Progesterone receptor gene inactivation and CpG island hypermethylation in human leukemia cancer cells

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Abstract Previous studies showed that progesterone receptor (PR), one of the hormone receptor superfamily, was only connected with the sex-correlated cancers such as breast cancer, endometrial cancer, prostate cancer, etc. This article deals with the PR gene in leukemia. We investigated the methylation status and the expression of the two different PR isoforms, PRA and PRB, in three leukemia cancer cell lines using methylation-specific polymerase chain reaction (MSP-PCR) and reverse transcription-PCR. The correlation of PR methylation and expression together with DNA methyltransferase (DNMT1) was further studied. We found that DNMT1 is required to maintain CpG methylation and aberrant gene silencing of PR gene in human leukemia cancer cells. The activity of 5-aza-2′-deoxycytidine in demethylation and gene reactivation may be through depleting cellular DNMT1 levels. In addition, extensive methylation of PRA and PRB was also observed in leukemia samples. Our results suggest that PR CpG island aberrant hypermethylation could be one molecular and genetic alteration in leukemia.

Keywords: Progesterone receptor; Promoter methylation; Leukemia; DNA methyltransferase; Methylation-specific PCR

1. Introduction

Progesterone receptor (PR) is a transcription factor of the steroid hormone receptor superfamily. The human *PR* gene encodes two isoforms, hPRA (79 kDa) and hPRB (109 kDa), which differ in both their N-terminal sequences and biological activities. The hPRB transcript is preferentially induced by estrogen receptor (ER). Since ligand-bound ER is a major transcriptional activator of hPRB gene expression, the presence of PR is indicative of functional ER [1]. The physiological action of PRA and PRB and their regulation of gene expression in target tissues are summarized in [2,3]. PR has been described in many normal and neoplastic tissues [4–7]. It correlates with cellular proliferation and endocrine treatment [2,8].

Tumors with inactivated PR gene lack PR transcription, but no mutations within the DNA-binding domain of the ER gene or major polymorphisms or deletions within the PR gene have been identified [9,10]. Acquired loss of transcription of these hormone receptor genes is a potential mechanism of resistance to hormonal therapies. One mechanism by which gene expression can be silenced is aberrant methylation of CpG islands in the 5′-regulatory region and first exon of genes [11]. Methylation of these islands has been shown to inhibit transcription directly or stabilize chromatin in a conformation that prevents transcription [12].

Normal hematopoietic cells appear to be partly regulated by endocrine factor ER [13]. Issa et al. investigated the methylation state of the ER gene CpG island in normal and neoplastic bone marrow cells and observed that it is methylated in nearly 90% of human leukemias and lymphomas [13]. This molecular alteration points to a potentially important role for loss of ER function in the pathogenesis of human leukemias.

In this study, by using methylation-specific polymerase chain reaction (MSP-PCR) and reverse transcription PCR (RT-PCR) assay method, we have tested the hypothesis that the PR gene might also be involved in leukomogenesis. We analyzed the expression and methylation status of two PR isoforms gene in leukemia cell lines. The results indicate that the aberrant methylation could be one molecular and genetic alteration in leukemia and might provide a clinically useful, tumor-specific marker.

2. Materials and methods

2.1. Cell culture and treatment with 5-Aza-CdR

The leukemia cell lines, Jurkat, K562 and HL-60, are obtained from the Southwest Hospital (Chongqing, PRC). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum. The cells were treated with a freshly prepared solution of 5-aza-2′-deoxycytidine (5-Aza-CdR, Sigma). On day 1, a final concentration of 2 μM 5-Aza-CdR in PBS was added to the flask. The next day, the medium was changed to RPMI 1640. On days 3 and 5, the cells were treated with 4 μM 5-Aza-CdR two times. On days 4 and 6, the medium was changed to RPMI 1640 two times. On day 7, the cells were harvested [14].

2.2. Tissue samples

44 cases of bone marrow samples from patients with leukemia and 10 cases of bone marrow samples from normal persons were obtained from Laboratory of Hematology, Southwest Hospital.

2.3. DNA and RNA preparation

Total DNA and RNA were extracted from various cancer cell lines and bone marrow samples using Tirpure-Isolation-Reagent (Roche, USA).

