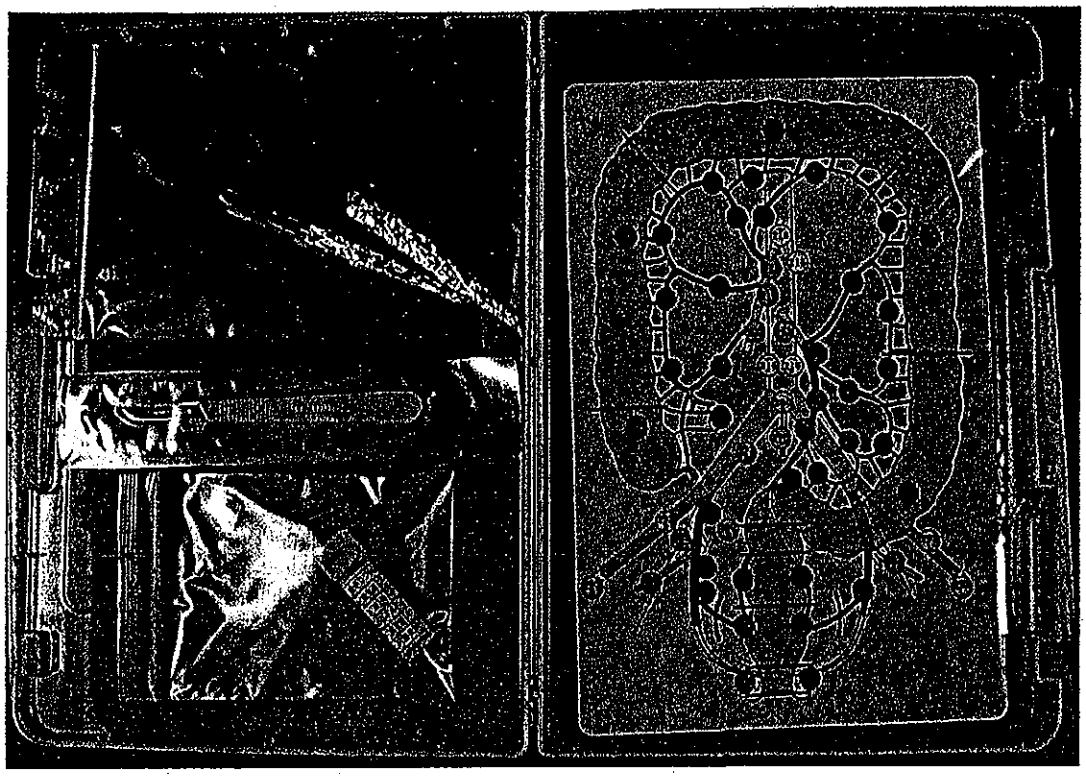

Figure 1. Tissue sampling kit. The kit contained a set of scissors, forceps, scalpel, a tube containing special liquid for RNA preservation (RNA later) and a board for arrangement of LNs. The kit was pre-treated with RNase-away to eliminate RNase activity.

0.2 μM of each primer, 1x Light Cycler-Fast start DNA Master SYBR Green I (Roche Diagnostics), 4 mM $MgCl_2$, and 2 μl of cDNA as template. PCR conditions were as follows: one cycle of denaturing at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 62°C for 10 sec and 72°C for 18 sec. Fluorescence was acquired at the end of each 72°C extension phase. The melting curves of final PCR products were analyzed after 40 cycles of PCR amplification by cooling the samples to 65°C, increasing the temperature up to 99°C at a rate of 0.1°C/sec, and monitoring fluorescence at each 0.1°C. Quantification data were analyzed using Light Cycler analysis software (Roche Diagnostics GmbH) as recommended by the manufacturer. The standard curves for quantification of CEA or PBGD mRNAs were drawn using 10-fold dilutions of cDNA from MKN45 cells. The sequences of CEA and PBGD primers were the same as those used in the conventional PCR.

Results

Tissue sampling kit. To avoid contamination of cancer cells, a disposable tissue sampling kit was developed (Fig. 1). The box was 30.0x23.0x3.8 cm in size, containing a set of scissors, forceps, blade, a tube containing special liquid for RNA preservation (RNA later) and a board for arrangement of LNs. RNA later can be stored at room temperature. The box and all tools were rendered RNase-free by pretreatment with RNase away (Molecular BioProducts, inc., San Diego, CA).

Inquiry to related hospitals about sampling practices. We asked surgeons working at related hospitals (n=17) about the time they usually collect LNs from the resected specimens after surgery. As shown in Table I, LN sampling was performed within 3 h after surgery in most hospitals (17 of 18 hospitals including our institute).

Table I. When do surgeons collect lymph nodes after surgery?

Hours	Hospitals
<1	6
<2	9
<3	2
<24	1
Total	18

Stability of RNA in LNs. LNs from 7 CRC patients were left at room temperature for 1, 2 and 3 h after surgery, at which times RNA was extracted. RNA quality was assessed by electrophoresis. Ribosomal RNAs at 28S and 18S were quite well preserved even after 3 h in all LN samples tested (Fig. 2A), while RNA extracted from tumor tissues was preserved at good quality at 3 h in 5 of 7 cases (data not shown). We then examined how long RNA in LNs could be preserved when LNs were stored in RNA later at -20°C. As shown in Fig. 2B, RNA was of good quality even after 3 weeks of storage.

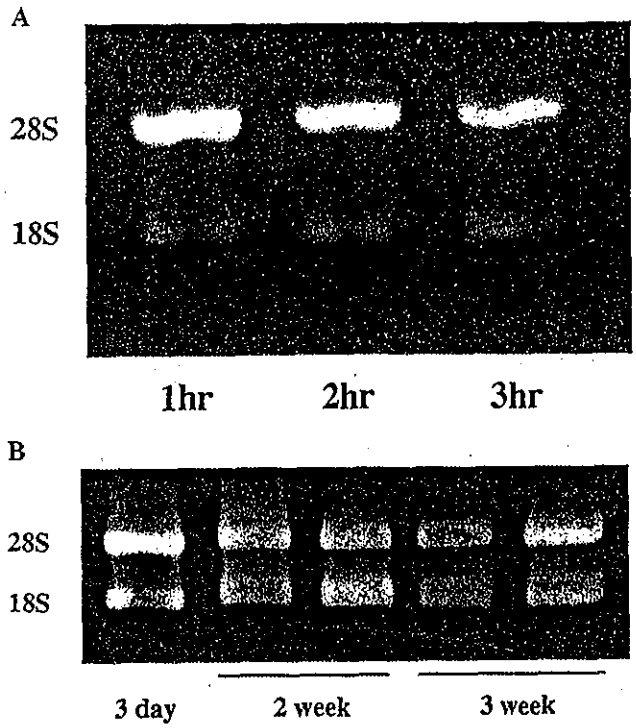

Figure 2. Stability of RNA in LNs. (A), Assessment of timing of LN sampling. LNs were left at room temperature for 1, 2 and 3 h after surgery and then RNA was extracted. Prominent doublet bands of 28S and 18S ribosomal RNAs were well preserved even in the 3-h samples. RNA extracts from a representative CRC patient are shown here. (B), Assessment of LN storage. LNs were collected from the same CRC patient. They were stored in RNA later at -20°C for 3 days, 2 weeks and 3 weeks and then RNA was extracted. RNA quality was sufficient even after 3 weeks of storage.

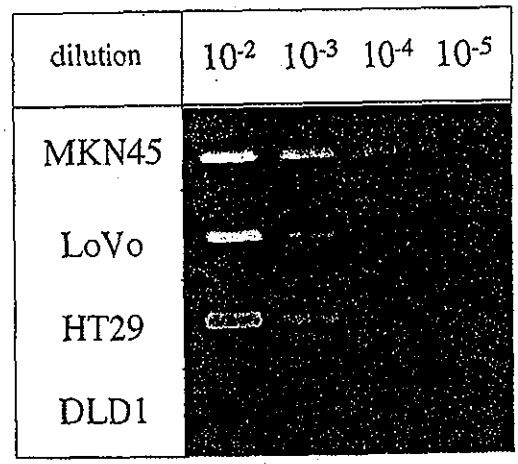

Figure 3. Control panel using CEA-expressing cells. A control panel was constructed as positive controls for conventional PCR. Using CEA mRNA as a marker, RT-PCR yielded bands for dilutions of MKN45, LoVo, HT29, but not DLD1 cells. MKN45 and LoVo cells exhibited good reproducibility.

Control panel using CEA-expressing cells. A control panel was constructed as positive control for conventional PCR. Using CEA mRNA as a marker, PCR yielded bands for dilutions of MKN45, LoVo, HT29, but not DLD1 cells (Fig. 3). Experiments were repeated 22 times using the LoVo cells at dilutions of 10^{-2}-10^{-4}, and the MKN45 cells at dilutions of 10^{-2}-10^{-5}, including all steps, that is, from RNA extraction to reverse transcription to PCR, and reproducible results were obtained 19 times. In one repetition, the highest dilutions (MKN45 at dilution of 10^{-5}, LoVo at dilution of 10^{-4}) yielded visible bands that should not have emerged. In the remaining two repetitions, there were no visible bands at all dilutions, indicating simple technical error. The unusual patterns in these three experiments reverted to the typical pattern by a single repeat experiment, indicating that these control dilutions were highly stable and ensured reproducibility. On the other hand, HT29 cells showed unstable results. Thus, at the highest dilution (10^{-4}), a visible band appeared in 6 of 10 repeat experiments (data not shown).

Modification of real-time PCR for CEA mRNA measurement. To measure CEA mRNA levels, we modified a real-time PCR method previously reported by our laboratories (17). First, human peripheral lymphoid cells were used in the original study to prepare serial dilutions of MKN45 cells when the standard curves were drawn. However, human blood is not suitable as a diluent for clinical workup, so we instead used rat spleen as a diluent. cDNA from rat spleen was not amplified with the primers for human CEA gene expression (data not shown). Since we have noted that the RT reaction itself occasionally produces inter-experimental deviation (data not shown), cDNA derived from 1 μg RNA of MKN45, rather than the RNA itself, was serially diluted with cDNA of rat spleen in making standard curves (Fig. 4A). In 40 separate experiments, the modified system rendered the standard curves highly stable and reproducible within the range of 1.0×10^{-1} to 1.0×10^{-4} (Fig. 4B). The standard deviation was <10% in each dilution in this range, but the system did not assure the measurement value at a dilution of 1×10^{-5} (data not shown).

The Light Cycler provides another benefit in that the melting temperature indicates whether the PCR product is real or not. This utility is based on the finding that the melting temperature is determined by the content of individual PCR products. When the bands for CEA were very faint by conventional PCR, it was sometimes difficult to distinguish them from non-specific bands (Fig. 5). The melting temperature determined by the MKN45-positive control sample revealed that sample no. 1 expressed a real CEA band, whereas samples nos. 2-4 displayed non-specific bands because their melting temperature was significantly different from that of the positive control.

