RAPID COMMUNICATION Jpn. J. Cancer Res. **90**, 485–489, May 1999

Silencing of the *CD44* Gene by CpG Methylation in a Human Gastric Carcinoma Cell Line

Sunao Sato,[1,2] Hiroshi Yokozaki,[1] Wataru Yasui,[1] Hiromasa Nikai[2] and Eiichi Tahara[1,3]

[1]*First Department of Pathology, Hiroshima University School of Medicine and* [2]*Department of Oral Pathology, Hiroshima University School of Dentistry, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553*

We analyzed 8 human gastric carcinoma cell lines for the expression of *CD44* by northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR), and identified 1 cell line MKN-28 that did not express *CD44*. In an attempt to clarify the mechanism responsible for the inactivation of *CD44* gene expression in this cell line, we investigated the methylation status around the promoter region of *CD44* gene by digestion of the DNA with the methylation-sensitive restriction enzyme *Hpa*II. The promoter region of CD44 in MKN-28 revealed hypermethylation, whereas other CD44-positive cell lines did not. Furthermore, treatment of MKN-28 with the demethylating agent 5-azacytidine restored the expression of the gene. These results suggest that *CD44* expression is controlled by a DNA hypermethylation mechanism in MKN-28.

Key words: *CD44* — Methylation — Gastric carcinoma

Metastasis is one of the most life-threatening aspects of cancer progression and is closely associated with cellular properties, including cell-to-cell adhesiveness. CD44 is one of the cell surface molecules that plays an important role in cancer metastasis.[1–3] We have also confirmed that an aberrant transcript of *CD44* gene, including exon 11 as well as intron 9, is overexpressed in most gastric carcinomas.[4]

In the present study, we conducted Southern blot, northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses on 8 human gastric carcinoma cell lines to identify genetic abnormalities of the *CD44* gene. We discovered a cell line that does not express *CD44* mRNA. To elucidate the mechanism of its disappearance, we performed a methylation assay.

Eight cell lines derived from human gastric carcinoma were used. The TMK-1 cell line was established from poorly differentiated adenocarcinoma in our laboratory.[5] Five gastric carcinoma cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28, and MKN-74, well-differentiated adenocarcinoma; MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical University, Fukushima). The KATO-III and HSC-39 cell lines, which were established from signet ring cell carcinoma, were kindly provided by Dr. M. Sekiguchi (University of Tokyo, Tokyo) and by Dr. K. Yanagihara (Hiroshima University, Hiroshima),[6] respectively. All the cell lines were routinely maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) containing 10% fetal bovine serum (FBS) (Whittaker, Walkersville, MA) under humidified 5% CO_2 in air at 37°C.

High-molecular-weight DNAs were prepared from the cell lines, digested with *Hin*dIII restriction enzyme and subjected to Southern blot analysis as described previously.[7] A probe for the detection of *CD44* was prepared as follows: the region between exon 3 and exon 18 was RT-PCR-amplified from normal human spleen total RNA with the following primers: P1 (5′-GAC ACA TAT TGC TTC AAT GCT TCA GC-3′) and P2 (5′-GAT GCC AAG ATG ATC AGC CAT TCT GGA AT-3′), as described by Matsumura and Tarin.[8]

RNAs were extracted by the standard guanidium isothiocyanate/cesium chloride method.[9] Five micrograms of poly(A)+ selected RNA was electrophoresed on 1.0% agarose/formaldehyde gel and blotted onto a nitrocellulose filter membrane. Filters were baked for 2 h at 80°C under vacuum. Hybridization using a ^{32}P-labeled probe and washing were performed as described previously[10] and filters were exposed to X-ray film. The probe for the detection of *CD44* mRNA was the same as used for Southern blot analysis.

