Epigenetic Regulation of the KAI1 Metastasis Suppressor Gene in Human Prostate Cancer Cell Lines

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Expression of the KAI1 gene, a metastasis-suppressor for prostate cancer, is reduced in all foci of prostatic metastasis. The altered regulatory mechanism is not strongly related to mutations or allelic losses of the KAI1 gene in prostate tumors. Since transcriptional silencing of genes has been found to be caused by epigenetic mechanisms, we have investigated the involvement of this epige-netic regulation of KAI1 expression in prostate cancers. The methylation status of the KAI1 promoter region was examined by restriction-enzyme digestion and sequencing, after amplifying a 331-bp fragment in the GC-rich promoter region from 4 human prostate cancer cell lines treated with bisulfite. The same 4 cell lines were also exposed to various concentrations of the demethylating agent, 5-aza-2′-deoxycytidine (5-AzaC) and/or the histone deacetylase inhibitor, trichostatin A (TSA). To clarify the influence of epigenetic modification on reduced KAI1 mRNA expression in the tumor cells, RT-PCR and northern blot analyses were performed. Bisulfite-sequencing data showed a few methylated CpG islands in the promoter. RT-PCR analysis of 5-AzaC and/or TSA-treated cells indicated reversal of suppression of KAI1 transcription in two cell lines (PC-3 and DU-145), although the expression could not be detected by northern blots. From these results, it is suggested that epigenetic change is not the main mechanism of KAI1 down-regulation, though there remains a possibility that methylation in a more upstream region might be associated with this regulation.

Key words: KAI1 — Metastasis suppressor gene — Methylation — Histone deacetylation — Prostate cancer

The KAI1 gene, located in the p11.2 region of human chromosome 11, is a metastasis-suppressor for prostate cancer and possibly for cancers of breast, lung, and bladder.1–4 The KAI1 gene was originally isolated from the Dunning rat prostate cancer system after introduction of human chromosome 11 by micro-mediated cell transfer.5 Although several studies have suggested that the product of this gene is involved in various physiological and pathological processes including interactions between neighboring cells and between cells and extra-cellular matrix, the biological functions of KAI1 protein have not been defined conclusively.

The mechanism that alters expression of the KAI1 gene in prostate tumors is not strongly related to either mutation or loss of an allele.5) Recently, transcriptional silencing of genes without genetic alterations has been found to be caused by DNA methylation and histone acetylation/deacetylation, so-called epigenetic mechanisms.6) Methylation might directly inhibit the binding of transcription factors or methyl-cytosine binding proteins (MeCP1, 2), which interact with other structural compounds of the chromatin, making the DNA inaccessible to transcription factors through histone deacetylation and stabilizing structural changes in chromatin.7 We and others have shown that DNA hypermethylation plays an important role in the inactivation of VHL, p16 (MTS1/CDKN2), E-cadherin, and androgen receptor in human cancers.8–11)

To clarify the involvement of epigenetic regulation in KAI1 expression, we analyzed the methylation status of the KAI1 5′ regulatory region using the bisulfite PCR method12, 13 in four prostate cancer cell lines. Additionally, we analyzed KAI1 expression in these four cell lines after treatment with a demethylating agent, 5-aza-2′-deoxycytidine (5-AzaC) and/or the histone deacetylase inhibitor, trichostatin A (TSA).

MATERIALS AND METHODS

Cell culture and reagents Four cell lines derived from
metastatic human prostate cancers, LNCaP, PC-3, DU-145, and TSU-Pr1, were used for this study. The origins and characteristics of these cell lines have been described previously.14–18 All cells were cultured in RPMI 1640 supplemented with 1% ampicillin and 10% fetal bovine serum (FBS), in a 5% CO₂ atmosphere at 37°C. Each culture was treated for 7 days with freshly prepared demethylating agent, 5-AzaC (Sigma Chemical, St. Louis, MO), at concentrations of 0.5–2.0 µM and/or histone deacetylase inhibitor, TSA, at the concentration of 50 ng/ml.

### Bisulfite modification
Genomic DNA was extracted from all cell lines using standard protocols. Four micrograms of each DNA in a 50 µl volume of water was denatured with NaOH (final concentration, 0.2 M) and incubated at 37°C for 15 min. A freshly prepared solution containing sodium bisulfite (final concentration, 3.1 M, pH 5.0) (Sigma) and hydroquinone (final concentration, 0.5 mM) (Sigma) was added to the denatured DNA. This mixture was incubated at 55°C for 16 h in darkness. After the reaction, DNA was desalted with a Wizard DNA clean-up system (Promega, Madison, WI), desulfonated by addition of NaOH (final concentration, 0.3 M), and incubated at 37°C for 15 min. The solution was neutralized by addition of ammonium acetate (final concentration, 3.0 M), and the DNA was ethanol-precipitated, dried, and re-suspend in 20 µl of TE buffer (pH 8.0).

