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Report

DNA methylation of retinoic acid receptor β in breast cancer and possible therapeutic role of 5-aza-2′-deoxycytidine

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The retinoic acid receptor β (RARβ), a putative tumor suppressor gene, has been reported to be poorly expressed in breast cancer. In this report using the methylation-specific PCR reaction we observed DNA methylation in the promoter region of RARβ in several primary breast tumors. DNA sequence analysis showed that the positions of 5-methylcytosine in the RARβ promoter region was almost identical to that reported previously by our laboratory for human DLD-1 colon carcinoma cells (*Anti-Cancer Drugs* 1998; 9: 743). Several other cancer-related genes have been also reported to be silenced by DNA methylation, including the p16 tumor suppressor gene, E-cadherin, an invasion suppressor gene and the estrogen receptor gene in breast cancer cell lines. Since breast cancer cells have several potential target genes for the DNA methylation inhibitor, 5-aza-2′-deoxycytidine (5-Aza-CdR), we investigated the *in vitro* antineoplastic activity of this analog on the human breast cancer cell line MDA-MB-231. We report that 5-Aza-CdR is a potent growth inhibitor and a potent cytotoxic agent against the breast carcinoma cells. These results suggest that 5-Aza-CdR may be an interesting agent to investigate in patients with breast cancer resistant to conventional chemotherapy. [© 1999 Lippincott Williams & Wilkins.]

Key words: 5-Aza-2′-deoxycytidine, 5-methylcytosine, breast cancer, DNA methylation, retinoic acid receptor β.

Introduction

About 30% of women diagnosed with breast cancer ultimately die from this disease.[1] Postmenopausal women with breast tumors that are estrogen receptor-positive respond initially to therapy with antiestrogens.[2] However, many patients eventually will become resistant to hormonal therapy. When metastic breast tumors arise, these patients may respond initially to cytotoxic drug therapy, but eventually tumor progression occurs and their survival is limited. It is urgent to consider new approaches for the therapy of this disease. Recent results suggest that tumor suppressor genes may be considered as interesting targets for the therapy of breast cancer.

Lack of expression of the retinoic acid receptor β (RARβ), a putative tumor suppressor gene, has been reported for breast cancers[3,4] and other types of cancer.[5–8] The RARβ gene is located at chromosome 3p24, a site that shows frequent loss of heterozygosity in breast cancer.[9] Additional evidence that RARβ is a tumor suppressor gene includes the reports that transfection of RARβ cDNA into some tumor cells induced terminal differentiation[8] and reduced their tumorigenicity in nude mice.[10]

It is possible that the loss of expression of RARβ in breast cancer is due to aberrant methylation of CpG islands in its promoter region. We reported previously that RARβ is methylated and not expressed in DLD-1 human colon carcinoma cells.[11] Recently, we identified the specific sites of 5-methylcytosine in the promoter region of RARβ in these tumor cells.[12] In this study, using the methylation-specific PCR (MSP) assay,[13] we detected methylation of RARβ in several breast tumor biopsies. In addition, using DNA sequence analysis we observed similar positions of 5-methylcytosine in the promoter region of RARβ in two primary human breast tumors as reported previously for the DLD-1 colon tumor cells.

It is interesting to note that a large number of cancer-related genes have been found to be methy-

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lated in breast cancer, including BRCA1,[14] the invasion suppressor gene, E-cadherin,[15] the estrogen receptor gene,[16] the tumor suppressor genes, p16,[17] MDGI (mammary-derived growth inhibitor)[18] and HIC-1 (hypermethylated in cancer).[19] In most of these cases, gene methylation results in the silencing of their expression. 5-Aza-2′-deoxycytidine (5-Aza-CdR), a potent inhibitor of DNA methylation,[20] has been reported to activate the *in vitro* expression of most of these genes. We reported previously that 5-Aza-CdR activates the expression of RARβ in DLD-1 colon carcinoma cells.[12] This analog may be a novel agent to investigate against breast cancer considering the many interesting target genes for its chemotherapeutic action. In clinical studies 5-Aza-CdR has shown activity in patients with leukemia[21–23] and promising activity in patients with advanced lung cancer.[24] In this study we observed that 5-Aza-CdR produced a potent growth inhibition and a profound reduction in the clonogenicity of MDA-MB-231 human breast carcinoma cells, supporting the hypothesis that this analog has some potential in the therapy of breast cancer.

