

A Highly Sensitive Method for the Detection of *p16* Methylation in the Serum of Colorectal Cancer Patients

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Abstract. *Background: For the purpose of early detection of carcinomas, detection of p16 hypermethylation by methylation-specific polymerase chain reaction (MSP) in the serum of many kinds of malignancies has been introduced. An attempt to increase the sensitivity of this assay is reported for colorectal cancer. Materials and Methods: DNA samples were subjected to limiting dilution before being divided into ten smaller samples. Subsequently, hemi-nested MSP was performed on those ten samples. The limiting dilution-MSP (LD-MSP) provided a 10-fold increase in sensitivity of the detection of methylated DNA compared with conventional MSP. Results: Of 44 colorectal cancer patients, 30 (68%) exhibited abnormal promoter methylation of p16 in their serum DNA by LD-MSP, while 13 (30%) exhibited it by conventional MSP. As a control, the serum DNA of 50 patients with colorectal carcinomas whose corresponding tumor DNA had no methylation in the p16 promoter was screened for aberrant methylation. No methylation was found in the serum DNA of this control group by LD-MSP or conventional MSP. Conclusion: The high sensitivity of LD-MSP makes it possible to detect smaller amounts of tumor DNA in the serum than conventional MSP. This technique also has great specificity and no abnormal methylation in serum has yet been observed if the corresponding tumor does not exhibit methylation. This observation supports the idea that LD-MSP could be applied in clinical use for the detection of tumor DNA in serum.*

Previous studies have proposed that enriched circulating DNA can be found in the serum of cancer patients (1, 2).

Abbreviations: MSP, methylation-specific PCR; LD-MSP, limiting dilution-MSP.

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On the basis of these studies, many attempts have been made to achieve the early detection of tumor-related aberrant DNA in the serum of patients with various malignancies (3, 4). Our studies have also shown that it is possible to detect tumor-specific DNA in the serum of various cancer patients using a mismatch ligation assay for K-ras and mitochondrial DNA mutations (5-8).

Recent studies have indicated that promoter methylation is an important mechanism for gene transcriptional inactivation. Several genes such as *p16* (9), *p14* (10), *HLTF* (11), *SOCS-1* (12) and *CDH13* (13) have been found to harbor promoter hypermethylation associated with a loss of gene expression in digestive tract carcinomas. The presence of epigenetic methylation might be useful as a molecular target for the detection of tumor DNA. In particular, hypermethylation of the *p16* tumor suppressor gene has been detected in the serum of many kinds of malignancies such as esophageal cancer, colorectal cancer, non-small cell lung cancer, liver cancer, and head and neck cancer (14-18).

Previously, we examined the methylation status of *p16* in colorectal carcinomas using a methylation-specific polymerase chain reaction (MSP) (16). Forty seven percent of the cancer DNAs exhibited abnormal promoter methylation of the *p16* gene and in 30 percent of those cases abnormal methylation was detected in the serum DNA. However, there is still room for improvement in the MSP technique since this methylation marker was not detected in the serum of the other 70% of cases.

To increase the sensitivity of detection of *p16* promoter methylation in the serum of cancer patients, we have developed a limiting dilution-MSP (LD-MSP). This method was derived from the concept of digital PCR, which aims to isolate single DNA molecules by dilution and amplify them by PCR (19).

Materials and Methods

Sample collection and DNA preparation. Tumor and corresponding normal tissue samples were obtained at the time of surgery from 94 patients with primary colorectal carcinoma at Nagoya University

