treatment, to have at least one bidimensionally measurable lesion, and to be treated with chemotherapy in clinical trials.

Patients treated in clinical practice were considered to be unsuitable and excluded from this study as tumor response evaluation in the clinical practice of oncology is not always carried out according to predefined criteria, but rather is made by subjective medical judgment based on clinical and laboratory data. In addition, tumor response evaluation is not always carried out by CT examination, and the intervals between tumor evaluations can be irregular.

Image analysis

Almost all images were acquired with a TCT-900S Superhelix (Toshiba Medical, Tokyo, Japan), with the remainder scanned on an X-Vigor helical CT scanner (Toshiba Medical). Helical CT was carried out with fixed scanning parameters, including a table speed of 15 mm/s, a pitch ratio of 1:1.5 per rotation time 1 s, and the same contrast agent for both baseline and follow-up evaluations. Image reconstruction was carried out at intervals of 10 mm.

On chest CT obtained during baseline examination before the initiation of chemotherapy, target lesions up to a maximum of five lesions per patient with longest and perpendicular diameters that could be measured accurately were selected by one diagnostic radiologist. In addition, one follow-up chest CT examination, indicating tumors with the greatest response to chemotherapy, was selected retrospectively. Target lesions included primary lung lesion, pulmonary metastases and lymph nodes.

For the target lesions, the two parameters consisting of the longest diameter and the diameter perpendicular to it were measured with electronic calipers on digitized images. Five observers of different backgrounds, blinded to patient profiles, reviewed all patients independently and no attempt was made to arrive at a consensus. These observers included one diagnostic radiologist, one thoracic physician, two medical oncologists and one thoracic surgeon.

Tumor response evaluation

The sum of the longest diameters for all target lesions was calculated for pretreatment and post-treatment UD. Similarly, the sum of the products of the longest diameters and their perpendicular diameters for all target lesions was calculated for pretreatment and post-treatment BD. If there were two or more lesions, the sum of all target lesions was calculated. The baseline sum was used as the reference from which objective tumor response could be calculated. The percentage changes were calculated as post-treatment value divided by pretreatment value for both UD and BD.

Percentage changes were then classified using the current RECIST guidelines and the previous WHO criteria tumor response classification system. Tumor response was categorized into CR, PR, SD and PD based on both RECIST guidelines and WHO criteria. The RECIST PR was defined as a 50% decrease in the percentage changes for UD, and the WHO PR was defined as a 30% decrease in the percentage changes for BD. The RECIST PD was defined as a 20% increase in the sum of the longest diameters, and the WHO PD was defined as a 25% increase in the sum of the products of the two diameters of all lesions or in the product of the diameters of one lesion. For the present study, no minimum interval was required for the confirmation of either CR or PR.

Analysis of intercriteria reproducibility

To examine intercriteria reproducibility, the mean and ranges of differences in the response rate between UD and BD were calculated. We then estimated those between UD-MLS and BD. Interobserver differences among the five observers yielded 20 pair comparisons. Intraobserver differences of the same observer yielded five pair comparisons.

Analysis of interobserver reproducibility

First, to examine the interobserver reproducibility of the percentage changes according to the two different dimensional measurements, we estimated the Spearman's correlation coefficient of the percentage changes among the five observers, calculated for each pair observed (five observers yielded 10 pair comparisons).

Second, to examine the interobserver reproducibility for two tumor response criteria, we estimated the proportion of agreement to the categories of CR, PR, SD and PD for both UD and BD among the five observers (10 pair comparisons). We then calculated the kappa statistics, a measure of agreement in which agreement is taken into consideration by chance, to assess interobserver reproducibility for tumor response categories.[9]

Third, we examined the influence of MLS on the number of eligible cases and target lesions. The same analyses on interobserver reproducibility were conducted applying the MLS. MLS was introduced into the RECIST guidelines, which specify a minimum lesion size of less than double the slice thickness on images. The slice thickness was 10 mm in the present study, so the MLS was set at no less than 20 mm at baseline evaluation before treatment. Cases that only had tumors smaller than the MLS were excluded from the present study. We defined the RECIST guidelines as the evaluation by UD for measurable cases and the WHO criteria as the evaluation by BD for all cases.

SAS version 8.02 (SAS Institute, Cary, NC, USA) was used for all analyses.

Results

Patient population

The characteristics of the 110 patients were as follows: male/female = 80/30, median age = 59 years (range 36–72 years), stage IIIB/IV = 33/77. Chemotherapy regimens are listed in Table 1. A total of 220 CT images were reviewed, comprising 110 CT images each from the baseline study (pretreatment) and from the follow-up (post-treatment) study.

Tumor response evaluation between UD and BD

The tumor response evaluation was categorized into CR, PR, SD and PD without MLS. The response rate results are shown in Table 2. None of the patients were rated CR. The use of UD resulted in response categories by observers A, B, C, D and E of 35, 28, 26, 34 and 36 PR, 73, 79, 81, 73 and 71 SD, and 2, 3, 3, 3 and 3 PD, respectively. The response rate ranged from 23.6 to 32.7%. For BD, the corresponding response categories were 37, 30, 33, 36 and 36 PR, 67, 73,

Table 1. Characteristics of the 110 patients enrolled in the present study

Characteristic	*n*
No. patients	110
Age (years)	
Median	59
Range	36–72
Sex	
Male	80
Female	30
Disease stage at study entry	
IIIB	33
IV	77
Tumor histology	
Adenocarcinoma	78
Squamous	22
Large-cell	1
Unclassified non-small cell	9
Regimen	
Cisplatin and gemcitabine	21
Cisplatin and paclitaxel	18
Nedaplatin and paclitaxel	15
Cisplatin and vinorelbine	14
Carboplatin and paclitaxel	14
Cisplatin and vindesine	13
Cisplatin, docetaxel and ifosfamide	7
Cisplatin and docetaxel	8

Table 2. Response rate (%) using four different measurements among five observers

Measurement	Observer					Mean
	A	B	C	D	E	
UD	31.8	25.5	23.6	30.9	32.7	28.9
BD†	33.6	27.3	30.0	32.7	32.7	31.3
UD-MLS‡	33.0	27.2	32.0	30.1	32.0	30.9
BD-MLS	35.0	32.0	33.0	33.0	34.0	33.4

†WHO criteria; ‡RECIST guidelines. BD, bidimensional measurement; MLS, minimum lesion size; UD, unidimensional measurement.

68, 68 and 68 SD, and 6, 7, 9, 6 and 6 PD, respectively. The response rate ranged from 27.3 to 33.6%.

Tumor response evaluation between UD-MLS and BD-MLS

When the MLS criteria were applied, the number of eligible cases decreased by 6.4% from 110 to 103, and the number of target lesions decreased by 44.6% from 402 to 223.

The response rate results are shown in Table 2. None of the patients were rated CR. When UD was used with MLS, the respective response evaluations made by observers A, B, C, D and E were 34, 28, 33, 31 and 33 PR, 68, 73, 67, 72 and 68 SD, and 1, 2, 3, 0 and 2 PD. The response rates of UD applying MLS ranged from 27.2 to 33.0%, showing a reduction in interobserver difference compared with those of UD not applying MLS. With BD using the MLS, the corresponding response categories were 36, 33, 34, 34 and 35 PR, 63, 66, 65, 63 and 64 SD, and 4, 4, 4, 6 and 4 PD. The response rate ranged from 32.0 to 35.0%.

Intercriteria reproducibility

The intercriteria reproducibility in the response rates is shown in Table 3. Between UD and BD, the intraobserver difference in the response rates ranged from 0 to 6.4% with a mean of 2.36%, and the interobserver difference ranged from 0 to 10.0% with a mean of 4.25%. Between UD-MLS and BD, the intraobserver difference in the response rates ranged from 0.1 to 2.6% with a mean of 1.26%, and the interobserver difference ranged from 0.1 to 6.4% with a mean of 2.76%.

Correlations between UD and BD

The mean and ranges of interobserver reproducibility among five observers using the two dimensional measurements are shown in Table 4. The mean value of the Spearman rank correlation coefficient for the percentage changes when using UD (0.81) was lower than that using BD (0.85), and the same tendency was observed for the mean value of proportion of agreement for the tumor response categories (82.5%, 908/1100 vs 84.4%, 928/1100) and the mean kappa statistics for the tumor response categories (0.61 vs 0.69). The lowest kappa statistics among the 10 pair comparisons were 0.49 with UD and 0.61 with BD. The kappa statistics obtained with BD were higher than those with UD in nine out of 10 pair comparisons (Fig. 1).

Correlations between UD and UD-MLS

The mean values and ranges of interobserver reproducibility when applying the MLS are shown in Table 4. The mean value of Spearman's correlation coefficient for UD-MLS (0.84) was higher than that for UD (0.81), and the same tendency was observed for the mean value of proportion of agreement for the tumor response categories (84.2%, 867/1030 vs 82.5%, 908/1100) and the mean kappa statistics for the tumor response categories (0.65 vs 0.61). The lowest kappa statistics among the 10 pair-based comparisons was 0.57 with MLS and 0.49 without. When MLS was used together with UD, the kappa statistics increased in eight out of 10 pair comparisons (Fig. 2).

Table 3. Intercriteria reproducibility: difference in the response rate (%) among five observers

Category	UD and BD†		UD-MLS‡ and BD†	
	Mean	Range	Mean	Range
Overall (25 comparisons)	3.87	0–10	2.45	0.1–6.4
Interobserver (20 comparisons)	4.25	0–10	2.76	0.1–6.4
Intraobserver (5 comparisons)	2.36	0–6.4	1.26	0.1–2.6

†WHO criteria; ‡RECIST guidelines. BD, bidimensional measurement; MLS, minimum lesion size; UD, unidimensional measurement.

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Table 4. Interobserver reproducibility (10 pair comparisons) using four different measurements among five observers

Category	No. patients	Spearman's correlation coefficient		Proportion of agreement (%)		Kappa statistic	
		Mean	Range	Mean	Range	Mean	Range
UD	110	0.81	0.76–0.86	82.5	77.3–89.1	0.61	0.49–0.75
BD[†]	110	0.85	0.79–0.89	84.4	80.0–89.1	0.69	0.61–0.78
UD-MLS[‡]	103	0.84	0.75–0.89	84.2	80.6–88.3	0.65	0.57–0.73
BD-MLS	103	0.86	0.80–0.89	84.0	78.6–89.3	0.68	0.58–0.78

[†]WHO criteria; [‡]RECIST guidelines. BD, bidimensional measurement; MLS, minimum lesion size; UD, unidimensional measurement.