Multi-institution group study. Using the above system, we commenced a multi-institutional clinical study in November 2001 to assess the actual incidence of micrometastasis of stage II CRC. Paracolic LNs were collected within 3 h after surgery, stored in RNA later at -20°C, and then moved to our laboratory once a week in a dry ice package. Homogenized LNs were mixed and RNA was extracted. All samples were first examined for RNA quality, and classified into four levels according to degradation pattern of ribosomal RNAs at 28S and 18S by electrophoresis; A, Excellent (28S>18S, without degradation); B, Fairly good (28S≥18S, only partial degradation, if any); C, Poor (18S>28S, with large degradation); D, Unequivocal (no visible ribosomal RNA, all

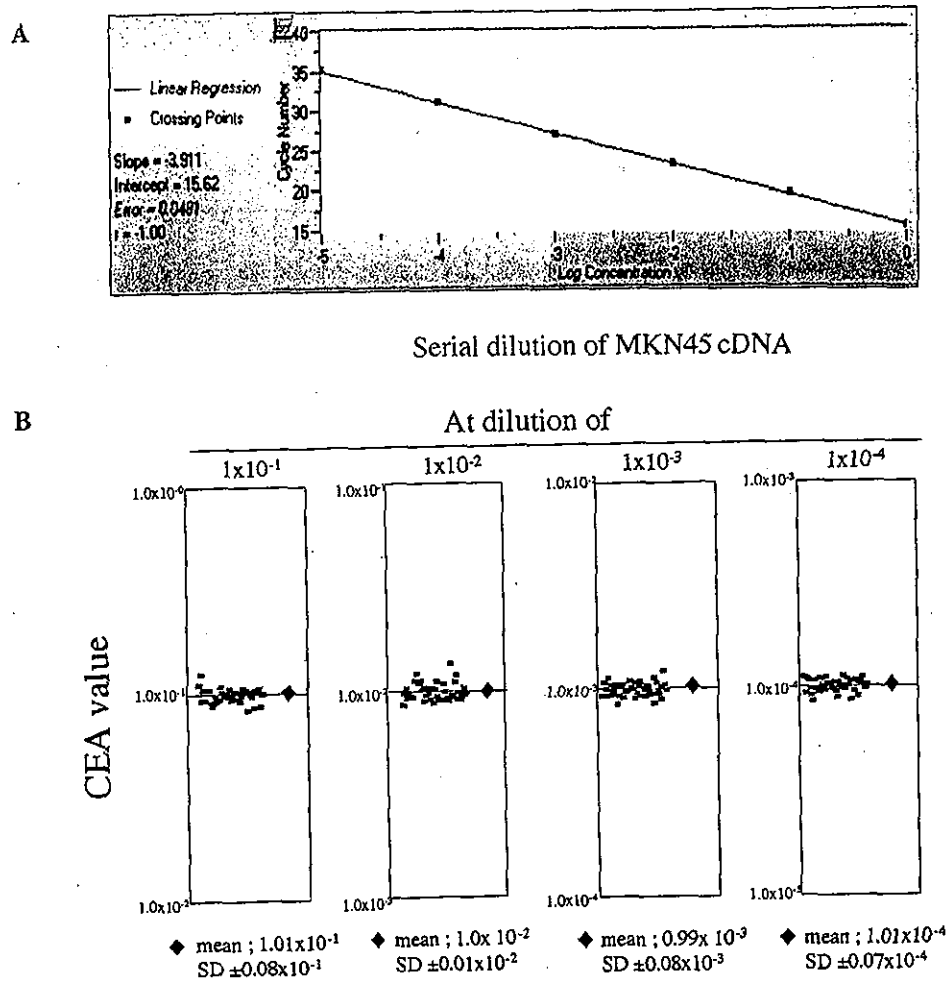

Figure 4. Real-time PCR for measurement of CEA mRNA. (A), Standard curve. cDNA derived from 1 μg RNA of MKN45 was serially diluted with cDNA of rat spleen at dilutions of 10^{0}-10^{-5}. Standard curves were drawn by plotting the PCR cycle number at which each diluted sample started making PCR product. (B), The modified RT-PCR system rendered the standard curves highly stable and reproducible within the range of $1.0x10^{-1}$ to $1.0x10^{-4}$. Standard deviation was <10% in each dilution within this range.

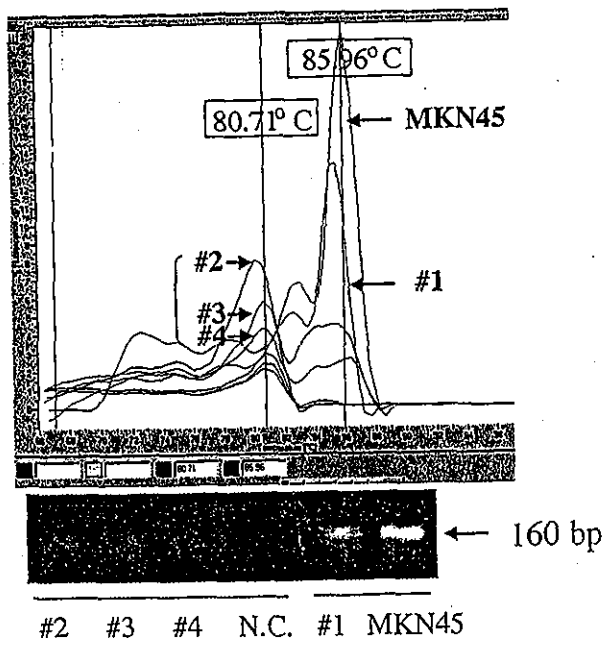

Figure 5. Melting temperature of the PCR product. The melting temperature determined by the MKN45-positive control sample was 85.9°C and sample no. 1 had a similar melting temperature, indicating that it expressed a real CEA band, whereas sample nos. 2-4 produced non-specific bands because their melting temperature was around 80.7°C. NC., negative control.

RNA degraded) (Fig. 6). Most of the samples (98 of 100 cases; 98%) were classified into levels A and B. Expression of the PBGD gene was evident in these samples (data not shown), and only samples of levels A and B were subject to further RT-PCR assay by both conventional PCR and modified real-time PCR. Fig. 7 shows two representative cases. Case 1 expressed a CEA band compatible with that of MKN45 dilution at 10^{-4} and yielded a CEA value of $1.9x10^{-4}$ by real-time PCR. Case 2 did not express the CEA band and the CEA value was $3.7x10^{-5}$, which was under the measurable range of $1.0x10^{-4}$ to $1.0x10^{-1}$. Analyses of a total of 98 CRC cases indicated that they were successfully divided into two groups, band-positive with a high quantity of CEA (24 of 98 cases: 24.5%) and band-negative with a low quantity of CEA (74 of 98: 75.5%) (Fig. 8). Only one case with ambiguous faint bands were judged as negative by analysis of melting temperature (data not shown).

Discussion

Even among node-negative CRC patients, approximately 10-20% suffer from relapse within 5 years (18). Since the majority of stage II CRCs are thought to still be localized and not yet systemic disease, there is a debate about appropriate

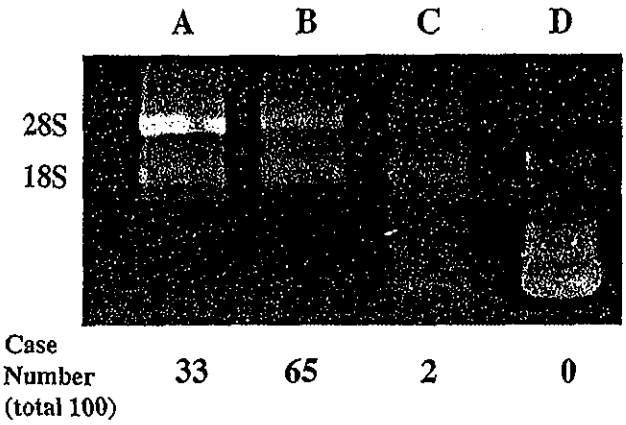

Figure 6. Assessment of RNA quality in clinical samples. According to the degradation pattern of ribosomal RNAs, clinical samples were classified as follows; (A), Excellent (28S>18S, without degradation); (B), Fairly good (28S≥18S, only partial degradation, if any); (C), Poor (18S>28S, with large degradation); (D), Unequivocal (no visible ribosomal RNA, all RNA degraded). Ninety-eight of 100 (98%) cases were classified into levels A and B.

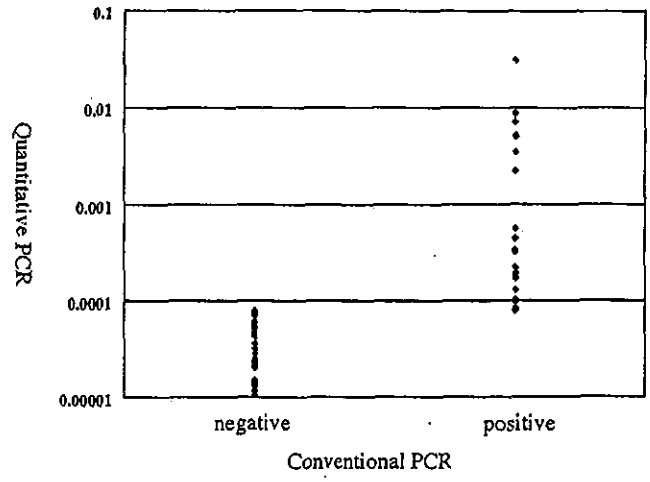

Figure 8. Analyses of a total of 100 CRC cases for micrometastasis. The patients were divided into two groups: i) band-positive with a high CEA quantity (24 of 98 cases: 24.5%) and ii) band-negative with a low CEA quantity (74 of 98: 75.5%).

post-operative chemotherapy for stage II CRC patients. Indeed, among our related hospital groups, more than half (12 of 18; 67%) presently use no post-operative chemotherapy, whereas the other 6 hospitals often use 5-FU-based chemotherapy for this group. Since there are no clear indicators whether or not to treat, it is therefore important to discriminate those at high risk for disease recurrence among stage II CRCs. For this purpose, we found that although information on clinical and pathological parameters was not helpful, our retrospective study showed that CEA-based RT-PCR was useful (3).

