CK/CD133 positivity who were considered to be at high risk were separated out on the basis of factors such as perforation or obstruction and/or lymphatic invasion, they demonstrated significantly worse OS and DFS than patients who were negative for these markers (Figs 1C and 1D). In patients with Dukes' stage C cancer, the OS and DFS of the group with CEA/CK/CD133 positivity were significantly worse than those of patients who were negative for these markers (Figs 1E and 1F). In the validation set, similar results were demonstrated for patients with Dukes' stage A (data not shown), B, and C (Figs 2A to 2F). These results suggest that the expression of CEA/CK/CD133 is associated with recurrence and poor prognosis in patients with Dukes' stage B and C cancer.

To clarify the clinical significance of CD133 addition in patients with Dukes' stage B and C cancer, we analyzed the OS and DFS in the general CTC markers (CEA, CK19, and/or CK20: CEA/CK), and in the CD133 single marker. In patients with Dukes' stage B cancer, no significant differences in OS and DFS were found between those who were positive for CEA/CK and those who were negative for CEA/CK, nor were significant differences seen between patients who were positive for CD133 as compared with those who were negative for CD133 (Data Supplement). In contrast, in patients with Dukes' stage C, significant differences were found in OS and DFS between those who were positive for CEA/CK and those who were negative for CEA/CK in both the training and the validation sets (Data Supplement). However, in the CD133 single-marker analysis, no significant differences in OS and DFS were found between the patients who were positive for

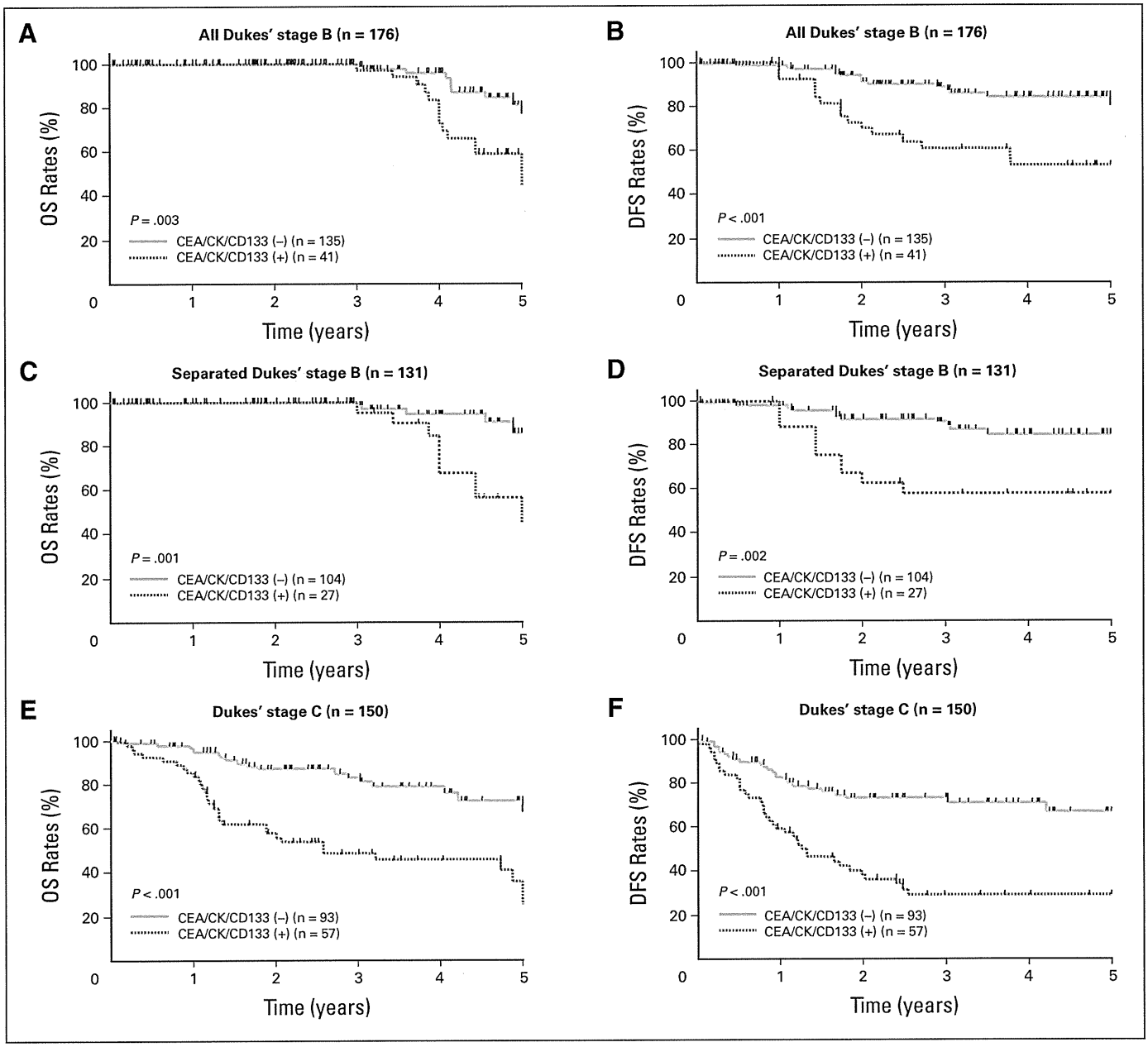

Fig 1. Kaplan-Meier survival curves for overall survival (OS) and disease-free survival (DFS) according to the CEA/CK/CD133 status in the training set. Patients with Dukes' stage B were separated into two groups as follows: all patients with Dukes' stage B and patients who were separated out on the basis of perforation or obstruction and/or lymphatic invasion (separated patients with Dukes' stage B). (A, B) OS and DFS of all patients with Dukes' stage B, (C, D) separated patients with Dukes' stage B, and (E, F) patients with Dukes' stage C were analyzed in the training set according to CEA/CK/CD133 mRNA levels.

CD133 and those who were negative for CD133 in either set. These results suggest that the addition of CD133 to general CTC markers is important for the determination of patients who are at high risk for recurrence and poor prognosis in Dukes' stage B cancer.

OS and DFS in Patients With Colon and Rectal Cancer

In patients with colon cancer, groups positive for CEA/CK/CD133 showed a significantly worse OS and DFS than those negative for CEA/CK/CD133 in the training and validation sets (Figs 3A, 3B, 3E, and 3F). In patients with rectal cancer, similar results were demonstrated in the training and validation sets (Figs 3C, 3D, 3G, and 3H). These results suggest that the prognostic value of CEA/CK/CD133 is the same in both colon and rectal cancer.