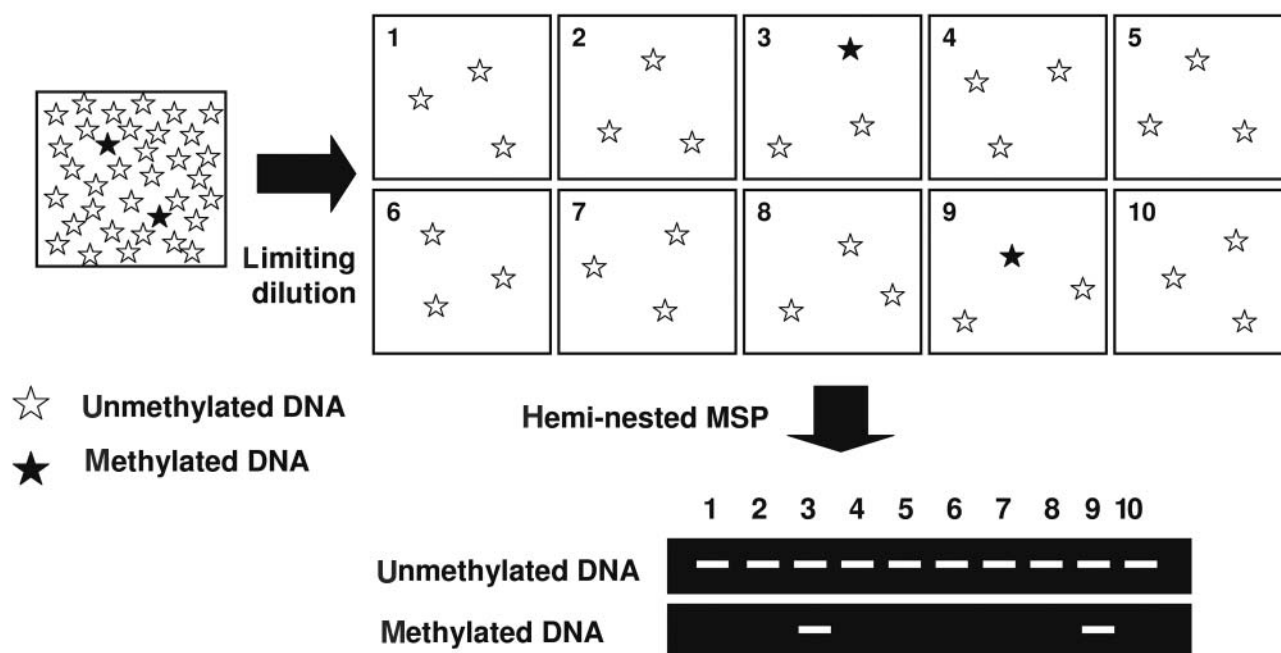


Figure 1. Schematic representation of LD-MSP.

Hospital, Nagoya, Japan. Corresponding serum samples had been obtained one week prior to surgery. Written informed consent, as indicated by the institutional review board, was obtained from all patients. The tumor, normal tissue and serum samples were immediately frozen and stored at -80°C until the DNA was extracted. The samples were digested with proteinase K and DNA was prepared as described previously (20).

Bisulfite modification. The DNA samples were modified by sodium bisulfite as previously described (14). Briefly, 1 μg of DNA was denatured with NaOH and modified by sodium bisulfite. The DNA samples were then purified using Wizard purification resin (Promega Corp., Madison, WI, USA), treated again with NaOH, precipitated with ethanol and resuspended in water.

Conventional MSP. The methylation status of the CpG island of the *p16* gene was first determined by MSP. The modified DNA samples were amplified by primers specific for both methylated and unmethylated sequences. These primer sequences have been described previously (14). The PCR amplification of the modified samples consisted of 1 cycle of 95°C for 5 min; 1 cycle of 78°C for 10 min, 33 cycles of 95°C for 30 sec, 69°C for 1 min, and 72°C for 1 min; and 1 cycle of 70°C for 10 min. DNA from L132 (embryonic lung cell line) and H1299 (lung cancer cell line) was used as positive control for unmethylated and methylated alleles, respectively. Controls without DNA were performed for each PCR set. Ten μl of each PCR product was directly loaded onto non-denaturing 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination. Each MSP was repeated at least three times.

LD-MSP. Conventional MSP can detect methylated DNA in 1,000-fold molar unmethylated DNA, which is sensitive enough to detect tumor DNA in the serum of the cancer patients described previously

(16). However, it is difficult to detect methylated DNA in 10,000-fold molar unmethylated DNA using this method. In LD-MSP, a limiting dilution was performed on the DNA samples so as to yield about one or two copies of methylated DNA among 10,000 copies of unmethylated DNA. This diluted sample was then divided into ten smaller samples. Statistically, at least one or two samples would include about one copy of methylated DNA among 1,000 copies of unmethylated DNA, whereas the other samples would only include unmethylated DNA. Subsequently, hemi-nested MSP was performed on these ten samples so that PCR products for methylated DNA could be shown in the samples with a copy of methylated DNA among 1,000 copies of unmethylated DNA. As a result, LD-MSP provided a 10-fold increase in sensitivity in the detection of methylated DNA compared with conventional MSP. A schematic representation of the LD-PCR process is shown in Figure 1.