Total RNA was prepared from cell lines using the GLASSMAX RNA Microisolation Spin Cartridge System (Gibco-BRL, Gaithersburg, MD). Total RNA of 1 μg was used for the first-strand cDNA synthesis using a First-Strand cDNA Synthesis Kit (Pharmacia-LKB, Uppsala, Sweden). The RT reaction was subjected to PCR using P1 and P2, which can amplify the extracellular domain of CD44 including the insertion point of alternative spliced exons.[8] RT-PCR was performed for one cycle of 94°C for 10 min followed by 35 cycles of 94°C for 2 min, 55°C for 2 min, and 72°C for 3 min, followed by

[3] To whom requests for reprints should be addressed.
E-mail: etahara@mcai.med.hiroshima-u.ac.jp

one cycle of 72°C for 10 min, using AmpliTaq Gold (Perkin Elmer, Norwalk, CT). Buffer contained 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl, 50 m*M* KCl, and 200 μM of each deoxynucleotide triphosphate (dNTP). The resulting amplification products were then analyzed by 1.2% agarose gel electrophoresis with ethidium bromide and examined under UV light. β-Actin-specific PCR products from the same RNA samples were amplified and served as internal controls.

Genomic DNA samples were digested with restriction endonucleases in 100 μl volumes of restriction endonuclease buffer containing 5 μg of genomic DNA. Reaction mixtures contained either no enzyme, 25 units of *Hpa*II or 25 units of *Msp*I for 7 h at 37°C. To analyze cleavage of the CD44 promoter region, 1 ng of DNA from each digest was analyzed by PCR using primer M1 (5′-CAG CCC CGA TTA TTT ACA GC-3′) and M2 (5′-GTG CCA CCA AAA CTT GTC CA-3′) designed to amplify nucleotides −337 to 21 of the *CD44* gene. PCR was performed for one cycle of 95°C for 9 min followed by 35 cycles of 94°C for 2 min, 55°C for 2 min, and 72°C for 3 min, followed by one cycle of 72°C for 10 min, using AmpliTaq Gold (Perkin Elmer). The buffer contained 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl, 50 m*M* KCl, and 200 μM of each dNTP. The resulting amplification products were then analyzed by 1.2% agarose gel electrophoresis with ethidium bromide and examined under UV light.

The CD44-negative gastric carcinoma cell line was treated with 10 μM 5-azacytidine (Sigma Chemical Co., Ltd., St. Louis, MO). After culture for 14, 30, 40, 53 and 56 days, total RNA was extracted as described previously.

We used northern blot analysis and RT-PCR to examine the expression of *CD44* mRNA in 8 human gastric carcinoma cell lines. In MKN-28, no expression of *CD44* mRNA was detectable by northern blot or RT-PCR analysis (Fig. 1, B and C). Other cell lines expressed *CD44* mRNA to various extents. These 8 human gastric carcinoma cell lines showed polymorphism of the *CD44* gene, as found by Dadi *et al*. by Southern blot analysis (Fig. 1A).[11] In MKN-7 and TMK-1, an allele near 8 kb was detected, whereas MKN-1, -28, -45, -74 and TMK-1 had another allele near 2 kb. In addition, HSC-39 displayed amplification of the gene as compared with the other cell lines.

Hypermethylation status of the *CD44* promoter region in the gastric cancer cell lines was examined by using a PCR assay. The results of this analysis are shown in Fig.