Univariate and Multivariate Cox Analysis

Table 2 shows the results of multivariate Cox analysis of various factors for OS and DFS in all of the patients. Multivariate analysis was performed for factors that showed significance in univariate analysis. In the training set, CEA/CK/CD133 and Dukes' stage showed significance for OS, and CEA/CK/CD133, Dukes' stage, and serum CEA protein showed significance for DFS. In the multivariate analysis of the validation set, CEA/CK/CD133 and Dukes' stage showed significance

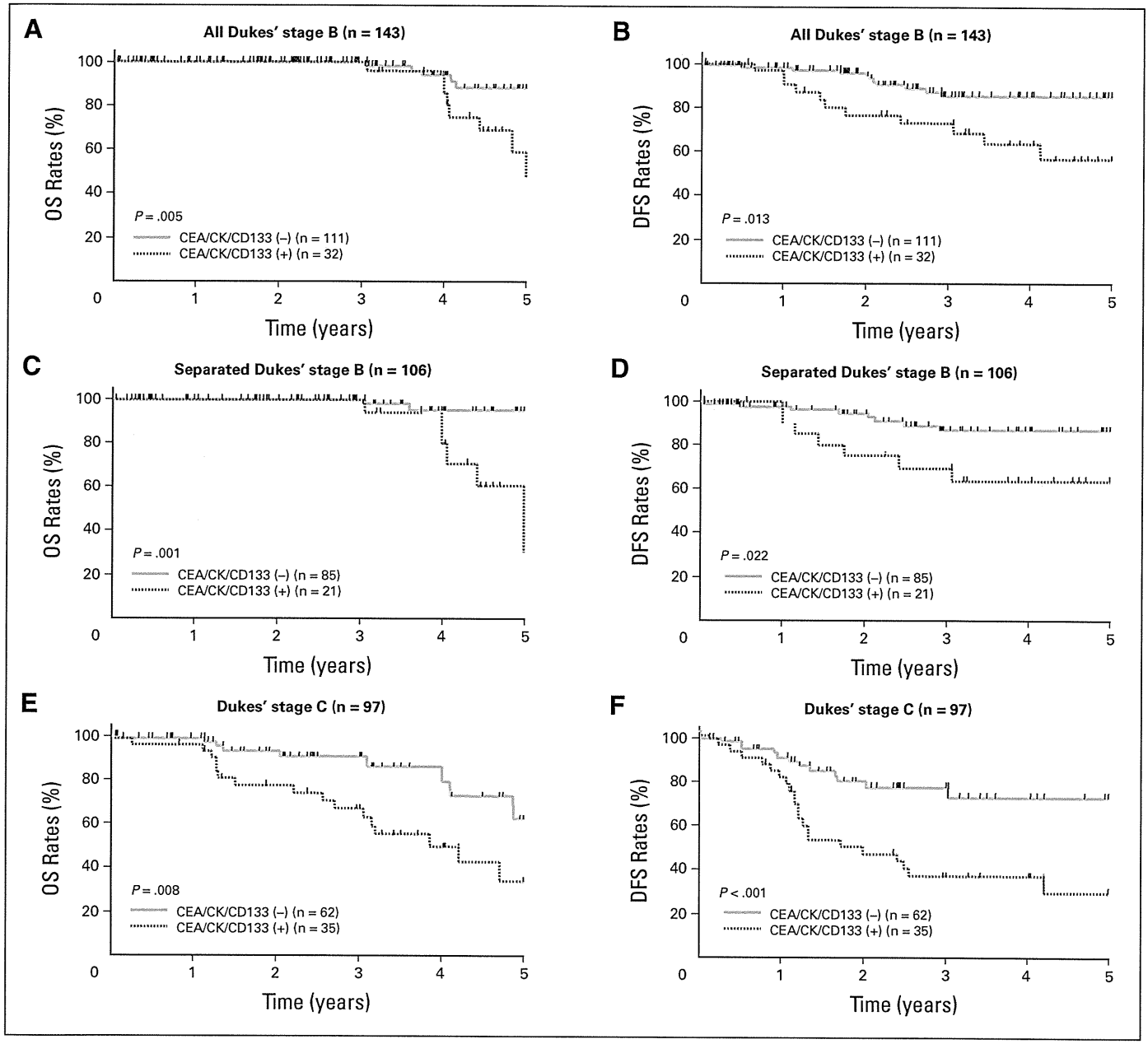

Fig 2. Kaplan-Meier survival curves of overall survival (OS) and disease-free survival (DFS) on the basis of the CEA/CK/CD133 status in the validation set. (A, B) OS and DFS of all patients with Dukes' stage B, (C, D) patients with Dukes' stage B who were separated out on the basis of factors such as perforation or obstruction and/or lymphatic invasion, and (E, F) patients with Dukes' stage C were analyzed in the validation set according to CEA/CK/CD133 mRNA levels.

for OS, and CEA/CK/CD133, Dukes' stage, and serum CEA showed significance for DFS.

Table 3 shows the results of multivariate Cox analysis of various factors for OS and DFS in the patients with Dukes' stage B and C cancer. In the analysis of all patients with Dukes' stage B and patients with Dukes' stage B who were separated out on the basis of perforation or obstruction and/or lymphatic invasion, the CEA/CK/CD133 showed significance for OS and DFS in the training and validation sets. In patients with Dukes' stage C cancer, CEA/CK/CD133 levels showed significance for OS, and CEA/CK/CD133 and serum CEA levels showed significance for DFS in the multivariate analysis of the training set. In the validation set, similar results were demonstrated. These results suggest that CEA/CK/CD133 is a significant predictor for poor prognosis and recurrence in patients with Dukes' stage B and C.

DISCUSSION

Our multi-institutional validation study demonstrates that the detection of CEA/CK/CD133 mRNA in PB samples has prognostic value in patients with Dukes' stage B and C colorectal cancer.

