Promoter Hypermethylation of RASSF1A in Esophageal Squamous Cell Carcinoma[1]

Tamotsu Kuroki, Francesco Trapasso, Sai Yendamuri, Ayumi Matsuyama, Hansjuerg Alder, Masaki Mori, and Carlo M. Croce[2]

Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania, Pennsylvania 19107 [T. K., F. T., S. Y., A. M., H. A., C. M. C.], and Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu 874-0838, Japan [M. M.]

ABSTRACT

Purpose: **The RAS association domain family 1A (*RASSF1A*) gene, a candidate tumor suppressor gene, is frequently inactivated by hypermethylation of its promoter region in several human cancers. The aim of this study was to evaluate the promoter methylation status of the RASSF1A in esophageal squamous cell carcinoma.**

Experimental Design: **We analyzed the methylation status of RASSF1A promoter by methylation-specific PCR in 23 esophageal squamous cell carcinoma cell lines and 48 primary tumors.**

Results: **Hypermethylation of RASSF1A was found in 74% of cell lines and 52% of primary tumors. The presence of hypermethylation was statistically associated with loss of RASSF1A mRNA expression in both cell lines ($P = 0.007$) and primary tumors ($P = 0.003$). There was a statistically significant correlation between the presence of hypermethylation and tumor stage ($P = 0.009$).**

Conclusions: **Our findings suggest that epigenetic silencing of *RASSF1A* gene expression by promoter hypermethylation could play an important role in primary esophageal squamous cell carcinogenesis.**

INTRODUCTION

The genesis of human cancers, including those of the esophagus, is a multistep process involving cumulative genetic alterations that include activation of oncogenes or inactivation of tumor suppressor genes. Although multiple genetic and epigenetic changes have been detected in esophageal squamous cell carcinoma (1–3), the precise molecular mechanisms of development and/or progression of esophageal squamous cell carcinoma still remain unknown.

Examples of genetic alterations involving homozygous deletions and LOH[3] of chromosome 3p have been reported in many different human tumors, including esophageal cancer (4, 5). The *fragile histidine triad* gene, located at 3p14.2, is a tumor suppressor gene that is predominantly altered by deletions, and it plays an important role in the development of esophageal cancers with exposure to environmental carcinogens (1, 6, 7). LOH analyses revealed that 3p21.3 is another region commonly deleted in several cancers (8, 9). Furthermore, we recently reported abnormalities in the *WWOX* gene, a putative tumor suppressor gene, involving LOH and point mutation (3). Recently, RASSF1A has been proposed as a candidate tumor suppressor gene, isolated within the minimal homozygous deletion at 3p21.3 in lung cancer (10). Recent studies have shown that the hypermethylation of CpG island into regulatory sequences is another mechanism that could account for silencing genes involved in cancer (11–13). The most recurrent mechanism of inactivation of RASSF1A is the hypermethylation of a CpG island in its promoter sequences as reported in lung, breast, prostate, and kidney cancers (14–16). To investigate whether the promoter hypermethylation of RASSF1A could play a role in human esophageal tumorigenesis, we performed a MSP on esophageal primary tumors and cell lines. We investigated the frequency and potential clinical implications of hypermethylation of RASSF1A promoter in human esophageal squamous cell carcinomas.

MATERIALS AND METHODS

Cell Lines and Tissues. Twenty-three human esophageal carcinoma cell lines and 13 TE series were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer at Tohoku University (Tohoku, Japan), and 10 KYSE series were gifts from Dr. Shimada of Kyoto University (Kyoto, Japan). The tumor samples and their corresponding noncancerous tissues were obtained from 48 Japanese patients who underwent surgery for esophageal squamous cell carcinoma (45 male and 3 female; median age, 60.7 years; range, 48–74 years). Four of them were stage I, 5 were stage II, 37 were stage III, and 2 were stage IV, according to the Tumor-Node-Metastasis classification.

DNA and RNA Extraction. The tissue samples were excised and immediately stored at −80°C. DNA and RNA were

Received 9/12/02; revised 12/3/02; accepted 12/5/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by USPHS Grants CA39860, CA56036, CA77738, and CA83698 from the National Cancer Institute.
[2] To whom requests for reprints should be addressed, at Kimmel Cancer Institute, Jefferson Medical College, Thomas Jefferson University, BLSB Room 1050, 233S 10th Street, Philadelphia, PA 19107-5799. Phone: (215) 503-4645; Fax: (215) 923-3528; E-mail:carlo.croce@mail.tju.edu.