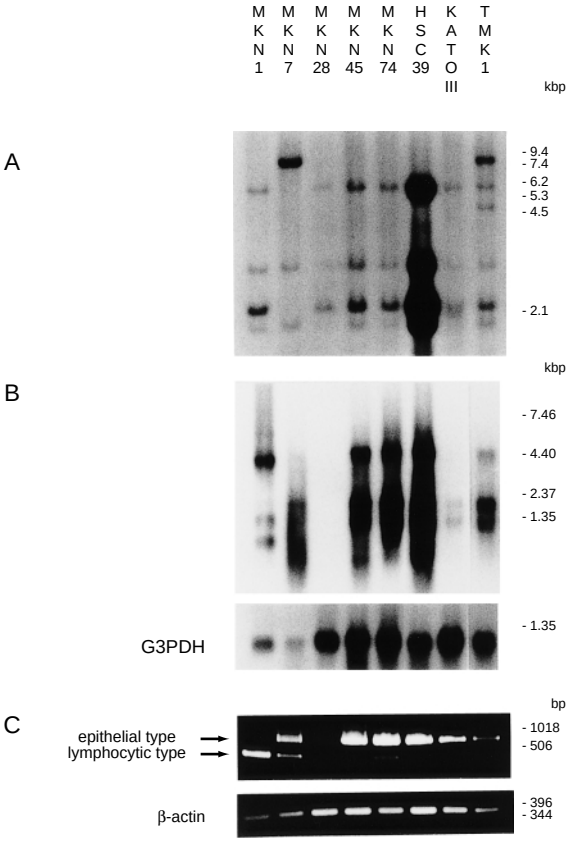

Fig. 1. Analysis of *CD44* gene. A: Southern blot analysis revealed *Hin*dIII polymorphism of *CD44* gene in 8 human gastric carcinoma cell lines. In addition, HSC-39 showed amplification of *CD44*. B: Northern blot analysis showed *CD44* mRNA expression in human gastric carcinoma cell lines with the exception of MKN-28. C: RT-PCR analysis of *CD44* mRNA expression in human gastric carcinoma cell lines. There was no expression of *CD44* mRNA in MKN-28. These results are consistent with those of northern blot analysis.

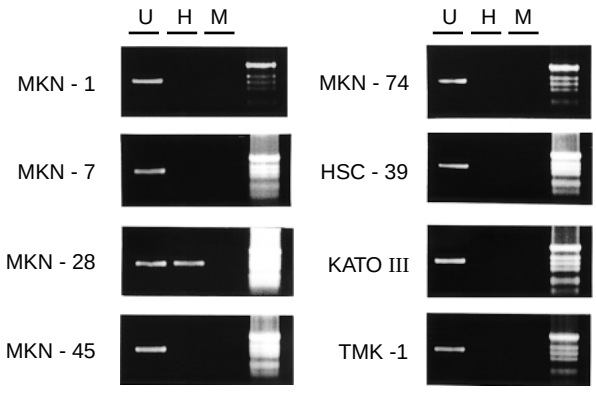

Fig. 2. Analysis of hypermethylation status of *CD44* gene promoter region. U, undigested; H, *Hpa*II-digested; M, *Msp*I-digested. The *CD44* promoter region of MKN-28 was resistant to digestion by *Hpa*II and sensitive to digestion by *Msp*I. In other cell lines, the promoter region of the cell lines was sensitive to digestion by both *Hpa*II and *Msp*I.

2. The *CD44* promoter region of MKN-28 was resistant to digestion by *Hpa*II and sensitive to digestion by *Msp*I, while the promoter region of the other cell lines was sensitive to digestion by both *Hpa*II and *Msp*I. These results indicate the existence of methylation at CpG sites of the *CD44* promoter region in MKN-28 cells that do not express *CD44* mRNA.

The CD44-negative MKN-28 cells were treated with 10 μM 5-azacytidine to see whether the silencing of *CD44* expression could be reversed by this DNA demethylating agent. After culture for 14 days with 5-azacytidine, methylation of MKN-28 cells had disappeared (data not shown). Fig. 3 shows the chronological changes of *CD44* mRNA expression level after treatment with 5-azacytidine, as determined by RT-PCR analysis. After culture for 40 days with 5-azacytidine, MKN-28 cells expressed *CD44* mRNA. These results suggest that hypermethylation of the *CD44* promoter region may be the mechanism of CD44 inactivation in the MKN-28 gastric carcinoma cell line and that treatment of CD44-inactivated cells with a demethylation agent may restore gene expression. The delay of *CD44* mRNA expression after disappearance of DNA methylation was interesting. Homman *et al*.[12] reported that the synthesis of new proteins required some time after DNA demethylation, since it is necessary to synthesize enough enzyme to allow growth under the new conditions or to change the chromatin conformation for stable reexpression.