Although many assays have been developed to detect CTCs, the two main approaches that are used involve either cytology that is based on immunocytochemical staining or PCR-based analysis.[18-21] Using

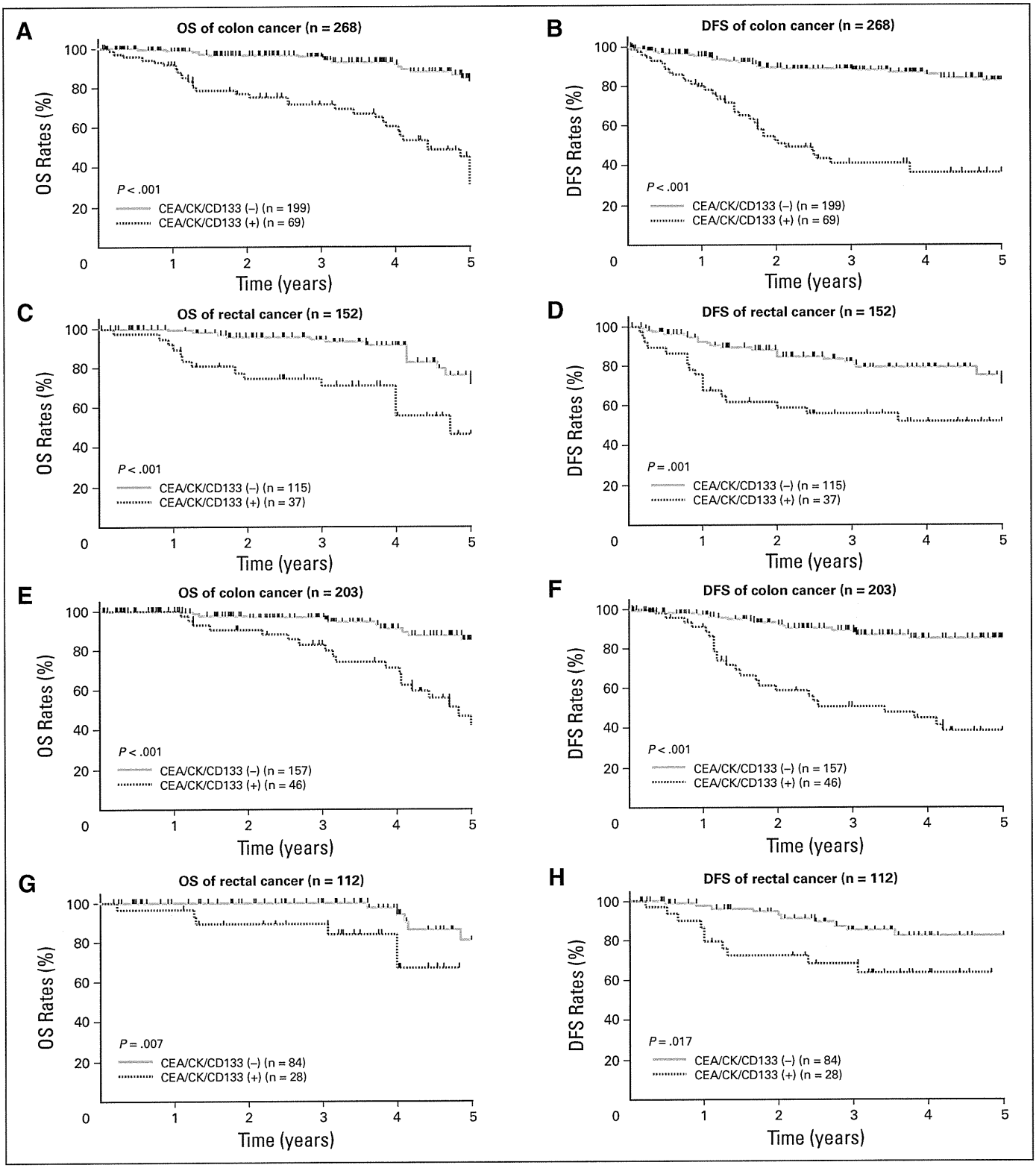

Fig 3. Kaplan-Meier survival curves of overall survival (OS) and disease-free survival (DFS) in patients with colon and rectal cancer. (A, B, E, F) OS and DFS of patients with colon and (C, D, G, H) rectal cancer in training and validation sets were analyzed according to CEA/CK/CD133 mRNA levels.

the US Food and Drug Administration–approved CellSearch system, the usefulness of CTCs as a predictive marker for survival has been reported in metastatic breast cancer and metastatic CRC.[9,10] This automatic cell analysis system is based on immunocytochemical staining, and the antibodies against EpCAM and cytokeratin are used to capture and detect the CTCs. However, the fact that epithelial-mesenchymal transition–transformed tumor cells and cancer stem cells are often characterized by downregulation of epithelial markers

Table 2. Multivariate Analysis for OS and DFS of All Patients in Training and Validation Sets

Characteristic	OS			DFS		
	HR	95% CI	*P*	HR	95% CI	*P*
Training set						
CEA/CK/CD133	3.84	2.41 to 6.22	< .001*	3.02	1.83 to 5.00	< .001*
Tumor size	1.06	0.66 to 1.71	.798	0.97	0.58 to 1.58	.899
Depth of invasion	1.92	0.88 to 4.82	.103	2.01	0.97 to 4.73	.061
Dukes' stage	3.23	2.00 to 5.34	< .001*	2.87	1.75 to 4.76	< .001*
Lymphatic invasion	1.35	0.82 to 2.22	.240	1.22	0.71 to 2.08	.475
Venous invasion	1.05	0.62 to 1.82	.857	0.79	0.46 to 1.38	.410
Histologic type	0.30	0.13 to 1.71	.071	0.49	0.30 to 1.83	.088
Serum CEA	1.71	0.71 to 4.23	.230	1.96	1.20 to 3.22	.007*
Validation set						
CEA/CK/CD133	3.20	1.67 to 6.31	< .001*	3.04	1.79 to 5.22	< .001*
Tumor size	1.08	0.56 to 2.08	.828	1.02	0.60 to 1.73	.933
Depth of invasion	0.99	0.41 to 2.67	.992	1.57	0.73 to 3.74	.259
Dukes' stage	3.39	1.64 to 7.27	< .001*	2.13	1.20 to 3.8	.010*
Lymphatic invasion	1.24	0.65 to 2.78	.536	1.09	0.62 to 1.92	.769
Venous invasion	1.34	0.62 to 2.78	.373	1.30	0.74 to 2.34	.362
Histologic type	0.41	0.21 to 1.81	.081	0.63	0.33 to 1.05	.105
Serum CEA	1.64	0.85 to 3.20	.142	1.85	1.09 to 3.19	.023*

Abbreviations: OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CEA, carcinoembryonic antigen; CK, cytokeratin.
*$P < .05$.

that include EpCAM and cytokeratin suggests that general CTC markers are not able to detect the most aggressive tumor cells in circulation.[22-24] These results may be related to the difficulty of using the CellSearch system as a screening tool for detection of new primary cancer or early tumor cell dissemination. In this study, we used a real-time RT-PCR assay, which has the advantage of improved sensitivity compared with cell-based assays. The limitation of this technique lies in the fact that it does not allow for individual cell analysis. However, we think that it has the potential to detect primitive tumor-initiating cells that may be EpCAM negative because this assay does not depend on the expression of EpCAM.

