Elevated expression of coactivator-associated arginine methyltransferase 1 is associated with early hepatocarcinogenesis

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Abstract. Aberrant expression of regulators for epigenetics is involved in tumorigenesis. There is an urgent need to identify and characterize regulators concerned with epigenetics in the early stages of hepatocarcinogenesis. In the present study, we found that the expression of coactivator-associated arginine methyltransferase 1 (CARM1), a histone methyltransferase that functions as a cofactor for nuclear hormone receptors and several transcription factors, was elevated in adenomas and aberrant in carcinomas during hepatocellular carcinogenesis. In addition to RNA expression, immunohistochemical staining of liver sections revealed that CARM1 was highly expressed in the nucleus of tumor marker glutathione *S*-transferase placental form (GST-P)-positive foci. Neoplastic transformation of GST-P-positive foci guides the formation of hepatocellular carcinomas. CARM1 expression was not elevated in GST-P-negative regions. Furthermore, a luciferase reporter analysis revealed that CARM1 activated the *Gst-p* promoter in H4IIE, a hepatocellular carcinoma cell line. This activation was mediated by the enhancer element responsible for the carcinogenic-specific expression of *Gst-p* and nuclear factor E2-related factor 2. Knockdown of *Carm1* by shRNA in H4IIE cells inhibited cell proliferation. These findings suggest that aberrantly expressed CARM1 in tumor marker-positive cells promotes tumorigenesis in the early stages of hepatocarcinogenesis.

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Introduction

Cancer is caused by mutations in oncogenes and tumor-suppressor genes. In addition to the identification of genetic mutations, progress has been made in understanding cancer epigenetics in oncology (1). DNA methylation and covalent histone modifications are prominent components of epigenetic regulation. Aberrant hypermethylation of regulatory regions in tumor-suppressor genes and global hypomethylation are found in cancerous cells. Perturbation of histone modification patterns is another hallmark of cancer. Loss of acetylation at lysine 16 in histone H4 (H4K16) and trimethylation of H4K20 is reported in many tumor types (2). Therefore, regulators of epigenetics have received attention as molecular targets, and several inhibitors of DNA methyltransferase and histone deacetylase have been approved for cancer treatment (3). However, regulators of epigenetics and their roles in the early stages of hepatocarcinogenesis remain poorly understood.

To identify factors concerned with tumorigenesis in the early stages of hepatocarcinogenesis, hepatic preneoplastic lesions were chemically induced in rats (4-6). We found that several histone modification enzymes were upregulated in livers highly expressing the tumor marker glutathione *S*-transferase placental form (GST-P) (7-9). Neoplastic transformation of GST-P-positive foci guides the formation of hepatocellular carcinomas. GST-P expression is completely repressed in normal rat liver. As specific induction of the *Gst-p* gene in the pre-neoplastic lesions is induced by almost all chemical carcinogens, analysis of the regulatory mechanism of *Gst-p* expression will lead to a better understanding of the early stages of hepatocarcinogenesis (10,11). Analyses of transgenic rats harboring the regulatory region of *Gst-p* gene and *in vitro* studies revealed that a strong enhancer element, GST-P enhancer 1 (GPE1), located 2.5 kb upstream from the transcription start site, was responsible for the hepatocarcinogenic specific expression and was recognized by a nuclear factor E2-related factor 2 (Nrf2)/musculoaponeurotic fibrosarcoma oncogene homolog K (MafK) heterodimer (11).

We previously found that the expression of coactivator-associated arginine methyltransferase 1 (CARM1), also termed

protein arginine methyltransferase 4 (PRMT4), was elevated in the early stages of hepatocarcinogenesis in microarray experiments (5). CARM1 was originally identified as a factor associated with p160 coactivators of nuclear receptor (12). CARM1 also interacts with several transcription factors including cAMP-response element-binding protein-binding protein (CBP), β-catenin, and the nuclear factor-κB (NF-κB) subunit p65 (13-15). CARM1 functions as a secondary cofactor and activates transcription by methylating arginine residues 17 and 26 of histone H3, cofactors and itself (16-18). CARM1 contributes not only to transcription but also RNA splicing (19). However, the functions of CARM1 in the early stages of hepatocarcinogenesis currently are unclear.

