

***p16^{INK4a}* Methylation in Serum as a Follow-up Marker for Recurrence of Colorectal Cancer**

GORO NAKAYAMA, YASUHIRO KODERA, NORIFUMI OHASHI, MASAHIKO KOIKE,
MICHITAKA FUJIWARA and AKIMASA NAKAO

Gastroenterological Surgery, Nagoya University Graduate School of Medicine Nagoya, Aichi 466-8560 Japan

Abstract. *Background:* *p16^{INK4a}* methylation present in the tumors of colorectal cancer (CRC) patients can be detected in their serum using quantitative methylation-specific PCR (Q-MSP). To investigate the possibility that this technique could be applied to the monitoring for cancer recurrence in CRC patients, *p16^{INK4a}* methylation in the serum of CRC patients during their follow-up period was evaluated. *Materials and Methods:* Using Q-MSP on serum samples from 21 CRC patients undergoing surgery for primary CRC, the *p16^{INK4a}* methylation score (*p16^{INK4a}* MS) was evaluated one day before surgery and during the follow-up period. *Results:* In the serum samples collected before primary resection, *p16^{INK4a}* methylation was detected in 8 out of the 13 patients with same methylation in the tumor. The *p16^{INK4a}* MS decreased within 2 weeks after surgery. Only two patients, who had the potential for recurrence, exhibited *p16^{INK4a}* methylation in their serum. One month after surgery, in the patients with recurrence of tumor, a dramatic increase in *p16^{INK4a}* MS was observed, while in the disease-free patients no methylation was seen continuously. *Conclusion:* *p16^{INK4a}* MS could sensitively reflect the recurrence status and may be useful for identifying the presence of recurrence during the follow-up of CRC patients.

Colorectal cancer (CRC) is one of the most common and fatal carcinomas in the world (1). The earlier detection of recurrence would help to reduce deaths from this disease. Although much progress has been made in the identification and characterization of the genetic changes in CRC, there are few reports of the detection of recurrence using molecular biological techniques. Previous studies have proposed that tumor DNA is released into the circulation and is present in

plasma and serum (2-5). The precise mechanism of the release of DNA into the bloodstream remains to be proven, however the major sources of circulating DNA were suggested to be apoptotic and necrotic cancer cells (5-7). Accordingly, it is possible to detect tumor-specific DNA, such as genetic or epigenetic alterations identified in the primary tumor DNA, in the serum of patients with various cancers (8).

We previously proved that *p16^{INK4a}* promoter methylation, present in the tumors of CRC patients, can be detected in the serum of those same patients using quantitative methylation-specific PCR (Q-MSP) (9), which indicated that this approach could potentially be useful for screening and monitoring the disease. To investigate the possibility that this technique could be applied to the monitoring of cancer recurrence during the follow-up of CRC patients, the *p16^{INK4a}* methylation level in the serum of patients with CRC collected before surgery and during the follow-up period was examined using Q-MSP. Since CEA in the serum has been used as the orthodox tumor marker for monitoring CRC occurrence, CEA was also measured in this study.

Materials and Methods

Sample collection and DNA preparation. Peripheral blood samples were collected from 20 healthy donors and from 21 sporadic CRC patients who underwent surgical resection at Nagoya University Hospital, Nagoya, Japan. The CRC patient blood samples were obtained on the day before surgery and during the follow-up period at 2 weeks and 1, 3 and 6 months after surgical intervention. Tumor and corresponding normal tissue samples were obtained at the time of surgery from the 21 primary CRC and from two metastatic CRC. Written informed consent, as approved by the institutional review board, was obtained from all the 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 the DNA was extracted.

Bisulfite modification. The DNA samples (1 μg) were denatured by NaOH and modified by sodium bisulfite as described elsewhere (10). The DNA samples were then purified using Wizard purification resin (Promega Corp, Madison, WI, USA), treated again with NaOH, precipitated with ethanol, then resuspended in water.

Correspondence to: Goro Nakayama, Gastroenterological Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan. Tel: +81 527442250, Fax: +81 527442252, e-mail: goro@med.nagoya-u.ac.jp

Key Words: *p16^{INK4a}*, methylation, colorectal cancer.

Table I. *p16^{INK4a}* methylation score (MS) according to tumor stage.

