[CANCER RESEARCH 60, 6236–6242, November 15, 2000]

Advances in Brief

TMS1, a Novel Proapoptotic Caspase Recruitment Domain Protein, Is a Target of Methylation-induced Gene Silencing in Human Breast Cancers[1]

Kerry E. Conway,[2] Beth B. McConnell,[2] Claire E. Bowring, Carlton D. Donald, Stephen T. Warren, and Paula M. Vertino[3]

Department of Radiation Oncology and the Winship Cancer Institute [K. E. C., B. B. M., C. E. B., C. D. D., P. M. V.], Departments of Biochemistry, Genetics, Pediatrics, and the Howard Hughes Medical Institute [S. T. W.], Emory University School of Medicine, Atlanta, Georgia 30322

Abstract

Gene silencing associated with aberrant methylation of promoter region CpG islands is an acquired epigenetic alteration that serves as an alternative to genetic defects in the inactivation of tumor suppressor and other genes in human cancers. The hypothesis that aberrant methylation plays a direct causal role in carcinogenesis hinges on the question of whether aberrant methylation is sufficient to drive gene silencing. To identify downstream targets of methylation-induced gene silencing, we used a human cell model in which aberrant CpG island methylation is induced by ectopic expression of DNA methyltransferase. Here we report the isolation and characterization of *TMS1* (*t*arget of *m*ethylation-induced *s*ilencing), a novel CpG island-associated gene that becomes hypermethylated and silenced in cells overexpressing DNA cytosine-5-methyltransferase-1. We also show that *TMS1* is aberrantly methylated and silenced in human breast cancer cells. Forty percent (11 of 27) of primary breast tumors exhibited aberrant methylation of *TMS1*. *TMS1* is localized to chromosome 16p11.2–12.1 and encodes a 22-kDa predicted protein containing a COOH-terminal caspase recruitment domain, a recently described protein interaction motif found in apoptotic signaling molecules. Ectopic expression of *TMS1* induced apoptosis in 293 cells and inhibited the survival of human breast cancer cells. The data suggest that methylation-mediated silencing of *TMS1* confers a survival advantage by allowing cells to escape from apoptosis, supporting a new role for aberrant methylation in breast tumorigenesis.

Introduction

Within a genome that is generally heavily methylated at sparsely distributed CpG dinucleotides, about one-half of human genes are marked at the 5′ end by a CpG-dense "island" (1). Normally unmethylated in adult cells, CpG islands have been shown to acquire methylation *de novo* during aging and carcinogenesis (2, 3). Aberrant methylation is associated with inappropriate gene silencing, and this epigenetic alteration has been shown to serve as an alternative to genetic defects in the inactivation of tumor suppressor genes in human cancers (2, 4). Methylation-associated silencing underlies the DNA repair defect in microsatellite unstable sporadic colon and endometrial cancers, and thus can contribute to carcinogenesis by promoting genome instability (5, 6). Methylation-associated silencing has also been implicated in tumor progression, through the inactivation of genes thought to suppress invasion and metastasis (7, 8). Accordingly, the identification of gene targets of methylation-associated silencing could lead to novel genes involved in the initiation and progression of human neoplasia.

Despite the strong correlation between aberrant CpG island methylation and lack of gene expression at tumor suppressor loci in cancer, it remains an open question whether aberrant methylation plays a direct causal role in carcinogenesis. At present, it is not clear how genes progress from an unmethylated, active state to a methylated and inactive one, and it is not known whether it is the methylation *per se* that drives gene silencing *in vivo*. Recent studies linking methylation-specific DNA-binding proteins to repressor complexes containing histone deacetyleases and other chromatin-remodeling proteins suggest that aberrant methylation may precipitate gene silencing through induced changes in chromatin structure (9–12). Indeed, genes that are aberrantly methylated and repressed cannot be reactivated by inhibition of histone deacetylase alone without prior demethylation, suggesting that methylation may be the primary determinant in the establishment of transcriptional silencing at these loci (13).

In this study, we sought to identify potential targets of methylation-induced gene silencing by directly screening for genes whose expression is down-regulated in response to aberrant methylation. Although the mechanism underlying aberrant methylation is not currently understood, this event can be induced in human cells by ectopic expression of *DNMT1*[4] (14). We report the identification of *TMS1*, a novel CpG island-associated gene that becomes aberrantly methylated and silenced in cells overexpressing *DNMT1*. We further show that *TMS1* is silenced in association with aberrant CpG island methylation in human breast cancer. *TMS1* encodes a novel CARD protein, and promotes apoptosis in human cells. The data suggest that aberrant methylation can drive gene silencing *in vivo* and support a new role for methylation-mediated silencing in human breast tumorigenesis: the silencing of genes that act as positive mediators of cell death.

Materials and Methods

Cell Lines and Primary Tissue. IMR90 normal human diploid fibroblasts and SV40-immortalized IMR90 cells (here called 90SV) were obtained from the National Institute on Aging Cell Repository and maintained in EMEM with 2 mM glutamine and 10% FCS. The isolation and maintenance of 90SV derivative cell lines stably overexpressing human DNMT1 (HMT.1E1) has been reported (14). The MCF10A cell line was obtained from the Karmanos Cancer Institute (Detroit, MI) and maintained in DMEM/F12 plus 5% FCS, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 2 mM glutamine. Passage 7 HMECs were obtained from Clonetics (Walkersville, MD). Other human breast cell lines and 293 human embryonic kidney cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM (4.5 g/liter glucose) plus 10% FCS and 2 mM glutamine. For Hs578Bst, Hs578t, T47D, and MDA

Received 7/10/00; accepted 10/3/00.

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] This work was supported by Grants 1RO1-CA77337 (to P. M. V.) and F32-CA83289 (to B. B. M.) from the National Cancer Institute. P. M. V. is an Avon Scholar in Breast Cancer Genomics. S. T. W. is an investigator of the Howard Hughes Medical Institute.

[2] These authors contributed equally to this work

[3] To whom requests for reprints should be addressed, at Winship Cancer Institute, Room B5119, 1365-B Clifton Road NE, Atlanta, GA 30322. Phone: (404) 778-3119; Fax: (404) 778-3965; E-mail: pvertin@emory.edu.

[4] The abbreviations used are: DNMT1, DNA cytosine-5-methyltransferase-1; BAC, bacterial artificial chromosome; CARD, caspase recruitment domain; EST, expressed sequence tag; HMEC, human mammary epithelial cell; MSP, methylation-specific PCR; RT-PCR, reverse-transcriptase PCR.

MB435 cells, medium was supplemented with 10 μg/ml insulin. SKBR3 was maintained in McCoy's 5A medium plus 10% FCS and 2 mM glutamine.

DNA isolated from primary breast tissues and tumors was generously provided by Dr. Sara Sukumar (The Johns Hopkins University, Baltimore, MD). Primary breast tissue from reduction mammoplasties or from breast tumors was obtained immediately after surgical resection at Johns Hopkins University Hospital or at Duke University and frozen at −80°C. Tumors were estimated to contain at least 50% tumor cells by microscopic examination of representative tissue sections.