In the present study, we demonstrated that CARM1 was highly expressed in GST-P-positive foci and activated the *Gst-p* promoter. Furthermore, knockdown of *Carm1* by shRNA in a hepatocellular carcinoma cell line inhibited cell proliferation. These findings suggest that aberrantly expressed CARM1 in GST-P-positive cells promotes tumorigenesis in the early stages of hepatocarcinogenesis.

Materials and methods

Animal experiments. Five-week-old male F344 rats were obtained from Charles River Japan, Inc. (Atsugi, Japan). At the age of 6 weeks, they were randomly divided into 2 groups, which continuously received either 0 or 50 ppm diethylnitrosamine (DEN) (Tokyo Kasei Co. Ltd., Tokyo, Japan) in their drinking water for up to 18 weeks. Rats were sacrificed at 12 or 18 weeks into the DEN treatment (6). The livers were immediately excised for analysis. Slices were fixed in 10% buffered formalin for immunohistochemical examination and hematoxylin and eosin staining, and the remaining liver tissue was immediately frozen in liquid nitrogen and stored at -80˚C until processed for RNA extraction. All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of the Nagoya City University School of Medical Sciences.

RNA preparation and quantitative reverse transcription coupled PCR (qRT-PCR). Total RNA was isolated with TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions and converted to cDNA using a random primer and ReverTra Ace (Toyobo, Osaka, Japan). Three adenomas (from 3 treated rats at 12 weeks), 3 carcinomas (from 3 treated rats at 18 weeks) and normal livers from 3 rats drinking water without DEN for 18 weeks were used. For qRT-PCR, the pre-designed primers and probe sets for *Carm1* and 18S rRNA were obtained from Applied Biosystems (Foster City, CA, USA). Data collection was performed with an ABI PRISM 7000 sequence detection system (Applied Biosystems).

Immunohistochemistry. Paraffin-embedded specimens were sectioned (3 μm) and stained with rabbit anti-rat GST-P antibody (MBL, Nagoya, Japan), CARM1 antibody (Millipore, Billerica, MA, USA) or Ki67 antibody (SP6; Acris Antibodies GmbH, Herford, Germany), and then with anti-mouse or anti-rabbit secondary antibody and avidin-biotin complex (Vectastatin Elite ABC kit; Vector Laboratories, Burlingame, CA, USA), and binding sites were visualized with diaminobenzidine. The sections were then counterstained lightly with hematoxylin for microscopic examination. The number of CARM1- and Ki67-labeled cells in at least 500 liver cells was counted to determine a labeling index. The staining intensity of CARM1 in the nucleus was quantitatively assessed with an Image Processor for Analytical Pathology (IPAP-WIN; Sumika Technoservice, Takarazuka, Japan) to provide the optical densities.

Luciferase reporter assay. Reporter plasmids, -2.5GST-luciferase containing the regulatory region of the *Gst-p* gene, -2.5 kb to +59 bp, and -2.15GST-luciferase, were described previously (9). A reporter plasmid including the GPE1 element, GPE1-50GST-luciferase, was kindly provided by Dr M. Sakai (Hokkaido University) (20). The Myc-tagged Nrf2 expression plasmid (pCMV-Myc-Nrf2) was constructed by subcloning of the rat Nrf2 open reading frame into the *Sal*I-*Not*I site of pCMV-Myc (Clontech Laboratories, Mountain View, CA, USA). The Flag-tagged CARM1 expression plasmid (Flag-CARM1) was kindly provided by Dr N. Ohkura (Osaka University) (19).

Rat hepatoma H4IIE cells, purchased from Dainippon Sumitomo Pharma (Osaka, Japan), were maintained in α-medium supplemented with 10% (v/v) fetal bovine serum. Transfection of H4IIE cells in 24-well plates was performed using HilyMax (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. The cells were transfected with 50 ng of reporter plasmid, 50 ng of pCMV-Myc-Nrf2, and 400 ng of the Flag-tagged Carm1 expression plasmid. The amount of plasmid during transfection was kept constant by using an empty vector. Fifty nanograms of the *Renilla* luciferase reporter plasmid phRL-TK (Promega Corporation, Madison, WI, USA), was used as the internal control. At 40 h after transfection, cells were harvested and assayed for luciferase activity by using the Dual-Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's recommendations.