TNM stage	Rec	n	<i>p16^{INK4a}</i> methylation in tumor	<i>p16^{INK4a}</i> MS in serum (mean value/number of <i>p16^{INK4a}</i> MS≥1.0%)							
				Before surgery	After surgery						
					2 weeks	1 month	3 months	2 weeks	1 month	3 months	
2	-	10	6	1.9	2	0.1	0	0	0	0	0
3	-	9	5	4.8	4	0.5	0	0	0	0	0
	+	1	1	19.8	1	9.1	1	6.1	1	6.4	1
4	+	1	1	5.4	1	-	1	3.2	1	5.3	1
Total		21	13	4.2 ^a	8	0.8 ^b	2	0.4 ^a	2	0.6 ^a	2

Rec, recurrence; ^an=21; ^bn=20.

Quantitative methylation-specific PCR. Q-MSP was performed in an ABI sequence detection system 7000 using two amplification primers and a dual-labeled fluorogenic hybridization probe (Applied Biosystems, Foster City, CA, USA). The *p16^{INK4a}* primers for RTQ-PCR have been described elsewhere (11). The sense and antisense primers and fluorogenic probe for the methylated sequence were *p16^{INK4a}* MS (5'-TTATTAGAGGGTGGGGCGGATCGC-3'), *p16^{INK4a}* MAS (5'-GACCCCGAACCGCGACCGTAA-3') and *p16^{INK4a}* MT [5'-(FAM)-AGTAGTATGGAGTCGGCGGGCGGG-(TAMRA)-3'], respectively. The sense and antisense primers and fluorogenic probe for the unmethylated sequence were *p16^{INK4a}* US (5'-TTATTAGAGGGTGGGGTGGATTGT-3'), *p16^{INK4a}* UAS (5'-CAACCCCAAACCACAACCATAA-3') and *p16^{INK4a}* UT [5'-(FAM)-AGGTAGTGGGTGGGGAGTAGTATGGAGμTTG-(TAMRA)-3'], respectively.

Thermocycling was performed in a final volume of 50 μl containing 5.0 μl of bisulfite-converted DNA from 180 μl of serum sample, 900 nM each of the *p16* primers (sense and antisense), 300 nM of the corresponding fluorogenic probe, 5.0 μl PCR buffer, 2.5 μl dNTP mix, and 1.5 units of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). The PCR amplification consisted of 50 cycles (95°C for 15 sec, 60°C for 60 sec, and 72°C for 18 sec) after an initial denaturation step (95°C for 10 min). Multiple negative water blanks were included in each analysis.

The amplification data were analyzed using the Sequence Detection System software developed by Applied Biosystems. The standard curve was established for quantifying the DNA copy number, using 9 known copy numbers of serially diluted (1 to 10⁸ copies) plasmids containing *p16^{INK4a}* methylated or unmethylated DNA. The Q-MSP assays were conducted in triplicate for each sample and the mean value (Q) was used for further calculation. The concentration (C) of *p16* methylated or unmethylated DNA in the serum (genome-equivalents/μl) should be Q/180 because the 5.0 μl of bisulfite-converted DNA applied to RTQ-PCR was derived from 180 μl of serum. *p16^{INK4a}* methylation score (*p16^{INK4a}*MS) was defined as: *p16^{INK4a}* MS (%) = CM/(CM+CU) × 100, where CM=concentration of *p16^{INK4a}* methylated DNA in the serum and CU=concentration of *p16^{INK4a}* unmethylated DNA in the serum.

Results

p16^{INK4a} methylation in tumor tissues. Out of the 21 primary CRC tumor samples, 13 (62%) exhibited *p16^{INK4a}* promoter

Table II. Clinicopathological features and *p16* methylation scores in serum DNA collected one day before surgery.

Clinicopathological feature	Variable	No. of cases	<i>p16^{INK4a}</i> MS (mean)	<i>p</i> -value ^a
Gender	male	12	4.01	0.71
	female	9	6.67	
Age	≤65	8	3.95	0.27
	65<	13	5.96	
Location of tumor	colon	12	2.38	0.63
	rectum	9	4.25	
Size (max)	≤5cm	11	4.83	0.91
	5cm<	10	4.37	
Lymph node metastasis	-	10	1.93	0.29
	+	11	4.10	
TNM stage	2	10	1.93	0.29
	3,4	11	4.10	

p16^{INK4a} MS, *p16^{INK4a}* methylation score; ^aMann-Whitney *U*-test.

hypermethylation (Table I) while no corresponding normal tissue samples exhibited *p16^{INK4a}* methylation.