[3] The abbreviations used are: LOH, loss of heterozygosity; RASSF1A, RAS association domain family 1A; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 Methylation status of RASSF1A and expression of RASSF1A in ESCC[a] cell lines

RASSF1A methylation status			
Methylated allele	Unmethylated allele	ESCC	RASSF1A expression (%)
+	−	14	0 (0)[b]
+	+	3	1 (33)
−	+	6	4 (67)

[a] ESCC, esophageal squamous cell carcinoma.
[b] $p = 0.007$; Fisher's exact test.

extracted from each of the 23 cell lines and 48 paired esophageal tissues, according to methods described previously (17).

RT-PCR Analysis of the RASSF1A Transcript. cDNAs were synthesized from 2 μg of total RNA, and the reverse transcription was performed as described previously (1). GAPDH amplification was used for cDNA normalization. The locations of primers used for RT-PCR analyses were used according to a previous report (14). One μl of cDNA was used for PCR amplification with RASSF1A-specific primers (forward primer, 5′-GGCGTCGTGCGCAAAGGCC-3′; reverse primer, 5′-GGGTGGCTTCTTGCTGGAGGG-3′) in a volume of 50 μl containing 20 pmol of each primer, 2.5 mM $MgCl_2$, 1.5 mM deoxynucleoside triphosphate mix, 1 × PCR buffer, and 2 units of AmpliTaq Gold (Perkin-Elmer Corp., Foster City, CA). PCR cycles included 1 cycle of 95°C for 10 min followed by 35 cycles each of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min and a final extension step of 72°C for 5 min in a Perkin-Elmer GeneAmp PCR system 9600. The amplified products were analyzed by electrophoresis on a 2% agarose gel. DNA bands of RASSF1A transcripts were excised from the gel, purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA), and sequenced on the Applied Biosystems Prism 377 DNA sequencing system (Perkin-Elmer).

Methylation Analysis. The methylation status in the promoter region of the RASSF1A was determined by MSP as described previously (14). Briefly, 1 μg of the genomic DNA was denatured by 2 M NaOH and then incubated in 3 M sodium bisulfite and 10 mM hydroquinone for 17 h at 55°C. Bisulfite-treated DNA was extracted using a genomic DNA cleanup kit (Promega, Madison, WI). Modified DNA was amplified by two different set of primers specific for unmethylated and methylated RASSF1A sequences as described previously (14). Human genomic DNA (Clontech, Palo Alto, CA) treated *in vitro* with SssI methylase (New England Biolabs, Inc., Beverly, MA) was used as a positive control. PCR products were analyzed on 2% agarose gels with ethidium bromide and visualized under UV illumination. To verify the methylation status determined by MSP, both unmethylated and methylated products were excised from the gel, purified using the QIAquick gel extraction kit (Qiagen), and sequenced on the Applied Biosystems Prism 377 DNA sequencing system.

Statistical Analysis. Fisher's exact test was used for statistical analysis; *P*s < 0.05 were regarded as statistically significant.

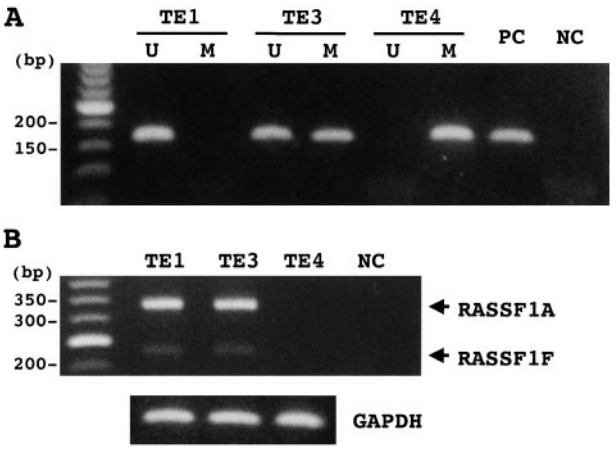

Fig. 1 Representative MSP (*A*) and RT-PCR (*B*) analysis in esophageal squamous cell carcinoma cell lines (*TE1*, *3*, and *4*). *A*, MSP of the RASSF1A promoter by using both unmethylated (*U*) and methylated (*M*)-specific primers. *In vitro* methylated DNA was used as a positive control (*PC*) for amplification with methylated-specific primers. *NC*, negative control. *B*, RT-PCR analysis of RASSF1A. *Arrows*, location of transcripts. GAPDH amplification served as a control for cDNA quality.