Representational Difference Analysis. Total RNA was isolated from 90SV cells and HMT.1E1 cells by lysis in guanidium isothiocyanate before acid/phenol extraction and isopropanol precipitation. Poly(A)+ RNA was selected on oligo-dT cellulose and used to synthesize double-stranded cDNA using a modified oligo-dT primer and the cDNA CHOICE system (Life Technologies). Double stranded cDNA was digested with *Dpn*I, ligated to linkers, and amplified by PCR using linker-specific primers to generate the tester (90SV) and driver (HMT.1E1) cDNA amplicons. The cDNA pools were then subjected to representational difference analysis as described (15). Three rounds of subtraction and enrichment of tester-only sequences were performed using tester:driver ratios of 1:80, 1:400, and 1:80,000. A predominant 350-bp difference product was isolated, digested with *Dpn*I, and subcloned into the *Bam*HI site of pBluescript SK+.

TMS1 Expression Constructs. The complete TMS1 coding region was subcloned from EST H16108 into pcDNA3.1 (Invitrogen) to generate pcDNATMS1. pcDNAmycTMS1 was generated from pcDNATMS1 using PCR to fuse a myc-epitope tag to amino acid 2 of TMS1.

BAC Isolation and Radiation Hybrid Mapping. A pooled human BAC library (Release III; Research Genetics) was screened by PCR using primers in the 3′ untranslated region of TMS1 (5′-GCACTTTATAGACCAGCA-3′ and 5′-ATTTGGTGGGATTGCCAG-3′), and four positive BACs were identified. A 6-kb *Hin*dIII subclone containing *TMS1* was identified by shotgun cloning and colony hybridization to the *TMS*1 cDNA. The same primers were used in a PCR-based screen of the human/rodent somatic cell hybrid mapping panel 2 (Coriell Cell Repository) to localize TMS1 to human chromosome 16. Fine mapping was carried out using radiation hybrid panels (Stanford RH Panel G3 and Stanford RH panel TNG4; Research Genetics). Results were analyzed using the Stanford Human Genome Center RHserver[5] and indicated linkage to markers SHGC-35326 on the G3 panel (lod score, 7.16) and SHGC-61092 on the TNG4 panel (lod score, 4.91).

Methylation-sensitive Restriction and Southern Blot Analysis. Ten μg of DNA isolated from 90SV or HMT.1E1 cells were digested with 200 units of the methylation-sensitive restriction enzyme *Sac*II or *Eag*I for 16 h, and then digested for an additional 16 h with 100 units of the methylation-insensitive enzyme *Hin*dIII. Digested DNA was separated by electrophoresis on a 1% agarose gel, transferred to a nylon filter (Zeta-Probe, BioRAD), and hybridized overnight with a random prime-labeled, 1.8-kb *Eco*RI TMS1 genomic probe. Blots were washed to a final stringency of 0.1× SSC-0.1% SDS at 65°C and exposed to X-ray film using intensifying screens (BioMAX-MS; Kodak).

Northern Blot Analysis. One μg poly(A)+ selected RNA was fractionated on a 1.5% agarose/formaldehyde gel, transferred to nylon filters, and hybridized with a random-prime-labeled full-length TMS1 cDNA fragment. Blots were washed to a final stringency of 0.1× SSC-0.1% SDS at room temperature and exposed to X-ray film using an intensifying screen (BioMAX-MS; Kodak). Blots were stripped and rehybridized with a human β-actin cDNA probe.

RT-PCR. Six μg of total RNA were pretreated with DNase I (Life Technologies) and reverse-transcribed using random hexamer primers and MMLV-reverse transcriptase (Life Technologies). One-thirtieth of the reverse-transcriptase reaction (200 ng of starting RNA) was used directly in a PCR reaction. The PCR reaction conditions were: 67 mM Tris-HCl (pH 8.8), 16.6 mM NH_4SO_4, 6.7 μM EDTA, 10 mM β-mercaptoethanol, 4.7 mM $MgCl_2$, 10% DMSO, and 400 nM each primer in a 25-μl reaction. A hot start was performed (5 min, 95°C), followed by the addition of 0.5 unit of *Taq* polymerase (Life Technologies) and 35 cycles of PCR (95°C, 30 s; 50–55°C, 60 s; and 72°C, 60 s). TMS1 primers (5′-TGG GCC TGC AGG AGA TG-3′ and 5′-ATT TGG TGG GAT TGC CAG-3′) were used at an annealing temperature of 50°C. β-actin primers (5′-CCT TCC TGG GCA TGG AGT CCT G-3′ and 5′-GGA GCA ATG ATC TTG ATC TTC-3′) were used at an annealing temperature of 55°C. Reaction products were separated by electrophoresis on a 6% polyacrylamide/Tris-borate-EDTA gel, stained with ethidium bromide, and photographed.

Bisulfite Modification and MSP. Bisulfite modification and MSP were performed as described previously (16). Approximately 50 ng of bisufite-modified DNA was amplified by PCR with the following reaction conditions: 67 mM Tris-HCl (pH 8.8), 16.6 mM NH_4SO_4, 6.7 μM EDTA, 10 mM β-mercaptoethanol, 6.7 mM $MgCl_2$, and 1 μM each primer in a 25 μl reaction. A hot start was performed (5 min, 95°C) followed by the addition of 0.5u *Taq* polymerase (Life Technologies) and 35 cycles of PCR (95°C, 30 s; 58°C, 30 s; and 72°C, 30 s). Reaction products were separated by electrophoresis on a 6% polyacrylamide/Tris-borate-EDTA gel, stained with ethidium bromide, and photographed. Primers were designed from the interpolated sequence after bisulfite conversion assuming the DNA was either methylated or unmethylated at CpG sites. To ensure maximal discrimination of unmethylated and methylated DNA, primers were designed to overlap three potential methylation sites. Primers used were 5′-GGT TGT AGT GGG GTG AGT GGT-3′ and 5′-CAA AAC ATC CAT AAA CAA CAA CAC A-3′ for the unmethylated reaction, and 5′-TTG TAG CGG GGT GAG CGG C-3′ and 5′-AAC GTC CAT AAA CAA CAA CGC G-3′ for the methylated reaction.

Colony Formation Assays. Breast cancer cells (3×10^5) were transfected with 1 μg of pcDNA3.1 or TMS1 expression constructs using 5 μl of Lipofectamine reagent (Life Technologies). Twenty-four h after transfection, cells were diluted and seeded into medium containing 400 μg/ml G418. After 14 days of selection, stable G418-resistant colonies were fixed and stained with 0.25% crystal violet in 50% methanol and counted. The total number of colonies recovered per transfection was extrapolated from counting at least 300 G418-resistant colonies per experiment.