### PCR amplification and restriction-enzyme digestion
Primers were designed to discriminate between methylated and unmethylated alleles following bisulfite treatment, and to discriminate between DNA modified by bisulfite treatment and DNA that was not modified.

In the first step, bisulfite-treated DNA was amplified using as a sense primer 5'-AGGGTAGGTTAGATTAGGAA-3' (KAI1-1S; -494 to -474) and as an antisense primer 5'-CTCCCTTTTACCCCAACTACT-3' (KAI1-1AS; +206 to +228). Thermal cycling was performed in 35 cycles of 94°C for 30 s, 50°C for 60 s, and 72°C for 60 s, followed by a final extension at 72°C for 5 min. Next, a 331-bp fragment was amplified using 2 µl of the first-round reaction mixture and the following primers under similar conditions, except for annealing at 55°C. In this case the primers were KAI1-2S: 5'-AGGTGTTGTTGGG-TAYGTTAT-3' (Y=C or T; -179 to -156) and KAI1-2AS: 5'-AAAAACXXAAACTAAACTAATTTACC-3' (X=A or G; +126 to +152). Locations of the primers were determined on the basis of the published KAI1 promoter sequence.19

After ethanol precipitation, PCR products were digested by BstUI, TaqI, and EcoRI, electrophoresed on 2% agarose, and visualized with ethidium bromide.

### Genomic sequencing
The 331-bp PCR products amplified from bisulfite-treated genomic DNA were subcloned into T-easy vector (Promega) and sequenced with vectorspecific primer (Sp6 and T7) by means of the ABI prism 310 sequencer. Ten clones of each product were sequenced. Then, the methylation rates of individual CpGs (1st to 33rd) in the four cell lines were calculated.

#### RT-PCR analysis and Southern blotting
Total RNA was purified from cells cultured with or without 5-AzaC and/or TSA. First-strand cDNA synthesis was performed with 1 µg of the total RNA, using a cDNA synthesis kit (QIAGEN, Chatsworth, CA) according to the manufacturer’s protocol. For PCR amplification we used a 1 µl aliquot of each reaction mixture. To obtain reproducible quantitative performance of the RT-PCR assay for KAI1, we titrated the amount of starting cDNA and the number of amplification cycles. All subsequent assays were carried out using the parameters that yielded amplification of both KAI1 and β-actin DNA within a linear range. On the basis of the nucleotide sequence of KAI1, 5'-AGTCTCTC-CCTGCTGCTGGTG-3' was used as the sense primer and 5'-TCAGTCAGGGTGGCAAGAGG-3' as the antisense primer. These primers amplified a 1031-bp fragment (nucleotides 65–1095). The reaction mixture was subjected to 30 cycles of PCR amplification consisting of 40 s at 94°C, 40 s at 60°C, and 90 s at 72°C. Amplification of β-actin DNA under the same conditions served as the internal PCR control; the sense primer for β-actin was 5'-CAAGAGATGCCAGCGTCGCT-3' and the antisense primer was 5'-TCTTTTCACTCATCTGTCGTA-3'. Complementary DNA obtained from benign hyperplastic prostate tissue served as a positive control. The amplified DNA samples were electrophoresed on 1% agarose gels, and bands were visualized with ethidium bromide. The same gel was used for Southern blotting, with the 1031-bp fragment labeled with digoxigenin serving as a probe.

#### Immunohistochemistry
All cell lines, treated with 5-AzaC or not, were cultured in chamber slides and were immunostained with mouse monoclonal anti-human C33 antibody. Normal kidney tissue was used as a positive control. The amplified DNA samples were electrophoresed on 1% agarose gels, and bands were visualized with ethidium bromide. The same gel was used for Southern blotting, with the 1031-bp fragment labeled with digoxigenin serving as a probe.

#### RESULTS

### Methylation status of the KAI1 promoter region in prostate cancer cell lines
To determine if methylation in the promoter region was heterogeneous, we amplified a 331-bp fragment of each DNA after bisulfite treatment. This fragment represents the most GC-rich area within the 5’ regulatory region of KAI1. The sequence contains 33 CpG dinucleotides, as well as the transcription-initiation site and several putative binding sites for transcription factors (Fig. 1A).

We digested the 331-bp PCR fragments with BstUI, TaqI, and EcoRI, but no digestion products were observed in any bisulfite-treated cell line (Fig. 1B). We sequenced the 331-bp fragments to detect methylated CpGs and found a few methylated CpGs in four cell lines. Methyla-
tion rates of individual CpGs were at most 20% (Fig. 1, C and D).