Many investigators have reported that the expression of CD44 variants was associated with tumor progression and metastasis of breast cancer,[13–15] colorectal cancer,[8] cancer of the uterine cervix,[16] renal cancer[17] and non-Hodgkin's lymphoma.[18, 19] We have also confirmed overexpression of *CD44* variants in human gastric and colorectal carcinomas.[4, 20] In contrast, decreased expression of CD44 has been reported in certain other malignancies. Progressive decrease in both the standard form (CD44H) and the v6 isoform of CD44 was observed in deeply invasive aneuploid transitional cell carcinoma of the urinary bladder.[21] Significant loss of CD44H expression associated with high-grade atypia as well as aneuploidy was demonstrated in prostate cancer.[22] Gross *et al*. found a highly significant negative relationship between N-myc amplification and CD44H expression in stage IV neuroblastomas, whereas most of the stage I to III tumors overexpressed CD44H.[23] Moreover, lymph-vascular space involvement was observed in CD44-negative endometrial cancers as opposed to the positive cancers.[24] Recently, Ue *et al*. reported a positive correlation between down-regulation of CD44 variant(s) expression and metastatic potential in oral squamous cell carcinomas.[25] These previous observations may indicate that the reduced expression of CD44 and its variants also contributes to the metastatic potential of cancers, depending on their origin. However, the precise mechanism of CD44 down-regulation in these malignancies has not been elucidated yet. In the present study, most of the human gastric cancer cell lines overexpressed *CD44* variants to various extents as detected by northern blot or RT-PCR analysis, with the exception of MKN-28. No gross genomic alteration of the *CD44* gene was detected in MKN-28. Moreover, we demonstrated CpG methylation in the *CD44* promoter region of genomic DNA and succeeded in restoring the expression of *CD44* by treatment of MKN-28 cells with 5-azacytidine. Therefore, it can be concluded that the transcriptional inactivation of *CD44* in MKN-28 was due to hypermethylation of CpG islands around the 5′-regulatory areas of the gene. It should also be noted that MKN-28 was established from the lymph node metastasis of a gastric cancer and might be a clone with high metastatic potential.[26] As we have confirmed frequent overexpression of *CD44* variants in human gastric carcinomas,[4, 20] the lack of expression of the gene in MKN-28 is a rare event in stomach cancer. However, this finding may provide a clue to explain the epigenetic mechanism of CD44 down-regulation in other malignancies as listed previously.

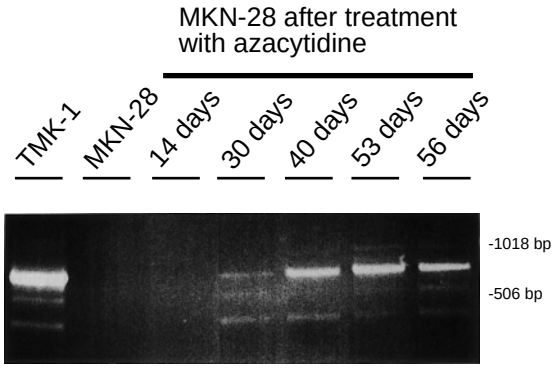

Fig. 3. Demethylation of the *CD44* promoter region of MKN-28 with 5-azacytidine. CD44-negative gastric carcinoma MKN-28 cells were treated with 10 μM 5-azacytidine. After culture for 40 days with 5-azacytidine, the MKN-28 cells expressed *CD44* mRNA.

