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 Nikai2 and Eiichi Tahara1, 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 HpaII. 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% CO2 in air at 37°C.

High-molecular-weight DNAs were prepared from the cell lines, digested with HindIII 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 32P-labeled probe and washing were performed as described previously 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

1To 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 mM MgCl₂, 10 mM Tris-HCl, 50 mM 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 HpaII or 25 units of MspI 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 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 mM MgCl₂, 10 mM Tris-HCl, 50 mM 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. 2.
2. The CD44 promoter region of MKN-28 was resistant to digestion by HpaII and sensitive to digestion by MspI, while the promoter region of the other cell lines was sensitive to digestion by both HpaII and MspI. 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 \( \mu M \) 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,\(^{16} \) cancer of the uterine cervix,\(^{17} \) colorectal cancer,\(^{18} \) and non-Hodgkin’s lymphoma.\(^{19} \) We have also confirmed overexpression of CD44 variants in human gastric and colorectal carcinomas.\(^{5, 20} \) 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.\(^{2} \) 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.\(^{2} \) 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,\(^{5, 20} \) 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.

Silencing of the genes encoding cellular adhesion molecules, such as E-cadherin, has also been reported.\(^{27, 28} \) Yoshiura et al.\(^{2} \) 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.

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