In hemi-nested MSP, primer sequences for the first amplification of unmethylated DNA were *p16* UM2S (sense), 5'-TTATTAGAGGGTGGGGTGG ATTGT; and *p16* UMAS (antisense), 5'-CAACCCCAAACCACA ACCATAA. The primers for the first amplification of methylated DNA were *p16* M2S (sense), 5'-TTATTAGAGGGTGGGGCG GATCGC; and *p16* MAS (antisense), 5'-GACCCCGAACCGCGA CCGTAA. The composition of the reactions was as described previously (19). The first PCR amplification of the modified DNA samples consisted of 1 cycle of 95°C for 5 min; 33 cycles of 95°C for 30 sec, 69°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. The primers and amplification conditions for the second PCR were described above as in conventional MSP.

Statistical analysis. The correlation between the *p16* methylation status of the serum samples and the clinicopathological characteristics of the patients was evaluated. Statistical significance was evaluated by Fisher's exact test.

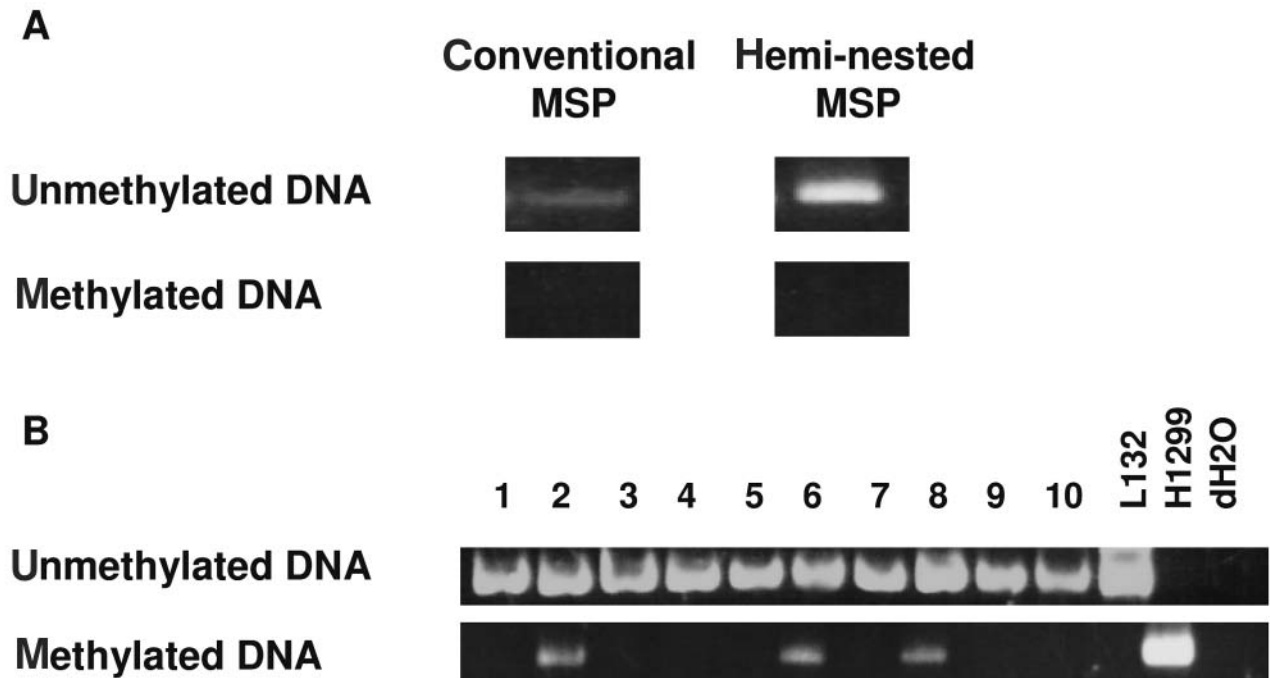


Figure 2. LD-MSP control study. 0.5 pg of methylated DNA derived from a H1299 cell line was mixed with 5 ng of unmethylated DNA derived from an L132 cell line. A, Conventional MSP or hemi-nested MSP could not amplify methylated DNA in the mixture at this concentration. B, The mixture was divided into 10 samples that were then amplified by LD-MSP. Methylated DNA was successfully amplified in 3 of the divided samples.