Whether CTCs have stem cell features is of interest. The cancer stem cell concept hypothesizes that tumors arise from a small population of stem cells, and it has been assumed that the founder cells of metastases may also be stem cells disseminated from the primary tumor to a distant metastatic site. This hypothesis is supported by the similarities between the properties of CTCs and cancer stem cells, which suggests that the founder cells of metastases arise from the CTC

Table 3. Multivariate Analysis for OS and DFS of Patients With Dukes' Stage B and C Cancer in Training and Validation Sets

Variables	Training Set						Validation Set					
	OS			DFS			OS			DFS		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
All Dukes' stage B												
CEA/CK/CD133	3.21	1.44 to 7.46	.005*	3.35	1.66 to 6.74	< .001*	3.75	1.25 to 12.47	.019*	3.10	1.33 to 7.24	.010*
Venous invasion	1.41	0.62 to 3.48	.418	1.30	0.64 to 2.74	.469	1.05	0.35 to 3.26	.933	1.29	0.56 to 3.13	.556
Separated Dukes' stage B												
CEA/CK/CD133	5.00	1.73 to 16.32	.003*	3.59	1.49 to 8.54	.005*	9.31	2.07 to 65.41	.003*	3.19	1.11 to 8.92	.032*
Venous invasion	1.28	0.44 to 3.90	.648	1.25	0.53 to 3.01	.605	0.91	0.21 to 3.88	.894	1.45	0.52 to 4.16	.472
Dukes' stage C												
CEA/CK/CD133	2.62	1.14 to 4.20	.024*	3.26	1.74 to 6.25	< .001*	2.47	1.06 to 6.25	.040*	2.97	1.44 to 6.40	.003*
Venous invasion	2.31	0.77 to 4.21	.087	0.72	0.35 to 1.53	.380	2.39	0.87 to 8.42	.095	1.54	0.68 to 3.96	.313
Serum CEA	1.79	0.78 to 4.49	.173	2.49	1.16 to 5.25	.020*	0.81	0.38 to 1.83	.597	2.21	1.07 to 4.88	.031*

NOTE. In all patients with Dukes' stage B cancer, six factors (tumor size, lymphatic invasion, venous invasion, histologic type, serum CEA, and CEA/CK/CD133 mRNA) were used for the univariate analysis. In the patients with Dukes' stage B cancer who were separated out, five factors (tumor size, venous invasion, histologic type, serum CEA, and CEA/CK/CD133 mRNA) were used for the univariate analysis. In groups with Dukes' stage B, CEA/CK/CD133 and venous invasion, which showed significance for overall survival and disease-free survival in the univariate analysis, were then used for the multivariate analysis. In patients with Dukes' stage C, seven factors (tumor size, depth of invasion, lymphatic invasion, venous invasion, histologic type, serum CEA, and CEA/CK/CD133 mRNA) were used for the univariate analysis. CEA/CK/CD133, venous invasion, and serum CEA, which showed significance for overall survival and disease-free survival in the univariate analysis, were then used for the multivariate analysis.
Abbreviations: OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CEA, carcinoembryonic antigen; CK, cytokeratin.
*$P < .05$.

population. It has been reported that stem cell markers are frequently overexpressed in the CTCs of patients with metastatic breast cancer, and most CTCs have stem cell phenotypes that are nonproliferating and resistant to chemotherapy.[25-28] These facts suggest that a new marker is necessary for the detection of cytokeratin downregulated aggressive CTCs, which may include cancer stem cells. The surface markers of colon cancer stem cells, CD133, CD44, CD166, Musashi-1, CD29, CD24, leucine-rich repeat-containing G-protein-coupled receptor 5, and aldehyde dehydrogenase 1 molecules have been reported,[29-32] and the CD133 molecule has emerged as the key marker for enriching cancer stem cells in many types of cancer.[14,15,32] In contrast, other publications have suggested that CD44 may be a more specific marker for colon cancer stem cells.[33] It is known that CD44 is expressed not only in cancer stem cells but also in a variety of other cells including blood cells.[33] Because our study did not separate the cancer cells from blood cells, it was difficult to measure the CD44 that originated in the cancer cells. Therefore, we selected CD133 for the genetic marker of cancer stem cells. CD133 belongs to the prominin family of proteins, the function of which remains unknown. It has a novel isoform that follows the detection of a small exon of 27 nucleotides by alternative mRNA splicing. Conversely, CD133 is known as a marker of endothelial progenitor cells, which are capable of expanding more than 1,000-fold and initiating the premetastatic niche.[34-36] Although the present assays are not designed to differentiate CD133-positive cancer stem cells from endothelial progenitor cells, we assume that cytokeratin-negative aggressive CTCs may be included in the CD133-expressing cells.