Apoptosis Assays. The 293 human embryonic kidney cells (1×10^5) plated on glass coverslips in 24-well dishes were transfected with 0.4 μg of pcDNA3.1 or the TMS1 expression constructs plus 0.1 μg of β-galactosidase expression vector (pCMVβgal; Clonetech) using the calcium phosphate precipitation method. Where indicated, 40 μM zVADfmk (Enzyme System Products, Livermore, CA) was included during the recovery period. Forty-eight h after transfection, coverslips were fixed in 4% paraformaldehyde and stained for β-galactosidase activity using X-gal and for nuclear morphology using Hoechst 33528 dye. Apoptotic cells were distinguished on the basis of morphological features typical of adherent cells undergoing apoptosis, including becoming condensed, rounded, and detached from the growth surface, as described (17). At least 200 β-galactosidase-positive cells from randomly selected fields were counted from each transfection.

Results

We have shown previously that human fibroblasts overexpressing *DNMT1* undergo progressive *de novo* methylation of endogenous CpG island sequences (14). To identify potential downstream targets of methylation-mediated gene silencing, we used cDNA representational difference analysis (15) to isolate genes that were down-regulated in cells overexpressing *DNMT1* (referred to as HMT.1E1) relative to the immortalized human fibroblasts (90SV) from which they were derived (14). After three rounds of subtraction, a predominant 350-bp fragment (RDA-2.15) was isolated and shown to be differentially represented in cDNA derived from 90SV and HMT.1E1 cells (Fig. 1*A*). Sequence analysis and BLAST homology searches of the GenBank and dbEST databases indicated that RDA-2.15 was derived from a novel gene represented by multiple human ESTs defining tentative human consensus (THC253346; TIGR Human Gene Index) and a unique Unigene cluster (Hs.71869; NCBI Unigene project). A 770-bp cDNA[6] was assembled from the complete sequence of two EST clones (H16108 and AI368975) that overlapped the entire Unigene cluster. The assembled cDNA sequence contained a 588-bp open reading frame with 5′ and 3′ UTRs of 74 bp and

[5] Internet address: www-shgc.stanford.edu/RH/rhserver_form2.

[6] Sequences appearing herein have been deposited in GenBank under accession nos. AF184072, AF184073, and AF255794.

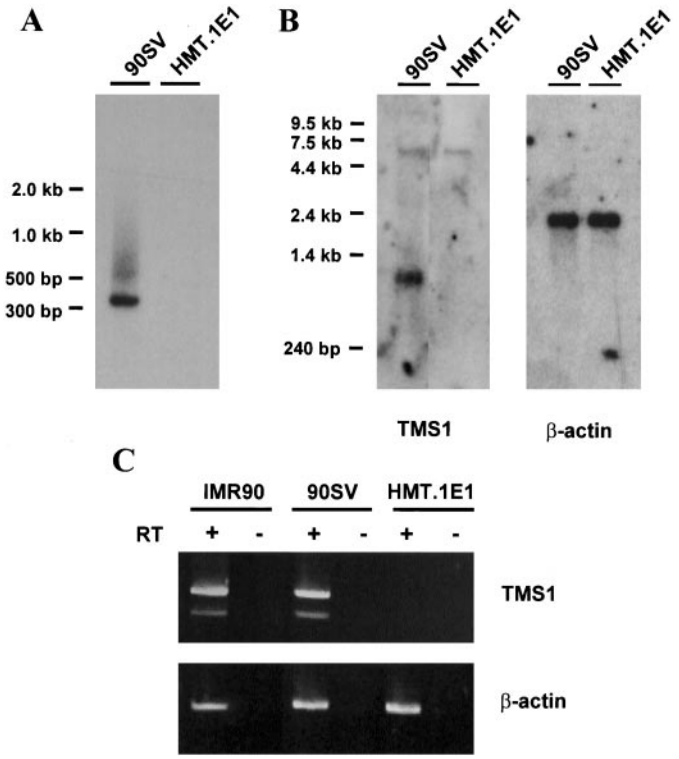

Fig. 1. Down-regulation of *TMS1* in fibroblasts overexpressing DNMT1. *A*, Southern blot prepared from 2 μg of the amplified tester (*90SV*) and driver (*HMT.1E1*) cDNA pools used to perform the RDA subtraction. Hybridization shows clone RDA-2.15 is differentially represented in cDNA derived from 90SV and HMT.1E1 cells. *B*, Northern blot of poly(A)+ selected RNA (2 μg) from 90SV and HMT.1E1 cells hybridized with the *TMS1* cDNA probe. The same blot was stripped and rehybridized with a human β-actin probe. The exposure times for TMS1 and β-actin were 3 days and 4 h, respectively. *C*, total RNA isolated from IMR90 human diploid fibroblasts, 90SV fibroblasts, and HMT.1E1 cells was reverse-transcribed (+*RT*) and amplified with primers specific to the *TMS1* transcript (*top*) or β-actin (*bottom*). Control reactions in which reverse transcriptase was omitted (−*RT*) were amplified under the same conditions.

97 bp, respectively, and included the poly(A) tail. No additional 5′ sequence was obtained using a 5′ rapid amplification of cDNA ends protocol (data not shown), indicating that the cDNA sequence was full-length. The cDNA hybridized to an ~0.8-kb message, which was expressed at low, but clearly detectable, levels in IMR90 normal human diploid fibroblasts and their immortalized derivatives, the 90SV cells (Fig. 1, *B* and *C*). The same message was undetectable by Northern blot or by RT-PCR analysis in the DNMT1-overexpressing cell line, HMT.1E1, confirming that the gene identified by RDA was silenced in HMT.1E1 cells (Fig. 1, *B* and *C*). A less abundant mRNA species was also detected by RT-PCR (see Fig. 1*C*) and was found to be an alternatively spliced form lacking exon 2 (see below). In all studies, the expression status of the two mRNA forms were concordant. We named the gene *TMS1* for "target of methylation-induced silencing-1."

Restriction mapping and Southern blot analysis of the *TMS1* locus confirmed that the absence of *TMS1* expression in the HMT.1E1 cells was not attributable to gene deletion or to gross chromosomal rearrangement (see below, and data not shown). To determine whether loss of *TMS1* expression in the HMT.1E1 cells was related to methylation, we first determined the genomic structure of *TMS1* by partial sequencing of a human genomic BAC clone. The *TMS1* locus is composed of three exons (449 bp, 58 bp, and 355 bp) spanning ~1.4 kb of genomic DNA and was localized to chromosome 16p11.2–12.1 by radiation hybrid mapping (Fig. 2*A*). An ~600-bp CpG island was identified in the 5′ end of *TMS1* surrounding exon 1. This region showed a high C + G content (69%), a CpG:GpC ratio of 0.82, and the presence of multiple sites for methylation-sensitive, CpG-recognizing restriction enzymes that often cluster within CpG islands (*Sac*II, *Eag*I, *Bss*HII, *Sma*I; Ref. 1; Fig. 2*A*). Examination of the methylation status of the CpG island by methylation-sensitive restriction analysis in *TMS1*-expressing (IMR90 and 90SV) and -nonexpressing (HMT.1E1) cells indicated that silencing of *TMS1* correlated with hypermethylation of the CpG island (Fig. 2*B*). IMR90 normal diploid fibroblasts were unmethylated at *Sac*II and *Eag*I sites within the *TMS1* CpG island. The *TMS1* CpG island exhibited a partially methylated pattern in the 90SV cells, with both methylated and unmethylated alleles present in the population (Fig. 2*B*). This result was similar to some other CpG islands we have studied in this model system which have accumulated some methylation as a result of immortalization before insertion of *DNMT1* (14). In contrast, HMT.1E1 cells exhibited complete methylation of *Sac*II and *Eag*I sites in the *TMS1* CpG island (Fig. 2*B*). The methylation status of the *TMS1* CpG island was analyzed additionally by a more sensitive MSP technique in which methylated and unmethylated alleles can be specifically amplified after chemical modification of DNA with sodium bisulfite (16). Data obtained from this analysis (Fig. 2*C*) and from direct sequencing of bisulfite-modified DNA (data not shown) agreed well with that obtained by methylation-sensitive restriction analysis and confirmed that the IMR90 cells were unmethylated and the 90SV