RESULTS

Hypermethylation Analysis of Esophageal Carcinoma Cell Lines. To evaluate the methylation status of the promoter region of RASSF1A in esophageal cancers, we used an MSP approach. Seventeen of 23 (74%) esophageal cancer cell lines showed methylated bands of RASSF1A, and 9 (39%) cell lines showed unmethylated bands. Both methylated and unmethylated bands were detected in 3 (13%) cell lines. The frequency of the methylated and unmethylated *RASSF1A* alleles is summarized in Table 1. Representative MSP results of cell lines are shown in Fig. 1*A*.

The mRNA expression of RASSF1A was analyzed in 23 esophageal cancer cell lines by RT-PCR. Only 5 of 23 cell lines (TE 1, TE 3, TE 11, TE14, and KYSE 140) still expressed the RASSF1A mRNA. RT-PCR amplification of RASSF1A in these 5 cell lines revealed a shorter transcript; sequencing analysis of the shorter transcript showed a RASSF1F transcript with a deleted exon 1C of RASSF1A as described previously (14). Representative RT-PCR results of cell lines are shown in Fig. 1*B*. In 4 of 5 cell lines (TE1, TE11, TE14, and KYSE140), RASSF1A was expressed in the absence of methylated *RASSF1A* allele. In 1 cell line (TE 3), RASSF1A was expressed in the presence of both methylated and unmethylated *RASSF1A* alleles. In 2 cell lines (TE 9 and TE 15), RASSF1A was not expressed in the absence of methylated allele (Table 1). There was a significant correlation between RASSF1A promoter hypermethylation and RASSF1A expression in cell lines ($P = 0.007$; Fisher's exact test).

Hypermethylation Analysis of Primary Esophageal Squamous Cell Carcinomas. Hypermethylation of the promoter region of RASSF1A was detected in 25 of 48 (52%) esophageal cancers. Unmethylated bands were detected in 37 of 48 (77%) tumor samples. In primary tumors, the presence of the remaining stromal cells may contribute to the presence of un-