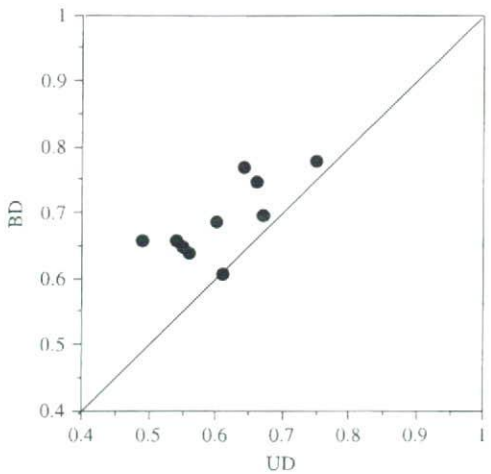

Fig. 1. Scattergram showing the kappa statistics for the use of unidimensional (UD) and bidimensional (BD) measurements. The kappa values for BD were higher than those for UD in nine of 10 pair comparisons.

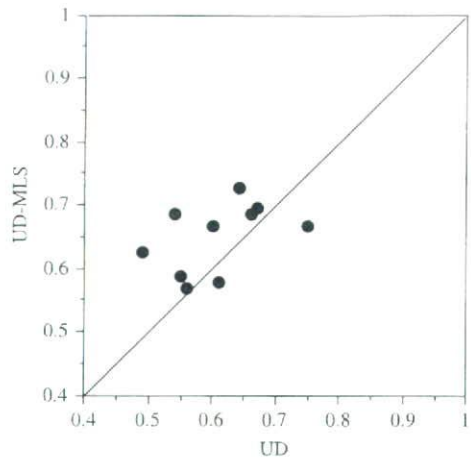

Fig. 2. Scattergram showing the kappa statistics for the use of unidimensional measurement (UD) and UD with minimum lesion size (MLS). When MLS was applied to UD, the kappa values increased in eight of 10 pair comparisons.

Discussion

Standardized tumor response evaluation systems are considered reliable in clinical trials when they are valid and reproducible among different observers. Although the intercriteria reproducibility between the new RECIST guidelines and the previous WHO criteria had been investigated,[2–7] little information was available concerning interobserver reproducibility of tumor response evaluation. In addition, statistical analysis results regarding the effect of MLS on interobserver reproducibility had not been provided in previous reports. This is the first study to investigate interobserver reproducibility of the RECIST guidelines evaluating the MLS.

The importance of interobserver reproducibility for any classification scheme has been discussed previously for other grading systems.[10–12] Clinical investigators must take into account interobserver reproducibility in tumor response evaluation, which can greatly affect the results in clinical trials. Our findings demonstrated that interobserver variability exists for bidimensional measurements, as in studies published previously.[13,14] For example, Hopper *et al.* showed considerable interobserver variability in CT tumor measurements between radiologists interpreting thoracic and abdominal/pelvic CT scans.[13] In another report, the impact of an evaluation committee on patients' overall response status in a large multicenter trial in oncology was evaluated.[14] Major disagreements occurred in 40% of cases and minor disagreements occurred in 10.5% of the cases reviewed. The number of responders was reduced by 23.2% after review by the evaluation committee.

The range of response rates among five observers was clearly narrowed by the MLS (Table 2). The response rates assessed by UD varied from 23.6 to 32.7%. When assessed by BD, the response rates ranged from 27.3 to 33.6%. Response rates assessed with UD-MLS ranged from 27.2 to 33.0%, which was almost identical when BD was used.

The results of the present study also suggested that BD was more reproducible than UD. When MLS was applied to UD, the mean values and ranges of Spearman's correlation coefficient, proportion of agreement and the kappa statistics improved (Table 4). In order to ensure comparable interobserver reproducibility (as was originally achieved with the WHO criteria) it is essential that the MLS be used in combination with UD when using RECIST.

Because of the need to retain some ability to compare results of future therapies with those available currently, no major discrepancy should exist between the old (WHO) and

new (RECIST) criteria, although measurement criteria would be different. The mean values and ranges of intercriteria reproducibility in the response rates between UD-MLS and BD were lower and narrower than those between UD and BD (Table 3). The introduction of MLS to UD improved the intercriteria reproducibility between WHO and RECIST.

As for intercriteria reproducibility, the mean values and ranges for intraobserver reproducibility were better than those for interobserver reproducibility (Table 3). Erasmus *et al.* have suggested that consistency can be improved if the same reader carries out serial measurements for any one patient.[15]

When MLS is included in the eligibility criteria, the number of patients with measurable lesions is less than that obtained with the previous WHO criteria because patients with only small lesions are excluded from measurement. In the present study, when MLS criteria were used the number of eligible cases decreased by 6.4% from 110 to 103 and the number of target lesions by 44.6% from 402 to 223. This reduction could affect the number of patients enrolled in clinical trials.

The present study had several limitations. First, the study cohort comprised NSCLC patients only and the application of the measurement modalities was limited to chest CT. Second, intraobserver variability between evaluations with different intervals was not investigated. Third, our reference was a 10 mm slice thickness and therefore the minimum lesion size was defined as 20 mm. However, RECIST guidelines allow for a minimum lesion size of 10 mm as a slice thickness of 5 mm measured by helical CT is used. Recently, multidetector CT, which creates a thinner slice thickness, has been developed and is being used in daily clinical practice. Therefore, the addition of the outcomes of patients ineligible for our study as a result of using a thinner slice thickness might change our results and should be evaluated in a further study.

In conclusion, the results of the present study suggest that UD yields poorer interobserver reproducibility of tumor response evaluation than BD; however, if MLS is applied to UD, interobserver reproducibility can improve and become the same as that obtained with BD. The introduction of MLS to UD could also improve intercriteria reproducibility between WHO and RECIST. It is therefore essential that investigators include MLS when using RECIST guidelines to ensure interobserver reproducibility comparable with the WHO criteria.

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Randomized, double-blind, placebo-controlled trial of bovine lactoferrin in patients with chronic hepatitis C

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Several studies have suggested that lactoferrin administration may decrease the serum level of hepatitis C virus (HCV) RNA in patients with chronic hepatitis C. The aim of the present study was to confirm the efficacy of orally administered bovine lactoferrin (bLF) in patients with chronic hepatitis C. The patients with chronic hepatitis C randomly received either oral bLF at a dose of 1.8 g daily for 12 weeks, or an oral placebo. The primary endpoint was the virologic response, defined as a 50% or greater decrease in serum HCV RNA level at 12 weeks compared with the baseline. The secondary endpoint was the biochemical response, which was defined as a 50% or greater decrease in the serum alanine aminotransferase (ALT) level at 12 weeks compared with the baseline. One hundred and ninety-eight of 199 patients were evaluable for efficacy and safety. bLF treatment was well tolerated and no serious toxicities were observed. A virologic response was achieved in 14 of 97 patients (14.4%) in the bLF group, and 19 of 101 (18.8%) in the placebo group. There was no significant difference in virologic response rates between the two groups (−4.4%, 95% confidence interval −14.8, 6.1). In addition, bLF intake did not have any favorable effect on the serum ALT level. The virologic responses were not different between two groups in any subgroup analysis. In conclusion, orally administered bLF does not demonstrate any significant efficacy in patients with chronic hepatitis C. (*Cancer Sci* 2006; 97: 1105–1110)

Hepatitis C virus is a leading cause of chronic liver disease in Japan, and nearly two million people are estimated to be infected.[1] It is well known that HCV infection frequently causes chronic hepatitis, and that chronic hepatitis eventually progresses to liver cirrhosis and HCC approximately 30 years after HCV infection.[2] In Japan, more than 30 000 people die of HCC annually, and approximately 80% of HCC patients are infected with HCV.[3] Therefore, effective anti-HCV therapy is necessary to reduce the number of patients suffering from cirrhosis or HCC. To date, interferon-based therapy is the only effective treatment used clinically for chronic hepatitis C. A sustained complete virologic response (loss of detectable serum HCV RNA) occurs in 15–20% of patients with chronic hepatitis C after interferon therapy.[4] Moreover, recent studies have demonstrated that interferon with ribavirin or peginterferon with ribavirin improves the sustained complete virologic response rate by up to 40–50%.[5,6] However, because more than half of patients do not respond to interferon therapy, and because interferon therapy sometimes induces strong adverse effects, further developments in the treatment of chronic hepatitis C are required.

Lactoferrin, a member of the transferrin family of iron-binding glycoproteins, is present mainly in breast milk and other exocrine secretions. Several biological activities of lactoferrin have been demonstrated, including regulation of iron absorption in the intestine and modulation of immunoreactions.[7] Lactoferrin also plays an important role in human innate defense mechanisms against bacteria, fungi and viruses.[8] *In vitro* studies to date have shown that lactoferrin has antiviral effects against human immunodeficiency virus-1 and human cytomegalovirus.[9] Recent experimental studies have suggested that lactoferrin has antiviral effect against HCV.[10–12] Yi *et al.* have reported that lactoferrin binds to HCV envelope proteins *in vitro*.[10] Ikeda *et al.* have reported that lactoferrin prevents HCV infection in cultured human hepatocytes, and suggested that the anti-HCV activity of lactoferrin might be related to its direct binding to viral surfaces.[11,12] In addition, recent clinical studies have demonstrated the potential efficacy of lactoferrin against chronic hepatitis C.[13,14] Tanaka *et al.* reported that 8-week oral administration of bLF at a dose of 1.8 or 3.6 g/day decreased the serum level of HCV RNA markedly in three of four patients with a low pre-treatment HCV RNA level (<100 Kcopy/mL).[13] Iwasa *et al.* administered bLF (3.6 g/day) orally to 15 patients with high viral loads (≥100 KIU/mL), and reported that the mean serum HCV RNA level decreased significantly from 1106 KIU/mL at entry to 612 KIU/mL after 6 months of treatment ($P < 0.01$).[14] Based on these promising findings, we planned to investigate the efficacy of orally administered bLF in patients with chronic hepatitis C. First, we conducted a dose-finding study in 45 patients with chronic hepatitis C.[15] In that study, three dose levels of bLF (1.8, 3.6 and 7.2 g/day) were scheduled, and 15 patients at each dose level received the determined dose of bLF for 8 weeks. bLF treatment was well tolerated up to 7.2 g/day, and no serious adverse events were observed. Although no relationship between bLF dose and efficacy was recognized, a 50% or greater decrease in the serum HCV RNA level was seen in four of 45 patients (8.9%). Furthermore, the HCV RNA level was decreased by 50% or more in eight patients (17.8%) at week 8 after the end of treatment. These results encouraged us to conduct further investigations, and the present randomized

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Abbreviations: ALT, alanine aminotransferase; bLF, bovine lactoferrin; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IL, interleukin; NK, natural killer.

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trial was designed to clarify the anti-HCV activity of bLF in patients with chronic hepatitis C.