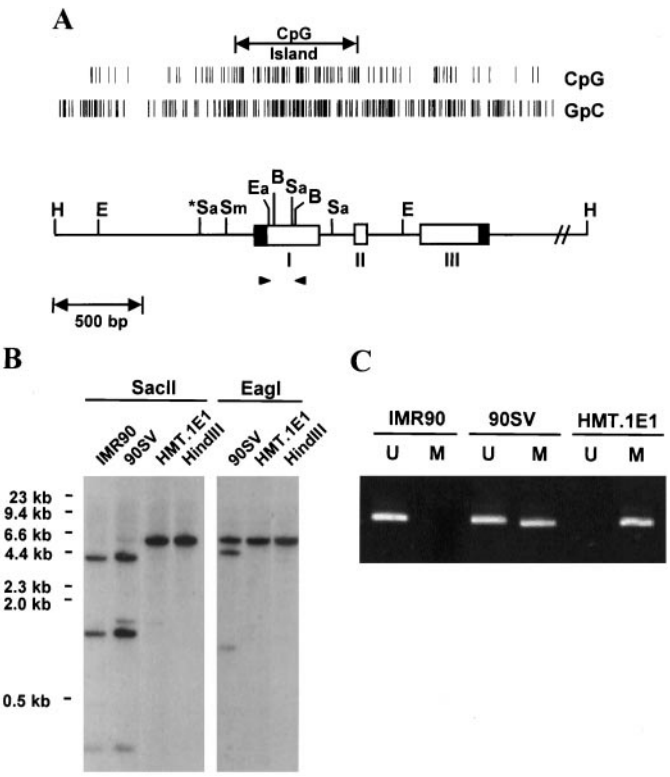

Fig. 2. Absence of *TMS1* expression correlates with hypermethylation of a CpG island in the *TMS1* gene. *A*, genomic structure of the *TMS1* locus. Intron/exon boundaries were determined by comparison of the cDNA and genomic sequences. The *TMS1* coding region spans 1.8 kb and three exons (*I*, *II*, and *III*). The 5′ and 3′ untranslated regions are *shaded*. A CpG island in the 5′ end of the gene is evident from the density of CpG sites relative to GpC (0.88) and the clustering of multiple restriction enzyme sites (*Sa*, *Sac*II; *Ea*, *Eag*I; *B*, *Bss*HII; *Sm*, *Sma*I). *H*, *Hin*dIII; *E*, *Eco*RI. *Closed arrows*, primers used for MSP. *B*, methylation of the *TMS1* CpG island was examined by digestion of DNA from the indicated cell line with *Hin*dIII alone or *Hin*dIII plus the methylation-sensitive restriction enzyme *Sac*II or *Eag*I (*all other lanes*), followed by Southern blot analysis using a 1.8 kb *Eco*RI *TMS1* genomic fragment as a probe. *C*, methylation at the *TMS1* locus determined by MSP. DNA from IMR90, 90SV, and HMT.1E1 cells was modified with sodium bisulfite, which deaminates unmethylated cytosines to uracil and leaves methylated cytosines unaltered. Bisulfite-modified DNA was used as a template for parallel PCR amplification reactions using primers designed to anneal specifically to unmethylated (*U*) or methylated (*M*) DNA. The "methylated" product was 191 bp and the "unmethylated" product was 196 bp because of differences in the length of the primers.

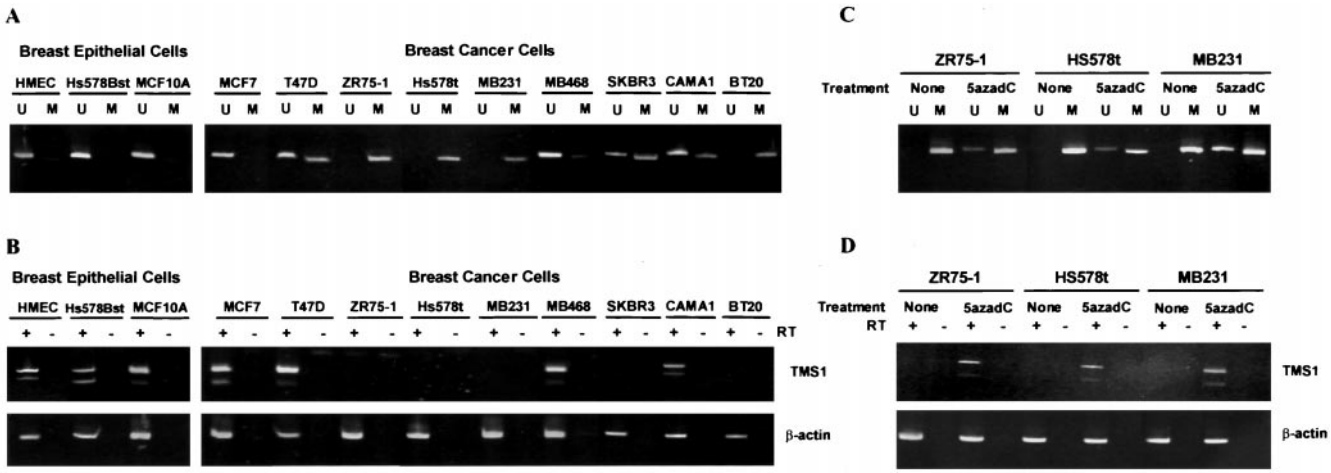

Fig. 3. Methylation-associated silencing of TMS1 CpG island in human breast cancer cells. *A*, MSP analysis of bisulfite-modified DNA from primary HMECs and two immortal, nontumorigenic breast epithelial cell lines (MCF10A and Hs578Bst) compared with that of nine breast cancer cell lines (MCF-7, T47-D, ZR-75-1, Hs578t, MDA MB231, MDA MB468, SKBR3, CAMA1, and BT-20). Parallel amplification reactions were performed using primers specific to methylated (*M*) or unmethylated (*U*) DNA. *B*, expression of *TMS1*. Total RNA isolated from the breast epithelial cells and breast cancer cells was reverse transcribed (*+RT*) and amplified with primers specific to the *TMS1* transcript (*top*) or human β-actin transcript (*bottom*). Control reactions in which reverse transcriptase was omitted (*−RT*) were amplified under the same conditions. *C* and *D*, demethylation of the *TMS1* CpG island restores expression in *TMS1*-negative breast cancer cells. ZR-75-1, MDA MB231, and Hs578t cells were not treated (*None*) or treated (*5azadC*) for 3 days with 0.5 μM 5-aza-2′-deoxycytidine and methylation of the *TMS1* CpG island was analyzed by MSP (*C*), and expression of *TMS1* was analyzed by RT-PCR (*D*).

cells were partially methylated, whereas the HMT.1E1 cells were fully methylated at the *TMS1* CpG island. These data indicated that overexpression of *DNMT1* in the HMT.1E1 cells promoted both hypermethylation and silencing of *TMS1*.