There is a fair amount of single-institution data available on the prognostic significance of CTCs in patients with CRC.[37] However, the number of large-scale multicenter studies is limited. Although Cohen et al,[11] in an analysis of 430 patients with metastatic CTCs, reported that the number of CTCs before and during treatment is an independent predictor of survival, the prognostic value of CTCs in patients with early-stage cancer and without overt metastasis remains unclear. In contrast, we evaluated a total of 735 patients with CRC, and the prognostic value of genetic CTC markers was analyzed at each tumor stage. To examine the clinical significance of CTCs with various characteristics, we selected general markers (CEA and CK) and the cancer stem cell marker (CD133) as the genetic markers of CTCs. Interestingly, our data demonstrated that CEA/CK/CD133, but not CEA/CK, is an independent prognostic factor that surpasses other existing clinicopathologic risk factors in patients with Dukes' stage B CRC. In contrast, not only CEA/CK/CD133 but also CEA/CK showed prognostic value in patients with Dukes' stage C cancer. As for the reason for the different properties of CTCs, we speculate that general marker-positive CTCs in Dukes' stage B cancer may be less aggressive than those of Dukes' stage C, which are capable of lymph node metastasis. To date, the determination of patients with Dukes' B who are at high risk for recurrence is difficult, and efforts to find a tool to select them are ongoing. Because of the limitations of the PCR method, it was difficult to examine whether individual cells expressed all markers or not. We speculate that CTCs that include the CD133-positive cells may enable identification of a certain subgroup that includes aggressive cancer stem-like cell properties and that this may increase their prognostic value in patients with Dukes' stage B cancer. With respect to the reason that the CD133 single marker did not show prognostic value in patients with Dukes' stage B and C cancer, we speculate that it may be a result of the small sample size or the potential limitations of CD133 as a cancer stem cell marker. To the best of our knowledge, this is the first multicenter validation study to clarify the prognostic value of CEA/CK/CD133 multimarkers in patients with Dukes' stage B and C cancer. However, these multimarkers did not show prognostic value in patients with Dukes' stage A cancer, as indicated by the low number of patients with recurrence.

In this study, we demonstrated that a multigenetic marker that includes the cancer stem cell in PB could be a useful tool for the determination of patients with Dukes' stage B and C who are at high risk for recurrence and poor prognosis. We hope that this information brings forth new possibilities for the clinical application of CTCs in patients with CRC.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Provision of study materials or patients: Toshiaki Watanabe, Koshi Mimori, Miki Adachi, Naoko Hayashi, Keiji Matsuda, Kota Okinaga, Mitsuru Sasako, Masaki Mori
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Final approval of manuscript: All authors

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Glossary Terms

Cancer stem cells: A cancer cell that has the potential to transfer disease or to form tumors after transplantation. Cancer stemlike cells have the potential to self-renew (see self-renewal), forming additional tumorigenic cancer cells of similar phenotype, and to give rise to phenotypically diverse cancer cells with more limited potential.

CD133: CD133, encoded by the Prominin-1 gene, is used as a marker for stem cells of normal tissues such as neural and hematopoietic stem cells, but also as a marker for tumor stem-like cells of distinct origin such as brain tumors and breast and colon cancer.

CTC (circulating tumor cell): Demonstration of isolated tumor cell circulation/ dissemination in the peripheral blood.

VOLUME 29 · NUMBER 33 · NOVEMBER 20 2011

JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Five-Year Outcomes of a Randomized Phase III Trial Comparing Adjuvant Chemotherapy With S-1 Versus Surgery Alone in Stage II or III Gastric Cancer

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See accompanying editorial on page 4348; listen to the podcast by Dr Mayer at www.jco.org/podcast

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Submitted April 19, 2011; accepted June 30, 2011; published online ahead of print at www.jco.org on October 17, 2011.

Written on behalf of the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer group.

Supported by Taiho Pharmaceutical, Tokyo, Japan.

Presented in part at the 35th European Society for Medical Oncology Congress, Milan, Italy, October 8-12, 2010.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical Trials repository link available on JCO.org.

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0732-183X/11/2933-4387/$20.00

DOI: 10.1200/JCO.2011.36.5908

ABSTRACT

Purpose
The first planned interim analysis (median follow-up, 3 years) of the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer confirmed that the oral fluoropyrimidine derivative S-1 significantly improved overall survival, the primary end point. The results were therefore opened at the recommendation of an independent data and safety monitoring committee. We report 5-year follow-up data on patients enrolled onto the ACTS-GC study.

Patients and Methods
Patients with histologically confirmed stage II or III gastric cancer who underwent gastrectomy with D2 lymphadenectomy were randomly assigned to receive S-1 after surgery or surgery only. S-1 (80 to 120 mg per day) was given for 4 weeks, followed by 2 weeks of rest. This 6-week cycle was repeated for 1 year. The primary end point was overall survival, and the secondary end points were relapse-free survival and safety.

Results
The overall survival rate at 5 years was 71.7% in the S-1 group and 61.1% in the surgery-only group (hazard ratio [HR], 0.669; 95% CI, 0.540 to 0.828). The relapse-free survival rate at 5 years was 65.4% in the S-1 group and 53.1% in the surgery-only group (HR, 0.653; 95% CI, 0.537 to 0.793). Subgroup analyses according to principal demographic factors such as sex, age, disease stage, and histologic type showed no interaction between treatment and any characteristic.

Conclusion
On the basis of 5-year follow-up data, postoperative adjuvant therapy with S-1 was confirmed to improve overall survival and relapse-free survival in patients with stage II or III gastric cancer who had undergone D2 gastrectomy.

J Clin Oncol 29:4387-4393. © 2011 by American Society of Clinical Oncology

INTRODUCTION

In 2008, there were 737,000 deaths from gastric cancer worldwide. Gastric cancer is the second leading cause of cancer-related death, with the highest mortality rates in East Asia, including Japan, Korea, and China (28.1 per 100,000 in males; 13.0 per 100,000 in females).[1] Approximately 60% of gastric cancers in the world are diagnosed in this area. The mainstay of treatment for gastric cancer is surgery. However, in stages II (excluding T1 disease) and III (moderately advanced), an appreciable proportion of patients have recurrence, even after curative resection. Consequently, various regimens for adjuvant chemotherapy have been implemented to prevent postoperative recurrence.

Although the results of many randomized, controlled studies conducted to verify the effectiveness of postoperative adjuvant chemotherapy for gastric cancer were negative on an individual study basis, meta-analyses of these results have suggested that postoperative adjuvant chemotherapy is therapeutically useful in patients with gastric cancer.[2-7] However, no regimens have been clearly recommended for adjuvant chemotherapy after gastrectomy with D2 lymphadenectomy (D2 gastrectomy), established as the standard procedure for advanced gastric cancer in East Asia.