Silencing of the genes encoding cellular adhesion molecules, such as E-cadherin, has also been reported.[27, 28] Yoshiura *et al*. reported that E-cadherin mRNA expression-negative cancer cells derived from various human cancers, including those of stomach, urinary bladder and liver exhibited hypermethylation, whereas positive cell lines did not. They also demonstrated the restoration of E-cadherin expression in MKN-1 gastric cancer cell line by treatment with 5-azacytidine.[27] This epigenetic regulation of gene expression, especially genes for cellular adhesion molecules such as E-cadherin, as well as CD44, may participate in the complex biological and morphological

alterations of cancer cells during tumor progression. Although the mechanisms of gene methylation in cancer cells remain to be precisely elucidated, detailed analysis of the hypermethylation status of the *CD44* promoter region in cancers with decreased expression of the gene may provide useful information about the role of CD44 in the processes of invasion and metastasis. Moreover, the change of the *CD44* expression status in MKN-28 with 5-azacytidine treatment may be a good experimental model in studies on the role of CD44 in tumor progression and metastasis.

(Received February 3, 1999/Revised March 24, 1999/Accepted March 30, 1999)

REFERENCES

1) Cooper, D. L., Dougherty, G., Harn, H. J., Jackson, S., Baptist, E. W., Byers, J., Datta, A., Phillips, G. and Isola, N. R. The complex CD44 transcriptional unit; alternative splicing of three internal exons generates the epithelial form of CD44. *Biochem. Biophys. Res. Commun.*, **182**, 569–578 (1992).
2) Heider, K. H., Hofman, M., Hors, E., van den Berg, F., Ponta, H., Herrlich, P. and Pals, S. T. A human homologue of the rat metastasis-associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. *J. Cell Biol.*, **120**, 227–233 (1993).
3) Tanabe, K. K., Ellis, L. M. and Saya, H. Expression of CD44R adhesion molecule in colon carcinomas and metastases. *Lancet*, **341**, 725–726 (1993).
4) Higashikawa, K., Yokozaki, H., Ue, T., Taniyama, K., Ishikawa, T., Tarin, D. and Tahara, E. Evaluation of CD44 transcription variants in human digestive tract carcinomas and normal tissues. *Int. J. Cancer*, **66**, 11–17 (1996).
5) Ochiai, A., Yasui, W. and Tahara, E. Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. *Jpn. J. Cancer Res.*, **76**, 1064–1074 (1985).
6) Yanagihara, K., Seyama, T., Tsumura, M., Kamada, N. and Yokoro, K. Establishment and characterization of human signet ring cell gastric carcinoma cell lines with amplification of the c-myc oncogene. *Cancer Res.*, **51**, 381–386 (1991).
7) Sano, T., Tsujino, T., Yoshida, K., Nakayama, H., Haruma, K., Ito, H., Nakayama, Y., Kajiyama, G. and Tahara, E. Frequent loss of heterozygosity on chromosomes 1q, 5q, and 17p in human gastric carcinomas. *Cancer Res.*, **51**, 2926–2931 (1991).
8) Matsumura, Y. and Tarin, D. Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet*, **340**, 1053–1058 (1992).
9) Yoshida, K., Yokozaki, H., Niimoto, M., Ito, H., Ito, M. and Tahara, E. Expression of TGF-β and procollagen type I and type III in human gastric carcinomas. *Int. J. Cancer*, **44**, 394–398 (1989).
10) Yoshida, K., Kyo, E., Tsujino, T., Sano, T., Niimoto, M. and Tahara, E. Expression of epidermal growth factor transforming growth factor-α and their receptor genes in human gastric carcinomas; implication for autocrine growth. *Jpn. J. Cancer Res.*, **81**, 43–51 (1990).
11) Dadi, H. K., Greaves, A. M., Cox, D. W. and Letarte, M. Identification of a polymorphism in human CD44. *Nucleic Acids Res.*, **19**, 969 (1991).
12) Homman, N., Heuertz, S. and Hors-Cayla, M. C. Time dependence of X gene reactivation induced by 5-azacytidine: possible progressive restructuring of chromatin. *Exp. Cell Res.*, **172**, 481–486 (1987).
13) Kaufmann, M., Heider, K. H., Sinn, H., von Minckwitz, G., Ponta, H. and Herrlich, P. CD44 variant exon epitopes in primary breast cancer and length of survival. *Lancet*, **345**, 615–619 (1995).
14) Dall, P., Heider, K. H., Sinn, H. P., Skroch-Angel, P., Adolf, G., Kaufmann, M., Herrlich, P. and Ponta, H. Comparison of immunohistochemistry and RT-PCR for detection of CD44v-expression, a new prognostic factor in human breast cancer. *Int. J. Cancer*, **60**, 471–477 (1995).
15) Tokue, Y., Matsumura, Y., Katsumata, N., Watanabe, T., Tarin, D. and Kakizoe, T. CD44 variant isoform expression and breast cancer prognosis. *Jpn. J. Cancer Res.*, **89**, 283–290 (1998).
16) Kainz, C., Kohlberger, P., Tempfer, C., Sliutz, G., Gitsch, G., Reinthaller, A. and Breitenecker, G. Prognostic value of CD44 splice variants in human stage III cervical cancer. *Eur. J. Cancer*, **31A**, 1706–1709 (1995).
17) Terpe, H. J., Storkel, S., Zimmer, U., Anquez, V., Fischer, C., Pantel, K. and Gunthert, U. Expression of CD44 isoforms in renal cell tumors. Positive correlation to tumor differentiation. *Am. J. Pathol.*, **148**, 453–463 (1996).
18) Stauder, R., Eisterer, W., Thaler, J. and Gunthert, U. CD44 variant isoforms in non-Hodgkin's lymphoma: a new independent prognostic factor. *Blood*, **85**, 2885–2899 (1995).
19) Ristamaki, R., Joensuu, H., Soderstrom, K. O. and Jalkanen, S. CD44v6 expression in non-Hodgkin's lymphoma: an association with low histological grade and poor prognosis. *J. Pathol.*, **176**, 259–267 (1995).
20) Yokozaki, H., Ito, R., Nakayama, H., Kuniyasu, H., Taniyama, K. and Tahara, E. Expression of CD44 abnormal transcripts in human gastric carcinomas. *Cancer Lett.*, **83**, 229–234 (1994).
21) Ross, J. S., del Rosario, A. D., Bui, H. X., Kallakury, B. V., Okby, N. T. and Figge, J. Expression of the CD44 cell adhesion molecule in urinary bladder transitional cell carcinoma. *Mod. Pathol.*, **9**, 854–860 (1996).
22) Kallakury, B. V., Yang, F., Figge, J., Smith, K. E., Kausik, S. J., Tacy, N. J., Fisher, H. A., Kaufman, R., Figge, H. and Ross, J. S. Decreased levels of CD44 protein and mRNA in prostate carcinoma. Correlation with tumor grade and ploidy. *Cancer*, **78**, 1461–1469 (1996).