Several CpG island loci that are subject to *de novo* methylation in the *DNMT1* overexpression model are also methylated and silenced in human tumors (7, 14, 18, 19). To determine whether *TMS1* was a target for methylation-associated silencing in human cancer, we determined the expression of *TMS1* and the methylation status of the *TMS1* CpG island in human breast cell lines. *TMS1* was unmethylated and expressed in primary HMECs and two immortalized, nontumorigenic breast epithelial cell lines, Hs578Bst and MCF10A (Fig. 3, *A* and *B*). In contrast, four of nine breast carcinoma cell lines were completely methylated at the *TMS1* CpG island locus and did not express *TMS1* (Fig. 3, *A* and *B*). As seen in the fibroblast system, breast cancer cell lines that were either completely unmethylated (MCF-7) or only partially methylated (T47D, MDA MB468, and CAMA-1) at the *TMS1* CpG island retained *TMS1* expression (Fig. 3, *A* and *B*). The only exception was SKBR3 cells which, although only partially methylated, did not express *TMS1*, suggesting that these cells are above a critical threshold level of methylation necessary to affect gene expression. Alternately, there may be another mechanism for *TMS1*-silencing in this cell line. Sequencing of the *TMS1* promoter and coding region in SKBR3 cells did not reveal any mutations (data not shown).

The ZR75-1, Hs578t, and MDA MB231 breast cancer cell lines exhibited complete methylation of *TMS1* and failed to express the *TMS1* message. If the loss of *TMS1* expression is directly related to methylation of the CpG island, then expression of *TMS1* should be restored after treatment with a demethylating agent. Treatment of ZR75-1, Hs578t, and MDA MB231 cells with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine resulted in the partial demethylation of the *TMS1* CpG island and reexpression of *TMS1* (Fig. 3, *C* and *D*). The absence of *TMS1* expression in these cell lines is therefore not attributable to abnormalities at the gene level or to the inability to express *TMS1* (*e*.g. because of a lack of necessary transcription factors), but rather is directly related to the methylation of *TMS1*.

To determine whether aberrant methylation also played a role in primary tumors, we examined primary breast tissues for *TMS1* methylation. The CpG island of *TMS1* was unmethylated in normal breast tissue derived from reduction mammoplasty (Fig. 4*A*). Likewise, short-term cultures of breast epithelial organoids derived from reduction mammoplasty (four of four) or benign fibrocystic disease (two of two) were also completely unmethylated at *TMS1* (data not shown). In contrast, 11 of 27 (40%) primary breast carcinomas showed aberrant *de novo* methylation of the *TMS1* CpG island (Fig. 4*B*). In some cases the degree of methylation was substantial, particularly considering that resected breast tumors consist of a heterogeneous mixture of tumor cells and normal stromal and lymphocytic cells. Adjacent normal-appearing breast tissue was also available for 18 of the tumor samples. Eight of 18 pairs showed hypermethylation of *TMS1* in the tumor relative to adjacent normal tissue (*e.g.*, see samples 1–7 in Fig. 4*C*). Although a few of the adjacent normal tissue samples showed some methylation of TMS1, these represented normal-appearing tissue adjacent to a methylated tumor, and methylated DNA was enriched in the tumor sample relative to the normal (*e.g.*, see samples 5–8 in Fig. 4*C*). Tumors that were unmethylated at *TMS1* were also unmethylated in the corresponding normal tissue (samples 9–12, Fig. 4*C*). Therefore, aberrant methylation of the *TMS1* CpG island in the breast cancer cell lines reflects aberrant methylation occurring in primary tumors, and methylation of *TMS1* is a frequent event in human breast cancer.

Because the breast tumor tissue samples contained a significant proportion of normal stromal and lymphocytic elements (which express TMS1), we were unable to determine directly the relationship between *TMS1* methylation and gene silencing in primary breast tumor cells specifically. However, using bisulfite genomic sequencing, we found that tumor-derived DNA consisted of two distinct populations of alleles: those that were methylated at nearly every CpG site (53 total) across 500 bp of the CpG island; and those that were completely unmethylated (data not shown). Similar analyses of breast cancer cell lines showed that cells lacking *TMS1* expression contain only fully methylated alleles. These data argue that there is a subset of cells in the tumor that are completely methylated, and that these cells are unlikely to express *TMS1*.

That *TMS1* was aberrantly methylated and silenced in a substantial proportion of human breast cancer cells suggested that *TMS1* may

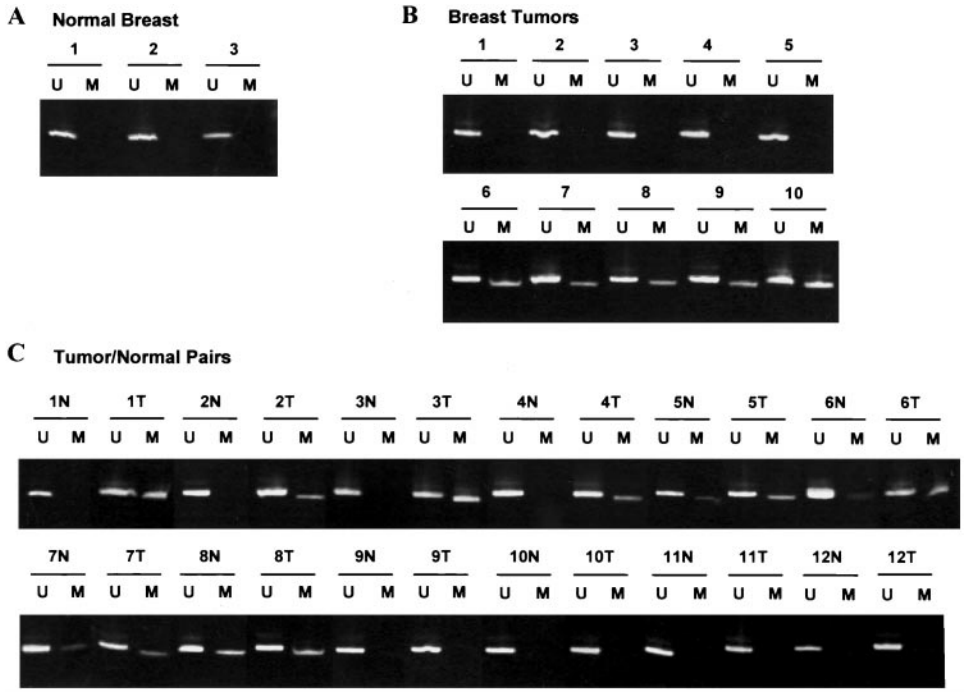

Fig. 4. Methylation of TMS1 in primary breast tissues. Methylation-specific PCR analysis of bisulfite-modified DNA from normal breast tissue from reduction mammoplasty (*A*), and primary resected breast carcinomas (*B*). Shown are five representative tumor samples that lacked methylation at the *TMS1* CpG island and five representative tumor samples exhibiting hypermethylation of the *TMS1* CpG island. Parallel amplification reactions were performed using primers specific to methylated (*M*) or unmethylated (*U*) DNA. *C*, methylation-specific PCR analysis of TMS1 in paired normal-appearing tissue (*N*) adjacent to breast tumors (*T*). Shown are 12 representative pairs of a total of 18 analyzed.