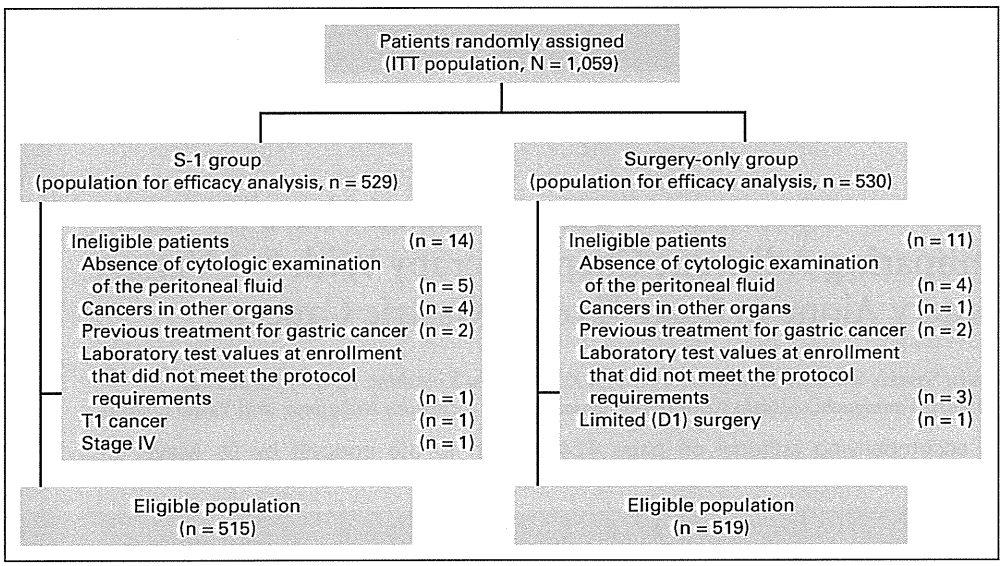

Fig 1. CONSORT diagram. D1 gastrectomy; ITT, intent-to-treat.

The Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) is a randomized phase III trial to confirm the effectiveness of 1-year postoperative treatment with S-1 compared with surgery alone in patients with stage II or III gastric cancer who underwent D2 gastrectomy. S-1 (TS-1; Taiho Pharmaceutical, Tokyo, Japan) is a dihydropyrimidine dehydrogenase inhibitory fluoropyrimidine preparation combining tegafur, gimeracil, and oteracil potassium in a molar ratio of 1:0.4:1.[8,9] Two phase II studies[10,11] in patients with advanced or recurrent gastric cancer obtained high response rates exceeding 40%. Postoperative adjuvant chemotherapy with S-1 was thus expected to be effective.

In this phase III trial, 1,059 patients with histologically confirmed stage II or III gastric cancer who underwent D2 gastrectomy were enrolled. A protocol-based interim analysis performed 1 year after the completion of enrollment (median follow-up, 3 years) confirmed that S-1 was effective. Because statistical analysis indicated that there was minimal probability that the results of this study would turn out to be negative after 5 years of follow-up, an independent data and safety monitoring committee recommended that the results should be disclosed at that time. An analysis of the results available at that time showed that the 3-year overall survival (OS) was 80.1% in the S-1 group compared with 70.1% in the surgery-only group. S-1 was demonstrated to reduce the risk of death by 32% (hazard ratio [HR], 0.68; 95% CI, 0.52 to 0.87; $P = .003$).[12] Although the study results were disclosed early because of these promising results, we considered it important to have 5-year follow-up data available. Such data would facilitate a comparison of our results for 5-year OS and other outcomes with those of previous trials. Moreover, this analysis may justify

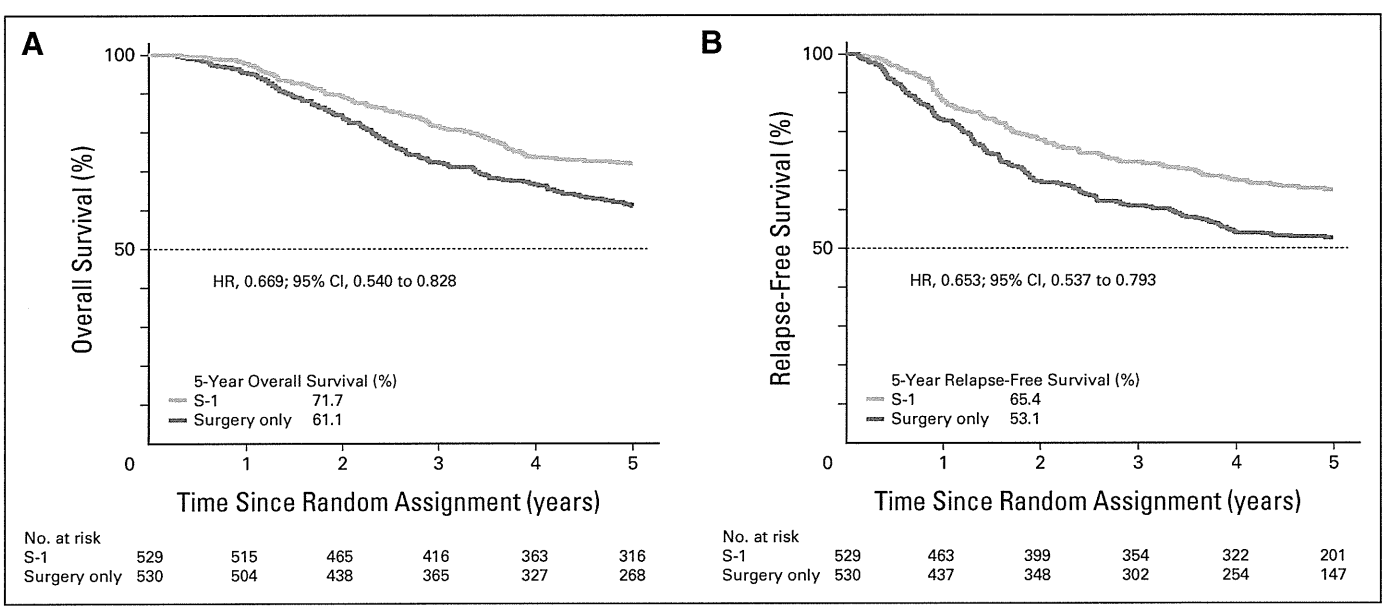

Fig 2. Kaplan-Meier estimates of (A) overall survival and (B) relapse-free survival for all randomly assigned patients. HR, hazard ratio.