23) Gross, N., Beretta, C., Peruisseau, G., Jackson, D., Simmons, D. and Beck, D. CD44H expression by human neuroblastoma cells: relation to MYCN amplification and lineage differentiation. *Cancer Res.*, **54**, 4238–4242 (1994).
24) Fujita, N., Yaegashi, N., Ide, Y., Sato, S., Nakamura, M., Ishiwata, I. and Yajima, A. Expression of CD44 in normal human versus tumor endometrial tissues: possible implication of reduced expression of CD44 in lymph-vascular involvement of cancer cells. *Cancer Res.*, **54**, 3922–3928 (1994).
25) Ue, T., Yokozaki, H., Kagai, K., Higashikawa, K., Yasui, W., Sugiyama, M., Tahara, E. and Ishikawa, T. Reduced expression of the CD44 variant exons in oral squamous cell carcinoma and its relationship to metastasis. *J. Oral Pathol. Med.*, **27**, 197–201 (1998).
26) Motoyama, T., Hojo, H. and Watanabe, H. Comparison of seven cell lines derived from human gastric carcinomas. *Acta Pathol. Jpn.*, **36**, 65–83 (1986).
27) Yoshiura, K., Kanai, Y., Ochiai, A., Shimoyama, Y., Sugimura, T. and Hirohashi, S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc. Natl. Acad. Sci. USA*, **92**, 7416–7419 (1995).
28) 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).