play a novel tumor suppressor function. *TMS1* exhibited widespread low-level expression in human tissues, with the greatest expression in colon, spleen, small intestine, lung, and peripheral blood leukocytes (Fig. 5*A*). Interestingly, *TMS1* was also silent in other human tumor cell lines, including Molt 4 lymphocytic leukemia cells and HeLa cervical carcinoma cells (Fig. 5*A*). The cDNA sequence predicted that *TMS1* encodes a protein of 195 amino acids and 22,000 M_r. Through a combination of BLAST homology searches of GenBank and the SMART domain recognition database (20), we found that TMS1 contained a COOH-terminal CARD (Fig. 5*B*). Similar in structure and function to the death domain and the death effector domain, the CARD is a homotypic protein interaction domain found in proteins that function in the regulation and execution of apoptosis (21). An alignment of TMS1 with other human CARDs indicated that the TMS1 CARD was most similar to that of the serine/threonine kinase RICK/CARDIAK/RIP2 (25% identity, 51% similarity; Refs. 17, 22) and the CED-4/apoptotic protease activating factor-1 family member CARD4/Nod1 (24% identity, 44% similarity; Refs. 23, 24; Fig. 5*B*).

The presence of a CARD strongly suggested that *TMS1* plays a role in apoptosis. To test this hypothesis, we determined the effect of ectopic *TMS1* expression on 293 cells. Cells transfected with *TMS1* became condensed, rounded, and eventually lifted off the culture surface, indicative of apoptosis (Fig. 5*C*). Expression of wild-type, or myc-tagged *TMS1* induced a 6-fold increase in apoptosis (Fig. 5*C*). The apoptotic activity of *TMS1* was blocked by the general caspase inhibitor zVAD, indicating that *TMS1*-induced cell death requires caspase activation (Fig. 5*C*). We investigated further the effects of *TMS1* on breast cancer cells. Stable expression of wild-type or myc-tagged *TMS1* in the *TMS1*-negative Hs578t cells or MDA MB231 cells reduced the number of surviving colonies by 3–4-fold (Fig. 5*D*). Not surprisingly given the proapoptotic activity of TMS1, overexpression of *TMS1* had similar growth-inhibitory effects on both *TMS1*-negative (Hs578t and MB231) and *TMS1*-positive (MCF7) breast cancer cells (Fig. 5*D*). Taken together, these data indicate that *TMS1* functions in the promotion of caspase-dependent apoptosis and overexpression of *TMS1* inhibits the growth of breast cancer cells.

Discussion

Aberrant methylation of gene-associated CpG islands is well recognized as a mechanism associated with the loss of tumor suppressor gene expression in human cancers. However, it has been unclear whether the aberrant CpG island methylation associated with carcinogenesis is sufficient to precipitate the silencing of an active, endogenous gene. *TMS1* is the first gene to be identified in a functional screen for such methylation-induced gene silencing events. The finding that *TMS1* is aberrantly methylated and silenced in response to overexpression of *DNMT1* is interesting for several reasons. First, it suggests that aberrant methylation can drive gene-silencing *in vivo*. This may occur indirectly, through the binding of methylated DNA-binding proteins and the recruitment of histone deacetylase complex (9), or directly, through the recruitment of histone deacetylase by DNMT1 itself (25). Secondly, increased expression of *DNMT1* promotes transformation, and is an early downstream effector of oncogene-induced transformation (26, 27). Taken together, these data support the hypothesis that aberrant methylation plays a direct role in carcinogenesis by precipitating the silencing of critical regulatory genes.

TMS1 belongs to a growing family of apoptotic signaling molecules that contain a CARD. The CARD is found within the prodomain of a number of caspases, and oligomerization with upstream CARD-containing regulatory proteins mediates their cleavage and activation (21). Other CARD-containing proteins with known roles in apoptosis include the *Caenorhabditis elegans* CED-3 and CED-4, the human homologue of CED-4, apoptotic protease activating factor-1, the cellular and viral inhibitors of apoptosis, the cellular homologue of herpesvirus EHV2 E10 protein, BCL10, and several proteins involved in the activation of NF-κB (28). By analogy to other CARDs, TMS1 is likely to function as an adapter protein, acting in the initiation phase of an apoptotic pathway by coupling death receptors at the cell surface or intrinsic death signals to the activation of the caspase cascade. Consistent with this hypothesis, we show here that *TMS1* promotes apoptosis directly, and that this apoptosis requires the activation of downstream caspases. Our additional studies indicate that apoptosis is triggered by a CARD-mediated cytoplasmic redistribution of *TMS1*