Materials and methods

Cell line and tumor samples

Human MDA-MB-231 breast cancer cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium (Life Technologies, Burlington, Ontario, Canada) containing 10% heat-inactivated fetal calf serum (Wisent, St Bruno, Quebec, Canada) and 1 mM sodium pyruvate in a 5% CO_2 incubator at 37°C. Genomic DNA was obtained from a tumor bank of breast tumors, and purified by proteinase K digestion and phenol chloroform extraction.

Bisulfite treatment of genomic DNA

Bisulfite treatment of single-stranded genomic DNA deaminates all the cytosines into uracil, with no change in 5-methylcytosine. This reaction was carried out as described by Frommer *et al.*[25] and Herman *et al.*[13] with minor modifications. Briefly, 2 μg of genomic DNA was digested with 10 U of *Mbo*II (Life Technologies). The DNA was denaturated with freshly prepared 0.6 M NaOH for 20 min at 45°C. Freshly prepared bisulfite (Sigma, Oakville, Ontario, Canada), pH 5, and hydroquinone (Sigma) were added to each sample to a final concentration of 3 M and 5 mM respectively, and incubated in the dark at 55°C for 16–20 h. The bisulfite-converted DNA was desalted using the Prep-A-Gene DNA purification kit (BioRad Laboratories, Mississauga, Ontario, Canada) according to the manufacturer's instructions. The DNA was desulfonated by addition of fresh 0.3 M NaOH by incubation at 40°C for 15 min. The sample was then neutralized with 2 M ammonium acetate (pH 7) and precipitated with ethanol. The DNA was resuspended in 10 mM Tris, 1 mM EDTA (pH 7.5).

DNA cloning and sequencing

Bisulfite-converted DNA specific primers were used to amplify a 395 bp fragment of RARβ promoter region (from position 738 to 1131) prior to cloning in the pCR2.1 TOPO vector (InVitrogen, Carlsbad, CA): sense primer 5′-GGA GTT GGT GAT GTT AGA TTA GTT G-3′ (position 738–762) and antisense primer 5′-TCC AAA TAA TCA TTT ACC ATT TTC C-3′ (position 1107–1131). PCR amplifications were performed in 25 μl reaction mixture containing 1–25 ng of bisulfite-treated DNA, 2.5 mM dNTPs, 0.2 μM primers, 1 mM $MgCl_2$, 8% glycerol, PCR buffer and 1.25 U Taq Polymerase (Pharmacia, Baie d'Urfé, Quebec, Canada), in a thermal minicycler (MJ Research, Watertown, MA) under the following conditions: 94°C for 2 min; 94°C for 30 s, 50°C for 30 s, 72°C for 1 min for 45 cycles; and 72°C for 5 min. The 395 bp amplified fragment was cloned into pCR2.1-TOPO vector (InVitrogen) according to the manufacturer's instructions. Plasmid DNA was purified using the Qiagen Plasmid Mini kit, and sequenced using the AutoRead 1000 sequencing kit and ALF automatic sequencer (Pharmacia).

MSP assay

MSP is a technique that takes advantage of DNA sequence differences existing between methylated and unmethylated DNA after bisulfite treatment.[13] Primer pairs for the RARβ promoter region (RARβ-M and RARβ-U) were described by Côte *et al.*[12] These primers were used to amplify a 146 bp fragment of the RARβ promoter region. MSP amplification was performed as described previously.