**Restored expression of KAI1 mRNA in prostate cancer cell lines treated with 5-AzaC and/or TSA** It remained possible that methylation elsewhere in the promoter region might be inhibiting KAI1 expression in the tumor cells. To address this possibility and to examine the influence of histone deacetylation, the four cell lines were exposed to various concentrations of the methylase inhibitor 5-AzaC and/or the histone deacetylase inhibitor TSA. RT-PCR analysis of 5-AzaC-treated cells demonstrated restored expression of the KAI1 gene in the PC-3 and DU-145 cell lines, but no activation of KAI1 mRNA expression occurred in LNCaP and TSU-Pr1 (Fig. 2A). Restored expression of KAI1 was evoked by TSA treatment in DU-145 cells, but not in PC-3 cells. Combined 5-AzaC/TSA treatment increased reactivation for KAI1 in both PC-3 and DU-145 cells (Fig. 2B). In LNCaP and TSU-Pr1, KAI1 expression was not increased by 5-AzaC and/or TSA treatment. However, northern blot analysis and immuno-
histochemical study failed to demonstrate expression of KAI1 in any cell line after treatment with 5-AzaC (data not shown). No morphological changes were seen in any cell line after treatment with 5-AzaC and/or TSA.

DISCUSSION

KAI1 is widely expressed in human tissues, and down-regulation of this gene is associated with the metastatic phenotype of several human malignancies including carcinomas of the prostate, lung, breast, bladder, pancreas, and colon. In normal prostatic tissue, high levels of KAI1 protein are expressed on the plasma membranes of epithelial but not stromal cells, whereas its expression is reduced in 70% of primary prostatic tumors and in all metastatic foci. A previous report from our laboratory indicated that both Gleason grade and clinical stage are inversely correlated with the percentage of KAI1-positive cancer cells in a prostate tumor. Furthermore, we demonstrated in the Dunning prostate cancer system that down-regulation of KAI1 is associated with the acquisition of high metastatic ability. The combined evidence indicated to us that loss of KAI1 protein is involved in the progression of human prostate cancer.

Although the KAI1 gene was isolated originally from the Dunning rat prostate cancer system after introduction of human chromosome 11, the mechanism causing down-regulation of the KAI1 gene in prostate cancers is not clear. Mutation in, or allelic loss of, the KAI1 gene is not frequent in prostate-cancer tissues. The present study revealed that expression of KAI1 was re-activated by 5-AzaC in two of the four prostate cancer cell lines examined (PC-3 and DU-145). However, northern-blot and immunohistochemical analyses showed no activated expression of KAI1 mRNA or increase in KAI1 protein in any cell line after treatment with 5-AzaC. This discrepancy may be explained by the comparatively higher sensitivity of the RT-PCR experiments.

A recent report by Jackson et al. provided only negative evidence for hypermethylation of KAI1 CpG islands in invasive bladder tumors or cell lines derived from such tumors; i.e., their RT-PCR analyses showed no activation of KAI1 mRNA after treatment with 5-AzaC. In the present study, we found restored expression of KAI1 in PC-3 and DU-145 cell lines treated with 5-AzaC alone and 5-AzaC/TSA by RT-PCR. Combined with bisulfite sequencing data, it is suggested that epigenetic change is not the main mechanism of KAI1 down-regulation, though we can not rule out the possibility that methylation in a more upstream region might be associated with this regulation.

Since Mashimo et al. reported that expression of KAI1 may be controlled, in part, by p53, further study will be needed to clarify the exact mechanism that regulates expression of KAI1 in prostate cells.

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REFERENCES

1) Dong, J. T., Lamb, P. W., Rinker-Schaeffer, C. W., Vukanovic, J., Ichikawa, T., Issacs, J. T. and Barrett, J. C. KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. *Science*, **268**, 884–886 (1995).

2) Yang, X., Welch, D. R., Phillips, K. K., Weissman, B. E. and Wei, L. L. KAI1, a putative marker for metastatic potential in human breast cancer. *Cancer Lett.*, **119**, 149–155 (1997).

3) Adachi, M., Taki, T., Ieki, Y., Huang, C. L., Higashiyama, M. and Miyake, M. Correlation of KAI1/CD82 gene expression with good prognosis in patients with non-small cell lung cancer. *Cancer Res.*, **56**, 1751–1755 (1996).

4) Yu, Y., Yang, J. L., Markovic, B., Jackson, P., Yardley, G., Barrett, J. and Russell, P. J. Loss of KAI1 messenger RNA expression in both high grade and invasive bladder cancers. *Clin. Cancer Res.*, **3**, 1045–1049 (1997).