Patients and Methods

Patients. Each patient was required to meet the following eligibility criteria: 20–74 years of age; positivity for anti-HCV antibody; an HCV RNA level of 0.5–850 KIU/mL evaluated within 1 month before entry; a sustained elevation of serum ALT level for at least 6 months; a serum ALT level of at least twice the upper normal limit evaluated within 1 month before entry; no evidence of HCC on the basis of ultrasonography or computed tomography carried out within 3 months before entry; and adequate bone marrow function (white blood cell count $\geq 4000/mm^3$, platelet count $\geq 100\,000/mm^3$, and hemoglobin level ≥ 11 g/dL), liver function (total bilirubin level ≥ 2.0 mg/dL, serum albumin level ≥ 3.5 g/dL, and serum aspartate aminotransferase and ALT level ≥ 200 IU/L) and renal function (normal serum creatinine and blood urea nitrogen levels).

The exclusion criteria were: positivity for hepatitis B surface antigen; interferon therapy within 6 months before entry; immunomodulatory or corticosteroid therapy within 3 months before entry; intravenous glycyrrhizin therapy within 1 month before entry; past or present history of bLF tablet intake; pregnant or lactating females; severe hepatic disease (e.g. autoimmune hepatitis and primary biliary cirrhosis); other serious medical conditions (e.g. gastrointestinal bleeding, active infection, severe pulmonary disease and psychiatric disorders).

Methods. This double-blind, placebo-controlled phase III trial was conducted at 11 centers in Japan. The study was approved by the institutional review board at each center, and all the participants provided written informed consent. Eligible participants were assigned randomly to one of two treatment groups in equal proportions using permutation blocks stratified by centers. A randomization list was drawn up using the SAS random number generator at the data center (Quintiles Transnational Japan K. K. Tokyo, Japan). The treatments consisted of bLF at a dose of 1.8 g/day or a placebo, administered orally twice daily for 12 weeks. In the current study, bLF at 1.8 g/day was selected on the basis of the previous dose-finding study, which indicated that there was no significant relationship between bLF dose (range, 1.8–7.2 g/day) and anti-HCV activity.[15] After the treatment allocation, the data center sent a numbered container of bLF or placebo tablets to a participant. During treatment, combined use of interferon, immunomodulatory therapy, corticosteroid and intravenous glycyrrhizin was prohibited. bLF (450 mg/tablet) and placebo tablets were provided by Morinaga Milk Industries (Tokyo, Japan).

In the current study, we tested the hypothesis that oral administration of bLF would: (1) reduce the serum HCV RNA level; and (2) reduce the serum ALT level in patients with chronic hepatitis C. In addition, we investigated the influence of orally administered bLF on systemic immune response in a small group of participants. The participants were evaluated every 4 weeks as outpatients until 4 weeks after completion of treatment. Serum HCV RNA level and serum ALT level were measured before treatment, during treatment at weeks 4, 8 and 12, and at 4 weeks after treatment. Serum HCV RNA level was determined by reverse transcription–polymerase chain reaction using the Amplicor-HCV monitor V 2.0 kit with a sensitivity of 0.5 KIU/mL (Roche Diagnostics, Tokyo, Japan). Anti-HCV antibody was determined by chemiluminescent enzyme immunoassay (Ortho-Clinical Diagnostics, Tokyo, Japan). HCV serotyping was carried out as described previously.[16] HCV serotype 1 corresponds to genotypes 1a and 1b of the Simmonds classification, and HCV serotype 2 corresponds to genotypes 2a and 2b.[17] Serum concentration of IL-18 was measured in participants at two institutions (National Cancer Center Hospital and Osaka Red Cross Hospital), and the percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ peripheral blood lymphocytes was measured in participants at the National Cancer Center Hospital. IL-18 and all lymphocytes were measured before treatment, during treatment at weeks 4, 8 and 12, and at 4 weeks after completion of treatment. Serum concentration of IL-18 was assayed with a human IL-18 enzyme-linked immunosorbent assay kit (Medical and Biological Laboratories, Nagoya, Japan). Lymphocyte surface phenotypes of CD4, CD8, CD16 and CD56 were determined by flow cytometry.

Adverse events were graded for severity according to the Japan Society for Cancer Therapy criteria,[18] which are similar to the National Cancer Institute Common Toxicity criteria. During treatment, participants were asked to record in a daily journal both compliance and any adverse events they experienced.

Assessment of efficacy and statistical analysis. Analyses were carried out on an intention to treat basis. The primary endpoint was a virologic response. In the current study, we defined a virologic response as a 50% or greater decrease in the serum HCV RNA level at 12 weeks compared with the baseline. Secondary endpoints were a biochemical response, as were changes in serum HCV RNA level and serum ALT level. If the serum ALT level at 12 weeks showed both a $\geq$50% decrease compared with the baseline and was $\leq$ twice the upper normal limit, we considered it a biochemical response. Response rate was calculated as the number of responders divided by the total number in each group. Participants whose HCV RNA (or ALT) data at 12 weeks were missing were included only in the denominator. Change in HCV RNA level (or ALT level) was calculated as the logarithm of the HCV RNA level (or ALT level) at 12 weeks minus the logarithm of these at the baseline. Differences in the virologic or biochemical response rates between two groups were analyzed using a test for the difference between two proportions. Differences in the change in HCV RNA level or ALT level between two groups were analyzed using a test for the difference between two means. In addition to the above planned analyses, subgroup analyses for virologic response were carried out based on pretreatment variables including age, serum HCV RNA level and HCV serotype. In a small group of participants, change in the serum concentration of IL-18 and changes in the percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ peripheral blood lymphocytes during the study period were investigated. Analyses were carried out using JMP4.0 and PC SAS Release v.8.02 (SAS Institute Japan Ltd, Tokyo, Japan). All P-values are two-tailed, and differences at $P < 0.05$ were regarded as statistically significant.

We estimated that a total of 250 participants would be the maximum to enroll for a 2-year enrollment period. Subsequent power analysis revealed that 125 participants per group would have 75% power to detect a 10% difference in the virologic response rate (15 vs 5%) at the 5% level of significance. An interim analysis by the independent data monitoring committee was planned after the first 125 participants had been enrolled. All trial personnel and participants were blinded to treatment assignment for the duration of the trial. Only the trial statistician and the independent data monitoring committee saw unblinded data. In the interim analysis of the primary endpoint, the O'Brien–Fleming method was used.[19]

Results

Patients. Enrollment began at seven institutions in April 2001. Because 250 participants were not enrolled for the 2 years planned originally, we extended the registration period for one more year and increased the number of participating institutions from seven to 11. An interim analysis was carried out in March 2004 with the data from the first 125 participants. Because the results of the interim analysis indicated that it was highly unlikely that a significant difference in treatment efficacy between

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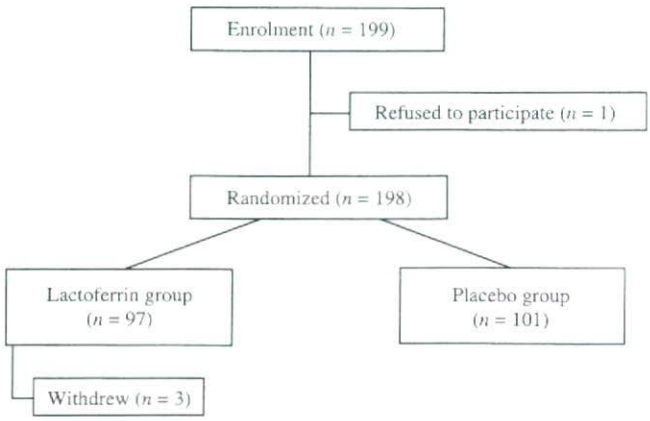

Fig. 1. Flow diagram of participant enrolment.

Table 1. Baseline characteristics of the patients

Characteristic	Bovine lactoferrin	Placebo
No. patients	97	101
Age (years)†	61 (29–74)	58 (31–74)
Sex (male/female)	53/44	55/46
History of interferon therapy	25	29
ALT level (IU/L)†	91 (41–340)	98 (27–250)
HCV RNA level (KIU/mL)†	378 (8.8–960)	452 (8.0–1560)
HCV serotype (1/2/ND)	78/17/1	76/22/3

†Median (range). ALT, alanine aminotransferase; HCV, hepatitis C virus; ND, not determined.

the two groups would be observed with the planned full enrollment of 250 participants, the data monitoring committee recommended discontinuation of further enrollment. Therefore, enrollment was stopped on 31 March 2004, at which point 199 participants had been enrolled. Because one patient refused to participate in the study before randomization, efficacy and safety were analyzed in the remaining 198 participants (97 bLF and 101 placebo) (Fig. 1). Although three participants in the bLF group discontinued treatment for reasons other than an adverse event, the remaining 195 participants completed the scheduled 12 weeks of treatment. The baseline characteristics of the 198 participants are shown in Table 1. There was no significant difference between the bLF and placebo groups regarding the pretreatment characteristics including age, sex, serum ALT level and serum HCV RNA level.

Virologic efficacy. Virologic response, the primary endpoint, was assessed in all 198 participants who received at least one dose of treatment. Virologic response was observed in 14 of 97 participants (14.4%) in the bLF group, and in 19 of 101 (18.8%) in the placebo group (Table 2). No complete virologic response (loss of detectable serum HCV RNA) was seen in either of the groups. There was no significant difference in the virologic response rate with bLF treatment in comparison with the placebo (–4.4%, 95% CI –14.8, 6.1). Change in the HCV RNA level at 12 weeks compared with the baseline was assessed in 190 participants (93 bLF group, 97 placebo group), excluding eight participants for whom HCV RNA data at 12 weeks were lacking. The change in the mean logarithm of the HCV RNA level was –0.09 in the bLF group and –0.09 in the placebo group, indicating no significant difference between the groups ($P = 1.00$).

Biochemical efficacy. Biochemical response was assessed in 198 participants. Biochemical response was seen in six of 97 participants (6.2%) in the bLF group, and in four of 101 participants (4.0%) in the placebo group (Table 2). No significant difference in the biochemical response rate was seen between the groups (2.2%, 95% CI –3.9, 8.3). Change in the serum AST level was assessed in 192 participants (93 bLF group, 99 placebo group), excluding six participants for whom ALT data at 12 weeks were lacking. The change in the mean logarithm of the ALT level was –0.085 in the bLF group and –0.080 in the placebo group, indicating no significant difference ($P = 0.93$).

Subgroup analysis. The rates of virologic response with respect to pretreatment variables are presented in Table 3. Among participants with a low HCV RNA level (<100 KIU/mL), the virologic response rate was 29.4% in the bLF group and 15.4% in the placebo group, indicating no significant difference between the groups (14.0%, 95% CI –15.2, 43.2). The virologic responses were also not different between two groups in other subgroup analyses such as age, sex and HCV serotype.