Transfection of shRNA expression plasmid and cell counting. To generate shRNA expression plasmids, rat *Carm1* shRNA was designed using the Qiagen siRNA online design tool. The selected region spanning base pairs 1339-1359 of Carm1 was subcloned with a 5'-TTCAAGAGA-3' loop into the *Apa*I-*Eco*RI site of the plasmid pSilencer 1.0-U6 (Ambion, Inc., Austin, TX, USA). H4IIE cells ($2.6x10^6$) were transfected with 6.5 μg of the shRNA expression plasmid using the Neon transfection system (Invitrogen Life Technologies, Carlsbad, CA, USA) and plated at $2x10^4$ cells in 24-well plates. Cells were trypsinized on days 1, 2, 3 and 4, and cell numbers were measured by a hemocytometer.

Results

Expression profile of Carm1 during hepatocarcinogenesis. *Carm1* was identified as one of the genes induced in GST-P-positive foci and involved in transcriptional regulation (5). To investigate the expression profile of *Carm1* in the early stages of hepatocarcinogenesis, adenomas and carcinomas were generated by giving rats DEN in their drinking water. After

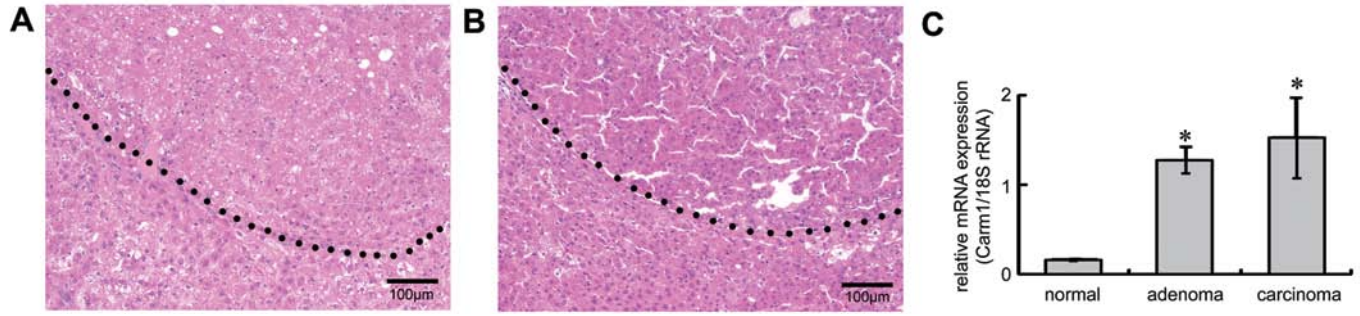

Figure 1. Histopathology and expression of *Carm1* in the early stages of hepatocarcinogenesis. Adenomas and carcinomas from the DEN-treated rats at 12 weeks (A) and 18 weeks (B), respectively, are indicated (right-upper side). (C) Expression profile of *Carm1* was determined by qRT-PCR. RNA was prepared from normal liver and regions of adenomas and carcinomas. RNA levels were normalized with 18S rRNA expression. Values are presented as the means ± SE (n=3). *p<0.05, significant differences as evaluated with Dunnett's multiple comparison test when compared with the values for normal liver.