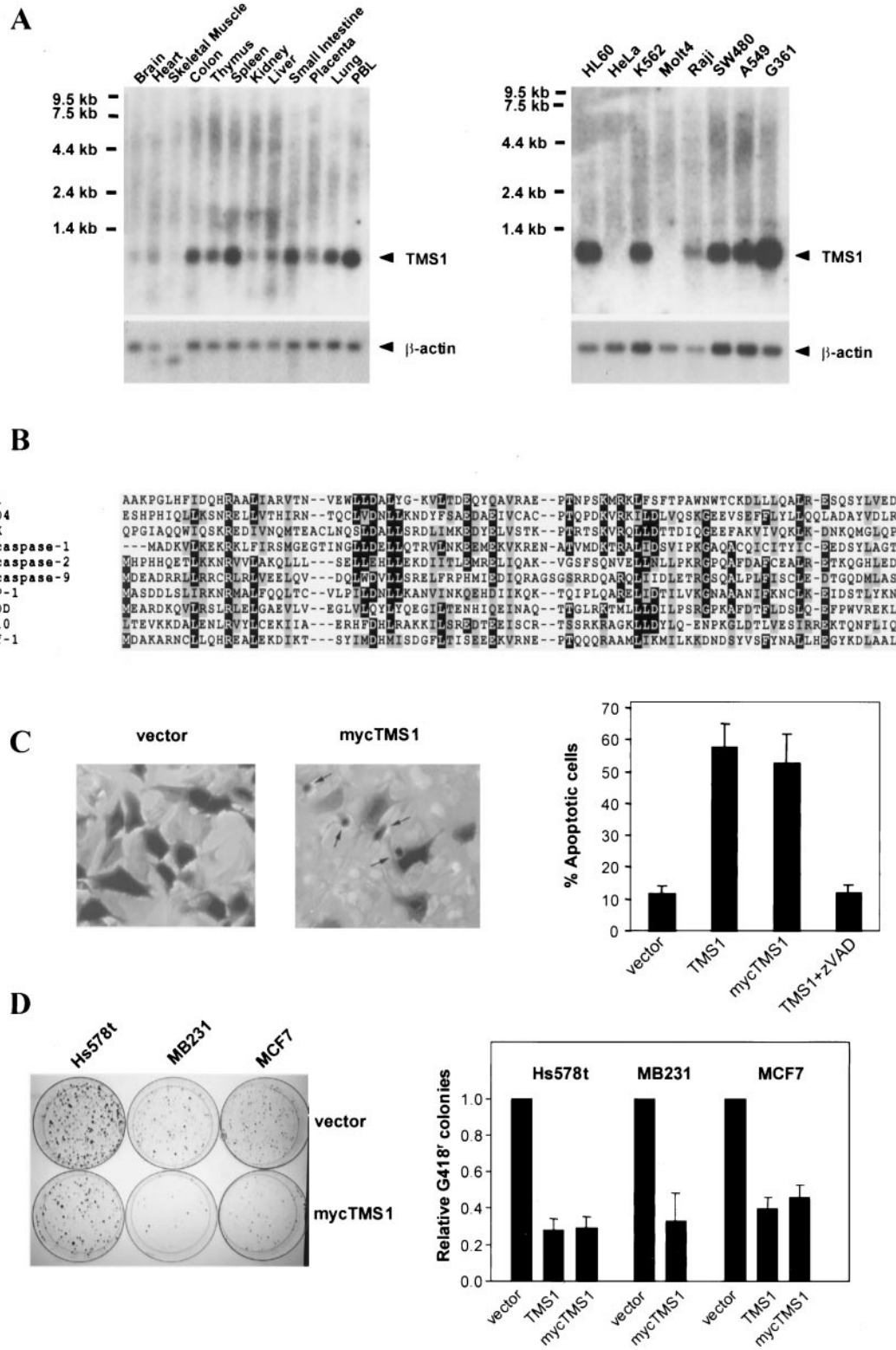

Fig. 5. TMS1 encodes a proapoptotic CARD protein. *A*, Northern blots of poly(A)+ RNA isolated from various normal human tissues and human tumor-derived cell lines hybridized to a TMS1 cDNA probe (*top*) or a human β-actin (*bottom*) probe. *B*, amino acid alignment of the TMS1 COOH-terminus with the CARD motif of other apoptotic signaling proteins. *Reverse type*, ≥50% amino acid identity; *gray shading*, ≥50% similarity through conserved amino acid substitutions. *C*, induction of apoptosis by TMS1. The 293 cells were cotransfected with 0.1 μg of pCMVβ-gal and 0.4 μg of vector (pcDNA3.1) or the indicated TMS1 expression construct. Where indicated, zVAD (40 μM) was added immediately after transfection. After 48 h, cells were stained with X-gal and examined by phase contrast microscopy (×400). β-galactosidase-positive cells undergoing apoptosis were distinguished by a more condensed, rounded appearance and showed evidence of detachment from the culture surface (*arrows*). Data are presented as the percentage of β-galactosidase-positive cells exhibiting apoptosis (mean ± SD) from three independent transfection experiments. *D*, colony-forming ability of breast cancer cells stably transfected with *TMS1* Hs578t, MDA MB231, or MCF 7 cells were transfected with the pcDNA3.1 vector or the indicated *TMS1* expression construct. After 24 h, cells were plated at clonal density in medium supplemented with G418. Stable G418-resistant colonies remaining after 14 days were fixed in methanol and stained with crystal violet. Data (mean ± SD) represent the number of G418-resistant colonies recovered for each test construct relative to that of the vector. At least three independent transfection experiments were performed.

and requires the activity of caspase-9 (B. McConnell and P. Vertino; see accompanying article). Thus, the data are most consistent with a role for TMS1 as a positive mediator of apoptosis.

Genetic alterations that lead to cellular resistance to apoptosis, such as mutational inactivation of proapoptotic genes such as *TP53* and *BAX*, or translocation and activation of antiapoptotic genes like *BCL2*, promote tumorigenesis by allowing damaged or unnecessary cells to persist and accumulate additional genetic insult (29). Our data suggest that epigenetic alterations resulting in the silencing of a proapoptotic gene such as *TMS1* could provide a similar survival advantage. Reintroduction of *TMS1* inhibited the growth of breast cancer cells, most likely as a result of its proapoptotic activity. Interestingly, this growth-suppressive activity appears to be p53-independent because p53-negative (Hs578t and MB231) and p53-positive (MCF7) cells were similarly affected. Together with the findings of others showing aberrant methylation of the death-associated protein kinase gene in hematological malignancies (30, 31) and methylation-associated silencing of caspase-8 in neuroblastomas (32), these data extend the role of aberrant methylation in carcinogenesis to include the silencing of genes that act as positive mediators of cell death.

There is accumulating evidence to suggest that altered function of CARD-containing regulatory molecules represents an important step in human carcinogenesis; two other CARD encoding genes, *API2* and *BCL10*, have been identified at translocation breakpoints in mucosal-associated lymphoid-tissue lymphomas (33–35). Recently, *TMS1* was independently identified (called "*ASC*") and shown to be involved in drug-induced apoptosis in HL-60 cells (36). Methylation-mediated silencing of *TMS1* may thus confer resistance to chemotherapeutic agents or other genotoxic stress. Interestingly, both our group and Masumoto *et al.* (36) noted that a number of human cell lines from multiple tumor types also lack *TMS1* expression (see Fig. 5*A*). Our data are the first to show the loss of function of a CARD protein through methylation-mediated silencing and to implicate *TMS1* as a novel tumor suppressor for breast, and perhaps other, cancers.

Acknowledgments

We thank Jane Iber for screening the human BAC library and radiation hybrid panels, Dr. Sara Sukumar for providing DNA from primary breast tissues and tumors, and Drs. Daniel Reines and Paul Doetsch for their critical review of the manuscript.