Analysis of IL-18 and lymphocytes. The serum concentration of IL-18 was measured in 73 participants enrolled at the National Cancer Center Hospital and Osaka Red Cross Hospital (36 bLF, 37 placebo). Figure 2 shows the changes in the mean IL-18 levels in the bLF group and placebo group. The mean IL-18 levels in the bLF and placebo groups were 293.9 pg/mL and 309.9 pg/dL at the baseline and 280.7 pg/mL and 291.5 pg/mL at 12 weeks, respectively. The corresponding changes in the mean IL-18 level at 12 weeks were –14.5 pg/mL and –15.9 pg/mL, respectively, indicating no significant difference between the groups ($P = 0.91$). Similarly, there were no significant differences between the groups at any other points during the study period. The percentage of lymphocyte was measured in 46 participants at the National Cancer Center Hospital (bLF 23, placebo 23), and the results are shown in Fig. 3. The percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ peripheral blood lymphocytes remained almost unchanged throughout the study in both groups, and the differences between them were not significant.

Safety. Safety was assessed in 198 participants who received at least one dose of bLF or placebo during the study. The bLF treatment was well tolerated, and no serious complications occurred during the treatment. Although minor adverse events including neutropenia, γ-GTP elevation and hyperglycemia were observed in participants treated with bLF, their frequency and intensity did not differ from those in the placebo group. HCC was detected in one participant in the bLF group and in one participant in the placebo group during the study period.

Discussion

The present study was carried out to confirm the anti-HCV activity of orally administered bLF in patients with chronic hepatitis C. A virologic response (a 50% or greater decrease in the serum level of HCV RNA at 12 weeks compared with the baseline) was observed in 14 of 97 participants (14.4%) in the bLF group, and 19 of 101 (18.8%) in the placebo group, the difference between the groups being non-significant. The virologic responses were not different between two groups in any subgroup analysis. Furthermore, bLF intake did not have any favorable effect on the serum ALT level. On the basis of these results, we concluded that orally administered bLF did not have any efficacy, including anti-HCV activity, in patients with chronic hepatitis C.

The virologic response rate of 14.4% observed in the bLF group was somewhat higher than that reported in the previous dose-finding study,[15] in which four of 45 patients (8.9%) showed a virologic response at the end of bLF treatment. Nevertheless, the current study failed to demonstrate any anti-HCV activity of bLF, because a similar virologic response rate to that in the bLF group was seen in the placebo group. Having designed this randomized study, we assumed that a virologic

Table 2. Virologic and biochemical efficacy

Characteristic	Bovine Lactoferrin	Placebo	Difference (95% CI)	*P*-value
Virologic efficacy				
Response rate (%)	14.4	18.8	−4.4 (−14.8, 6.1)	
Change in HCV RNA level†	−0.09	−0.09		1.00
Biochemical efficacy				
Response rate (%)	6.2	4.0	2.2 (−3.9, 8.3)	
Change in ALT level†	−0.085	−0.080		0.93

†Mean logarithm. ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus.

Table 3. Virologic response rate as a function of baseline variables

Variable	Bovine lactoferrin (*n* = 97)		Placebo (*n* = 101)		Difference	
	Response/total	%	Response/total	%	%	95% CI
Age						
<65 years	12/62	19.4	14/77	18.2	1.2	−11.9, 14.2
≥65 years	2/35	5.7	5/24	20.8	−15.1	−33.1, 2.9
Sex						
Male	10/53	18.9	10/55	18.2	0.7	−14.0, 15.3
Female	4/44	9.1	9/46	19.6	−10.5	−24.7, 3.8
ALT level						
<100 IU/L	6/57	10.5	7/51	13.7	−3.2	−15.6, 9.2
≥100 IU/L	8/40	20.0	12/50	24.0	−4.0	−21.1, 13.1
HCV RNA level						
<100 KIU/mL	5/17	29.4	2/13	15.4	14.0	−15.2, 43.2
≥100 KIU/mL	9/80	11.3	17/88	19.3	−8.0	−18.8, 2.7
HCV serotype†						
1	11/78	14.1	16/76	21.1	−7.0	−18.9, 5.0
2	3/18	16.7	2/22	9.1	7.6	−31.4, 28.6

†Hepatitis C virus serotype was not measured in four patients. ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus.

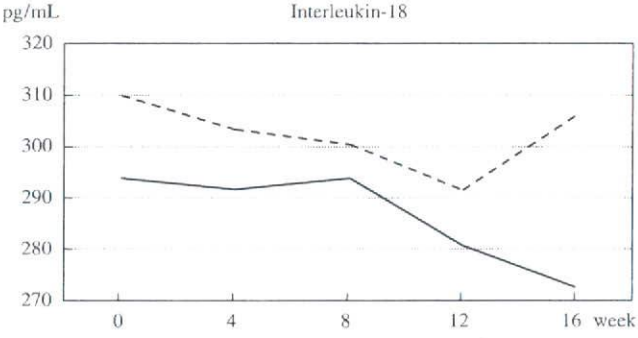

Fig. 2. Changes in the mean serum concentration of interleukin-18 in the bovine lactoferrin group (straight line, *n* = 36) and the placebo group (dotted line, *n* = 37).

response rate of around 5% would be seen in the placebo group due to spontaneous remission of viral activity. However, contrary to our expectation, 19 of 101 participants (18.8%) in the placebo group showed a ≥50% decrease in the HCV RNA level at 12 weeks, indicating that our assumption was inappropriate. Our results suggested that in order to assess the reduction of the HCV RNA level, periodic evaluation would be necessary to exclude the influence of spontaneous fluctuation of HCV RNA.

Several experimental studies have suggested that lactoferrin has some activity against HCV. Yi *et al.*[10] reported that lactoferrin binds to the HCV E1 and E2 envelope proteins *in vitro*, and Ikeda *et al.*[11,12] reported that lactoferrin prevents HCV infection in cultured human hepatocytes. They suggested that the anti-HCV activity of lactoferrin might be due to a neutralizing efficacy, in which the administered lactoferrin became bound directly to the HCV virion, thus inhibiting adsorption of the HCV-lactoferrin complex into human hepatocytes. Therefore, intravenous administration of lactoferrin might improve the viremic state in patients with chronic hepatitis C. However, for practical application, administration of lactoferrin directly into blood does not seem to be a suitable approach because lactoferrin is a large glycoprotein molecule (80 kDa) that may cause allergic reactions. Therefore, oral administration of bLF was selected for the present study, even though the metabolism and mechanism of ingested lactoferrin are yet to be clarified. As to absorption, it has been reported that intact lactoferrin and its fragments are present in the urine of human milk-fed preterm infants.[20] However, in adult rats, lactoferrin and its fragments are not detectable in portal blood after bLF ingestion,[21] and in adult humans, the serum lactoferrin level does not increase after oral administration of recombinant human lactoferrin.[22] However, several studies have suggested that orally administered lactoferrin might enhance immune responses via cytokine production.[23,24] It has been reported that oral administration of bLF to mice enhances the production of IL-18 and interferon-γ in the mucosa of the small intestine, and increases the number of $CD4^+$, $CD8^+$ and NK cells in the small-intestinal epithelium.[25,26] Varadhachary *et al.* reported that oral administration of recombinant human lactoferrin to mice stimulates IL-18 production from gut enterocytes, and augments the NK activity of spleen cells and production of blood $CD8^+$ cells.[27] Furthermore, a recent clinical study has demonstrated that oral administration of bLF (0.6 g/day) for 3 months in 36 patients with chronic hepatitis C increased the serum IL-18 level significantly compared with the

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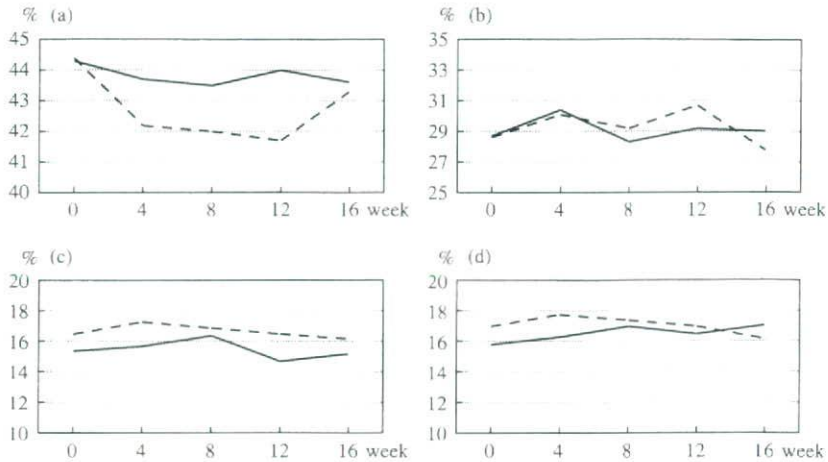

Fig. 3. Changes in the mean percentages of (a) $CD4^+$, (b) $CD8^+$, (c) $CD16^+$ and (d) $CD56^+$ peripheral blood lymphocytes in the bovine lactoferrin group (straight line, $n = 23$) and the placebo group (dotted line, $n = 23$).

baseline.[28] However, our study found no evidence that oral administration of bLF influences the serum concentration of IL-18 or the percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ lymphocytes. Further investigations are required to clarify the peripheral and systemic effects of orally administered lactoferrin. In addition, as many *in vitro* studies have suggested that lactoferrin has direct binding neutralizing efficacy against HCV,[29–31] further investigations are needed to devise a means of delivering lactoferrin or its fragment into the bloodstream safely and effectively.

Recently, several studies have investigated the value of adding lactoferrin to interferon therapy for chronic hepatitis C. Hirashima *et al.* randomly assigned 21 patients with chronic hepatitis C to either a consensus interferon plus oral lactoferrin (3.0 g/day) group or a consensus interferon monotherapy group.[32] Three of 10 patients in the consensus interferon plus lactoferrin group showed a sustained complete virologic response, as did four of 11 patients in the consensus interferon group, indicating no statistically significant difference between the groups. Ishibashi *et al.* conducted a randomized controlled trial to investigate the efficacy of interferon α-2b and ribavirin plus oral lactoferrin (0.6 g/day) compared with interferon α-2b and ribavirin plus placebo in 36 patients with chronic hepatitis C.[33] A sustained complete virologic response was seen in six of 18 patients in the lactoferrin group and in five of 18 patients in the placebo group, there being no statistically significant difference between the groups ($P = 0.7$). Although the numbers of patients recruited in the two randomized trials were small, these results suggested that the additional value of oral lactoferrin combined with interferon therapy would be negative for the treatment of chronic hepatitis C.