This finding was exciting, yet we should be cautious as we cannot exclude the possibility that LNs collected in the past might have been subject to contamination of cancer cells via scissors or blades used for tumor excision. Therefore, a prospective study using a strict sampling procedure was essential, ideally including a larger number of CRCs, to confirm the previous results. However, as mentioned in Introduction, many issues remain regarding the use of RT-PCR for clinical diagnosis. Osaka University Hospital is well supplied with surgeons and medical staff, and collection of LNs is usually completed in less than 1 h of excision. On the other hand, there are only a limited number of surgeons at related hospitals and often it is not feasible to collect LNs within 1 h. If the RNA in LNs was easily degraded, it would be a major technical problem for establishing RT-PCR for general use to detect micrometastasis. Fortunately, we determined the optimal LN sampling time to be within 3 h after resection, a time frame feasibly met by surgeons and during which the quality of RNA was well preserved (Table I, Fig. 2A). This result regarding the preservation time of RNA in LNs was far better than had been anticipated, which was encouraging as it indicates that RT-PCR assay for CEA mRNA in LNs could be practically applicable in most general hospitals. Moreover, LNs could be stored for at least 3 weeks (Fig. 2B), if stored in RNA later at -20°C. Storage

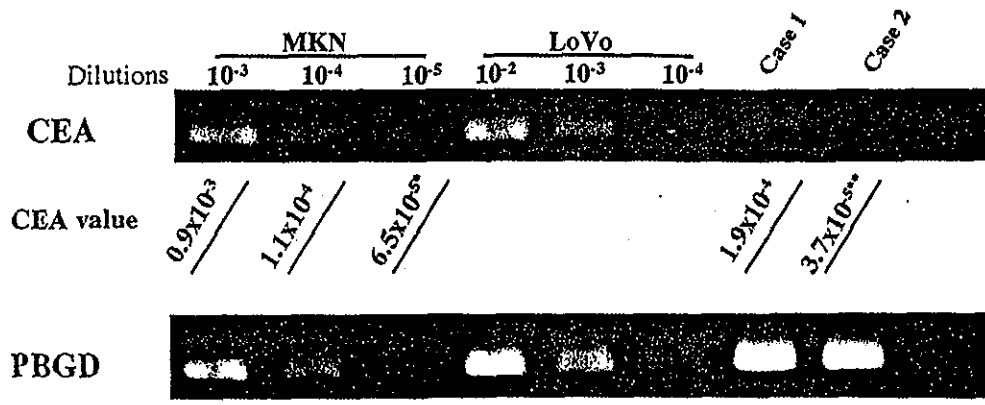

Figure 7. Two representative cases in the prospective study. Case 1 expressed a CEA band compatible with that of the MKN45 dilution at 10^{-4} and gave a CEA value of 1.9×10^{-4} by real-time PCR. Case 2 did not express a CEA band and the CEA value was 3.7×10^{-5}, which was under the measurable range of 1.0×10^{-4} to 1.0×10^{-1}. Both samples expressed intense bands for the housekeeping PBGD gene. *,**These CEA values were outside the measurable range.

at -80°C is not necessarily required. This result is convenient on a cost-benefit basis as it means that the RT-PCR assay could be applied solely to node-negative CRC patients after pathological analysis.

When RNA is utilized as the material for examination, special attention must be taken to avoid contamination by RNase in the LN sampling procedure. To address this issue we developed a tissue sampling kit. This kit was intended for busy surgeons working at related hospitals to facilitate LN sampling. When we first explained the method of LN sampling for RT-PCR to surgeons, the majority insisted that they would not be able to prepare RNase-free equipment for each procedure. After introducing the tissue sampling kit, they agreed to participate in the project of molecular detection of micrometastasis. The kit contains a disposable blade, scissors and forceps that are rendered RNase-free. After one or two educational demonstrations of LN sampling (mainly executed by Osamu Takayama, one of the main investigators), surgeons at each hospital could execute appropriate LN sampling, so that artificial contamination of cancer cells over LNs could be avoided.

False positive reactions and lack of reproducibility are the two major concerns with regard to the PCR technique. With respect to false positivity, in our earlier studies we assessed optimal PCR conditions in which LNs from non-cancer patients would never be amplified (2,3). Our results from these studies indicated that we should perform a single PCR amplification of 35 cycles for the CEA transcript, at least in our PCR system.

Reproducibility is a problem that remains to be fully addressed. This issue is especially important in detection of the minimal level of mRNA expression. A single intense positive control is not useful as a control for such minimal transcripts because a slight decrease in PCR efficiency could easily produce false negative results even with preservation of the intense control signal. To address this issue, we have constructed a control panel using CEA-expressing cells. Cancer cell lines express different levels of CEA mRNA and we tried to find dilutions of appropriate cell lines that would show reproducible results under our PCR conditions. Although the HT29 and DLD1 cell lines were not appropriate for this purpose, MKN45 and LoVo cells exhibited high reproducibility. With a panel of dilutions of these two cell lines, our prospective clinical study proceeded and the expected expression pattern was obtained in almost all PCR reactions (data not shown).

We previously showed that amount of micrometastasis of CRC varied quite widely (17). In a prospective study, it is important to quantify micrometastasis so that a critical cut-off level for the prediction of prognosis can be determined. For this purpose we modified our real-time PCR method. As diluents of MKN45 cells, we first used *E. coli*, but this was not feasible as the primers for the human CEA sequence unexpectedly produced PCR products from cDNA derived from *E. coli* (data not shown). We then employed rat spleen since it was used to measure PBGD mRNA in our previous study (17) and found that it was also appropriate for measuring CEA mRNA. Certain DNA plasmids may alternatively be applicable as diluents. The modified system can measure CEA transcripts accurately within the range of $1x10^{-1}$ to $1x10^{-4}$. The minimal detectable CEA band of MKN45 cells (at dilution of 10^{-4}) coincidentally corresponded to a CEA value of approximately $1.0x10^{-4}$ (Fig. 7), and thus, double confirmation was possible.

In this study, we performed a translational research analysis and established a clinically feasible RT-PCR system. To date, using this system, more than 98 stage II CRCs have been prospectively examined for micrometastasis. The percentage of band-positive cases (24.5%) was somewhat lower than that (29.6%) reported in our retrospective study (3). This could be due to differences in sampling procedures between the retrospective and prospective studies. The strict sampling procedure using the tissue sampling kit in our current study would not permit artificial contamination of cancer cells, whereas such contamination was likely in the retrospective study. The present study also indicates that RT-PCR for micrometastasis is applicable not only at specified institutes, but also at general hospitals. Therefore, it may become a powerful clinical tool, if our prospective study confirms that RT-PCR for micrometastasis is useful for predicting the prognosis of stage II CRC.

Acknowledgments

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Appendix

Participants in clinical study: K. Ikeda, M. Okuyama, T. Shimano: Department of Surgery, Toyonaka Municipal Hospital, Osaka; T. Kato, M. Tsujie, N. Kikkawa: Department of Surgery, Minoh City Hospital, Osaka; M. Fukunaga, N. Tomita: Department of Surgery, Kansai Rosai Hospital, Hyogo; H. Ishida, H. Furukawa: Department of Surgery, Sakai Municipal Hospital, Osaka; T. Kanou, S. Ohnishi, T. Monden: Department of Surgery, NTT West Osaka Hospital, Osaka; S. Tominaga, T. Kobayashi: Department of Surgery, Ikeda Municipal Hospital, Osaka; S. Morita, N. Shibata: Department of Surgery, Yao Municipal Hospital, Osaka; N. Tanaka, H. Maruyama: Department of Surgery, Kawanishi City Hospital, Hyogo; K. Murata, M. Kameyama: Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka; H. Ohta, K. Yamasaki: Department of Surgery, Saiseikai Senri Hospital, Osaka; Y. Uemura, K. Kobayashi: Department of Surgery, Kinki Central Hospital of the Mutual Aid Association of Public School Teachers, Hyogo; M. Amano, S. Ohshima: Department of Surgery, Nishinomiya Municipal Central Hospital, Hyogo; M. Watase, Y. Ogawa: Department of Surgery, Tane General Hospital, Osaka; F. Kimura, M. Yamamoto: Department of Surgery, Itami City Hospital, Hyogo; K. Fukuda, H. Hatanaka: Department of Surgery, Fujimoto Hospital, Osaka; Y. Watanabe, T. Kabuto: Department of Surgery, Osaka Seamen's Insurance Hospital, Osaka; N. Yamamoto, H. Oka: Department of Surgery, Moriguti Keijinnkai Hospital, Osaka, Japan.

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Cancer Genetics Report

The Novel Germline Mutation of the *hMLH1* Gene in a Case of Suspected Hereditary Non-polyposis Colorectal Cancer (HNPCC) in a Patient with No Family History of Cancer

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Hereditary non-polyposis colorectal cancer (HNPCC) is a very important clinical entity in oncology. In order to identify HNPCC, the international diagnostic criteria, named 'Amsterdam criteria', has been used. In this report, we present a patient with HNPCC who completely lacks a family history of cancer, thus does not meet the revised Amsterdam criteria and was finally confirmed as HNPCC by genetic testing which revealed a novel germline mutation of the *hMLH1* gene. The proband was a 52-year-old Japanese female with a diagnosis of advanced ascending colon cancer. She had a past history of Miles' operation for rectal cancer at the age of 40. A subtotal colectomy was performed and the subsequent microsatellite instability (MSI) analysis revealed high MSI in the resected tumor tissue. PCR/direct sequencing analysis of the genomic DNA revealed the base deletion 2006delAAAAG at codon 669 in exon 18 of the *hMLH1* gene, which was considered to be a pathogenic mutation. According to the Human Mutation Database and International Collaborative Group on HNPCC (ICG-HNPCC) Database, this is the first report of this type of deletion mutation in the *hMLH1* gene.

Key words: genetic testing – hereditary non-polyposis colorectal cancer (HNPCC) – hMLH1 – microsatellite instability (MSI) – revised Amsterdam Criteria

CASE REPORT AND GENETIC ANALYSIS

A genetic summary is included in Table 1.

Hereditary non-polyposis colorectal cancer (HNPCC) is a very important clinical entity because of its clinical characteristics such as relatively high frequency (1.0–5.0%) among all colorectal cancers and relatively early onset of malignancies in various organs, etc. (1). In order to identify HNPCC, the international diagnostic criteria, named 'Amsterdam minimum criteria' was proposed in 1991 and had been used in many countries, including Japan. However, considering the variety of malignancies other than colorectal cancer occurring in HNPCC families, some proportion of true HNPCC might be missed under this criteria, which needs at least three colorectal cancer patients in the pedigree. Thus, the revised version, Amsterdam criteria II, was proposed in 1999. This revised criteria allows diagnosis of the family containing at least three HNPCC-related cancer patients as HNPCC, however, there still remains the possibility of the so-called *de novo* HNPCC patient, in whom the initial mutation of mismatch repair gene might occur, could be missed.

In this report, we present an HNPCC-suspected case with no family history of cancer, who, thus, does not meet the revised Amsterdam criteria, and who was finally confirmed as HNPCC by genetic testing which revealed a novel germline mutation of *hMLH1*.