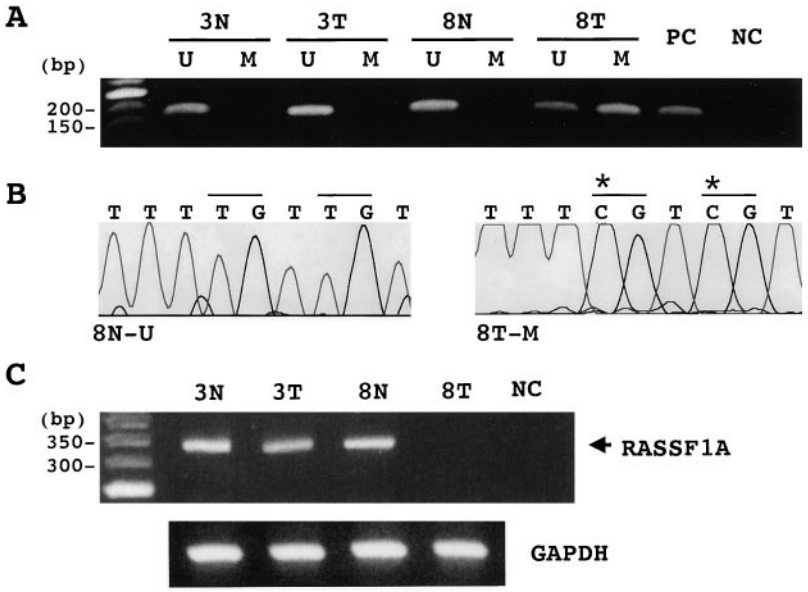

Fig. 2 Representative MSP (*A*), direct sequence of PCR products (*B*), and RT-PCR (*C*) analysis in primary esophageal squamous cell carcinomas. *A*, MSP analysis from two cases, cases 3 and 8. MSP of the RASSF1A promoter by using both unmethylated (*U*) and methylated (*M*)-specific primers. *PC*, positive control; *NC*, negative control; *T*, tumor; *N*, corresponding normal tissue. *B*, direct sequencing chromatogram of PCR products from case 8. The CpG sites are marked by a *bar* and methylated cytosine by an *asterisk*. *C*, RT-PCR analysis of RASSF1A from two cases, cases 3 and 8. *Arrow*, location of transcript. GAPDH amplification served as a control for cDNA quality.

Table 2 Clinicopathological correlation of RASSF1A promoter hypermethylation

	RASSF1A methylation status		
	Hypermethylation	Nonhypermethylation	P^a
Age (mean ± SD; yr)	60.4 ± 7.4	61.0 ± 7.6	
Differentiation			
Well	4	7	
Moderate	15	11	0.544
Low	6	5	
Stage			
I/II	1	8	0.009
III/IV	24	15	

[a] Fisher's exact test.

Table 3 Methylation status of RASSF1A and expression of RASSF1A in primary esophageal squamous cell carcinomas

RASSF1A expression	RASSF1A methylation status	
	Hypermethylation	Nonhypermethylation
+	5	9[a]
−	9	0

[a] $p = 0.003$; Fisher's exact test.

methylated bands in many cases. Unmethylated bands were detected in all of the 48 corresponding noncancerous tissues. Two of 48 noncancerous tissues (4%) showed methylated bands. Representative MSP results of esophageal squamous cell carcinomas are shown in Fig. 2*A*. The PCR products obtained with the unmethylated and methylated primers of three different tumors and corresponding noncancerous tissues were directly sequenced. In the PCR product from unmethylated primers, all unmethylated cytosine nucleotides, including those in the CpG dinucleotides, changed to thymines as a result of bisulfite modification. Cytosine nucleotides in the CpG dinucleotides were found to remain as cytosine, which indicated the presence of methylated DNA (Fig. 2*B*).

The results of statistical analysis for correlation of RASSF1A promoter methylation status in the tumor tissues with histological differentiation and tumor stage are shown in Table. 2. There were no significant correlations of RASSF1A promoter hypermethylation with age and histological differentiation. However, there was a significant correlation between RASSF1A promoter hypermethylation and tumor stage ($P = 0.009$; Fisher's exact test).

RASSF1A expression was examined by RT-PCR in 23 primary esophageal squamous cell carcinomas and corresponding noncancerous tissues. RT-PCR analysis demonstrated that no RASSF1A transcripts were amplified from 9 tumor tissues. In all of the 9 tumors with the RASSF1A promoter hypermethylation, RASSF1A was not expressed. The remaining 14 tumors and all 23 noncancerous tissues showed RASSF1A expression. In 9 of 14 tumors, RASSF1A was expressed with the nonhypermethylation of the RASSF1A promoter (Table 3). Overall, there was a significant correlation between RASSF1A promoter hypermethylation and loss of RASSF1A expression in clinical samples ($P = 0.003$; Fisher's exact test). Representative RT-PCR results of esophageal squamous cell carcinomas are shown in Fig. 2*C*.

DISCUSSION

Several studies have suggested that chromosome 3p21 contains a candidate tumor suppressor gene involved in various tumor types (4, 5, 8, 9). The *RASSF1A* gene is a novel candidate tumor suppressor gene that was isolated from this region (10). The RASSF1A protein shows high homology to the mouse Ras effector protein Nore1 (10). Nore1 directly interacts with Ras in a GTP-dependent manner and follows receptor activation (18). Promoter hypermethylation has been reported to be the major mechanism of RASSF1A inactiva-

tion in several cancers (14–16). In the present study, we found hypermethylation of RASSF1A in 52% of primary esophageal squamous cell carcinoma. In addition, there was a significant concordance between RASSF1A promoter methylation status and its expression. These findings indicate that hypermethylation of RASSF1A promoter may represent a common mechanism of inactivation of this candidate tumor suppressor gene in primary esophageal squamous cell carcinomas.