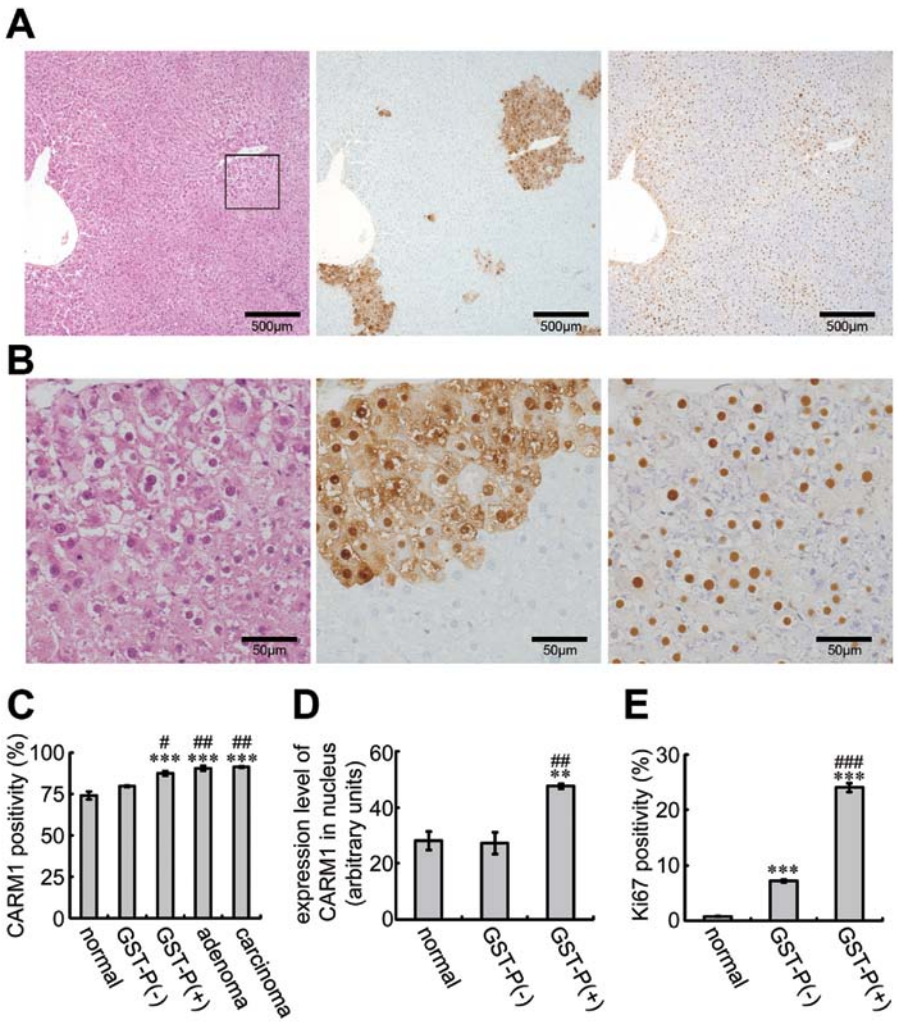

Figure 2. Immunohistochemistry of CARM1 and GST-P. (A) Sections derived from rats treated with DEN for 12 weeks were stained with anti-CARM1 antibody (right) and anti-GST-P antibody (middle). H&E staining (left) is shown (x40). (B) Higher magnification of the rectangle in A (x400). (C) Percentage of CARM1-positive cells, (D) expression level of CARM1 in the nucleus, and (E) percentage of Ki67-positive cells. Values are presented as the means ± SE (n=3). Significant differences were evaluated with Tukey's multiple comparison test. **p<0.01 and ***p<0.001 compared to normal liver. $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared to GST-P-negative cells.

12 weeks of DEN treatment, adenomas, round nodular lesions compressing adjacent normal hepatocytes, were generated (right-upper side, Fig. 1A). Carcinomas, which had aberrant trabeculae and were circumferentially infiltrated, formed after 18 weeks of treatment (right-upper side, Fig. 1B). RNA was prepared from dissected adenomas and carcinomas, and qRT-PCR was performed. Expression of *Carm1* was elevated in the adenomas when compared with expression in the

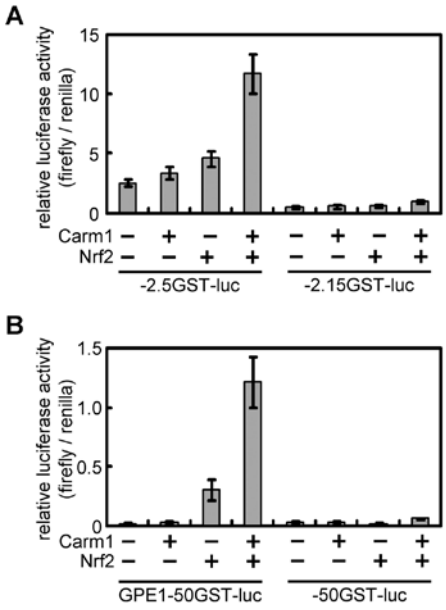

Figure 3. Effects of overexpression of CARM1 on the promoter activity of *Gst-p* in rat hepatoma H4IIE cells. (A) Effects of overexpression of CARM1 with Nrf2 on *Gst-p* promoter activity. Reporter plasmid, -2.5GST-luciferase containing the GPE1 enhancer, or -2.15GST-luciferase, was transfected with or without 400 ng of CARM1 or 50 ng of Nrf2 expression plasmid into H4IIE cells. (B) Effects of overexpression of CARM1 with Nrf2 on the GPE1 enhancer. Reporter plasmid containing the minimal promoter sequence of the rat *Gst-p* gene (-50/+37 bp) with or without the GPE1 enhancer was used. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Values are presented as the means ± SD. All transfection experiments were performed in triplicate. A typical result from at least 2 independent experiments is shown.