5) Dong, J. T., Suzuki, H., Pin, S. S., Bova, G. S., Schalken, J. A., Issacs, W. B., Barrett, J. C. and Issacs, J. T. Down-regulation of the KAI1 metastasis suppressor gene during the progression of human prostatic cancer infrequently involves gene mutation or allelic loss. *Cancer Res.*, **56**, 4387–4390 (1996).

6) Jones, P. L. and Wolff, A. P. Relationships between chromatin organization and DNA methylation in determining gene expression. *Semin. Cancer Biol.*, **9**, 339–347 (1999).

7) Singal, R. and Ginder, G. D. DNA methylation. *Blood*, **93**, 4059–4070 (1999).

8) Herman, J. G., Latif, F., Weng, Y., Lerman, M. I., Zbar, B., Liu, S., Samid, D., Duan, D. S., Gnarr, J. R., Linehan, W. M. and Baylin, S. B. Silencing of the VH1 tumor-suppressor gene by methylation in renal cell carcinoma. *Proc. Natl. Acad. Sci. USA*, **91**, 9700–9704 (1994).

9) Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Baylin, S. B. and Sidransky, D. 5′ CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. *Nat. Med.*, **1**, 686–692 (1995).

10) Graff, J. R., Herman, J. G., Lapidus, R. G., Chopra, H., Xu, R., Jarrard, D. F., Issacs, 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).

11) Nakayama, T., Watanabe, M., Suzuki, H., Toyota, M., Sekita, N., Hirokawa, Y., Mizokami, A., Ito, H., Yatani, R. and Shiraishi, T. Epigenetic regulation of androgen receptor gene expression in human prostate cancers. *Lab. Invest.*, **80**, 1789–1796 (2000).

12) 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).

13) Hayatsu, H., Watabe, Y., Kai, K. and Iida, S. Reaction of sodium bisulfite with uracil, cytosine, and their derivatives. *Biochemistry*, **9**, 2858–2865 (1970).

14) Horoszewicz, J. S., Leong, S. S., Chu, T. M., Wajsman, Z. L., Friedman, M., Papsidero, L., Kim, U., Chai, L., S., Kakati, S., Arya, S. K. and Sandberg, A. A. The LNCaP cell line—a new model for studies on human prostatic carcinoma. *Prog. Clin. Biol. Res.*, **37**, 115–132 (1980).

15) Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F. and Jones, L. W. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest. Urol.*, **17**, 16–23 (1979).

16) Mickey, D. D., Stone, K. R., Wunderli, H., Mickey, G. H., Vollmer, R. T. and Paulson, D. F. Heterotransplantation of a human prostatic adenocarcinoma cell line in nude mice. *Cancer Res.*, **37**, 4049–4058 (1977).

17) Ohnuki, Y., Marnell, M. M., Babcock, M. S., Lechner, J. F. and Kaighn, M. E. Chromosomal analysis of human prostatic adenocarcinoma cell lines. *Cancer Res.*, **40**, 524–534 (1980).

18) Iizumi, T., Yazaki, T., Kanoh, S., Kono, I. and Koiso, K. Establishment of a new prostatic carcinoma cell line (TSU-Pr1). *J. Urol.*, **137**, 1304–1306 (1987).

19) Dong, J. T., Issacs, W. B., Barrett, J. C. and Issacs, J. T. Genomic organization of the human KAI1 metastasis-suppressor gene. *Genomics*, **41**, 25–32 (1997).

20) Ueda, T., Ichikawa, T., Tamaru, J., Mikata, A., Akakura, K., Akimoto, S., Imai, T., Yoshie, O., Shiraishi, T., Yatani, R., Ito, H. and Shimazaki, J. Expression of KAI1 protein in benign prostatic hyperplasia and prostate cancer. *Am. J. Pathol.*, **149**, 1435–1440 (1996).

21) Suzuki, H., Dong, J. T., Gao, A. C., Barrett, J. C. and Issacs, J. T. Identification of the rat homologue of KAI1 and its expression in Dunning rat prostate cancers. *Prostate*, **37**, 253–260 (1998).

22) Jackson, P., Millar, D., Kingsley, E., Yardley, G., Ow, K., Clark, S. and Russell, P. J. Methylation of a CpG island within the promoter region of the KAI1 metastasis suppressor gene is not responsible for down-regulation of KAI1 expression in invasive bladder cancers or cancer cell lines. *Cancer Lett.*, **157**, 169–176 (2000).

23) Mashimo, T., Watabe, M., Hirota, S., Hosobe, S., Miura, K., Tegtmeyer, P. J., Rinker-Schaeffer, C. W. and Watabe, K. The expression of the KAI1 gene, a tumor metastasis suppressor, is directly activated by p53. *Proc. Natl. Acad. Sci. USA*, **95**, 11307–11311 (1998).