Results

To determine the amount of DNA corresponding to one genome equivalent, modified DNA samples were serially diluted and tested *via* hemi-nested MSP. The limiting amount that yielded amplified products was about 0.5 pg of DNA, which was used in the subsequent LD-MSP experiments. As a control study, a mixture of 0.5 pg of methylated DNA derived from the H1299 cell line and 5 ng of unmethylated DNA derived from the L132 cell line were prepared. Conventional and hemi-nested MSP could not amplify the methylated DNA in the control mixture (Figure 2A), when it was subsequently divided into 10 smaller samples and amplified by LD-MSP, as expected, the methylated DNA was successfully amplified in three of the divided samples (Figure 2B).

We next examined the methylation status of *p16* in the serum DNA of colorectal cancer patients whose corresponding tumor DNA exhibited *p16* methylation. Out of the 94 colorectal cancer patients, 44 had tumor DNA which exhibited methylation in the *p16* promoter. Of these, 30 (68%) exhibited abnormal promoter methylation of *p16* in their serum DNA by LD-MSP, while 13 (30%) exhibited it by conventional MSP (Table I). Representative results of LD-MSP for *p16* methylation are shown in Figure 3. The serum DNA of the 50 patients with colorectal cancers whose corresponding tumor DNA had no methylation in the *p16*

Table I. *p16* promoter methylation in tumor and serum DNA.

<i>p16</i> methylation in tumor	No. of cases	<i>p16</i> methylation in serum			
		Conventional MSP		LD-MSP	
		+	-	+	-
+	44	13 (30%)	31	30 (68%) ¹	14
-	50	0	50	0	50

¹Thirty cases detected by LD-MSP include the 13 detected by conventional MSP.

promoter was also screened for aberrant methylation. No methylation was found in the serum DNA of this control group by either LD-MSP or conventional MSP.

After completion of LD-MSP analysis in all specimens, clinicopathological data were correlated with the molecular analysis (Table II). No association of overall aberrant methylation in the serum DNA with age, sex, histologic type, depth of invasion, stage of disease, or presence of lymph node metastasis was found. Interestingly, abnormal methylation was found in the serum of patients at all clinical stages, suggesting that colorectal cancer could be detected from its early stages using the LD-MSP technique.