The proband was a 52-year-old Japanese female who was admitted to Kansai Rosai Hospital with a diagnosis of advanced ascending colon cancer. The initial history taking was done at the outpatient's clinic and revealed that she had a

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history of surgery of Miles' operation with sigmoid colostomy for advanced rectal cancer at the age of 40. There is no family history of cancer as shown in Figure 1. The patient's past history of rectal cancer at her relatively young age of 40, together with the present occurrence of the ascending colon cancer, made us suspect that this case might be of the HNPCC kindred, even without a family history of cancer. The preoperative abdominal computed tomography revealed the existence of uterine myoma, but no other disorders, including malignancies, could be found.

Table 1. Genetic summary

Disorder	Hereditary non-polyposis colorectal cancer
Ethnicity of patient	Japanese
Gene	*hMLH1*
GenBank accession number	U07343
Chromosomal assignment	3p21.3
Type of DNA variant	Germline deletion mutation
Mutation	base deletion 2006delAAAAG at codon 669 in exon 18 of the *hMLH1* gene
Method of mutation detection	RT-PCR/direct sequencing and PCR/direct sequencing

We gave a precise explanation to the patient and her family members, including her husband, about her possibility of HNPCC kindred and the choice of surgical procedures of either right hemicolectomy or total colectomy, and also about the optional surgery for uterus and ovary. Under her informed consent, total colectomy with ileostomy and simple total hysterectomy with bilateral salpingo-oophorectomy were carried out with the cooperation of the doctors of the gynecological department in May 2003. In the operation, direct invasion of the ascending colon cancer to the jejunum was found and part of the jejunum was resected, together with the main tumor. The laterally spreading tumor, 2.5 cm in size, was found in the cecum in the resected specimen and the existence of well differentiated adenocarcinoma localized in the mucosal layer in the tubular adenoma was later diagnosed histologically. A histopathological diagnosis of the main tumor was moderately differentiated adenocarcinoma without lymph node metastasis, therefore the clinical stage was determined as Dukes' B.

Microsatellite instability (MSI) analysis revealed that among five microsatellite markers used, four markers, D2S136, D3S1067, D18S51 and BAT26, were positive, resulting in high MSI in the tumor tissue (Fig. 2). This result, taken together with the patient's history, further suggested that the patient should be of the HNPCC kindred. Under written informed consent, genetic testing was carried out using

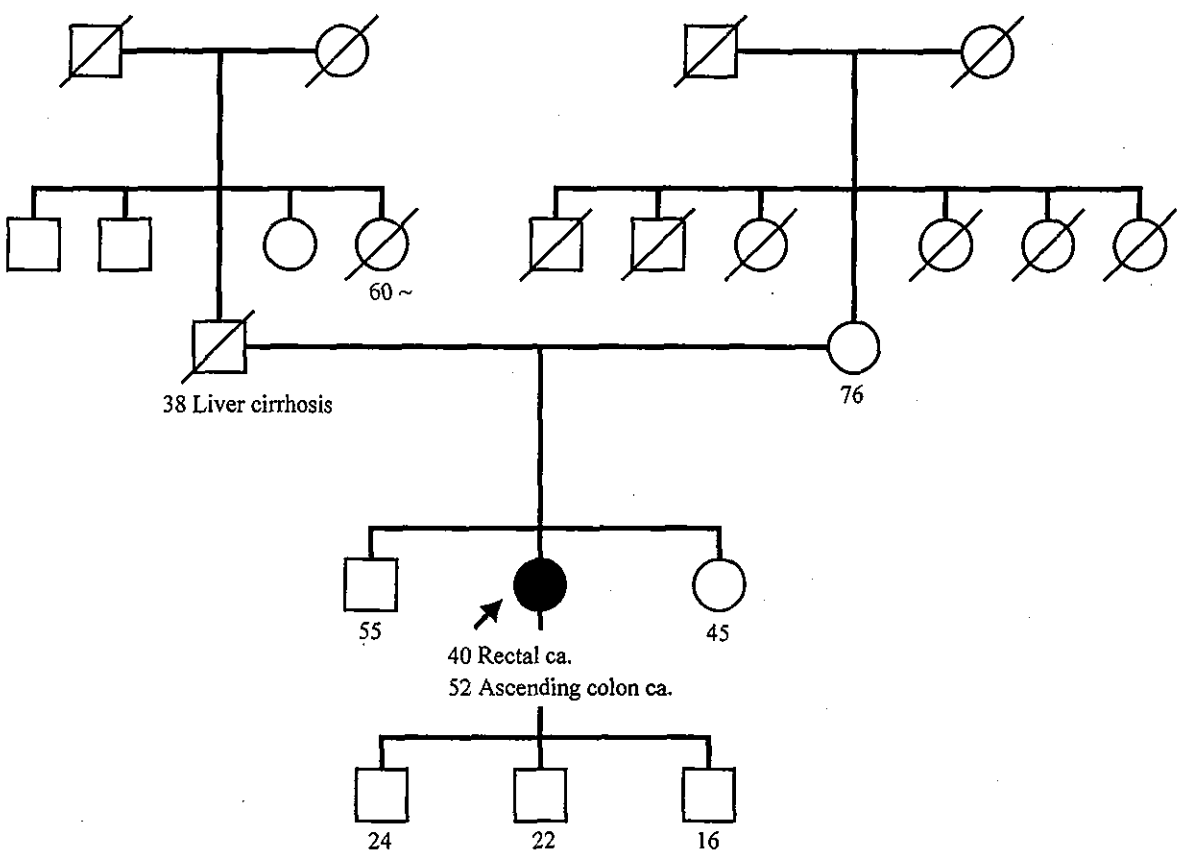

Figure 1. The pedigree of this family. An arrow indicates the proband.

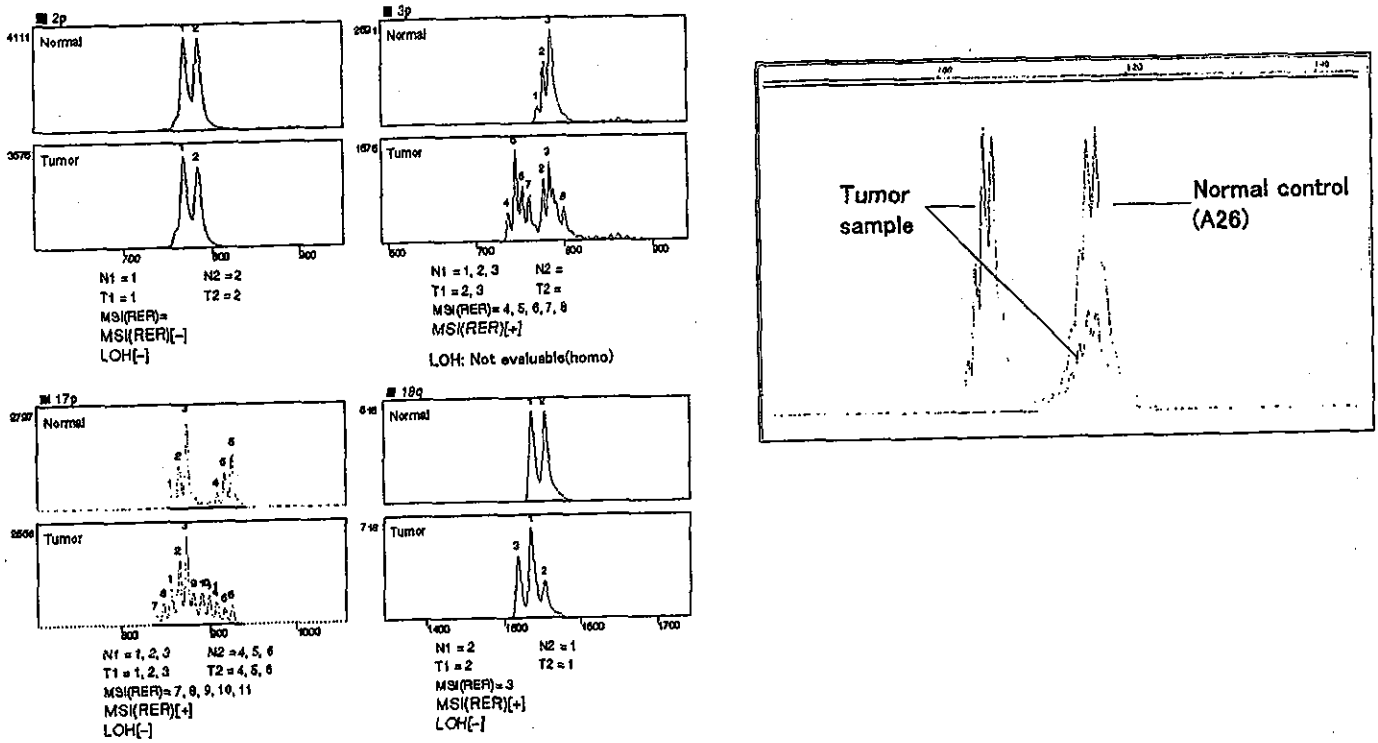

Figure 2. PCR profiles showing the DNA replication error at the D2S136 locus in chromosome 2p (left panel, 2p), the D3S1067 locus in chromosome 3p (left panel, 3p), the TP53 locus in chromosome 17p (left panel, 17p), the D18S51 in chromosome 18q (left panel, 18q) and the BAT26 locus in chromosome 2p (right panel).

DNA extracted from the proband's peripheral lymphocytes. As a result, the five base deletion 2006delAAAAG at codon 669 in exon 18 of the *hMLH1* gene was detected by long RT–PCR/direct sequencing analysis and was confirmed by PCR/direct sequencing analysis. This could lead to the stop codon of codon 672, and is thus considered to be a functional pathologic mutation. There was no mutation detected in the *hMSH2* gene. The representative profiles of germline DNA analysis are shown in Figures 3 and 4.

Our search of the Human Mutation Database and International Collaborative Group on HNPCC (ICG-HNPCC) Database, found that the base deletion 2006delAAAAG at codon 669 in exon 18 of the *hMLH1* gene had not been reported previously and this is considered to be the first case. The mutation detected in the proband was likely to be a *de novo* occurrence, which includes the possibility of the parent's gonadal mosaicism and that of the proband's post-zygotic mutation. Another possibility was that either the father or mother of the proband might be an asymptomatic carrier of the same mutation. In order to confirm that the mutation detected in this report had truly occurred *de novo*, further genetic analysis of the family members, especially the proband's parents, without history of cancer, was needed. And the confirmation that this mutation is truly the disease-causing mutation should be obtained by some functional assay. Also, career diagnosis in this family might be clinically useful and beneficial for each family member. However, since the proband did not want to approach other family members at the time of diagnosis, the further genetic analysis has not yet been undertaken.