	Total No. of Patients	Overall Survival HR	95% CI	Interaction P	Relapse-Free Survival HR	95% CI	Interaction P
Sex				.8147			.5032
Male	720	0.679	0.524 to 0.880		0.700	0.554 to 0.885	
Female	314	0.642	0.429 to 0.959		0.604	0.419 to 0.869	
Age, years				.4424			.5460
< 60	383	0.550	0.378 to 0.799		0.568	0.405 to 0.797	
60-69	404	0.678	0.467 to 0.983		0.726	0.523 to 1.008	
70-80	247	0.779	0.527 to 1.151		0.706	0.490 to 1.017	
Cancer stage (Japanese classification)				.2945			.2771
II	465	0.509	0.338 to 0.765		0.521	0.362 to 0.750	
IIIA	397	0.708	0.510 to 0.983		0.696	0.514 to 0.941	
IIIB	172	0.791	0.520 to 1.205		0.788	0.539 to 1.151	
Cancer stage (UICC 6th)				.4129			.6611
IIA	538	0.518	0.356 to 0.753		0.570	0.408 to 0.796	
IIIA	318	0.665	0.460 to 0.962		0.629	0.446 to 0.886	
IIIB	106	0.855	0.510 to 1.431		0.712	0.445 to 1.139	
IV	72	0.784	0.422 to 1.458		0.834	0.486 to 1.432	
Tumor stage (UICC 6th)				.7789			.9026
T2	565	0.652	0.471 to 0.902		0.658	0.489 to 0.886	
T3	444	0.690	0.511 to 0.932		0.655	0.500 to 0.859	
T4	25	0.412	0.098 to 1.733		0.868	0.264 to 2.858	
Nodal stage (Japanese classification)				.0882			.0729
N0	112	0.317	0.127 to 0.790		0.308	0.139 to 0.681	
N1	563	0.608	0.440 to 0.840		0.651	0.487 to 0.869	
N2	359	0.839	0.612 to 1.150		0.806	0.603 to 1.078	
No. of nodal metastasis (UICC 6th)				.2119			.2106
0	112	0.317	0.127 to 0.790		0.308	0.139 to 0.681	
1-6	642	0.606	0.444 to 0.828		0.677	0.511 to 0.897	
7-15	224	0.779	0.534 to 1.138		0.693	0.488 to 0.984	
> 16	56	0.927	0.477 to 1.799		0.874	0.486 to 1.570	
No. of nodal metastasis (UICC 7th)				.0861			.0431
0	112	0.317	0.127 to 0.790		0.308	0.139 to 0.681	
1-2	334	0.454	0.275 to 0.749		0.482	0.309 to 0.752	
3-6	308	0.740	0.494 to 1.108		0.877	0.606 to 1.268	
> 7	280	0.820	0.590 to 1.138		0.729	0.540 to 0.984	
Histrologic type				.9806			.7339
Differentiated	423	0.670	0.478 to 0.938		0.706	0.515 to 0.967	
Undifferentiated	608	0.673	0.506 to 0.896		0.657	0.510 to 0.847	

Fig 3. Subgroup analysis: overall survival and relapse-free survival for eligible population. In the surgery-only group, cancers in three patients could not be classified as differentiated or undifferentiated. HR, hazard ratio; UICC, International Union Against Cancer (UICC) TNM Classification of Malignant Tumours.

the present controversial use of 3-year relapse-free survival (RFS) as the primary end point in clinical trials of adjuvant chemotherapy for potentially curable gastric cancer.

PATIENTS AND METHODS

The trial was conducted in accordance with the World Medical Association Declaration of Helsinki and Japanese Good Clinical Practice guidelines. This protocol was approved by the institutional review board of each participating hospital (see Data Supplement). Written informed consent was obtained from all patients. Tumor stage classification and D classification were in accordance with the Japanese Classification of Gastric Carcinoma (Second English Edition).[13]

Patients and Treatment

Eligibility criteria were as follows: a histopathologically confirmed diagnosis of stage II (except for T1 disease), IIIA, or IIIB gastric cancer; R0 resection (with no tumor cells at the margin) with D2 or more extensive lymph node dissection; no evidence of hepatic, peritoneal, or distant metastasis; no tumor cells in peritoneal fluid on cytologic analysis; age 20 to 80 years; no previous treatment for cancer except for the initial gastric resection for the primary lesion; and adequate organ function. Patients were enrolled within 6 weeks after surgery over the telephone or by means of facsimile. Patients were randomly assigned to either the S-1 group or the surgery-only group. The assignments were made by the minimization method according to disease stage (II, IIIA, or IIIB) at the ACTS-GC data center.

Patients assigned to the S-1 group received S-1 in a daily dose of 80, 100, or 120 mg in two divided doses. The dose of S-1 was assigned on the basis of body surface area. S-1 was given for 4 weeks, followed by 2 weeks of rest. Treatment was continued for 1 year after surgery. Patients assigned to the surgery-only group received no anticancer treatment postoperatively until the confirmation of recurrence. The criteria for dose reduction and toxicity were described previously.[12]

Follow-Up

In the S-1 group, the results of blood tests and clinical findings were assessed at 2-week intervals during treatment with S-1. In the surgery-only group, patients came to the hospital for re-examination at least once every 3 months for the first year after surgery. From the second year onward, all patients were followed up in the same manner. Relapse was confirmed by imaging studies, including ultrasonography, computed tomography, and GI radiography, as well as endoscopy. Patients underwent at least one imaging study at 6-month intervals for the first 2 years after surgery and at 1-year intervals until 5 years after surgery. Individual patients were followed up for 5 years from the date of random assignment.

Statistical Analysis

The sample size was calculated as follows. Given that the 5-year survival rate would be 70% in the surgery-only group, with an HR of 0.70, $\alpha = .05$ (two-sided), and a statistical power of 80%, we estimated that 1,000 patients would be required. OS and RFS were estimated on the basis of all randomly assigned patients. The results in eligible patients were analyzed according to disease stage. OS was defined as the interval from the date of random assignment to the date of death from any cause. RFS was defined as the interval from the date of random assignment to the date of confirming recurrence or death from any cause, whichever came first. Data for up to 5 years from the date of random assignment were analyzed. Data obtained after 5 years were not included in this analysis. The survival rate was estimated by using the Kaplan-Meier method. The Cox proportional hazards model was used to calculate HRs. All statistical analyses were done with SAS, version 9.1 (SAS Institute, Cary, NC).