Growth inhibition and clonogenic assay

For growth inhibition evaluation, MDA-MB-231 cells were plated at 100 000 cells/well of a six-well dish in 2 ml of media. 5-Aza-CdR (Decitabine) was

obtained from Pharmachemie, (Haarlem, The Netherlands), dissolved in 0.45% NaCl containing 10 mM sodium phosphate (pH 6.8) and stored at −70°C. The cells were treated with different concentrations of 5-Aza-CdR ranging from 1 to 1000 ng/ml. Cells were counted at 48, 72 and 96 h after the beginning of drug treatment. The growth inhibition values are expressed as the cell count of the drug-treated cells relative to the cell count of the untreated control cells.

For the clonogenic assay, MDA-MB-231 breast carcinoma cells were plated at 100 cells/well on a six-well dish containing 2 ml of media and treated with 5-Aza-CdR as described above for the growth assay. The cells were incubated with 5-Aza-CdR at 37°C for 24-96 h. The colonies were counted on day 10-14 after drug treatment. The loss of clonogenicity is expressed as the number of colonies formed by drug-treated cells relative to the number of colonies formed by untreated control cells. Due to the chemical instability of 5-Aza-CdR, for both the growth inhibition and the clonogenic assay fresh drug was added to the medium every 24 h.

Results

MSP

After DNA isolation from breast tumor biopsies and corresponding normal tissue and bisulfite treatment, we assessed the methylation status of the RARβ promoter region by MSP. As shown in Figure 1, we detected significantly positive signs of methylation of RARβ in four of 13 (30%) breast tumor biopsies (T2, T5, T7 and T8). Using the unmethylation-specific primers we detected strong bands of unmethylation in 10 of 13 (77%) of the tumor biopsies. Two tumor biopsies (T11 and T12) showed very weak bands by MSP, presumably due to unsuitable DNA for PCR analysis. For the tumors that showed significant methylation, an amplified DNA band was also produced by the unmethylation-specific primers for tumor samples T2 and T8. It is likely that these tumor samples contained significant amounts of normal tissue. In normal corresponding tissue we detected significant signs of methylation of RARβ in sample N8, but only very weak bands in samples N7 and N9.