References

1. Bird, A. P. CpG-rich islands and the function of DNA methylation. Nature (Lond.), *321:* 209–213, 1986.
2. Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M., and Issa, J. P. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv. Cancer Res., *72:* 141–196, 1998.
3. Issa, J. P. Aging, DNA methylation and cancer. Crit. Rev. Oncol. Hematol., *32:* 31–43, 1999.
4. Jones, P. A., and Laird, P. W. Cancer epigenetics comes of age. Nat. Genet., *21:* 163–167, 1999.
5. Herman, J. G., Umar, A., Polyak, K., Graff, J. R., Ahuja, N., Issa, J. P., Markowitz, S., Willson, J. K., Hamilton, S. R., Kinzler, K. W., Kane, M. F., Kolodner, R. D., Vogelstein, B., Kunkel, T. A., and Baylin, S. B. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc. Natl. Acad. Sci. USA, *95:* 6870–6875, 1998.
6. Veigl, M. L., Kasturi, L., Olechnowicz, J., Ma, A. H., Lutterbaugh, J. D., Periyasamy, S., Li, G. M., Drummond, J., Modrich, P. L., Sedwick, W. D., and Markowitz, S. D. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. Proc. Natl. Acad. Sci. USA, *95:* 8698–8702, 1998.
7. Graff, J. R., Herman, J. G., Lapidus, R. G., Chopra, H., Xu, R., Jarrard, D. F., Isaacs, W. B., Pitha, P. M., Davidson, N. E., and Baylin, S. B. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res., *55:* 5195–5199, 1995.
8. Bachman, K. E., Herman, J. G., Corn, P. G., Merlo, A., Costello, J. F., Cavenee, W. K., Baylin, S. B., and Graff, J. R. Methylation-associated silencing of the tissue inhibitor of *metalloproteinase-3* gene suggest a suppressor role in kidney, brain, and other human cancers. Cancer Res., *59:* 798–802, 1999.
9. Bird, A. P., and Wolffe, A. P. Methylation-induced repression: belts, braces, and chromatin. Cell, *99:* 451–454, 1999.
10. Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A. P. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat. Genet., *19:* 187–191, 1998.
11. Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature (Lond.), *393:* 386–389, 1998.
12. Wade, P. A., Gegonne, A., Jones, P. L., Ballestar, E., Aubry, F., and Wolffe, A. P. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat. Genet., *23:* 62–66, 1999.
13. Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., and Baylin, S. B. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat. Genet., *21:* 103–107, 1999.
14. Vertino, P. M., Yen, R. W., Gao, J., and Baylin, S. B. *De novo* methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5-)-methyltransferase. Mol. Cell. Biol., *16:* 4555–4565, 1996.
15. Hubank, M., and Schatz, D. G. Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res., *22:* 5640–5648, 1994.
16. 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 islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.
17. McCarthy, J. V., Ni, J., and Dixit, V. M. RIP2 is a novel NF-κB-activating and cell death-inducing kinase. J. Biol. Chem., *273:* 16968–16975, 1998.
18. Fujii, H., Biel, M. A., Zhou, W., Weitzman, S. A., Baylin, S. B., and Gabrielson, E. Methylation of the *HIC-1* candidate tumor suppressor gene in human breast cancer. Oncogene, *16:* 2159–2164, 1998.
19. Ottaviano, Y. L., Issa, J. P., Parl, F. F., Smith, H. S., Baylin, S. B., and Davidson, N. E. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res., *54:* 2552–2555, 1994.
20. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. USA, *95:* 5857–5864, 1998.
21. Hofmann, K., Bucher, P., and Tschopp, J. The CARD domain: a new apoptotic signalling motif. Trends Biochem. Sci., *22:* 155–156, 1997.
22. Inohara, N., del Peso, L., Koseki, T., Chen, S., and Nunez, G. RICK, a novel protein kinase containing a caspase recruitment domain, interacts with CLARP and regulates CD95-mediated apoptosis. J. Biol. Chem., *273:* 12296–12300, 1998.
23. Bertin, J., Nir, W. J., Fischer, C. M., Tayber, O. V., Errada, P. R., Grant, J. R., Keilty, J. J., Gosselin, M. L., Robison, K. E., Wong, G. H., Glucksmann, M. A., and DiStefano, P. S. Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-κB. J. Biol. Chem., *274:* 12955–12958, 1999.
24. Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J., and Nunez, G. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-κB. J. Biol. Chem., *274:* 14560–14567, 1999.
25. Fuks, F., Burgers, W. A., Brehm, A., Hughes-Davies, L., and Kouzarides, T. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nat. Genet., *24:* 88–91, 2000.
26. Wu, J., Issa, J. P., Herman, J., Bassett, D. E. J., Nelkin, B. D., and Baylin, S. B. Expression of an exogenous eukaryotic DNA methyltransferase gene induces transformation of NIH 3T3 cells. Proc. Natl. Acad. Sci. USA, *90:* 8891–8895, 1993.
27. Bakin, A. V., and Curran, T. Role of DNA 5-methylcytosine transferase in cell transformation by fos. Science (Washington DC), *283:* 387–390, 1999.
28. Hofmann, K. The modular nature of apoptotic signaling proteins. Cell Mol. Life Sci., *55:* 1113–1128, 1999.
29. Pan, H., Yin, C., and Van Dyke, T. Apoptosis and cancer mechanisms. Cancer Surv., *29:* 305–327, 1997.
30. Kissil, J. L., Feinstein, E., Cohen, O., Jones, P. A., Tsai, Y. C., Knowles, M. A., Eydmann, M. E., and Kimchi, A. DAP-kinase loss of expression in various carcinoma and B-cell lymphoma cell lines: possible implications for role as tumor suppressor gene. Oncogene, *15:* 403–407, 1997.
31. Katzenellenbogen, R. A., Baylin, S. B., and Herman, J. G. Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. Blood, *93:* 4347–4353, 1999.
32. Teitz, T, Wei, T., Valentine, M. B., Vanin, E. F., Grenet, J., Valentine, V. A., Behm, F. G., Look, A. T., Lahti, J. M., and Kidd, V. J. Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of *MYCN*. Nat. Med., *6:* 529–535, 2000.
33. Zhang, Q., Siebert, R., Yan, M., Hinzmann, B., Cui, X., Xue, L., Rakestraw, K. M., Naeve, C. W., Beckmann, G., Weisenburger, D. D., Sanger, W. G., Nowotny, H., Vesely, M., Callet-Bauchu, E., Salles, G., Dixit, V. M., Rosenthal, A., Schlegelberger, B., and Morris, S. W. Inactivating mutations and overexpression of *BCL10*, a caspase recruitment domain-containing gene, in MALT lymphoma with t(1;14)(p22;q32). Nat. Genet., *22:* 63–68, 1999.
34. Willis, T. G., Jadayel, D. M., Du, M. Q., Peng, H., Perry, A. R., Abdul-Rauf, M., Price, H., Karran, L., Majekodunmi, O., Wlodarska, I., Pan, L., Crook, T., Hamoudi, R., Isaacson, P. G., and Dyer, M. J. Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. Cell, *96:* 35–45, 1999.
35. Dierlamm, J., Baens, M., Wlodarska, I., Stefanova-Ouzounova, M., Hernandez, J. M., Hossfeld, D. K., De Wolf-Peeters, C., Hagemeijer, A., Van den Berghe, H., and Marynen, P. The apoptosis inhibitor gene *API2* and a novel 18q gene, *MLT*, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. Blood, *93:* 3601–3609, 1999.
36. Masumoto, J., Taniguchi, S., Ayukawa, K., Sarvotham, H., Kishino, T., Niikawa, N., Hidaka, E., Katsuyama, T., Higuchi, T., and Sagara, J. ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. J. Biol. Chem., *274:* 33835–33838, 1999.

TMS1, a Novel Proapoptotic Caspase Recruitment Domain Protein, Is a Target of Methylation-induced Gene Silencing in Human Breast Cancers

Kerry E. Conway, Beth B. McConnell, Claire E. Bowring, et al.

Cancer Res 2000;60:6236-6242.