One of the disadvantages of the present Amsterdam criteria (Amsterdam criteria II) is that this criteria could not diagnose the *de novo* HNPCC patient due to the lack of a family cancer history, which might be the case in this report. Further extensive study on large numbers of HNPCC-suspected cases, with or without family history, coupled with their genetic analyses, are needed.

METHODS FOR MUTATION DETECTION

Fluorescence-based PCR was performed with the following conditions and parameters for identification of MSI and loss of heterozygosity (LOH) on four dinucleotide markers, as described previously (2,3).

PCR primers for chromosome 2p (D2S136 locus): forward, 5′-AGCTTGAGACCTCTTGTGTCC-3′; reverse, 5′-ATTCAGAAGAAACAGTGATGGT-3′; size of PCR product, 95 bp.

PCR primers for chromosome 3p (D3S1067 locus): forward, 5′-TCATCTATCTCCCAACTGTTGAG-3′; reverse, 5′-GAGCACTACCTGTTTAAGATAGG-3′; size of PCR product, 95 bp.

PCR primers for chromosome 17p (TP53 locus): forward, 5′-ACTGCCACTCCTTGCCCCATTC-3′; reverse, 5′-AGGGATACTATTCAGCCCGAG-3′; size of PCR product, 118 bp.

PCR primers for chromosome 18q (D18S51 locus): forward, 5′-CCGACTACCAGCAACAACAC-3′; reverse,

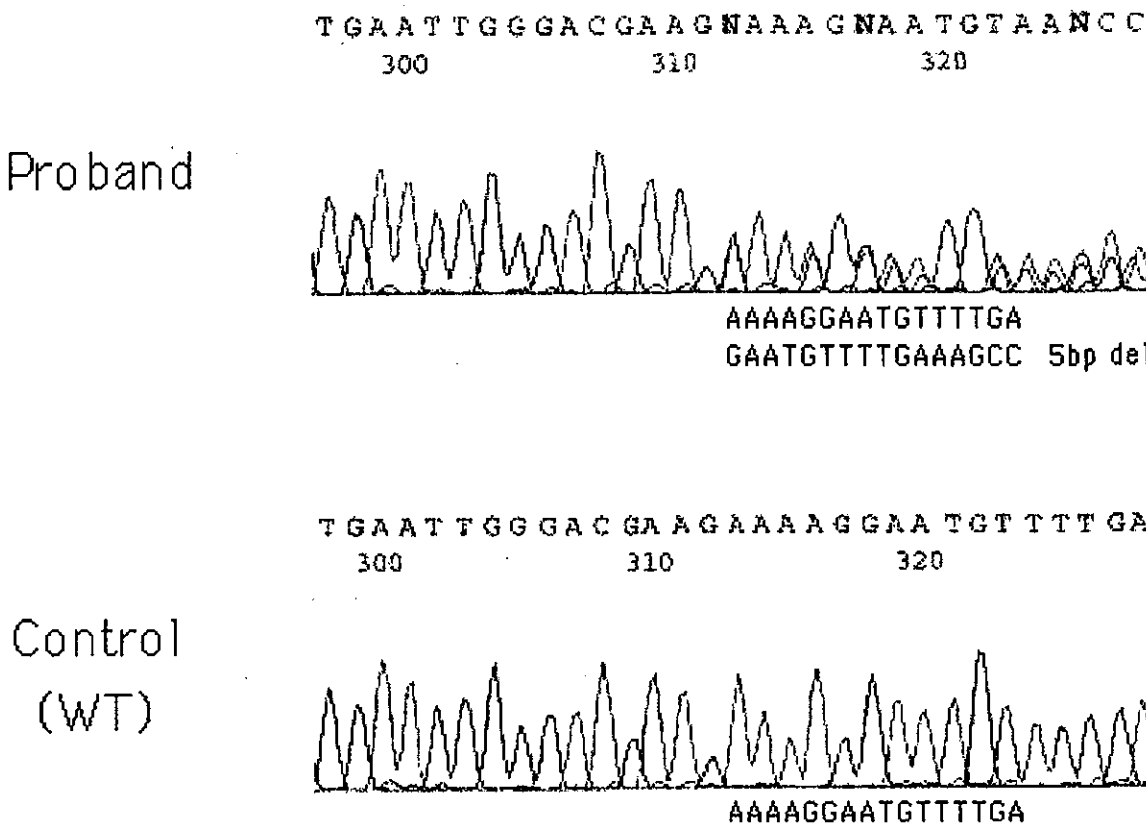

Figure 3. Long RT–PCR analysis of the *hMLH1* gene covering exon 18 suggesting the the existence of the mutation (base deletion 2006delAAAAG at codon 669 in exon 18 of the *hMLH1* gene). Upper lane is for the RT–PCR product from the specimen of the case. Lower lane is for the normal control.

5′-CATGCCACTGCACTTCACTC-3′; size of PCR product, 278 bp.

Reverse primers were labelled with 6-FAM (2p), TET (3p), HEX (17p) or TAMRA (18q) and non-labelled forward primers for the above four regions were synthesized.

Thermal cycle profile: initial denaturation, 94°C, 3 min; amplification, 35 cycles of 94°C, 45 s/58°C, 1 min/72°C, 1.5 min; final extention, 72°C, 5 min.

Fluorescence-based PCR was performed with the following conditions and parameters for identification of MSI on mononucleotide marker, BAT26. PCR primers for chromosome 2p (BAT26): forward, 5′-CTACTTTTGACTTCAGCC-3′; reverse, 5′-ACCAATCAACATTTTTAACCC-3′; size of PCR product, 117 bp. Forward primer was labelled with HEX (2p) and non-labelled reverse primer was synthesized. Thermal cycle profile was the same as that for the four dinucleotide markers except that the denaturation temperatue was 95°C. PCR products were then denatured for 5 min at 95°C in formamide dye and electrophoresed in 6% acrylamide gel (5.7% acrylamide, 0.3% *N,N′*-methylenebisacrylamide) containing 6 M urea using a ABI PRISM 377 DNA sequencer (Applied Biosystems, Perkin-Elmer).

Analysis using long RT–PCR and PCR using genomic DNA was performed as described previously (4).

Long RT–PCR was performed with the following conditions and parameters for identification of the *hMLH1* gene containing exon 18. Forward primer, 5′-CATCTAGACGTTTCCTTGGCTCTTC-3′; reverse primer, 5′-TAAAGGAATACTATCAGAAGGCAAGTATA-3′. Thermal cycle profile: initial denaturation, 94°C, 1 min; amplification, 40 cycles of 94°C, 15 s/65°C, 4 min; final extention, 65°C, 10 min.

Reverse transcription was carried out with 200 U of MMLV reverse transcriptase SuperScript™ II (Life Technologies, Inc., MD), 0.5 mM oligo (dT) primer and 1 μg of the total RNA in 20 μl of 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol and incubated for 50 min at 42°C. Primers used for long RT–PCR are RT-Lf1 and RT-Lr1 for *hMLH1*. Long PCR for cDNA was carried out using Advantage™ cDNA PCR kit (Clontech Laboratories, Inc., CA). Reagent conditions were subjected to the manufacturer's recommendation with a minor modification of adding 10% glycerol in the PCR mixture.

PCR using genomic DNA was performed with the following conditions and parameters for identification of the *hMLH1* gene exon 18. Forward primer, 5′-AATTCGTACCTATTTTG-AGG-3′; reverse primer, 5′-ATTGTATAGGCCTGTCCTAG-3′.

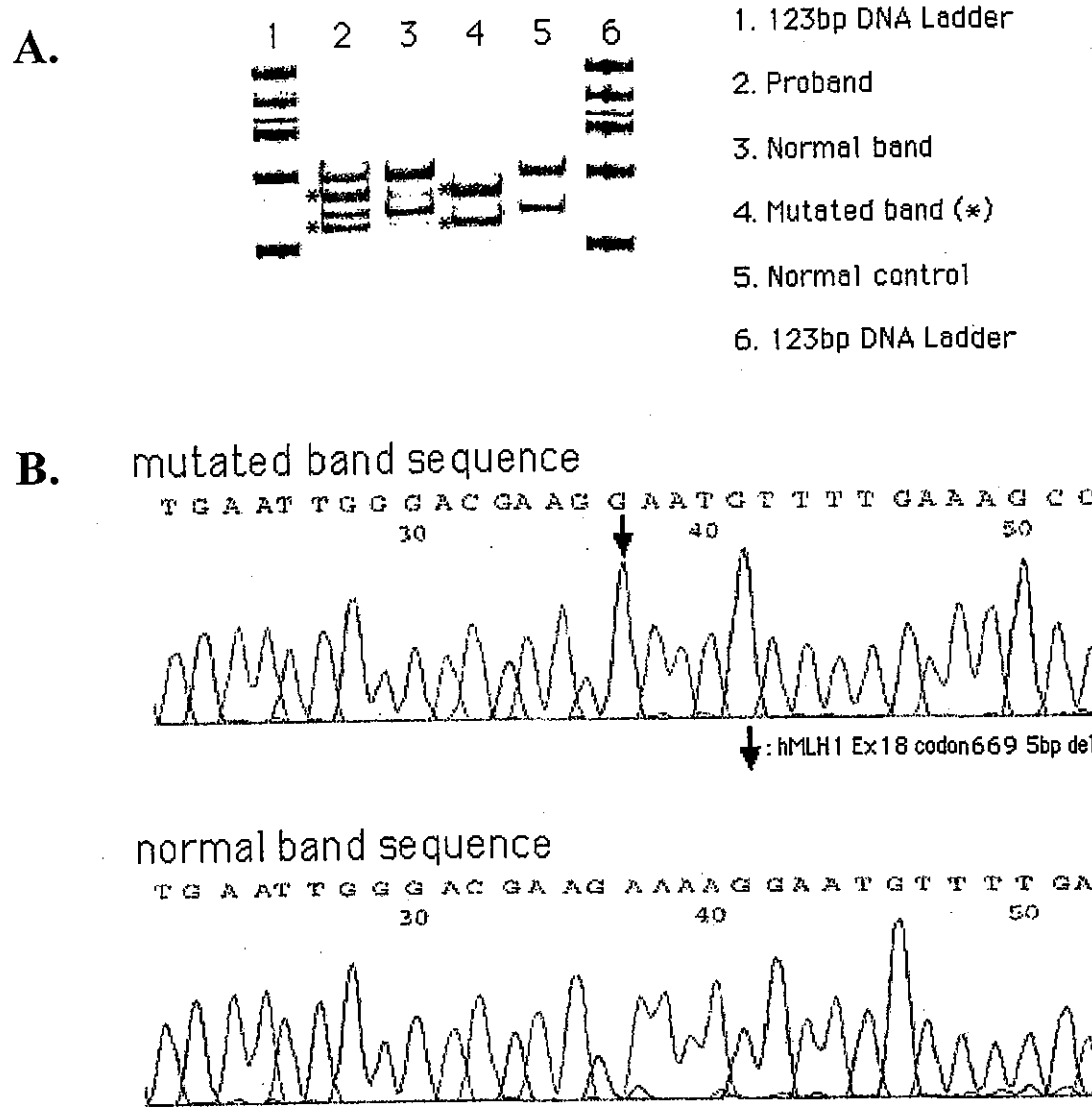

Figure 4. PCR–SSCP and PCR/direct sequencing analysis of the genomic DNA confirmed base deletion 2006delAAAAG at codon 669 in exon 18 of the *hMLH1* gene. (A) The PCR–SSCP analysis in which the genomic DNA of exon 18 was used as the template and the abnormal band was cut out from the electropheresis gel and the corresponding DNA fragment was re-confirmed in this electrophoresis. Finally, this DNA fragment was direct-sequenced and the five base deletion (2006delAAAAG) at codon 669 in exon 18 of the *hMLH1* gene was confirmed as shown (B). The sequencing of the normal control DNA was also shown in parallel.