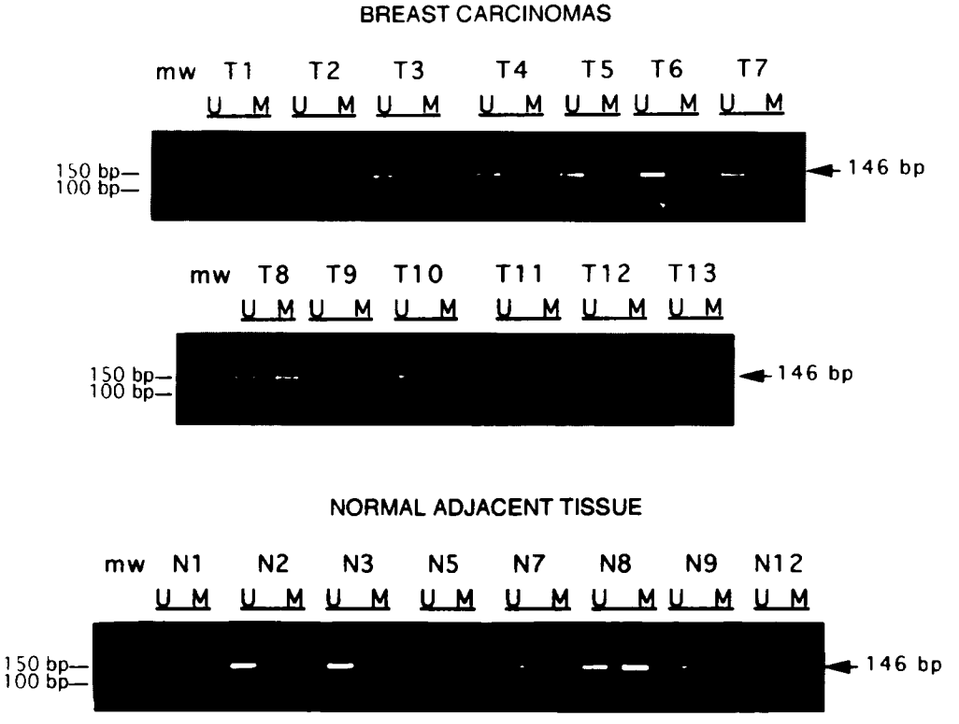

Figure 1. MSP analysis of the promoter region of RARβ gene in breast cancer biopsies (T) and normal adjacent breast tissue (N). Genomic DNA was isolated from breast tumors and normal adjacent tissue from different patients. After treatment with bisulfite the DNA was used in the MSP assay with methylation-specific primers (M) and unmethylation-specific primers (U) to amplify a 146 bp DNA fragment.

DNA sequence analysis

Table 1 shows the positions of 5-methylcytosine in the promoter region of RARβ of human DLD-1 colon carcinoma cells and two human breast tumor samples (T2 and T8). In the promoter region from position 738 to 1131, there were 17 sites of 5-methylcytosine present in the DLD-1 cells. Tumor biopsy T2 had the identical sites of 5-methylcytosine as DLD-1 cells. For tumor biopsy T8, all the positions of 5-methylcytosine were the same as the DLD-1 cells, except at positions 784, 798 and 810 at the 5′ end, and positions 1082 and 1095 at the 3′ end of the sequenced DNA fragment.

Growth inhibition and clonogenic assay

The effects of 5-Aza-CdR on the growth of MDA-MB-231 human breast carcinoma cells are shown in Figure 2. A 48 h treatment of 5-Aza-CdR at a concentration of 10 and 100 ng/ml produced 30 and 47% inhibition of growth, respectively. At this time point the lower concentration of 1 ng/ml produced only 14% growth inhibition. The growth inhibitory effects of 5-Aza-CdR increased with exposure time. At 72 h 5-Aza-CdR at 10 ng/ml produced 48.2% growth inhibition whereas 100 ng/ml produced 68.5% inhibition.

To evaluate the effects of this analog on the colony formation of the MDA-MB-231 breast carcinoma cells, we performed a clonogenic assay using different concentrations of 5-Aza-CdR at different exposure times (Figure 3). 5-Aza-CdR at 10 and 100 ng/ml for 48 h exposure produced 30.8 and 97.6% loss of clonogenicity, respectively. These concentrations of 5-Aza-CdR for a 24 h exposure produced 23 and 91.3% loss of clonogenicity, respectively (data not shown). The loss of clonogenicity produced by 5-Aza-CdR increased with the duration of exposure. For example, for the 96 h exposure 10 ng/ml of this analog produced a 73.5% loss of clonogenicity, whereas for 100 ng/ml the decrease in clonogenicity was greater than 99%.

Discussion

Tumorigenesis arises via the accumulation of specific genetic and epigenetic alterations that give tumor cells growth advantages over the normal cells. Aberrant methylation of CpG islands in the promoter regions of genes involved in the regulation of cell growth has been described in both tumor cell lines[26] and primary tumors.[27] Hypermethylation of promoter regions of these growth regulatory genes results in silencing of their expression.[11,28] Interestingly, many cancer-related genes have been found to be silenced by *de novo* methylation in breast cancer.[14-19]

We evaluated the methylation status of RARβ gene in tumor biopsies from 13 patients with breast cancer.

Table 1. Comparison of the positions of 5-methylcytosine in the promoter region of RARβ in DLD-1 colon carcinoma cells and human breast tumor biopsies

Position of 5-methylcytosine[a]	DLD-1 cells	Breast tumor #2	Breast tumor #8
784	+	+	−
798	+	+	−
810	+	+	−
927	+	+	+
951	+	+	+
957	+	+	+
959	+	+	+
963	+	+	+
968	+	+	+
990	+	+	+
993	+	+	+
1005	+	+	+
1010	+	+	+
1016	+	+	+
1077	+	+	+
1082	+	+	−
1095	+	+	−

[a]The positions of cytosine as designated by the DNA sequence of the RARβ_2 promoter region (GenBank accession no. X56849). +, 5-methylcytosine present; −, 5-methylcytosine absent.