In summary, oral administration of bLF at a dose of 1.8 g/day for 12 weeks showed an acceptable safety profile in patients with chronic hepatitis C. However, there was no significant difference in the virologic responses between patients who received oral bLF and those receiving placebo. In addition, bLF intake did not have any favorable effect on the serum ALT level. These findings do not support the practical use of oral bLF in patients with chronic hepatitis C.

Acknowledgments

This article is dedicated to the memory of the late Dr S. Okada, a principal investigator, and the late Dr N. Okazaki, the chairman of the data monitoring committee. We thank Drs M. Kato, Y. Inaba and K. Hayashi as members of the data monitoring committee. We are grateful to Dr H. Tsuda as a scientific contributor. We also thank Ms K. Kondo and Dr H. Nakajima for assistance in data management, and Morinaga Milk Industries for providing the bLF tablets and placebo. This work was supported by Grants-in-Aid for Cancer Research for the Second- and Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan.

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Short Communication

Higher Methylation Levels in Gastric Mucosae Significantly Correlate with Higher Risk of Gastric Cancers

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Abstract

Background: ***Helicobacter pylori* infection potently induces methylation of CpG islands in gastric mucosae, which is considered to decrease to a certain level after active *H. pylori* infection discontinues. Noncancerous gastric mucosae of *H. pylori*–negative cases with a gastric cancer had higher methylation levels than those of *H. pylori*–negative healthy individuals. Here, using cases with multiple gastric cancers, we analyzed whether the higher methylation levels correlated with the higher risk of gastric cancers.**

Methods: **Twenty-six healthy volunteers (HV), 30 cases with a single well-differentiated gastric cancer (S cases), and 32 cases with multiple well-differentiated gastric cancers (M cases) were recruited. *H. pylori* infection status was analyzed by the culture method. Methylation levels were quantified by real-time methylation-specific PCR of seven CpG islands.**

Results: **In *H. pylori*–negative individuals, significant increasing trends were present in the order of HV, S cases, and M cases for *FLNc* and *HAND1* methylation levels ($P < 0.01$, Spearman's rank-order test). Furthermore, the *FLNc* methylation level of M cases was significantly higher than that of S cases ($P < 0.01$, t test). Even adjusted by the extent of gastric atrophy, the *FLNc* methylation level retained a significant increasing trend ($P = 0.03$). In contrast, methylation levels in *H. pylori*–positive individuals were increased to various degrees in all the three groups.**

Conclusions: **In *H. pylori*–negative individuals, methylation levels in gastric mucosae significantly increased in cases with a single gastric cancer and more in cases with multiple gastric cancers. Quantitative analysis of methylation levels is a promising risk marker for gastric cancers.** (Cancer Epidemiol Biomarkers Prev 2006;15(11):2317–21)

Introduction

Gastric cancer is one of the most common malignancies worldwide (1). As a novel therapeutic procedure, endoscopic resection (ER), including both endoscopic mucosal resection and endoscopic submucosal dissection, is becoming common not only in Japan but also in western countries (2, 3). ER preserves a larger portion of the stomach than partial gastrectomy, and cumulative incidence of metachronous gastric cancers reaches as high as 8.5% to 14.0% (4, 5), in contrast with the incidence of 1.8% to 2.4% after partial gastrectomy (6, 7). Therefore, it is important to clarify whether specific individuals with a very high risk of gastric cancers develop multiple gastric cancers and, if so, to develop a marker to estimate the risk of individual cases.

The presence of individuals with a very high risk is indicated by the fact that cases with multiple gastric cancers have a much higher chance of developing another gastric cancer than those of cases with a single gastric cancer (8, 9). To identify these people, markers for multiple gastric cancers, such as gastric atrophy (10), microsatellite instability in cancers (11), and expression of brain-type glycogen phosphorylase in noncancerous gastric mucosae (12), have been developed. However, their sensitivity and specificity are not satisfactory, and a more sensitive marker whose value correlates with the degree of an individual's risk of cancer is awaited.

We showed recently that *Helicobacter pylori* infection induces methylation of various but preferential CpG islands (CGI) in gastric mucosae (13). Among *H. pylori*–negative individuals, methylation levels in noncancerous gastric mucosae were higher in gastric cancer cases than in healthy volunteers (HV). *H. pylori*–positive individuals, those who are with current, or active, *H. pylori* infection, had higher methylation levels than *H. pylori*–negative gastric cancer cases, most of whom are expected to have had past *H. pylori* infection (14). Therefore, it was indicated that current *H. pylori* infection potently induces methylation, that the high methylation level decreases to a certain level after active *H. pylori* infection discontinues, and that the final methylation level is associated with the risk of gastric cancers (15).

In this study, to develop a novel risk marker for multiple gastric cancers, we aimed to clarify that higher methylation levels in noncancerous gastric mucosae correlate with higher risks of developing gastric cancers. Risk levels in HV, cases with a single gastric cancer (S cases) with follow-up longer than 1 year, and cases with multiple gastric cancers (M cases) can be considered to be low, high, and very high, respectively (5, 8, 9), and their methylation levels were quantitatively analyzed.

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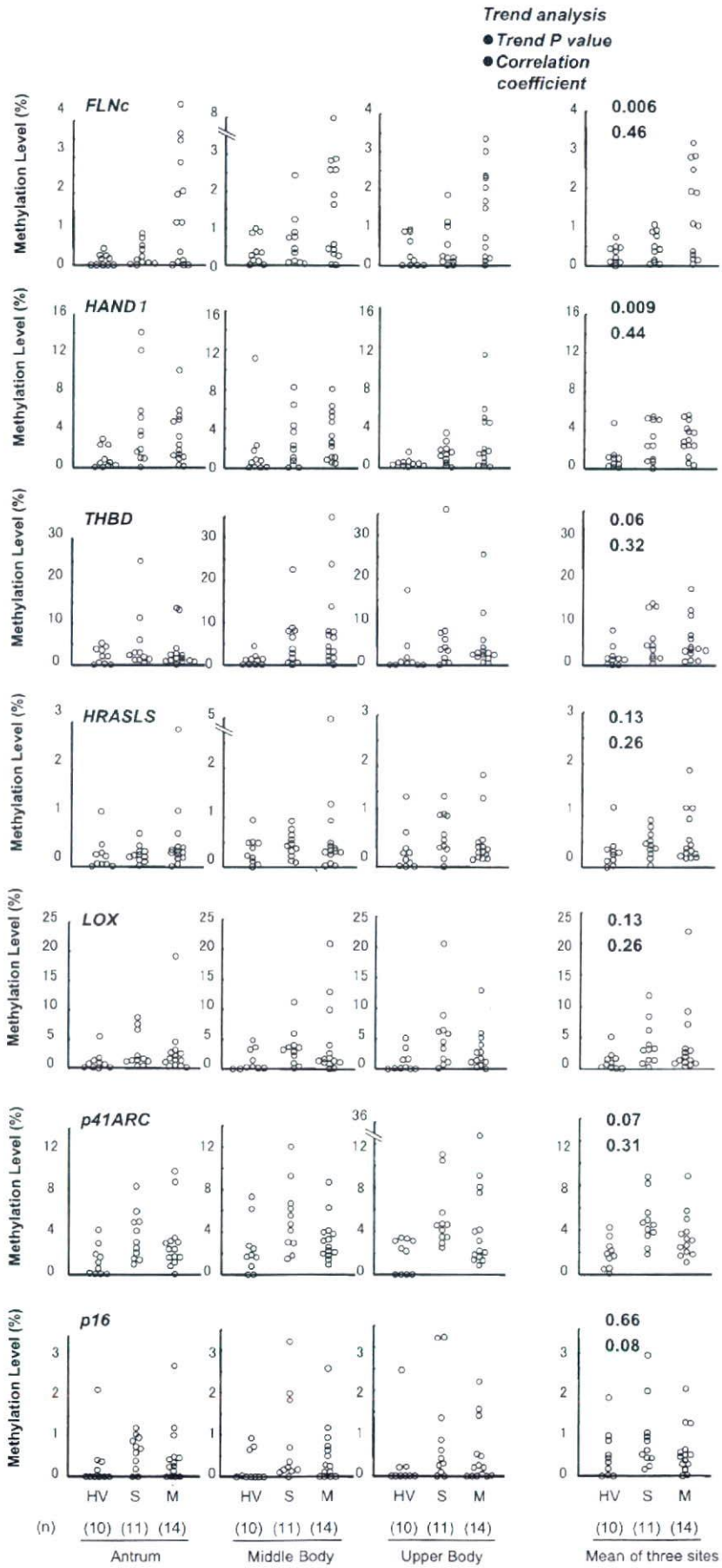

Figure 1. Methylation levels of the seven CGIs in *H. pylori*–negative HV, S cases, and M cases. Methylation levels in three sites within the stomach and their mean values. Trend *P* values and correlation coefficients are shown for the mean values, which were most closely associated with gastric cancer risk levels. Significant increasing trends were observed for *FLNc* and *HAND1* by the Spearman's rank-order correlation test (P = 0.006 and 0.009). Tendency of increasing trends was observed for *THBD* and *p41ARC*. Even when S and M cases are compared, *FLNc* methylation levels showed a significant increase in M cases (P = 0.009, t test).

Materials and Methods

Cases and Biopsy Materials. Twenty-six HV (male/female, 18/8), 30 S cases (23/7), and 32 M cases (23/9) were recruited from June 2004 to March 2005 at the National Cancer Center Hospital (Tokyo, Japan) under approval of the institutional review board and with written informed consents. The S and M cases previously underwent ER for well-differentiated early gastric adenocarcinoma according to the pre-ER indications, and curative resection was confirmed using expanded histologic criteria (16). M cases were defined according to the criteria of Moertel et al. (17): (*a*) each lesion is histopathologically malignant, (*b*) each lesion is separated from another, and (*c*) each lesion is not the result of a local extension or metastasis of another lesion. Because distinction between "real" metachronous gastric cancer and "missed" synchronous gastric cancer is difficult and has little meaning from the viewpoint of their risk levels, both of them were classified as M cases. S cases were defined as those who underwent ER and were followed up without a new gastric cancer for at least 1 year (median, 2.4 ± 1.9 years; range, 1.0-7.3 years, respectively). HV were recruited so that their mean age (66.0 years; range, 56-76 years) would match that of M cases (69.5 years; range, 55-77 years) and S cases (69.0 years; range, 55-76 years). Cases with hereditary cancer syndromes (hereditary nonpolyposis colorectal cancer, familial adenomatous polyposis, hereditary diffuse gastric cancers, and Li-Fraumeni syndrome), those with malignancy of other organs, and those who received chemotherapy or radiation therapy were excluded based on clinical history.