Recently, Maruyama *et al.* (19) reported that promoter hypermethylation of RASSF1A was significantly correlated with several parameters of poor prognosis of bladder cancers. Here, we demonstrated that RASSF1A hypermethylation is correlated with tumor stage in patients with esophageal squamous cell carcinoma. These findings suggest that in many cases, RASSF1A hypermethylation may occur as a late event during the progression of esophageal squamous cell carcinomas. Thus, RASSF1A hypermethylation may be a potential new prognostic marker of esophageal squamous cell carcinomas.

In this study, RASSF1A promoter hypermethylation of noncancerous tissues was detected in 4% (2 of 48) of patients with esophageal squamous cell carcinoma. This finding suggests that RASSF1A promoter hypermethylation could be an early event in the pathogenesis of only few cases of esophageal squamous cell carcinomas. Furthermore, the presence of the hypermethylation in corresponding noncancerous tissues may represent the appearance of premalignant lesions. We also found that 5 of 14 cases showing hypermethylation showed RASSF1A expression. This finding can be explained by the partial methylation of RASSF1A promoter. Several studies have reported that partial methylation could cause gene expression in the tumor cells despite promoter hypermethylation (20, 21). Another possibility for explaining this result is the high sensitivity of the MSP analysis. In fact, MSP can detect about one methylated gene copy in 1000 unmethylated copies in dilution experiments (22). MSP analysis is a sensitive, simple, and useful method, and MSP analysis of RASSF1A may thus contribute to an improved outcome of patients with esophageal squamous cell carcinoma.

In summary, hypermethylation of RASSF1A promoter region is observed frequently in esophageal squamous cell carcinomas and associated with loss of mRNA expression in many cases. Our results suggest that hypermethylation of RASSF1A may play an important role in RASSF1A inactivation in esophageal squamous cell carcinogenesis. The further study of RASSF1A involvement in esophageal squamous cell carcinoma and delineation of metabolic pathways in which this gene is involved may lead to therapeutic alternatives for this cancer.

REFERENCES

1. Mori, M., Mimori, K., Shiraishi, T., Alder, H., Inoue, H., Tanaka, Y., Sugimachi, K., Huebner, K., and Croce, C. M. Altered expression of Fhit in carcinoma and precarcinomatous lesions of the esophagus. Cancer Res., *60:* 1177–1182, 2000.

2. Ishii, H., Baffa, R., Numata, S., Murakumo, Y., Rattan, S., Inoue, H., Mori, M., Fidanza, V., Alder, H., and Croce, C. M. The *FEZ1* gene at chromosome 8p22 encodes a leucine-zipper protein, and its expression is altered in multiple human tumors. Proc. Natl. Acad. Sci. USA, *96:* 3928–3933, 1999.

3. Kuroki, T., Trapasso, F., Shiraishi, T., Alder, H., Mimori, K., Mori, M., and Croce, C. M. Genetic alterations of the tumor suppressor gene *WWOX* in esophageal squamous cell carcinoma. Cancer Res., *62:* 2258–2260, 2002.

4. Roth, M. J., Hu, N., Emmert-Buck, M. R., Wang, Q. H., Dawsey, S., Li, G., Guo, W. J., Zhang, Y. Z., and Taylor, P. R. Genetic progression and heterogeneity associated with the development of esophageal squamous cell carcinoma. Cancer Res., *61:* 4098–4104, 2001.

5. Shimada, M., Yanagisawa, A., Kato, Y., Inoue, M., Shiozaki, H., Monden, M., and Nakamura, Y. Genetic mechanisms in esophageal carcinogenesis: frequent deletion of 3p and 17p in premalignant lesions. Genes Chromosomes Cancer, *15:* 165–169, 1996.

6. Ohta, M., Inoue, H., Cotticelli, M. G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Mori, M., McCue, P., Druck, T., Croce, C. M., and Huebner, K. The *FHIT* gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Cell, *84:* 587–597, 1996.

7. Croce, C. M., Sozzi, G., and Huebner, K. Role of *FHIT* in human cancer. J. Clin. Oncol., *17:* 1618–1624, 1999.

8. Cheng, Y., Poulos, N. E., Lung, M. L., Hampton, G., Ou. B., Lerman, M. I., and Stanbridge, E. J. Functional evidence for a nasopharyngeal carcinoma tumor suppressor gene that maps at chromosome 3p21.3. Proc. Natl. Acad. Sci. USA, *95:* 3042–3047, 1998.