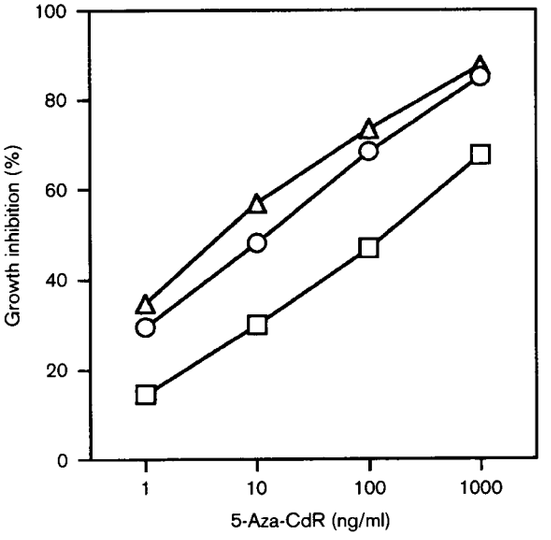

Figure 2. Effect of different concentrations of 5-Aza-CdR on the inhibition of growth of human MDA-MB-231 breast carcinoma cells. Cell counts were performed after 48 (□), 72 (○) or 96 (△) h of drug exposure. Mean values with SE$\leqslant$15% are shown, n=6.

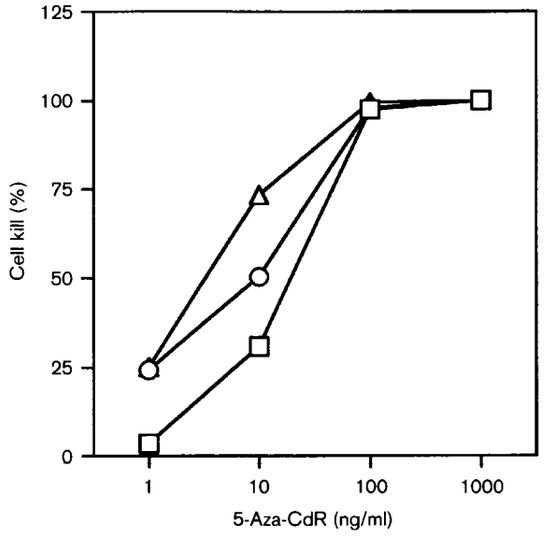

Figure 3. Effect of different concentrations of 5-Aza-CdR on colony formation by human MDA-MB-231 breast carcinoma cells. The cells were exposed to the indicated concentrations of 5-Aza-CdR for 48 (□), 72 (○) or 96 (△) h. The colonies were counted 10–14 days later. Cell kill (%) is defined as the number of colonies formed by drug-treated cells divided by the number of colonies formed by control cells. Mean values with SE $\leqslant$ 15% are shown, n=6.

Low expression of RARβ has been reported to occur frequently in breast cancer.[3,4] We reported previously that this putative tumor suppressor gene is silenced by methylation in DLD-1 human colon carcinoma cells.[29] Using DNA sequence analysis we identified the sites of methylation of the RARβ promoter region.[12] In this report using MSP analysis of breast cancer biopsies from patients, we observed that four of 13 samples analyzed (30%) showed methylation of the RARβ gene (Figure 1). With unmethylation specific primers (RARβ-U) we detected positive signals in most tumor samples which may have been due to their contamination with normal tissue. Normal tissue, adjacent to the breast tumor, was also screened for the methylation status of RARβ. For the normal sample N8 we observed a strong positive signal for DNA methylation. One possible explanation for this result is that the MSP reaction detected the presence of malignant cells in the normal tissue or that some normal cells contained a methylated RARβ which may represent an early neoplastic change that could eventually lead to their malignant transformation.