By endoscopic biopsy using sterilized biopsy forceps (Boston Scientific, Natick, MA), gastric mucosa were obtained from three standard sites: (*a*) the lesser curvature at 2 to 3 cm from the pyloric ring (antrum), (*b*) the lesser curvature at 2 to 3 cm proximal from incisura angularis (middle body), and (*c*) the lesser curvature at 2 to 3 cm distal to the cardia (upper body). Cases with any endoscopic abnormal appearance at biopsy points, such as ulceration, reddish or discolored areas, deformity, or elevation, were excluded. The samples were snap frozen immediately and stored at −80°C. *H. pylori* infection status was analyzed by culture of two sites, which are known to have a sensitivity of 94% and a specificity of 100% for current infection status (18-20). The endoscopic extent of gastric atrophy was assessed using the atrophic pattern system proposed by Kimura et al. (21), which well correlates with the histologic degree of atrophic gastritis.

CGIs Analyzed. Seven CGIs were selected for methylation analysis from the eight CGI regions previously analyzed (13). These included CGIs of *FLNc, HAND1, THBD, HRASLS, LOX*

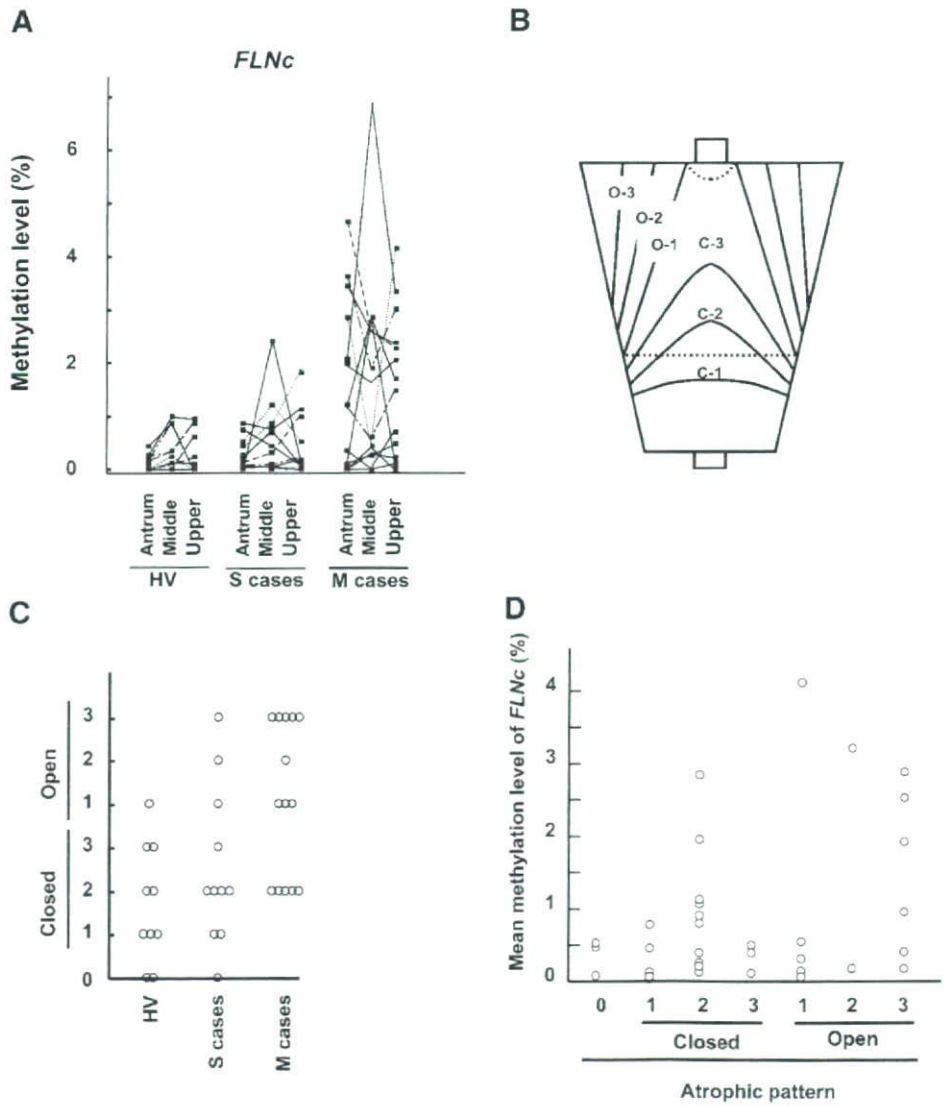

Figure 2. Methylation distribution and effect of atrophy. **A.** methylation levels in the three sites of the same *H. pylori*–negative individuals ($n = 35$). The *FLNc* methylation levels are shown for the three sites. Some individuals had relatively homogeneous methylation levels, but others, especially M cases, had divergent methylation levels. The occasional presence of cases with divergent methylation levels also supported that the mean methylation level best reflects the risk of gastric cancer of a specific individual. **B.** schematic representation of the spread of gastric atrophy through the stomach (21). Each line is a border between an endoscopically atrophic area and nonatrophic area. C and O are closed-type and open-type gastritis, respectively. In C-1, the atrophic area is localized in the antral region, and the area expands in C-2, C-3, O-1, O-2, and O-3. In O-3, almost the entire stomach has atrophy. **C.** correlation between the extents of atrophy and gastric cancer risk levels in *H. pylori*–negative individuals ($r = 0.52$; $P = 0.001$). **D.** correlation between the extent of atrophy and mean *FLNc* methylation level in *H. pylori*–negative individuals. A weak correlation was observed ($r = 0.36$; $P = 0.035$).

Table 1. Correlations between the methylation level and atrophy and between the methylation level and risk levels after adjustment by atrophy

	Extent of gastric atrophy		Gastric cancer risk levels after adjustment by the extent of gastric atrophy	
	Correlation coefficient	P_{trend}	Correlation coefficient*	P_{trend}
FLNc	0.36	0.035	0.37	0.029
HAND1	0.34	0.043	0.32	0.06
THBD	0.18	0.31	0.22	0.21
HRASLS	0.46	0.006	0.04	0.79
LOX	0.08	0.63	0.1	0.57
p41ARC	0.28	0.10	0.22	0.19
p16	0.09	0.61	−0.003	0.99

*Spearman's partial correlation coefficients adjusted by atrophy levels.

(up to here is in promoter regions), *p41ARC* (exon 8), and *p16* (exon 1). The *p16* promoter region was hardly methylated in our previous study and thus was excluded in this study.

Quantitative Methylation-Specific PCR. Methylation levels were quantified by real-time methylation-specific PCR as in our previous report (13). The methylation level of a sample for a CGI was calculated as the fraction of methylated molecules in the total DNA molecules (number of M molecules + number of U molecules). The standard DNA for real-time methylation-specific PCR is available on request.

Statistical Analysis. Methylation levels between S and M cases were compared by Welch's t test (two sided). Correlation between methylation levels and risk levels was analyzed by the Spearman's rank-order correlation coefficient (r), and the effect of gastric atrophy was adjusted by calculating Spearman's partial correlation coefficients.

Results

Correlation between Methylation Levels and Gastric Cancer Risk Levels in *H. pylori*–Negative Individuals. Methylation levels of the seven CGIs were analyzed for the three biopsy sites of each case. Because increased methylation levels in *H. pylori*–positive individuals are composed of both permanent and temporary components and do not necessarily reflect gastric cancer risk (13), we first focused on correlation between methylation levels and risk levels in *H. pylori*–negative individuals (Fig. 1). Methylation levels of *FLNc* and *HAND1* showed significant increasing trends ($r = 0.47$; $P_{trend} = 0.006$ for *FLNc*; $P_{trend} = 0.44$ and 0.009 for *HAND1*). The *THBD* and *p41ARC* methylation levels also showed increasing tendencies ($r = 0.32$; $P_{trend} = 0.06$ for *THBD*; $P_{trend} = 0.31$ and 0.07 for *p41ARC*). Even between S and M cases, the *FLNc* methylation level was significantly higher in M cases than in S cases ($P = 0.009$, Welch's t test). Interestingly, the methylation level of exon 1 of *p16* had no correlation with risk levels of gastric cancer. These data showed that the methylation levels of specific CGIs correlate with the risk levels of gastric cancers.

Comparison of the Three Biopsy Sites and Their Mean Value. Distribution of methylation levels in three biopsy sites was scrutinized using *FLNc* (Fig. 2A), which had the largest correlation coefficient and the smallest trend P value with gastric cancer risk levels. Some individuals had relatively similar methylation levels among the three sites, but others, especially M cases, had divergent methylation levels. This suggested that different sites of gastric mucosae have different methylation levels and possibly cancer risks, and gastric cancer risk of an individual was considered to be most accurately estimated by the mean values of these sites. This explained the finding that smaller trend P values were obtained using the mean methylation levels than those for individual sites.

Association between the Methylation Level and Endoscopic Extent of Gastric Atrophy and Their Independent Predictive Powers. The extent of gastric atrophy (Fig. 2B) is known to have a correlation with gastric cancer risk (10). Also in this study, the correlation between the extent of gastric atrophy and the gastric cancer risk level was confirmed ($r = 0.52$; $P = 0.001$; Fig. 2C).

The *FLNc* methylation level had a weak correlation with the extent of atrophy ($r = 0.36$; $P = 0.035$; Fig. 2D; Table 1). To examine the independent predictive power of the methylation level for the gastric cancer risk, we calculated Spearman's partial correlation coefficients between the methylation level and gastric cancer risk levels after adjustment by the extent of atrophy. The methylation level retained its correlation with the gastric cancer risk level ($r = 0.37$; $P = 0.029$; Table 1). The *HAND1* methylation level showed a similar tendency. This finding indicated that the methylation level of *FLNc*, and possibly *HAND1*, is a promising risk marker for gastric cancers, independent of gastric atrophy.

Effect of Active *H. pylori* Infection. In *H. pylori*–positive individuals, methylation levels in HV, S cases, and M cases were increased to various degrees, as in our previous study (13). The *FLNc* methylation level in *H. pylori*–positive HV individuals (1.9 ± 0.6 %) was almost comparable with those in *H. pylori*–negative M cases (1.6 ± 0.4). In contrast, *HAND1* (4.6 ± 0.9), *THBD* (21.1 ± 3.7), *HRASLS* (2.3 ± 0.5), *LOX* (13.2 ± 2.3), *p41ARC* (13.5 ± 2.0), and *p16* (3.6 ± 0.8) in *H. pylori*–positive HV individuals had much higher levels than those in *H. pylori*–negative M and S cases. This finding supported that current, or active, *H. pylori* infection potently induces methylation with both temporary and permanent components and that methylation levels in *H. pylori*–positive individuals do not necessarily correlate with gastric cancer risk levels.