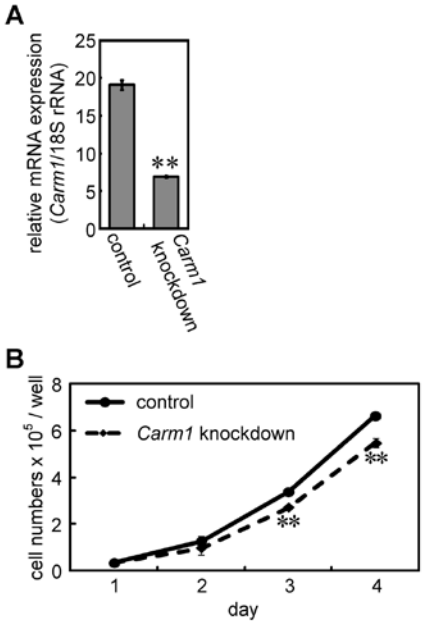

Figure 4. Effects of shRNA-mediated knockdown of *Carm1* expression on cell proliferation in H4IIE cells. (A) Total RNA was prepared from H4IIE cells transfected with the shRNA expression plasmid for *Carm1* or the control shRNA expression plasmid 3 days following electroporation. Expression levels were normalized to 18S rRNA expression. (B) Cells transfected with the shRNA expression plasmid were plated in 24-well plates, and the cell number was measured by a hemocytometer. Values are presented as the means ± SD (n=3). $^{**}p<0.01$, significant differences with the Student's t-test when compared with the values for the control.

normal liver, and expression of *Carm1* remained aberrant in the carcinomas (Fig. 1C).

Expression of CARM1 in GST-P-positive cells. To examine the expression of CARM1 immunohistochemically, sections from rats treated with DEN for 12 weeks were stained with the anti-CARM1 and anti-GST-P antibodies (Fig. 2A and B). CARM1 was expressed in the nucleus in many liver cells (Fig. 2B, right panel). Notably, CARM1 was detected in the nucleus in almost all GST-P-positive foci. CARM1 positivity in several types of liver cells revealed that CARM1-positive cells with GST-P staining were significantly induced compared to both normal liver cells and GST-P-negative cells (Fig. 2C). In addition, high CARM1 positivity was retained in the adenomas and carcinomas. To compare CARM1 expression in the nucleus, we measured the staining intensity of CARM1 in several types of cells (Fig. 2D). The expression level of CARM1 in the nucleus was higher in GST-P-positive cells than that in the normal liver cells and GST-P-negative-cells. We also examined Ki67, a marker of cell proliferation, in the GST-P-positive and -negative cells (Fig. 2E). Ki67 positivity was greater in the GST-P-positive cells than that in the negative cells. These results suggest that the elevated expression of CARM1 in GST-P-positive foci is associated with tumorigenesis and that CARM1 is involved in cell proliferation.

Effects of ectopic expression of CARM1 on Gst-p promoter activity. Immunohistochemical staining of liver sections revealed that nuclear CARM1 was overexpressed in GST-P-positive foci. Next, we investigated the effect of the ectopic expression of CARM1 on *Gst-p* promoter activity. The regulatory region of the *Gst-p* gene is well characterized, and GST-P enhancer 1 (GPE1), located -2.5 kb upstream from the cap site, is responsible for the hepatocarcinogenic specific gene expression (11). GPE1 is recognized by the Nrf2/MafK heterodimer. When the CARM1 expression plasmid was transfected into H4IIE cells with -2.5GST-luciferase, which has the entire *Gst-p* regulatory region and promoter, the promoter was not activated (Fig. 3A). However, *Gst-p* promoter activity was enhanced when the CARM1 expression plasmid was introduced with the Nrf2 expression plasmid. This activation was not observed when -2.15GST-lucifearse lacking GPE1, was introduced. To restrict the element responding to CARM1, a reporter plasmid containing the minimal promoter sequence of the *Gst-p* gene with or without GPE1 was used. CARM1 enhanced the *Gst-p* promoter activity dependent on GPE1 and Nrf2 (Fig. 3B).