The sequencing of a 395 bp fragment of bisulfite-treated DNA from the RARβ promoter region of two human breast cancer biopsies (T2 and T8) revealed that the positions of 5-methylcytosine were similar to those in DLD-1 human colon carcinoma cell line (Table 1). In particular, in patient T2 all the 5-methylcytosine positions correspond precisely to those detected in DLD-1 cells. A similar pattern was observed for patient T8, except for the cytosines at the 5′ and 3′ ends of the DNA fragment. In the latter case, it is possible that these unmethylated cytosines may not play an important role in the control of the expression of RARβ. It is intriguing to note that these results indicate that primary breast tumors can contain a methylation pattern of RARβ that is almost identical to that of the DLD-1 colon tumor cell line, suggesting the importance of this epigenetic change in different types of neoplastic cells.

Many genes involved in tumor progression have been shown to be silenced by DNA methylation and their expression activated by 5-Aza-CdR in breast cancer. RARβ is another target gene for this analog. These findings suggest that 5-Aza-CdR may be an interesting agent to investigate for the treatment of breast cancer. In order to explore this possibility we have studied the *in vitro* antineoplastic activity of 5-Aza-CdR on MDA-MB-231 human breast carcinoma cell line. Growth inhibition and clonogenic assays show that this analog is a potent inhibitor of cell growth and clonogenic potential in this breast carcinoma cell line. We observed that the growth inhibitory action and the loss of clonogenicity produced by 5-Aza-CdR increased with exposure time and drug concentration. 5-Aza-CdR shows clinical activity against leukemia[21] and advanced lung cancer.[24] Pharmacokinetic analysis shows that the plasma concentration of this analog that produced clinical responses[24] is in the range of the concentration that showed effective *in vitro* antineoplastic activity (Figure 3). Based on these findings, a pilot clinical study in patients with breast cancer resistant to conventional therapy has been initiated with 5-Aza-CdR.

References

1. Fisher B, Osborne CK, Margolese R, Bloomer W. Neoplasm of the breast. In: Holland J, *et al.*, eds. *Cancer medicine.* Philadelphia: Lea & Febiger 1993; **2**: 1706–78.
2. Hudis CA, Norton L. Adjuvant drug therapy for operable breast cancer. *Semin Oncol* 1996; **23**: 475–93.
3. Swisshelm K, Ryan K, Lee X, Tsou HC, Peacocke M, Sager R. Down-regulation of retinoic acid receptor β in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth Different* 1994; **5**: 133–41.
4. Widschwendter M, Berger J, Daxenbichler G, *et al.* Loss of retinoic acid receptor β expression in breast cancer and morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer. *Cancer Res* 1997; **57**: 4158–61.