Discussion

Significant increasing trends of *FLNc* and *HAND1* methylation levels in noncancerous gastric mucosae were observed in the order of HV, S cases, and M cases in *H. pylori*–negative individuals, those who are without current, or active, *H. pylori* infection. Even between the S and M cases, the *FLNc* methylation level was significantly higher in the M cases. In addition, when adjusted by the extent of gastric atrophy, *FLNc* methylation levels retained their increasing trend in the S and M cases. These findings showed that DNA methylation levels in the gastric mucosae correlate with gastric cancer risk levels and that they are good candidates for a risk marker of gastric cancers. In other words, the so-called "field defect" produced by past exposure to carcinogens could be detected and measured using DNA methylation levels as a marker. Now, a prospective study in gastric cancers is warranted, and application of the concept to other cancers seems promising.

Noncancerous gastric mucosae of individuals with active or current *H. pylori* infection, whether they had cancers or not, showed higher methylation levels than those of *H. pylori*–negative S and M cases, most of whom were considered to have had past exposure to *H. pylori* (14). This indicated that high methylation levels induced by *H. pylori* infection would decrease after the *H. pylori* infection discontinues, as in our previous study (13). The most likely mechanism was a decrease due to turnover of gastric epithelial cells with methylation. Methylation in the infiltrating lymphocytes was unlikely because peripheral lymphocytes did not have methylation of the CGI analyzed (data not shown). It is known that cells in a gastric gland are renewed every 3 days by cells supplied from progenitor cells, and progenitor cells themselves are replaced by those supplied from a single stem cell with a much longer cycle (22). If we assume that active *H. pylori* infection continually induces methylation at the level of progenitor cells, their replacement by fresh progenitor cells without methylation after discontinuation of *H. pylori* infection explains the decrease of methylation levels. The methylation level in *H. pylori*–negative individuals is considered to reflect the fraction of stem cells with methylation and to correlate with gastric cancer risk (15).

The seven CGIs analyzed showed different levels of methylation induced by *H. pylori* infection and association with risk levels in the S and M cases. The effect of *H. pylori* was prominent in *THBD*, *LOX*, and *p41ARC* but not clear in *FLNc*. In contrast, association between methylation levels and risk levels was clear in *FLNc* and *HAND1* followed by *THBD* and *p41ARC*. Especially, the difference between S and M cases was significant only for *FLNc*. This suggested that *H. pylori* infection induces methylation preferentially in some CGIs and that the degree of methylation induction in nonstem cells is also different among CGIs. Low levels of gene transcription are an important factor that promote methylation of promoter CGIs (23), and transcription levels are different among various genes and between stem cells and nonstem cells. Methylation of genes useful as a risk marker, such as *FLNc*, is considered to occur in association with that of genes critical for cancer development but to have a higher rate. If there is a CGI that is methylated preferentially in stem cells and rarely in progenitor cells due to low transcription in stem cells, such a CGI would be useful as a risk marker.

In summary, the higher DNA methylation levels in gastric mucosae correlated with the higher risk levels of gastric cancers, and the DNA methylation levels are a promising risk marker.

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新薬展望 2007

第Ⅰ部　治験を取り巻く環境変化

医師主導型治験の今後のあり方

後澤乃扶子*1)・笠井　宏委*2)・安藤　正志*3)・藤原　康弘*4)

平成15年7月，いわゆる改正薬事法の施行により，医師主導型治験の実施が可能となった。医師による厚生労働大臣への治験計画届の提出が許可されたことにより，これまで承認後の市場の小ささにより医薬品・医療機器メーカーが開発に着手しなかったものの医学的には必要性の高い治療法の導入を，医師が主導して行うことが可能となった。一方，治験計画届を提出する医師には薬事法上の責務も加わり，医療機関の負担も大きくなった。

本稿では，医師主導治験で新たに生じる責務と業務について概説し，医療機関における今後の課題について紹介する。

■キーワード：医師主導治験，GCP，薬事法，CRC

1 はじめに

従来の薬事制度においては，医薬品・医療機器メーカーが医療機関に依頼して治験（薬事法第2条に定義される用語）を行う場合のみ，「医薬品の臨床試験の実施の基準に関する省令（GCP：good clinical practice）」（平成9年厚生省令第28号）の遵守のもとで，未承認の医薬品や医療機器の医療機関への提供が認められていた。一方，旧厚生省は平成11年に「適応外使用に係る医療用医薬品の取扱いについて」（研第4号医薬審第104号：いわゆる2課長通知）の中で，「公的な研究事業の委託研究等により実施されるなどその実施に係る倫理性，科学性及び信頼性が確認し得る臨床試験の試験成績がある場合」は，その結果を承認申請の際に提出する資料とできるという姿勢を示しながらも，医師が臨床試験の計画の時点から未承認の医薬品や医療機器の承認申請を目指し，厚生労働大臣への治験計画届等を提出して自ら治験を実施することは認められなかった。

しかし，平成15年7月30日に「薬事法及び採血及び供血あつせん業取締法の一部を改正する法律（平成14年法律第96号：いわゆる「改正薬事法」）」が施行となり，「医師主導型治験」の実施が可能となった。つまり，医師自ら（以下，自ら治験を実施する者）による厚生労働大臣への治験計画届等の提出が認められ，「医薬品の臨床試験の実施の基準に関する省令」の一部を改正する省令（平成15年6月12日　厚生労働省令第106号）（以下，**改正GCP**）を遵守すれば，未承認の医薬品または医療機器の提供を受けて（あるいは購入して），国内未承認薬もしくは新たな効能・効果の追加を目的とした臨床試験が可能となったのである。

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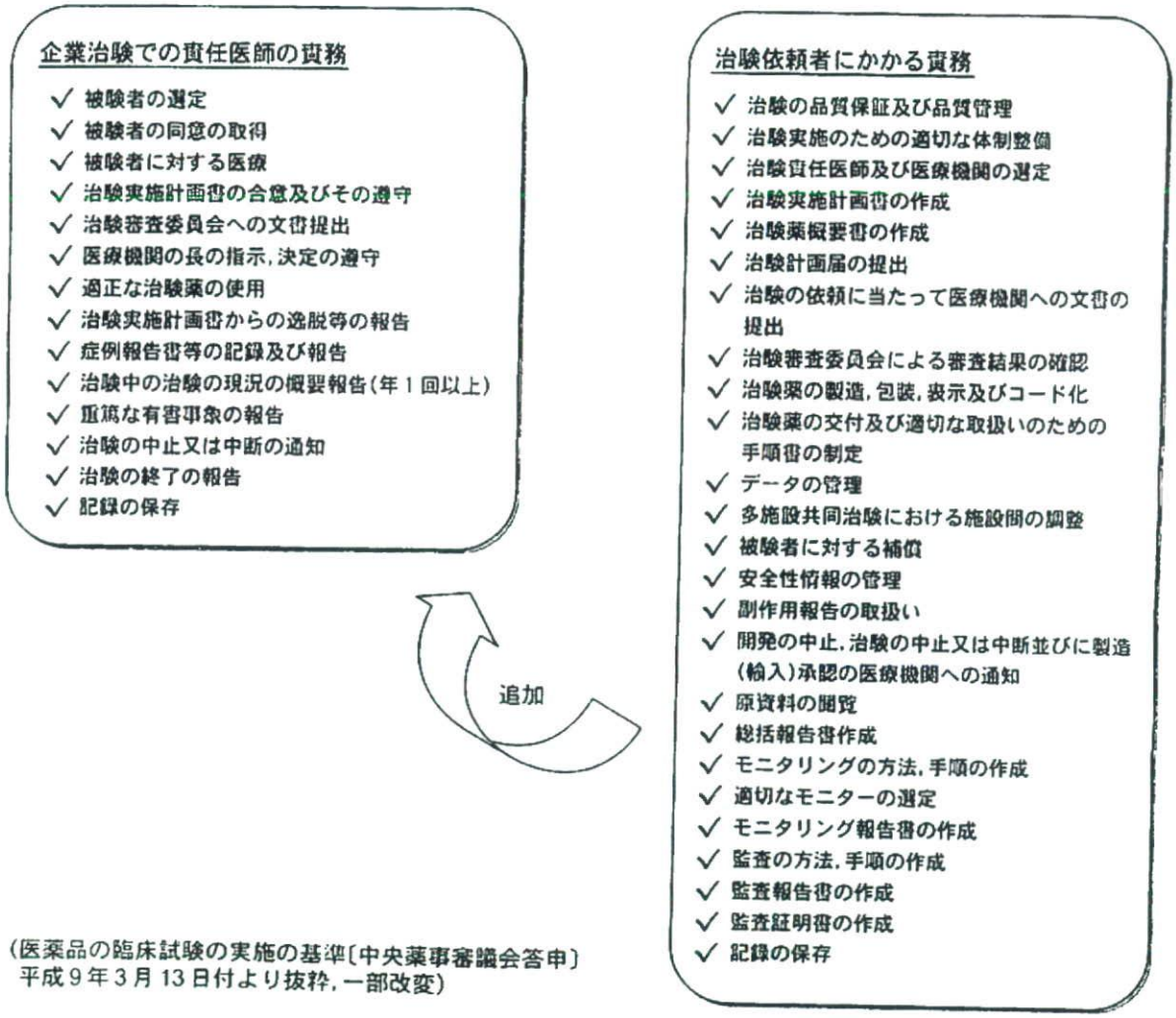

（医薬品の臨床試験の実施の基準〔中央薬事審議会答申〕平成９年３月13日付より抜粋，一部改変）

図1 自ら治験を実施する者の責務

自ら治験を実施する者の責務には，企業治験における責任医師の責務に加えて，治験依頼者の責務も加わる．

医師主導型治験が制度化され，それまで，承認後の市場の小ささなどにより医薬品・医療機器メーカーが開発に興味を示さなかった，がん領域などの罹患数の少ない領域にも，医学的には必要性の高い治療の導入を医師自らが推進していくことが可能となった。しかし，一方では，自ら治験を実施する者は薬事法上の責務を負うことになった。（図1）。

国立がんセンター中央病院の医師が自ら治験を実施する者として実施している医師主導型治験は，平成16年11月2日に治験計画届を提出した「再発あるいは治療抵抗性のc-kitあるいはPDGFR[注]陽性肉腫に対するイマチニブの第Ⅱ相試験」（以下，医師主導型治験イマチニブ）を始めとして，現在実施中の治験２本，準備段階の治験４本である。筆者らは，これら６本の医師主導型治験に，治験調整医師として，また治験調整事務局や自ら治験を実施する者をサポートする臨床研究コーディネーター（CRC）として携わっている。そこで，本稿では，筆者らが行っている医師主導型治験に関連する業務を紹介するとともに，医薬品あるいは医療機器メーカーから依頼を受けて実施する治験（以下，企業治験）と比べて新たに生じる医師の責務，それに伴う業務のいくつかについ