p16^{INK4a} methylation in serum samples before surgery. In the corresponding serum samples collected one day before surgery, *p16^{INK4a}* methylation was detected in 8 out of the 13 patients (62%) with *p16^{INK4a}* methylation in the tumor DNA (Table I). The mean value of *p16^{INK4a}* MS in the serum DNA was 4.2% (range 0-19.8%), with a median value of 5.0%. The *p16^{INK4a}* MS according to the clinicopathological background of the patients is summarized in Table II. These data were derived from our previous study of 168 primary CRC patients (9). As a control, no methylation was found in the serum DNA from 20 healthy donors and the 8 CRC patients whose corresponding tumor DNA had no *p16^{INK4a}* methylation.

p16^{INK4a} methylation in serum samples during the follow-up period after surgery. At 2 weeks after primary resection, the CRC patients displayed a noticeable decrease in *p16^{INK4a}*

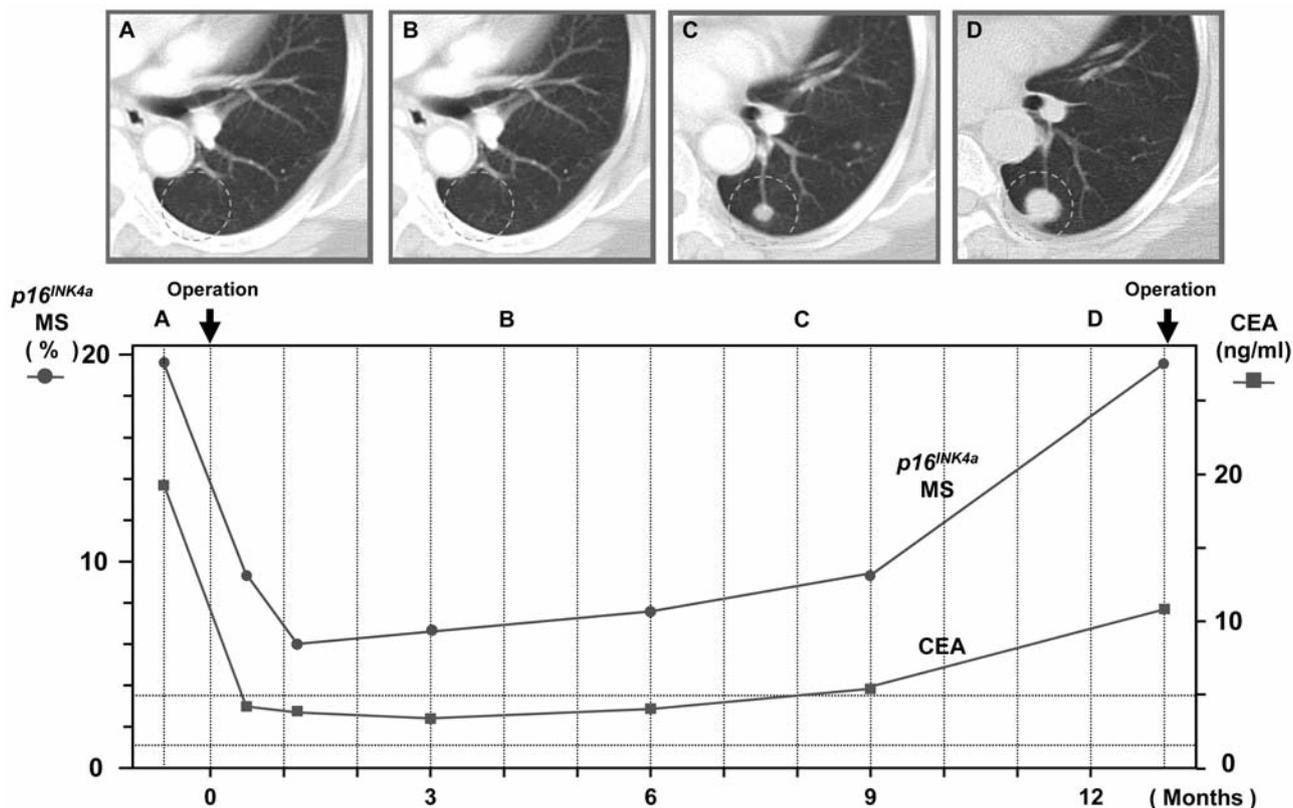


Figure 1. The clinical course of case 13 with colon cancer stage 3. Recurrence in the lung, $p16^{INK4a}$ methylation exhibited in the serum DNA from 2 weeks after surgical resection of the primary lesion and a dramatic increase in $p16^{INK4a}$ MS 1 month after surgery.

MS (Table I). The mean $p16^{INK4a}$ MS observed was 0.8% (range 0-9.1%). Only 2 patients, who had residual metastatic tumor or would have recurrence later, exhibited $p16^{INK4a}$ methylation in their serum DNA.