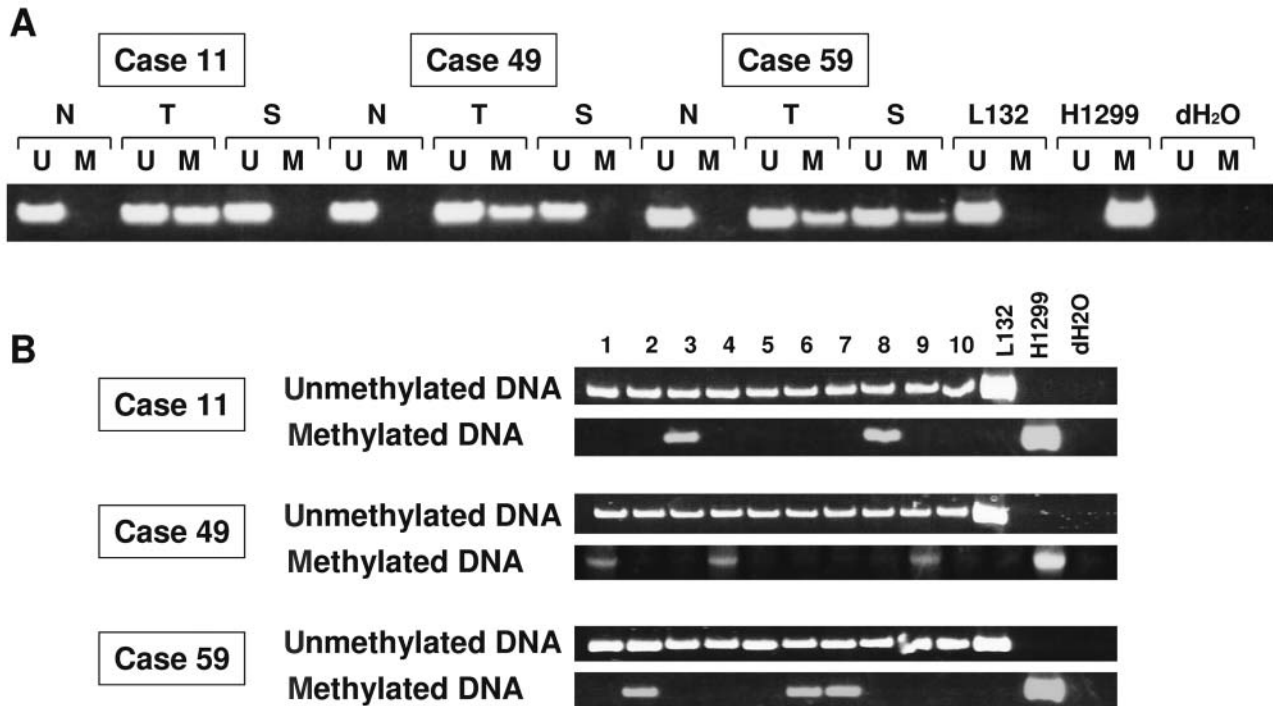


Figure 3. Representative LD-MSP and conventional MSP of *p16* promoter hypermethylation in the serum samples from primary colorectal cancers. *A*, Conventional MSP. A visible PCR product in lane U indicates the presence of unmethylated genes; a visible product in lane M indicates the presence of methylated genes in all cases. Only case 59 showed methylation in serum DNA by conventional MSP. In each case, modified DNAs from L132 and H1299 were used as positive controls for unmethylated and methylated alleles, respectively. *B*, LD-MSP. Cases 11, 49, and 59 showed methylation in serum DNA by LD-MSP. N: normal tissue, T: tumor tissue, S: serum; 1-10: samples for LD-MSP analysis.

Discussion

In previous studies, tumor-related aberrant DNA has been identified in the serum of cancer patients. In particular, hypermethylation of the *p16* tumor suppressor gene has been studied in various malignancies such as esophageal cancer, colorectal cancer, non-small cell lung cancer, liver cancer, and head and neck cancer (14-17, 21). The MSP technique is a beneficial procedure for the detection of promoter hypermethylation because of its high sensitivity and specificity. No abnormal methylation was found in serum DNA if this alteration was not present in the primary tumor. Moreover, MSP has sufficient sensitivity to detect even a very low level of tumor DNA in serum, having detected approximately one methylated gene copy per 1000 unmethylated copies in dilution experiments (15). In clinical use, however, a more sensitive method is required.

As shown in our study, abnormal promoter methylation of *p16* in serum DNA was detected in 68% of patients by LD-MSP, compared to 30% by conventional MSP, indicating that LD-MSP could detect methylated DNA of *p16* twice as often as conventional MSP. This improved sensitivity might be sufficient for clinical use, since not all

Table II. Clinicopathological features and *p16* promoter methylation in serum DNA.

Clinico-pathological feature	Variable	No. of cases	<i>p16</i> methylation		<i>p</i> -value ¹
			+	-	
Age	<60	17	11	6	0.99<
	60	27	19	8	
Gender	male	24	15	9	0.52
	female	20	15	5	
Histologic type	well ² , mod ³	40	27	13	0.99<
	poor ⁴ , muc ⁵	4	3	1	
Depth of tumor	≤mt ⁶	9	6	3	0.99<
	mt<	35	24	11	
Max. tumor size	≤5 cm	21	14	7	0.99<
	5 cm<	23	16	7	
TNM stage	0, 1, 2	20	13	7	0.75
	3, 4	24	17	7	
Lymph node metastasis	+	22	13	9	0.33
	-	22	17	5	
Total		44	30	14	

¹Fisher's exact test; ²well-differentiated adenocarcinoma; ³moderately-differentiated adenocarcinoma; ⁴poorly-differentiated adenocarcinoma; ⁵mucinous adenocarcinoma; ⁶muscular tunic.

colorectal cancer patients invariably produce tumor DNA in their serum. In addition, no methylation was found in the serum DNA of the fifty patients with colorectal carcinomas whose corresponding tumor DNA had no methylation in *p16*, suggesting that LD-MSP was specific for the detection of methylated DNA.

In conclusion, LD-MSP has several advantages in detecting tumor DNA in the serum of cancer patients. Its high sensitivity makes it possible to detect smaller amounts of tumor DNA in the serum than conventional MSP. This technique also has great specificity, and no abnormal methylation in serum has yet been observed if the corresponding tumor does not exhibit methylation. This observation supports the idea that LD-MSP could be applicable to clinical use in the detection of tumor DNA in serum. Although *p16* methylation of serum DNA is not specific for colorectal cancer, LD-MSP could be used to detect a wide variety of tumors with aberrant methylation in the promoters of genes. The methylation status of a primary tumor does not need to be known in advance of detecting circulating tumor DNA, suggesting that LD-MSP could be used as a screening method for cancer.

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