9. Lerman, M. I., and Minna, J. D. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res., *60:* 6116–6133, 2000.

10. Dammann, R., Li, C., Yoon, J. H., Chin, P. L., Bates, S., and Pfeifer, G. P. Epigenetic inactivation of a RAS association domain family protein from the lung tumor suppressor locus 3p21.3. Nat. Genet., *25:* 315–319, 2000.

11. Tanaka, H., Shimada, Y., Harada, H., Shinoda, M., Hatooka, S., Imamura, M., and Ishizaki, K. Methylation of the 5′ CpG island of the *FHIT* gene is closely associated with transcriptional inactivation in esophageal squamous cell carcinomas. Cancer Res., *58:* 3429–3434, 1998.

12. Nuovo, G. J., Plaia, T. W., Belinsky, S. A., Baylin, S. B., and Herman, J. G. In situ detection of the hypermethylation-induced inactivation of the *p16* gene as an early event in oncogenesis. Proc. Natl. Acad. Sci. USA, *96:* 12754–12759, 1999.

13. Oue, N., Shigeishi, H., Kuniyasu, H., Yokozaki, H., Kuraoka, K., Ito, R., and Yasui, W. Promoter hypermethylation of MGMT is associated with protein loss in gastric carcinoma. Int. J. Cancer, *93:* 805–809, 2001.

14. Burbee, D. G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., Sekido, Y., Latif, F., Milchgrub, S., Toyooka, S., Gazdar, A. F., Lerman, M. I., Zabarovsky, E., White, M., and Minna, J. D. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J. Natl. Cancer Inst. (Bethesda), *93:* 691–699, 2001.

15. Maruyama, R., Toyooka, S., Toyooka, K. O., Virmani, A. K., Zochbauer-Muller, S., Farinas, A. J., Minna, J. D., McConnell, J., Frenkel, E. P., and Gazdar, A. F. Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. Clin. Cancer Res., *8:* 514–519, 2002.

16. Dreijerink, K., Braga, E., Kuzmin, I., Geil, L., Duh, F. M., Angeloni, D., Zbar, B., Lerman, M. I., Stanbridge, E. J., Minna, J. D., Protopopov, A., Li, J., Kashuba, V., Klein, G., and Zabarovsky, E. R. The candidate tumor suppressor gene. RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. Proc. Natl. Acad. Sci. USA, *98:* 7504–7509, 2001.

17. Mori, M., Shiraishi, T., Tanaka, S., Yamagata, M., Mafune, K., Tanaka, Y., Ueo, H., Barnard, G. F., and Sugimachi, K. Lack of DMBT1

expression in esophageal, gastric and colon cancer. Br. J. Cancer, *79:* 211–213, 1999.

18. Vavvas, D., Li, X., Avruch, J., and Zhang, X. F. Identification of Nore1 as a potential Ras effector. J. Biol. Chem., *273:* 5439–5442, 1998.

19. Maruyama, R., Toyooka, S., Toyooka, K. O., Harada, K., Virmani, A. K., Zochbauer-Muller, S., Farinas, A. J., Vakar-Lopez, F., Minna, J. D., Sagalowsky, A., Czerniak, B., and Gazdar, A. F. Aberrant promoter methylation profile of bladder cancers and its relationship to clinicopathological features. Cancer Res., *61:* 8659–8663, 2001.

20. Foster, S. A., Wong, D. J., Barrett, M. T., and Galloway, D. A. Inactivation of p16 in human mammary epithelial cells by CpG island methylation. Mol. Cell. Biol., *18:* 1793–1801, 1998.

21. Shim, Y. H., Kang, G. H., and Ro, J. Y. Correlation of *p16* hypermethylation with p16 protein loss in sporadic gastric carcinomas. Lab. Investig., *80:* 689–695, 2000.

22. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG island. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.

Clinical Cancer Research

Promoter Hypermethylation of RASSF1A in Esophageal Squamous Cell Carcinoma

Tamotsu Kuroki, Francesco Trapasso, Sai Yendamuri, et al.

Clin Cancer Res 2003;9:1441-1445.