Thermal cycle profile: initial denaturation, 95°C, 12 min; amplification, 40 cycles of 95°C, 30 s/50°C, 30 s/72°C, 1 min; final extension: 72°C, 5 min.

Acknowledgements

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Phase II Study of Oral S-1 for Treatment of Metastatic Colorectal Carcinoma

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BACKGROUND. The goal of the current study was to evaluate the objective response rate and toxicity associated with the oral fluoropyrimidine S-1 (a combination of tegafur, 5-chloro-2,4-dihydroxypyridine, and potassium oxonate) in patients with previously untreated metastatic colorectal carcinoma.

METHODS. Thirty-eight patients were enrolled in the study. S-1 was administered orally at a dose of 40 mg/m^2 twice daily for 28 days, followed by a 14-day rest period. Treatment was repeated every 6 weeks unless disease progression was observed.

RESULTS. A combined total of 173 courses of S-1 were administered to the 38 enrolled patients. The median number of courses administered to a given patient was 3.5 (range, 1–18). Although no patient exhibited a complete response to treatment, 15 had partial responses (response rate, 39.5%; 95% confidence interval, 24.0–56.6%). In addition, 5 patients had minor responses, and 14 had stable disease. Four patients were found to have progressive disease after two courses of treatment. The median survival time was 358 days (95% confidence interval, 305–490 days), and the 1-year survival rate was 47.4%. The most common adverse reactions included myelosuppression and gastrointestinal toxicity; most cases involved Grade 1 or 2 toxicity, but Grade 3 toxicities (anemia [7.9% of patients], neutropenia [5.3% of patients], diarrhea [2.6% of patients], and abnormal bilirubin levels [7.9% of patients]) also were noted. Neither Grade 4 toxicity nor treatment-related death was observed during the study.

CONCLUSIONS. Orally administered S-1 is active against metastatic colorectal carcinoma and has an acceptable toxicity profile. This promising agent has the potential to become a valuable chemotherapeutic option. *Cancer* **2004;100: 2355–61.** © *2004 American Cancer Society.*

KEYWORDS: colorectal carcinoma, S-1, 5-fluorouracil derivative, oral fluoropyrimidine, Phase II study.

Colorectal carcinoma is one of the most common causes of malignancy-related death in the United States, Japan, and most European countries. The median survival duration for patients with metastatic colorectal carcinoma treated with supportive care alone is approximately 4–6 months.[1] Systemic chemotherapy with 5-fluorouracil (5-FU) recently was shown to prolong survival, with a median

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survival time of 17–21 months associated with such treatment.[2,3] The administration of irinotecan together with 5-FU and leucovorin (LV) as first-line treatment for metastatic disease also has been shown to produce a survival benefit,[2,4] but recently, concern has been raised regarding the toxicity of the weekly bolus combination of these agents.[5]

A randomized cooperative group study has yielded preliminary data supporting the role of 5-FU and LV administered via continuous intravenous infusion (CVI) as the backbone of treatment strategies for metastatic colorectal carcinoma.[6] Nonetheless, CVI performed using a portable pump and an indwelling catheter is challenging and may induce phlebitis or infection originating at the injection site and requiring long-term hospitalization; thus, oral anticancer agents have been developed to address this problem.[7] The results of large Phase III studies of oral capecitabine and the combination of tegafur + uracil (UFT) with LV were reported recently and demonstrated survival benefits that were equivalent to those achieved using intravenous 5-FU + LV.[8–11] Oral chemotherapy has major advantages over intravenously administered treatment in terms of pharmacoeconomic considerations and patient preferences, because oral treatment can be administered on an outpatient basis, thereby reducing the length of patients' hospital stays.[12] Over time, the role of oral chemotherapy in the treatment of malignant disease is expected to become increasingly significant.

Gastrointestinal side effects represent the dose-limiting toxicity associated with 5-FU in a long-term administration schedule (i.e., a CVI schedule).[7] Therefore, to maximize the therapeutic effects of 5-FU, prevention of gastrointestinal toxicity is of primary importance. A new oral fluoropyrimidine, S-1, has been developed by Taiho Pharmaceutical Co. (Tokyo, Japan) and adapted for use in the treatment of advanced gastric[13–15] and head and neck malignancies[16]; at present, this agent is used widely throughout Japan. S-1 consists of tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate in a molar ratio of 1:0.4:1.[17] Tegafur is a precursor of 5-FU and functions as an effector. As an enhancer of the antitumor activity of tegafur, CDHP is prescribed to potently and reversibly inhibit the 5-FU degradation enzyme dihydropyrimidine dehydrogenase (DPD); by inhibiting DPD, CDHP induced the long-term retention of an increased concentration of 5-FU in the blood.[18] Orally administered potassium oxonate is selectively distributed to the gastrointestinal tract with high concentration and inhibits orotate phosphoribosyltransferase, which phosphorylates 5-FU to yield the active metabolite form of 5-FU in humans.[19]

In rats bearing subcutaneous Yoshida sarcoma compared with UFT administered at an equally harmful dose to the rats, S-1 tended to maintain the concentration of 5-FU in plasma and tumor tissue for a longer duration and with less gastrointestinal toxicity.[20] Furthermore, compared with tegafur, UFT, and other fluoropyrimidines, S-1 exhibited greater therapeutic efficacy against various rat tumors and human xenografts.[21]

In a Phase I study involving Japanese patients, S-1 was administered orally for 28 days. The maximum allowed dose of S-1 was 150 mg once daily or 75 mg twice daily, and leukopenia was the resulting dose-limiting toxicity. The pharmacokinetic profile of S-1 revealed that twice-daily administration preserved therapeutic 5-FU levels without increasing the maximum 5-FU concentration in the blood.[22,23] Therefore, oral administration of S-1 at a dose of 75 mg twice daily for 28 consecutive days, with a subsequent 14-day rest period, was recommended. Two Phase II studies of twice-daily S-1 administered as a single agent for the treatment of metastatic gastric malignancy yielded response rates of approximately 50%, with minimal toxicity.[13–15]

Based on these results, two Phase II studies of S-1 in the treatment of metastatic colorectal carcinoma were initiated. Response rates of 17% and 35% were observed in these two trials.[13,24] To verify the reproducibility of these findings, we performed our own Phase II study of S-1 in the treatment of Japanese patients with metastatic colorectal carcinoma.

MATERIALS AND METHODS

Eligibility

Patients were entered into the study only if they fulfilled the following eligibility requirements: 1) histologically confirmed colorectal carcinoma; 2) inoperable metastatic disease or recurrent metastatic disease after surgery; 3) the presence of measurable or evaluable lesions; 4) age $\geq$ 20 years but $<$ 75 years; 5) Eastern Cooperative Oncology Group performance status (PS) $\leq$ 2; 6) no previous chemotherapy or radiotherapy for advanced disease (with any adjuvant chemotherapy for colorectal carcinoma required to have been completed $\geq$ 6 months before enrollment); 7) adequate bone marrow function (hemoglobin concentration $\geq$ 9.0 mg/dL, white blood cell count $\geq$ 4000/μL but $\leq$ 12,000/μL, and platelet count $\geq$ 100,000/μL); 8) adequate liver function (serum bilirubin levels $\leq$ 1.5 mg/dL, serum transaminase levels $\leq$ 100 international units per liter, and serum alkaline phosphatase levels $<$ 2 times the upper limit of normal); 9) adequate renal function (serum creatinine levels within normal limits); 10) no other severe med-

ical conditions; and 11) no other active malignancies. In addition, patients were required to provide written informed consent, and pregnant women were excluded from the study.

Treatment Schedule

S-1 was administered at a dose of 40 mg/m^2 twice daily for 28 consecutive days, with a subsequent 14-day rest period. Patients were assigned on the basis of body surface area (BSA) to receive one of the following doses twice daily: 40 mg (BSA < 1.25 m^2), 50 mg (BSA ≤ 125 to < 1.50 m^2), or 60 mg (BSA > 1.50 m^2). S-1 was supplied by Taiho Pharmaceutical Co. in the form of 20 and 25 mg capsules (i.e., 20 and 25 mg tegafur). A course of therapy was defined as 28 consecutive days of treatment followed by a 14-day rest period, and courses were repeated every 6 weeks until either disease progression or unacceptable toxicity was observed. Patients whose toxicities necessitated a rest period of more than 4 weeks were withdrawn from treatment. Prophylactic use of antiemetic agents was not allowed. For all patients, treatment compliance and receipt of treatment without hospitalization were verified by patient interviews conducted on a regular schedule.

Evaluation

Before entry into the study, patients were evaluated using appropriate investigational methods to determine the extent of disease. A complete blood cell count, liver function testing, renal function testing, and urinalysis were performed at least once every 2 weeks during treatment. Appropriate investigation was repeated as necessary to evaluate target lesion sites before every treatment course. Antitumor activity was evaluated in accordance with the general rules, based on the corresponding World Health Organization criteria, set forth by the Japanese Research Society for Colorectal Carcinoma.[25] Complete response (CR) was defined as the disappearance of all evidence of malignant disease for more than 4 weeks. Partial response (PR) was defined as a reduction (lasting longer than 4 weeks) of greater than 50% in the sum over all lesions of the product of the longest perpendicular tumor dimensions, with no evidence of new lesions or of the progression of any preexisting lesion. Stable disease (SD) was defined as a reduction of less than 50% or an increase of less than 25% in the sum over all lesions of the product of the longest perpendicular tumor dimensions, with no evidence of new lesions. Progressive disease (PD) was defined by increases of greater than 25% in sum overall lesions of the product of the longest perpendicular tumor dimensions or the appearance of new lesions. The toxicity criteria of the Japan Society for Cancer Therapy, which were based (with some modification) on the World Health Organization criteria, were used to evaluate treatment-related toxicity.[26] The eligibility and suitability of patients for assessment and the responses of patients to treatment were reviewed extramurally.