Effects of shRNA-mediated knockdown of Carm1 on cell proliferation in a hepatocellular carcinoma cell line. The ratio of Ki67-positive cells in the GST-P-positive foci was higher than that in the GST-P-negative cells (Fig. 2E). To investigate the effects of the depletion of *Carm1* on cell proliferation, we constructed shRNA expression plasmid for *Carm1*. Cells transfected with the plasmid were plated in 24-well plates, and the expression level of the target gene was measured 3 days following transfection (Fig. 4A). Furthermore, the cell numbers were counted using a hemocytometer (Fig. 4B). The expression

of the target gene was suppressed by the shRNA expression plasmid for *Carm1*. Under these conditions, the number of cells transfected with the shRNA expression plasmid for *Carm1* were reduced when compared with the number of cells in the control at days 3 and 4. These results suggest that *Carm1* is involved in the cell proliferation of H4IIE cells.

Discussion

We demonstrated here that CARM1 expression is upregulated in GST-P-positive foci during hepatocarcinogenesis. In addition, we demonstrated that CARM1 activated the *Gst-p* promoter through Nrf2. Furthermore, knockdown of *Carm1* by shRNA in H4IIE cells inhibited cell proliferation. These observations suggest that aberrantly expressed CARM1 in GST-P-positive cells promotes tumorigenesis in the early stages of hepatocarcinogenesis.

Analysis of the expression of CARM1 in human cancers using a tissue microarray containing various tumor types including brain tumors, melanoma, colorectal cancer, prostate cancer and breast cancer, but not liver cancer, revealed that CARM1 expression was elevated in colorectal cancer, but not in prostate or breast cancer (21). In contrast, elevated expression of CARM1 in prostate cancer was previously reported (22,23). These observations indicate that CARM1 is involved in the tumorigenesis of various types of cancers. Although CARM1 is known as a coactivator for nuclear receptors including the androgen receptor, our results and previous observations suggest that it is also involved in sex hormone-independent cancer.

We showed here that CARM1 activated Nrf2-mediated *Gst-p* promoter activity. As no interaction between Nrf2 and CARM1 or localization of CARM1 to GPE1 was detected in H4IIE cells in the immunoprecipitation and chromatin immunoprecipitation experiments, respectively (data not shown), the chromatin-based localization of CARM1 to GPE1 may be weak or transient. Nrf2 possesses a degron common to both Keap1-independent and -dependent degradation in the nucleus (24,25). Proteasome and ubiquitin-conjugated enzymes are distributed throughout the cytoplasm and nucleus and they are important for the regulation of gene expression (26). Nrf2 interaction with CARM1 may be rapidly degraded after it functions as a transcriptional activator. Nrf2 is crucial for the transactivation of the *Gst-p* promoter mediated by GPE1. It is known that Nrf2 interacts with several cofactors including CBP (27). Depletion of *Carm1* did not decrease the expression of *Gst-p* (data not shown). The loss of CARM1 may be compensated for by the recruitment of other Nrf2 cofactors including CBP on the *Gst-p* promoter.

Nrf2 upregulates expression of the anti-apoptotic gene *Bcl-2* and prevents apoptosis (28). NF-κB, one of the transcription factors targeted by CARM1, directly activates anti-apoptotic genes including the genes for tumor necrosis factor receptor-associated factor 1 (TRAF1), TRAF2 and the inhibitor-of-apoptosis (IAP) proteins c-IAP1 and cIAP2 (29). We demonstrated that anti-apoptotic genes, including *Bcl-2*, were upregulated in GST-P-positive foci (5). These observations indicate that CARM1 may promote tumorigenesis by enhancing cell proliferation as well as through its anti-apoptotic function. To better understand the molecular mechanism of CARM1-mediated tumorigenesis, the identification of CARM1 target gene(s) in GST-P-positive foci is required.

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