PDGFR：platelet-derived growth factor receptor：血小板由来増殖因子受容体

■表1　医師主導型治験に関連する法令・通知（抜粋）　（平成18年12月末現在）

1）薬事法
（昭和35年法律第145号）
2）薬事法施行規則
（昭和35年厚生省令第1号）
治験の届出，副作用報告等に関して薬事法の詳細を定めたもの
薬事法施行規則の一部を改正する省令
（平成17年12月28日付　厚生労働省令第178号）
医師主導治験の副作用等の報告に係る改正（薬事法施行規則第273条）
3）医薬品の臨床試験の実施の基準に関する省令
（平成9年厚生省令第28号）
いわゆる「GCP省令」
医薬品の臨床試験の実施の基準に関する省令の一部を改正する省令
（平成18年4月1日薬食発第0401001号）
いわゆる「改正GCP」と呼ばれているもの　「医師主導型治験」に関するGCPの規定，治験審査委員会の質及び機能の向上が記述されている。
4）医薬品の臨床試験の実施の基準の運用について
（平成18年9月21日付　薬食審査発第0921001号　厚生労働省医薬食品局審査管理課長通知）
改正GCPの運用に関する詳細な規定。
5）医薬品の臨床試験の実施の基準の運用における必須文書の構成について
（平成16年10月18日付厚生労働省医薬食品局審査管理課事務連絡）
GCPでの必須文書に関する説明と合理化の例を示したもの
6）薬事法及び採血及び供血あっせん業取締法の一部を改正する法律の一部の施行について
（平成15年5月15日付医薬発第0515017号厚生労働省医薬局長通知）
治験計画届書などの記載要領について説明
7）「薬物に係る治験の計画の届出等に関する取扱いについて」の一部改正について
（平成15年6月12日付医薬審発第0612004号厚生労働省医薬局審査管理課長通知）
上記6）の医薬発第0515017号の解説
8）自ら実施する薬物に係る治験の計画の届出等に関する取扱いについて
（平成15年6月12日付医薬審発第0612001号厚生労働省医薬局審査管理課長通知）
自ら治験を実施しようとする者の治験の計画の届出に関する規定
「自ら実施する薬物に係る治験の計画の届出等に関する取扱いについて」の一部改正について
（平成17年10月25日薬食審査発第1025001号　厚生労働省医薬食品局審査管理課長通知）
9）治験薬の製造管理及び品質管理基準及び治験薬の製造施設の構造設備基準（治験薬GMP）について
（平成9年3月31日付薬発第480号厚生省薬務局長通知）
いわゆる「治験薬GMP」
自ら治験を実施する者は，使用する治験薬が「治験薬GMP」に準拠したものであることを証明する必要がある。
10）独立行政法人医薬品医療機器総合機構に対する治験副作用等報告について
（平成16年3月30日付薬食発第0330001号厚生労働省医薬食品局長通知）
自ら治験を実施した者による治験副作用等報告の取扱いについて
（平成17年10月25日付薬食審査発第1025017号厚生労働省医薬食品局審査管理課長通知）
「独立行政法人医薬品医療機器総合機構設立後の自ら治験を実施した者による治験副作用等報告について」の改正について
（平成17年10月25日付薬食審査発第1025005号厚生労働省医薬食品局審査管理課長通知）
「独立行政法人医薬品医療機器総合機構に対する治験副作用等報告に関する報告上の留意点等について」の改正について
（平成17年10月25日付薬食審査発第1025013号厚生労働省医薬食品局審査管理課長通知）

表1 医師主導型治験に関連する法令・通知（抜粋）（つづき）

治験副作用等報告に関する報告上の留意点等について
（平成18年4月26日付 薬食審査発第0426001号 厚生労働省医薬食品局審査管理課長通知）

11) 薬物に係る治験に関する副作用等の報告に係る薬事法施行規則の一部を改正する省令の施行について
（平成17年12月28日付 薬食発第1228001号厚生労働省医薬食品局長通知）

12)「療担規則及び薬担規則並びに療担基準に基づき厚生労働大臣が定める掲示事項等」及び「選定療養及び特定療養費用に係る厚生労働大臣が定める医薬品等」の制定に伴う実施上の留意事項について」の一部改正について
（平成17年3月31日付保医発第0331011号厚生労働省保険局医療課長通知）

13) 自ら治験を実施する者による医薬品の臨床試験の実施の基準に関するQ&Aについて
（平成17年10月25日 付事務連絡）

14) 医薬品GCP実地調査の実施要領について
（平成18年1月31日付 薬食審査発第0131006号 厚生労働省医薬食品局審査管理課長通知）

15) 新医薬品の承認申請資料に係るGCP実地調査の実施手続きについて
（平成18年2月15日付 独立行政法人医薬品医療機器総合機構 信頼性保証部長 事務連絡）

16) 治験の総括報告書の構成と内容に関するガイドラインについて
（平成8年5月1日付薬審第335号厚生省薬務局審査課長通知）

て紹介してみたい。

2 医師主導治験における自ら治験を実施する者の責務

医師主導型治験では改正GCPの中で，自ら治験を実施する者に，企業治験における治験依頼者（医薬品・医療機器メーカー）と全く同じ事務手続きと品質保証（監査）・品質管理（モニタリング）を要求されており，実施に際しては表1に一部抜粋したような，医師主導型治験に関係する法律（薬事法），政令（薬事法施行令），省令（薬事法施行規則，改正GCP），通知（局長通知，課長通知），事務連絡に十分に目を通して，その内容を理解しておく必要がある。以下に医師主導型治験において新たに加わる責務の概略を紹介する。

1．治験薬の確保（改正GCP第26条の2）

医師主導型治験の法制上の整備はされたが，実際にひとつの治験を実施するためには当然のことながら対象となる治験薬（または医療機器）の確保が必須である。しかし，製造工場を持たない医師には製造管理及び品質管理規則（GMP）を遵守した治験薬の製造は不可能である。実際には，医薬品メーカーから提供（もしくは購入）をうけることが必要となる。医師主導型治験イマチニブでは，まず，プロトコールコンセプトの策定とイマチニブを製造・販売している日本法人との接触を開始し，海外本社のProtocol Review Committeeによるプロトコールコンセプトの審査をパスし，ようやく治験薬の提供を受けることができた。また，他の治験では，すでに当該医薬品の国内承認から長い年月が経過しており当該治験薬を保有する医薬品メーカー内でも企業治験は実施していないため，治験薬製造ラインが確保できず，改正GCP第26条の2の要求を満たす治験薬の提供（いわゆる白箱提供）を受けることができなかった。対応策として，市販薬の容器および被包を変更をして提供を受ける方法を検討中である。

2．治験の準備段階（改正GCP第2章第2節）

1）標準業務手順書（SOP）作成

準備段階で，最も大変な作業は，改正GCP第15条の2に規定される各種のSOPの作成である。医師主導型治験イマチニブ開始前に作成したSOP一覧を表2に示す。このSOPの作成は病院の体制に関するSOPから当該治験特有のSOPまで多岐にわたる。筆者の着任前であるが，当院においても医師主導型治験イマチニブ開始前に，当時の病院長である野村和弘院長の指示により「医師主導型治験に対する国立がんセンターの対応に関する検討会」が設置され（事務局長は西條長宏薬物療法部長〔当時〕が担当），運営部，看護部，

表2　医師主導治験イマチニブの研究申込前に作成した業務手順書一覧

医療機関における業務手順書（1 ～ 6）は，当院最初の医師主導治験実施前に作成し，その後の医師主導治験では共通の手順書として使用している。個々の治験のために作成する業務手順書は，治験実施計画書に合わせて作成する（7 ～ 22）。

研究申込前に作成した業務手順書一覧

【医療機関における業務手順書等】
①国立がんセンター医師主導治験取扱規程
②国立がんセンター医師主導治験標準業務手順書
③医師主導治験における監査の受入に関する標準業務手順書
④医師主導治験におけるモニタリングの受入に関する標準業務手順書
⑤医師主導治験における国立がんセンター治験審査委員会標準業務手順書
⑥国立がんセンター医師主導治験審査予備調査会規定

【医師主導治験イマチニブのために作成した業務手順書】
⑦治験実施計画書および症例報告書の作成に関する標準業務手順書
⑧治験薬概要書の作成に関する標準業務手順書
⑨安全性情報に関する標準業務手順書
⑩被験者の補償に関する標準業務手順書
⑪治験薬の取り扱い標準業務手順書
⑫モニタリングに係る標準業務手順書
⑬監査に関わる標準業務手順書
⑭自ら治験を実施する者に係る標準業務手順書
⑮治験調整医師に係る標準業務手順書
⑯効果安全性評価委員会に係る標準業務手順書
⑰効果判定委員会に係る標準業務手順書
⑱病理中央診断実施手順書
⑲登録業務に関する標準業務手順書
⑳データ取扱いに関する標準業務手順書
㉑記録の保管に関する標準業務手順書
㉒総括報告書の作成に関する標準業務手順書

薬剤部も含めたメンバーにより医師主導型治験実施をめぐる実務的な問題点の洗い出しと，それらへの対応策の検討を行ったと聞いている。次に続く医師主導型治験からは，これらのSOPを雛型とするそれぞれの治験特有の手順書作成に作業は絞られ負担は軽減した。とはいえ，自ら治験を実施しようとする者は，医師主導型治験の実施は初めての場合が多く，これらのSOPを臨床現場で働く医師自らが作成することは大変な作業である。

2）医療機関の長への研究申込

医師主導型治験の特徴のひとつに，薬事法第80条の2の2に規定されている厚生労働大臣への治験計画の届出に先だって，自ら治験を実施する者が，あらかじめ改正GCP第15条の7に規定された文書を実施医療機関の長に提出し，治験の実施の承認を得なければならない点がある。当院で，医療機関の長への研究申込の際に提出した資料の一覧を表3に示す。企業治験では，治験審査委員会とのやり取りにおいて，審議資料の作成から申請までを企業の臨床開発担当者に大きく依存してしまうのが常であるが，医師主導型治験では，医師やCRC，治験事務局等の医療機関で医師主導型治験実施に携わる者たち自身がすべてその業務を担うことになる。文書の作成が最も大変な作業であることは当然であるが，表3で示した文書を体裁を整えて一塊の資料を作成するにも，相当な作業が発生する。実際，当院では，研究申込時には，数名のCRCと事務担当者が1日がかり