TABLE 1
Patient Characteristics

Characteristic	No. of patients
No. of eligible patients	38
Median age in yrs (range)	58.5 (28–74)
Gender (%)	
Male	18 (47)
Female	20 (53)
ECOG PS (%)	
0	18 (47)
1	20 (53)
Primary lesion site (%)	
Colon	23 (61)
Rectum	15 (39)
Histology (%)	
Well/moderately differentiated	33 (87)
Poorly differentiated	5 (13)
Previous therapy (%)	
Surgery	23 (61)
Surgery + adjuvant chemotherapy	4 (11)
Surgery + radiotherapy	2 (5)
None	9 (24)
Mean body surface area in m^2 (range)	1.53 (1.26–1.85)

ECOG PS: Eastern Cooperative Oncology Group Performance Status.

Statistical Methods

Previous Phase II studies have reported a 35.5% response rate for metastatic colorectal carcinoma treated with S-1. The current study was designed to have a target activity level of 35% and a minimum activity level of 15%, with an α error of 0.05 and a β error of 0.2; thus, a minimum of 38 patients were required. Survival was calculated from the date of treatment initiation using the Kaplan–Meier method.

Ethical Considerations

The current trial was approved by the institutional review boards of the clinical oncology programs at all participating hospitals. Approval was based on the 1975 revision of the Helsinki Declaration. Oral and written statements of informed consent were acquired from all patients.

RESULTS

Thirty-eight patients (18 men and 20 women) with advanced metastatic colorectal carcinoma were en-

TABLE 2
Body Surface Area and Corresponding S-1 Dose

BSA (m^2)	S-1 dose[a] (mg)	No. of patients (%)
< 1.25	40	0
≤1.25 to < 1.50	50	15 (39)
≥1.50	60	23 (61)

BSA: body surface area.
[a] Dose administered twice daily.

TABLE 3
Objective Response Data

Response type	No. of patients
Complete response	0
Partial response	15
Minor response	5
Stable disease	14
Progressive disease	4
Overall response rate[a]	39.5% (15/38)
95% confidence interval	24.0–56.6%

[a] Includes complete responses and partial responses.

tered into the trial between June 1999 and December 2000. Patient characteristics are summarized in Table 1. The median patient age was 58.5 years (range, 28–74 years). Eighteen patients had PS 0, and the remaining 20 had PS 1. The primary tumor was located in the colon in 23 patients (61%) and in the rectum in 15 patients (39%). Thirty-three patients (87%) had well or moderately differentiated adenocarcinoma, whereas 5 (13%) had poorly differentiated adenocarcinoma. Of the 38 patients in the current study, 29 (76%) had undergone surgery before entry, 4 (11%) had received 5-FU-based adjuvant chemotherapy, and 2 had received pelvic radiotherapy.

The mean BSA in the current study population was 1.53 m^2 (range, 1.26–1.85 m^2). Daily S-1 doses according to BSA are shown in Table 2. The median S-1 dose was 60 mg administered twice daily. A combined total of 173 treatment courses were administered to the 38 patients enrolled in the study. The median number of courses per patient was 3.5 (range, 1–18), and the median cumulative S-1 dose per patient was 10,080 mg (range, 2660–44,660 mg).

Response

All 38 patients had measurable metastatic lesions. Although no patient experienced a CR, 15 patients had PRs (response rate, 39.5%; 95% confidence interval, 24.0–56.6%) (Table 3). Among these 15 patients, the median time required for a 50% reduction in tumor

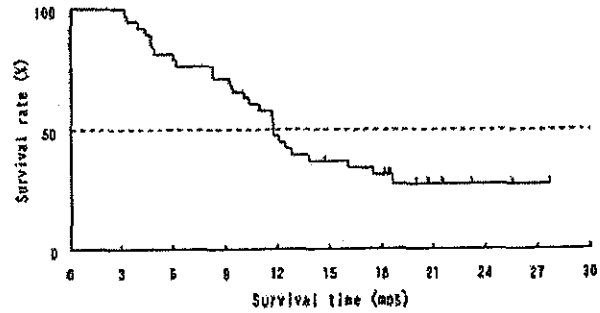

FIGURE 1. Overall survival of 38 patients treated with S-1 for previously untreated metastatic colorectal carcinoma. Median survival time, 358 days (95% confidence interval, 305–490 days).

size was 68 days (range, 29–130 days), and the median duration of response was 232 days (range, 96–679 days). Five patients had minor responses, and 14 had SD. The remaining four patients were found to have PD after two courses of treatment. Response rates according to metastatic site were as follows: liver, 38% (9 of 24 patients); lung, 27% (4 of 15 patients); and lymph nodes, 30% (3 of 10 patients). The response rate among patients with colon carcinoma was 44% (10 of 23 patients), and the response rate among patients with rectal carcinoma was 33% (5 of 15 patients). The response rate at the primary site as evaluated using the roentgenographic evaluation criteria proposed by the Japanese Society for Cancer of the Colon and Rectum was 43% (3 of 7 patients). One of the four patients who had a history of adjuvant chemotherapy achieved a PR.

At the close of the trial, the median time to evidence of disease progression was 162 days (range, 118–254 days). The median survival time from the beginning of treatment was 358 days (median follow-up, 666 days; 95% confidence interval, 305–490 days) for the overall study cohort, and the 1-year survival rate was 47.4% (Fig. 1).

Toxicity

For each toxicity, the patient distribution with respect to highest observed grade is summarized in Table 4. The most common adverse reactions included myelosuppression and gastrointestinal toxicity, although these events generally were mild, and no cumulative toxicity was noted. Neither Grade 4 toxicity nor treatment-related death was observed during the study. Toxicity incidence rates were as follows: anemia, 45% (17 of 38 patients); leukopenia, 45% (17 of 38 patients); neutropenia, 42% (16 of 38 patients); and thrombocytopenia, 13% (5 of 38 patients). Nonetheless, Grade ≥ 3 toxicities were noted in less than 8% of patients.

TABLE 4
Toxicity Data

Toxicity	Grade 1	2	3	4	Grade ≥3 (%)
Anemia	7	7	3	0	7.9
Leukopenia	7	10	0	0	0
Neutropenia	4	10	2	0	5.3
Thrombocytopenia	4	1	0	0	0
Diarrhea	5	8	1	0	2.6
Nausea/vomiting	8	7	0	0	0
Anorexia	15	4	0	0	0
Stomatitis	11	3	0	0	0
Hand-foot syndrome	2	0	0	0	0
Pigmentation	15	0	0	0	0
Malaise	17	2	0	0	0
Bilirubinemia	—[a]	14	3	0	7.9

[a] Grade 1 bilirubinemia is not defined in the toxicity criteria of the Japan Society for Cancer Therapy. (See: Japan Society for Cancer Therapy. Criteria for the evaluation of the clinical effects of solid cancer chemotherapy. *J Jpn Soc Cancer Ther.* 1993;28:101–130.[26])

The overall incidence rate for diarrhea was 37% (14 of 38 patients), with Grade 3 diarrhea noted in 3% of the study cohort (1 of 38 patients). The overall stomatitis incidence rate was 37% (14 of 38 patients); however, Grade ≥ 3 stomatitis was not observed. The incidence rate for hand-foot syndrome (palmar-plantar erythrodysesthesia) was 5% (2 of 38 patients); Grade 1 erythrodysesthesia was noted in both cases. Overall, abnormal bilirubin levels were noted in 45% of the study cohort (17 of 38 patients), with an incidence rate of 8% (3 of 38 patients) for Grade 3 bilirubin abnormalities. Nonetheless, no Grade ≥ 3 elevation of aspartate aminotransferase or alanine aminotransferase levels was observed in the current study.

Toxicity caused two patients to discontinue S-1 treatment. One of these two was hospitalized for abdominal pain (Grade 2), nausea with vomiting (Grade 2), and anorexia (Grade 2) during the third treatment course, and S-1 treatment subsequently was discontinued. The other patient withdrew from the study during the second treatment course due to diarrhea (Grade 3) and neutropenia (Grade 2). Discontinuation of treatment was not considered necessary for any of the other patients who experienced Grade 2 or Grade 3 toxicities; instead, these patients were able to continue receiving treatment after a brief interruption or after dose reduction. Thirty-five of 38 patients (92%) were treated as outpatients, a finding that indicates extremely good compliance. Of the 173 courses that were administered overall, 163 (94%) were administered at ≥ 75% of the protocol-defined dose.

DISCUSSION

The current study was conducted to evaluate the objective response rate and toxicity associated with an oral regimen of S-1 for patients with previously untreated metastatic colorectal carcinoma. We observed a response rate of 39.5%, which was equal to or greater than the corresponding response rates associated with 5-FU alone and with 5-FU + LV. In an earlier Phase II study of S-1, an overall response rate of 35% was reported for patients who had not previously received chemotherapy.[24] That earlier study and the current one were similar in terms of dosing and scheduling of S-1, eligibility criteria, and response criteria, and both studies also reported similar response rates and survival times; these similarities suggest that the activity of oral S-1 against metastatic colorectal carcinoma represents a reproducible finding.

In a previous Phase I study involving Japanese patients, S-1 was administered orally for 28 consecutive days.[22] The maximum allowable S-1 dose was 150 mg once daily or 75 mg twice daily, and myelosuppression (primarily leukopenia) was found to be the dose-limiting toxicity. This daily dose of 150 mg per day is equivalent to 100 mg/m^2 per day for the average Japanese patient, who has a BSA of 1.5 m^2. For the current study, we selected an S-1 dose of 80 mg/m^2 per day (40 mg/m^2 twice daily), which was slightly less than the maximum allowable dose identified by Phase I trials.[22] The most commonly observed adverse reactions in the current study were myelosuppression and gastrointestinal toxicity; these events generally were mild, with no Grade 4 toxicity noted. Although a small number of cases of Grade 4 myelosuppression have been reported in other Phase II studies in which a total daily dose of 80 mg/m^2 S-1 was used to treat malignant disease (gastric,[14,15] colorectal,[24] head and neck,[16] lung,[27] or breast[28]), the incidence and degree of toxicity observed in those studies did not differ substantially from what was documented in the current study.