At 1 and 3 months after surgical intervention, the mean $p16^{INK4a}$ MS was 0.4% (range 0-6.1%) and 0.6% (range 0-6.4%). In the patients with recurrence of tumor a dramatic increase of $p16^{INK4a}$ MS was observed, while in the disease-free patients no methylation was seen continuously. Figures 1 and 2 show the clinical course of the patients with recurrence. In each case the $p16^{INK4a}$ MS in the follow-up period reflected more sensitively the recurrence status than CEA.

Discussion

In our previous studies $p16^{INK4a}$ methylation was detected in 60% of CRC tumor tissues (12), the same abnormal change was detected in the serum of almost 40% of primary CRC patients and of 70% of recurrent CRC patients with $p16^{INK4a}$ methylation in the tumor (12), the $p16$ methylation level in the serum of CRC patients significantly increased with the tumor stage and was closely associated with the malignancy of the carcinoma (9) and patients with high $p16$ methylation

level in the serum had significantly worse survival rates than those with a low $p16$ methylation level (9).

In the present study, after surgical resection of the primary lesions, the $p16^{INK4a}$ methylation level in the serum DNA decreased within 2 weeks, except in those patients who had residual metastatic tumor or would have recurrence later. In the follow-up period, in the patients with recurrence of tumor a dramatic and sensitive increase in $p16^{INK4a}$ MS was observed, while in the disease-free patients no methylation was seen continuously.

At the time of tumor recurrence, the $p16^{INK4a}$ methylation level was elevated earlier than CEA in both cases. Tumor recurrence could also be detected earlier by $p16^{INK4a}$ methylation compared to CEA because $p16^{INK4a}$ methylation could be detected in the serum at an early tumor stage (TNM Stage I) whereas CEA elevation requires an advanced tumor mass (9, 10, 13). Although studies with a larger series of patients with CRC and with a longer follow-up period are required to confirm this possibility, $p16^{INK4a}$ MS could sensitively reflect the recurrence status and may be useful for identifying the presence of a recurrence or metastasis during the follow-up of CRC patients. $p16^{INK4a}$ MS may also be useful prospectively to identify high-risk individuals.