RESULTS

Patients

From October 2001 through December 2004, a total of 1,059 patients were enrolled at 109 centers throughout Japan; 529 were assigned to the S-1 group and 530 to the surgery-only group (intention-to-treat population; Fig 1). In both groups combined, 474 patients (44.8%) had stage II disease, 409 (38.6%) had stage IIIA disease, and 175 (16.5%) had stage IIIB disease. The numbers of patients with each stage of disease were similar in the two treatment groups. The groups were also well balanced with respect to the type of gastrectomy performed, the combined resection of other organs, and other factors. Details of the patient demographics and baseline characteristics have been reported previously.[12]

Fourteen patients in the S-1 group and 11 in the surgery-only group were ineligible, as shown in Figure 1. In the S-1 group, 12 patients did not receive S-1. In the surgery-only group, four patients received adjuvant treatment at their strong request, violating the protocol.

Safety

Details of the safety analysis have been reported previously.[12] In brief, except for anorexia (incidence, 6%), grade 3 or 4 adverse events occurred in less than 5% of the patients in the S-1 group.

OS and RFS in All Randomly Assigned Patients

Among 1,059 patients, 145 and 199 died, 32 and 42 patients are alive with recurrence, and 352 and 289 patients are alive without recurrence in the S-1 and the surgery-only groups, respectively. Data on 131 patients lost to follow-up within 5 years from the date of random assignment were censored.

OS and RFS were analyzed in all 1,059 randomly assigned patients. The 5-year OS rate was 71.7% (95% CI, 67.8% to 75.7%) in the S-1 group and 61.1% (95% CI, 56.8% to 65.3%) in the surgery-only group. The HR for death in the S-1 group compared with the surgery-only group was 0.669 (95% CI, 0.540 to 0.828), indicating that S-1 reduced the risk of death by 33.1% (Fig 2A). The 5-year RFS rate was 65.4% (95% CI, 61.2% to 69.5%) in the S-1 group and 53.1% (95% CI, 48.7% to 57.4%) in the surgery-only group. The HR for relapse in the S-1 group compared with that in the surgery-only group was 0.653 (95% CI, 0.537 to 0.793). Treatment with S-1 thus reduced the risk of relapse by 34.7% (Fig 2B).

Subgroup Analysis

OS and RFS in eligible patients were analyzed according to sex, age, disease stage (Japanese Classification, 13th edition), and histologic type. There was no interaction between treatment and any of these factors (Fig 3). Kaplan-Meier estimates of OS and RFS are shown according to disease stage, which was used as a stratification factor when patients were randomly assigned (Figs 4, 5, and 6).

The 5-year OS rates of the patients with stage II disease were 84.2% (95% CI, 79.5% to 89.0%) in the S-1group and 71.3% (95% CI, 65.3% to 77.2%) in the surgery-only group, with an HR of 0.509 (95% CI, 0.338 to 0.765; Fig 4A). Their 5-year RFS rates were 79.2% (95% CI, 73.8% to 84.6%) in the S-1 group and 64.4% (95% CI, 58.1% to 70.7%) in the surgery-only group, with an HR of 0.521 (95% CI, 0.362 to 0.750; Fig 4B). The 5-year OS rates of stage IIIA patients were 67.1% (95% CI, 60.4% to 73.8%) in the S-1 group and 57.3% (95% CI, 50.3% to 64.2%) in the surgery-alone group, with an HR of 0.708 (95% CI, 0.510 to 0.983; Fig 5A). Their 5-year RFS rates were 61.4% (95% CI, 54.5% to 68.4%) in the S-1 group and 50.0% (95% CI, 42.9% to 57.0%) in the surgery-alone group, with an HR of 0.696 (95% CI,

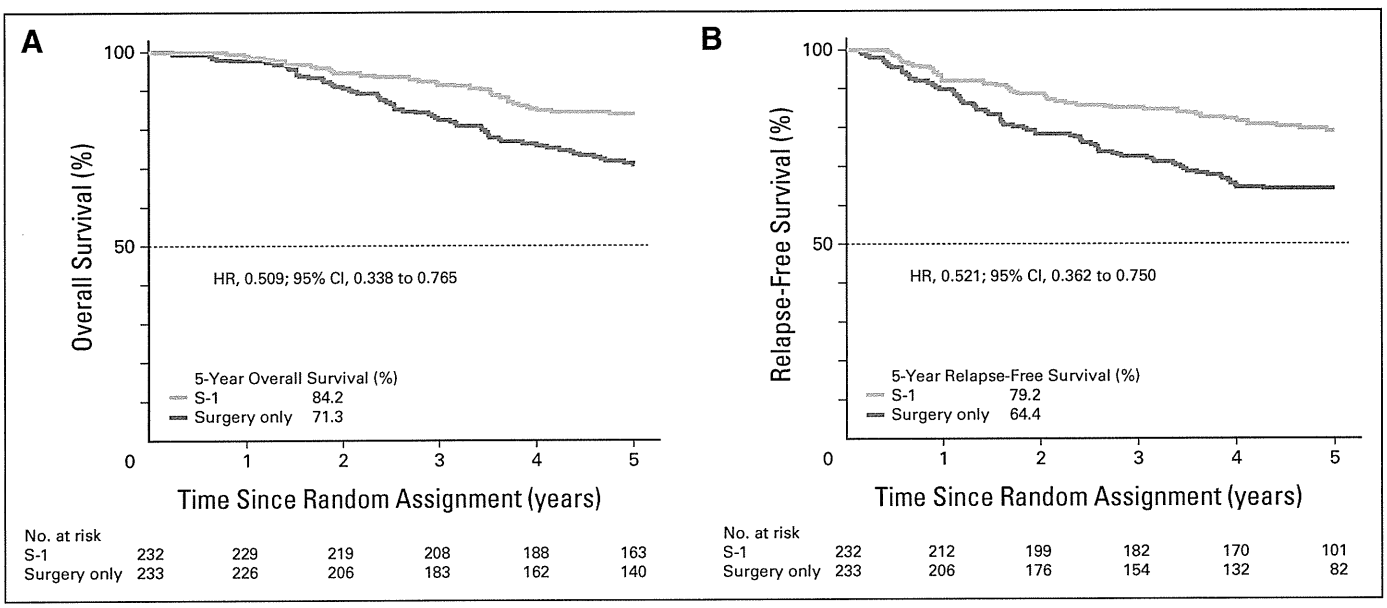

Fig 4. Kaplan-Meier estimates of (A) overall survival and (B) relapse-free survival for eligible patients with stage II gastric cancer. HR, hazard ratio.