The toxicity profile of 5-FU is schedule dependent. Myelosuppression is the primary toxic effect observed in patients receiving bolus 5-FU schedules, whereas hand-foot syndrome, stomatitis, neurotoxicity, and cardiotoxicity are associated with continuous infusion of 5-FU.[7] Hand-foot syndrome, in addition to being a typical side effect of prolonged 5-FU administration via CVI,[29] is commonly associated with the oral administration of other fluoropyrimidines, such as capecitabine.[10,11] The mechanism involved in the development of hand-foot syndrome has not been completely elucidated; however, some 5-FU catabolites are believed to be inducers of this condition.[30]

Thus, the low incidence of hand-foot syndrome associated with UFT use is consistent with the observation of low plasma levels of 5-FU catabolites in patients receiving UFT.[31] In the current trial, hand-foot syndrome was observed in only 5% of the study cohort (2 of 38 patients); furthermore, both of these cases involved reversible, Grade 1 hand-foot syndrome. In other trials, only mild S-1-induced hand-foot syndrome, which was not suggestive of dose-limiting toxicity, has been reported. These findings may reflect the inhibitory effect of CDHP on DPD.

The pharmacokinetic characteristics of prolonged S-1 administration were believed to be consistent with the use of CVI; however, the dose-limiting toxicity induced by S-1 was myelosuppression, which is associated with the bolus dose protocol. In a previous Phase I study, the maximum plasma 5-FU concentration was estimated to be approximately 230 ng/mL for Japanese patients who received S-1 at a dose of 75 mg per day.[22] This relatively high peak plasma 5-FU concentration may result in myelotoxicity, rather than gastrointestinal toxicity, in spite of the prolonged S-1 administration protocol. The low severity of gastrointestinal toxicity, even in the face of a relatively high peak plasma 5-FU concentration[22,23] and area under the plasma concentration–time curve, suggests the usefulness (previously noted in rats[19]) of potassium oxonate in humans. The toxicity observed in the current trial, in which S-1 was administered at a dose of 80 mg/m^2 per day (40 mg/m^2 twice daily), was mild and reversible, and yet the observed activity was remarkable, being equal to or greater than the activity of 5-FU alone.

Oral chemotherapy, for which only limited hospitalization is necessary, has major advantages over intravenously administered treatment in terms of pharmacoeconomic considerations and patient preference, as well as compliance.[12] In one study, it was reported that more than 90% of patients with advanced solid malignancies preferred oral agents over infusional agents when both types of treatment provided comparable efficacy.[32] Furthermore, a randomized crossover trial involving patients with advanced colorectal carcinoma found that oral UFT + LV compared favorably with intravenous 5-FU + LV in terms of toxicity and patient preference.[31]

In the current study, the S-1 regimen was administered successfully, with good treatment compliance, on an outpatient basis. Due to the absence of severe toxicity, especially with regard to symptoms such as nausea, vomiting, and diarrhea, almost all patients received ≥ 75% of the full protocol-defined S-1 dose; it is clear that good compliance increases the likelihood of favorable therapeutic responses. Thus, the findings of the current study indicate that S-1 is a promising agent that has the potential to become a valuable oral treatment option, along with capecitabine and UFT + LV, for patients with colorectal carcinoma. Clinical studies of S-1 in the treatment of metastatic colorectal and gastric malignancies[33,34] also suggest that S-1 possesses superior therapeutic activity compared with other regimens.

The combination of irinotecan or oxaliplatin with 5-FU + LV recently has been identified as a candidate regimen for the standard treatment of metastatic colorectal carcinoma. To determine which of these chemotherapeutic agents are most suitable for use in combination with S-1, clinical trials are essential. Three Phase I/II trials of S-1 with LV irinotecan or oxaliplatin for the treatment of metastatic colorectal carcinoma have been scheduled. In addition, a Phase III study of adjuvant chemotherapy (surgery alone vs. surgery followed by S-1) in the treatment of gastric tumors and a Phase III study comparing the use of S-1 alone with the use of S-1 + cisplatin in the treatment of metastatic gastric malignancies are ongoing. In another ongoing Phase III trial involving patients with gastric malignancies, the Japan Clinical Oncology Group is comparing 5-FU, which currently is the standard treatment agent, with single-agent S-1 and with cisplatin + irinotecan.

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Diseases of the Colon & Rectum

Oncologic and Functional Results of Total Mesorectal Excision and Autonomic Nerve-Preserving Operation for Advanced Lower Rectal Cancer

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PURPOSE: Total mesorectal excision contains two different procedures: autonomic nerve preservation, and autonomic nerve sacrifice. It is unclear whether autonomic nerve preservation is suitable curative procedure. We clarify the significance of autonomic nerve preservation for an advanced lower rectal cancer. METHODS: All 403 patients curatively resected between 1975 and 1999 were clinicopathologically studied. Between 1975 and 1984, all patients routinely received total mesorectal excision without autonomic nerve preservation (TME-P(−) group). Since 1985, total mesorectal excision with autonomic nerve preservation has been performed in 81 percent of patients (TME-P(+) group). The remaining patients received TME-P(−) because of suspicious invasion to autonomic nerve plexus. All clinical and pathologic data were entered into a computer database. Long-term follow-up was used to analyze the oncologic and functional results of TME-P(+) group compared with TME-P(−) group. RESULTS: The follow-up rate was 98.1 percent. In either Dukes A+B or Dukes C disease, the TME-P(+) group did not increase local recurrence or decrease ten-year disease-free survival compared with the TME-P(−) group of Period 1975 to 1984. The TME-P(−) group of Period 1985 to 1999 had the highest distant metastasis and the lowest survival rates than any other groups. Urinary or sexual function was well preserved in the TME-P(+) group. CONCLUSIONS: Autonomic nerve preservation is oncologically and functionally excellent and suitable for almost all patients with advanced lower rectal cancer. Intensive chemotherapy is needed for patients whose autonomic nerves were killed in suspicion of nerve invasion. [Key words: Autonomic nerve preservation; Total mesorectal excision; Local recurrence; Disease-free survival]

Lateral lymphadenectomy (LLA) sometimes termed pelvic lymphadenectomy or extended surgery, was performed for rectal cancer between the early 1970s and the mid 1980s in Japan. This surgical technique was accomplished with total mesorectal excision (TME), including resection of autonomic nerve plexus. It was widely practiced in Japan with the goal of ultimate radical cure. However, disadvantages such as urinary dysfunction or sexual functional disorder afflicted many patients after LLA.[1] During the mid 1980s, autonomic nerve preservation (ANP) was instituted to avoid postoperative functional disorders. This remarkable development of ANP was accomplished in Japan.[2,3] On the other hand, many surgeons in Western countries renounced LLA and moved toward favoring TME, first reported in 1986 by Heald.[4] It began to show a surprising improvement in survival and decrease in local recurrence (LR). Recently, the concept of TME gradually has changed.[5] It seems to be defined as complete removal of circumferential fatty tissue around rectum with ANP. However, there are some important problems concerning ANP. When the nerve plexus is preserved, there is a risk in which cancer cells remain around the nerves. Moreover, there is a possibility that the LR occur at a high rate in advanced rectal cancer, especially in Dukes C disease. Long-term follow-up after the operation is needed to investigate the oncologic results of ANP. Also, there is

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Table 1.
Number of Patients According to Operation Methods and Dukes Stage

	Dukes A+B	Dukes C	Total
Period 1975–1984[a]			
TME-P(–)	103	91	194
Period 1985–1999[b]			
TME-P(+)	102	67	169
TME-P(–)	22	18	40
Total	227	176	403

TME-P(–) = total mesorectal excision without autonomic nerve preservation; TME-P(+) = total mesorectal excision with autonomic nerve preservation.

[a]In the Period 1975 to 1984, 194 patients received TME-P(–).

[b]In the Period 1985 to 1999, 169 patients received TME-P(+) and 40 patients received TME-P(-).

a close relationship between ANP and urinary or/and sexual function. In the present study, we analyzed the oncologic and functional results and examined whether ANP is suitable for patients with advanced rectal cancer based on the long-term follow-up.

PATIENTS AND METHODS

Between 1975 and 1999, 403 patients underwent curative surgery for an advanced lower rectal cancer. In Japan, curative surgery means both macroscopically complete resection of the tumor and microscopically surgical margin negativity. Surgical margin-positive cases (29 cases; 5.4 percent of total advanced lower rectal cancer) were not included in this study. The detailed clinical data from the long-term follow-up of these patients was retrospectively investigated. The follow-up duration was divided into two periods: Period 1975 to 1984 and Period 1985 to 1999, because the operation method was not different except for ANP.

Operation

Period 1975 to 1984. During this period, inferior mesenteric artery (IMA) was ligated at the origin of the aorta. TME, including the resection of autonomic nerve plexus (hypogastric and pelvic nerves), was routinely performed for all patients (TME-P(–) group) without selection. Aortocaval and LLA was performed for most patients. The operation methods and Dukes stage are shown in Table 1. A total of 194 patients survived curative surgery, including 103 patients with Dukes A+B disease and 91 patients with Dukes C disease.

Period 1985 to 1999. The IMA was ligated at the origin of the aorta. TME with autonomic nerve preservation (ANP) was used for 169 of 209 (81 percent) patients (TME-P(+) group). LLA was enforced for most patients by the techniques similar to those in Period 1975 to 1984. As shown in Table 1, we had 102 patients with Dukes A+B disease and 67 patients with Dukes C disease. The other 40 patients had no ANP (TME-P(–) group; Dukes A+B, 22; Dukes C, 18), because cancer cells were suspected to have invaded the autonomic nerve tissue. Histologically, perineural invasion of autonomic nerves was found in 3 of 22 patients with Dukes A+B disease and in 8 of 18 patients with Dukes C disease.

Histologic Diagnosis

Histologic diagnosis of resected specimens was made by one of the authors (KS). The diagnosis before 1982 was reviewed by the author 20 years ago. Since 1982, whole tumor mass of resected specimens was sliced at 5-mm step section. The detailed histologic findings such as depth of tumor invasion, venous invasion, lymphatic permeation, perineural invasion, lymph node metastasis, state of surgical margin and others were sketched for each patient.[6–9]

Adjuvant Chemotherapy

Preoperative adjuvant chemotherapy was not given to any patients during either Period. Postoperative oral administration of fluorinated pyrimidine such as 5-fluorouracil, 1-hexylcarbamoyl-5-fluorouracil, or 1-(2-tetrahydrofuryl)-5-fluorouracil 2,4 (1H, 3H)-pyrimidinedione was routinely given to patients with Dukes B or C disease for at lease one year but was not given to patients with Dukes A disease.

Urinary and Sexual Function

The urinary and sexual function was evaluated by questionnaire survey. The grade of urinary disorder was classified as good, fair, and poor function. Good function meant possible self-urination, fair meant frequent urination, prolonged urination, or sense of residual urine, and poor meant necessity of urethral catheter or severe incontinence. Sexual disorder for erection and ejaculation was investigated in males younger than aged 60 years.