5. Gebert JF, Moghal N, Frangioni JV, Sugarbaker DJ, Neel BG. High frequency of retinoic acid receptor β abnormalities in human lung cancer. *Oncogene* 1991; **6**: 1859–68.
6. Hu L, Crowe DL, Rheinwald JG, Chambon P, Gudas LJ. Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell line. *Cancer Res* 1991; **51**: 3972–81.
7. Crowe DL. Retinoic acid receptor β induces terminal differentiation of squamous cell carcinoma lines in the absence of cyclin-dependent kinase inhibitor expression. *Cancer Res* 1998; **58**: 142–8.
8. Caliaro MJ, Marmouget C, Guichard S, *et al.* Response of four ovarian carcinoma cell lines to all-*trans* retinoic acid: relationship with induction of differentiation and retinoic acid receptor expression. *Int J Cancer* 1994; **56**: 743–8.
9. Deng G, Lu Y, Zlotnikov G, Thor AD, Smith HS. Loss of herterozygosity in normal tissue next to breast cancer. *Science* 1996; **274**: 2057–9.
10. Houle B, Rochette-Egly C, Bradley WEC. Tumor-suppressive effect of retinoic acid receptor β in human epidermoid lung cancer cells. *Proc Natl Acad Sci USA* 1993; **90**: 985–9.
11. Côté S, Momparler RL. Activation of the retinoic acid receptor β gene by 5-aza-2′deoxycytidine in human DLD-1 colon carinoma cells. *Anti-Cancer Drugs* 1997; **8**: 56–61.
12. Côté S, Sinnett D, Momparler RL. Demethylation by 5-aza-2′-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor β gene in human colon carcinoma cells. *Anti-Cancer Drugs* 1998; **9**: 743–50.
13. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**: 9821–6.
14. Dobrovic A, Simpfendorfer D. Methylation of the BRCA1 gene in sporadic breast cancer. *Cancer Res* 1997; **57**: 3347–50.
15. Oka H, Shiozaki H, Kobayashi K, Inoue M, *et al.* Expression of E-cadherin cell adhesion molecules in human breast tissue and its relationship to metastasis. *Cancer Res* 1993; **53**: 1696–701.
16. Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, Davidson NE. Methylation of the estrogen receptor gene CpG islands marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res* 1994; **54**: 2552–5.
17. Huschtscha LI, Noble JR, Neumann AA, *et al.* Loss of $p16^{INK4}$ expression by methylation is associated with lifespan extension of human mammary epithelial cells. *Cancer Res* 1998; **58**: 3508–12.
18. Phelan CM, Larsson C, Baird S, *et al.* The human derived growth inhibitor (MDGI) gene-genomic structure and mutation analysis in human breast tumors. *Genomics* 1996; **34**: 63–8.
19. Fujii H, Biel MA, Zhou W, Weitzman SA, Baylin SB, Gabrielson E. Methylation of the HIC-1 candidate tumor suppressor gene in human breast cancer. *Oncogene* 1998; **16**: 2159–64.
20. Jüttermann R, Li E, Jaenisch R. Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc Natl Acad Sci USA* 1994; **91**: 11797–801.
21. Rivard GE, Momparler RL, Demers J, *et al.* Phase I study on 5-aza-2′-deoxycytidine in children with acute leukemia. *Leuk Res* 1981; **5**: 453–62.
22. Momparler RL, Rivard GE, Gyger M. Clinical trial on 5-aza-2′-deoxycytidine in patients with acute myeloid leukemia. *Pharmacol Ther* 1985; **30**: 277–86.
23. Petti MC, Mandelli F, Zagonel V, *et al.* Pilot study of 5-aza-2′-deoxycytidine (Decitabine) in the treatment of poor prognosis acute myelogenous leukemia patients: preliminary results. *Leukemia* 1993; **7**: 36–41.
24. Momparler RL, Bouffard DY, Momparler LF, Dionne J, Bélanger K, Ayoub J. Pilot phase I–II study on 5-aza-2′-deoxycytidine (Decitabine) in patients with metastatic lung cancer. *Anti-Cancer Drugs* 1997; **8**: 358–68.
25. Frommer M, McDonald LE, Millar DS, *et al.* A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci USA* 1992; **89**: 1827–31.
26. Rideout WM, Eversole-Cire P, Spruck CH, *et al.* Progressive increase in the methylation status and heterochromatinization of the Myo D1 CpG island during oncogenic transformation. *Mol Cell Biol* 1994; **14**: 6143–52.
27. Vertino PM, Spillare EA, Harris CC, Baylin SB. Altered chromosomal methylation patterns accompany oncogene-induced transformation of human bronchial epithelial cells. *Cancer Res* 1993; **53**: 1684–9.
28. Merlo A, Herman JG, Mao L, *et al.* 5′ CpG island methylation is associated with transcriptional silencing of the tumor suppressor gene p16/CDKN2/MTS1 in human cancers. *Nat Med* 1995; **1**: 686–92.
29. Côté S, Momparler RL. Antineoplastic action of all-*trans* retinoic acid and 5-aza-2′-deoxycytidine on human DLD-1 colon carcinoma cells. *Cell Pharmacol* 1995; **2**: 221–8.

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