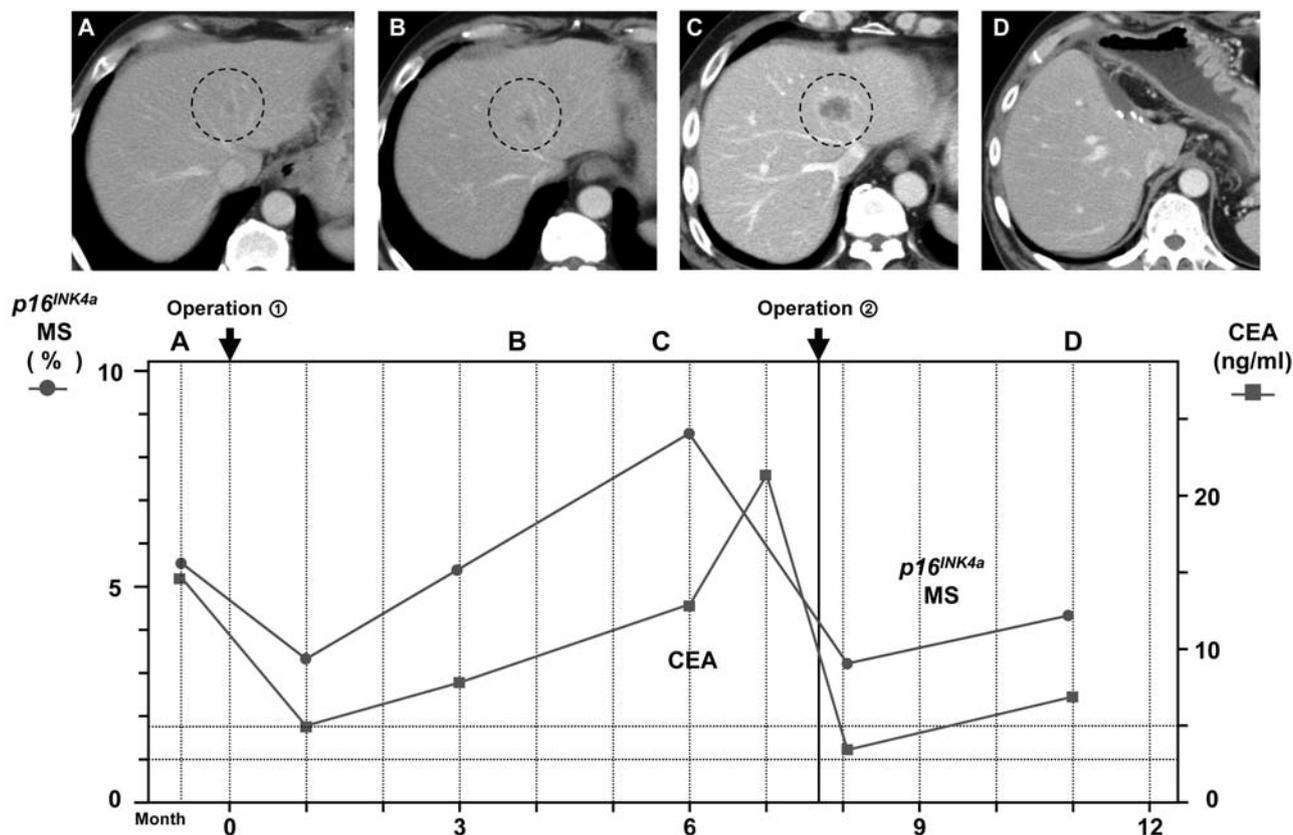


Figure 2. The clinical course of case 21 with rectal cancer stage 4. Residual metastatic liver tumor, p16^{INK4a} methylation exhibited in the serum DNA from 1 month after surgical resection of the primary lesion.

References

- 1 Greenlee RT, Murray T, Bolden S and Wingo PA: Cancer statistics. *CA Cancer J Clin* 50: 7-33, 2000.
- 2 Esteller M, Sanchez-Cespedes M, Rosell M, Sidransky D, Baylin SB and Herman JG: Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* 59: 67-70, 1999.
- 3 Wong IHN, Lo YMD, Zhang J, Liew CT, Ng MHL, Wong N, Lai PBS, Lau WY, Hjelm NM and Johnson PJ: Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. *Cancer Res* 59: 71-73, 1999.
- 4 Frattini M, Gallino G, Signoroni S, Balestra D, Lusa L, Battaglia L, Sozzi G, Bertario L, Leo E, Pilotti S and Pierotti MA: Quantitative analysis of plasma DNA in colorectal cancer patients: a novel prognostic tool. *Ann NY Acad Sci* 1075: 185-190, 2006.
- 5 Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH and Koch WM: Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res* 60: 892-895, 2000.
- 6 Ziegler A, Zangemeister-Wittke U and Stahel RA: Circulating DNA: a new diagnostic gold mine? *Cancer Treat Rev* 28: 255-271, 2002.
- 7 Linder S, Havelka AM, Ueno T and Shoshan MC: Determining tumor apoptosis and necrosis in patient serum using cytokeratin 18 as a biomarker. *Cancer Lett* 214: 1-9, 2004.

- 8 Johnson PJ and Lo YM: Plasma nucleic acids in the diagnosis and management of malignant disease. *Clin Chem* 48: 1186-1193, 2002.
- 9 Nakayama G, Hibi K, Kodera Y, Koike M, Fujiwara M and Nakao A: p16 methylation in serum as a potential marker for the malignancy of colorectal carcinoma. *Anticancer Res* 27: 3367-3370, 2007.
- 10 Hibi K, Taguchi M, Nakayama H, Takase T, Kasai Y, Ito K, Akiyama S and Nakao A: Molecular detection of p16 promoter methylation in the serum of patients with esophageal squamous cell carcinoma. *Clin Cancer Res* 7: 3135-3138, 2001.
- 11 Lo YMD, Wong IHN, Zhang J, Tein MSC, Ng MHL and Hjelm NM: Quantitative analysis of aberrant p16 methylation using real-time quantitative methylation-specific polymerase chain reaction. *Cancer Res* 59: 3899-3903, 1999.
- 12 Nakayama H, Hibi K, Takase T, Yamazaki T, Kasai Y, Ito K, Akiyama S and Nakao A: Molecular detection of p16 promoter methylation in the serum of recurrent colorectal cancer patients. *Int J Cancer* 105: 491-493, 2003.
- 13 Nakayama G, Hibi K, Nakayama H, Kodera Y, Ito K, Akiyama S and Nakao A: A highly sensitive method for the detection of p16 methylation in the serum of colorectal cancer patients. *Anticancer Res* 27: 1459-1463, 2007.

Received January 12, 2011

Revised April 11, 2011

Accepted April 12, 2011