2.4. Sodium bisulfite treatment

DNA bisulfite treatment was carried out using the reagents provided in the CpGenome DNA Modification Kit (Intergen, Purchase, NY,

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Abbreviations: 5-Aza-CdR, 5-Aza-2′-deoxycytidine; PCR, polymerase chain reaction; DNMT, DNA methyltransferase; MSP, methylation-specific PCR; RT-PCR, reverse transcription PCR

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USA). Briefly, 1 μg of DNA was denatured using NaOH and treated with sodium bisulfite for 18 h. Modified DNA was resuspended in 30 μl of TE (10 mM Tris/0.1 mM EDTA, pH 7.5) and stored immediately at −20 °C [15,16].

2.5. Methylation-specific PCR analysis conditions

PCR was performed using *Taq* polymerase (Promega) and the hot start procedure [17]. The PR specific primer sets are designed to locate on the 5′-upper region of each promoter for distinguishing PRA and PRB separately [18]. Primer set U will anneal to unmethylated DNA that has undergone a chemical modification. Primer set M will anneal to methylated DNA that has undergone a chemical modification. The unmethylated or methylated sequence of promoter A of this gene was detected with PRA-Uf and PRA-Ur or PRA-Mf and PRA-Mr, respectively. The unmethylated or methylated sequence of promoter B of this gene was detected with PRB-Uf and PRB-Ur or PRB-Mf and PRB-Mr, respectively (Table 1). The PCR mixture (50 μl) contained 2.0 mM $MgCl_2$, 0.2 mM each of 4 dNTPs, 0.8 mM each of oligonucleotide primers and 2.5 units of *Taq* DNA polymerase. PCR conditions are listed in Table 1. After PCR, 9 μl of PCR product was mixed with 1.5 μl of 10× loading dye and then run on 2% agarose gel. Electrophoresis was carried out at 100 V at ambient temperature. The bands on the gels were visualized by ethidium bromide staining. The PCR products for PRA and PRB were 99 and 200 base pairs, respectively.

2.6. RT-PCR

RT-PCR kits (TaKaRa, Japan) were used to synthesize cDNA from 2 μg of total RNA using random hexamer primers. cDNA synthesis was carried out as suggested by the kit protocol, using AMV reverse transcriptase. The RT was carried out for 45 min at 45 °C, after an incubation at 99 °C for 5 min to inactivate the reverse transcriptase. 5 μl of cDNA was used to amplify regions of PR common to both isoforms and region unique to PRB. β-Actin cDNA fragments was also amplified as a positive control (Table 1). Primers for β-actin were chosen specifically to cross two exons in the β-actin gene. In the presence of contaminating genomic DNA, additional larger bands would be amplified. The lack of amplification of any larger bands would indicate that there was no contamination with any genomic DNA [18]. The PCR conditions are as follows: pre-PCR incubation at 94 °C for 2 min; denature at 94 °C for 30 s; annealing at 62 °C for 30 s; extension at 72 °C for 30 s; 35 cycles; final extension at 72 °C for 8 min. After PCR, 8 μl product was mixed with 1 μl of 10× loading dye and then run on 2% agarose gel. Electrophoresis was carried out at 100 V at ambient temperature. The bands on the gels were visualized by ethidium bromide staining. Electrophoresis profiles were analyzed by the software, Gel-Pro3.1 (Media Cybernetics Inc., USA). The ratio of the integrated optical density (IOD) of PR and β-actin represented the mRNA level. PRA mRNA cannot be distinguished from PRB by conventional RT-PCR because PRA has no specific sequence to distinguish it from PRB mRNA. Therefore, we measured PRAB and PRB mRNA. The primes for DNA methyltransferase (DNMT1) were also listed in Table 1. The PCR product for DNMT1 was 361 base pairs [19].

2.7. Antisense methyltransferase oligo treatment

The antisense methyltransferase oligo was designed to hybridize to the 5′-untranslated region [19,20]. Antisense sequence MG88 (5′-AAGCATGAGCACCGTTCTCC-3′); Control sequence MG208 (5′-AACGATCAGGACCCTTGTCC-3′) with six mismatch bases. The two oligos were 2′-O-methyl modification at the first and the last four bases. Cells were treated with these oligos at doses from 0 to 70 nM in the presence of DOSPER Liposomal Transfection Reagent (Roche). In the first day, cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (5% CO_2), then transfected with antisense or control oligo for 6 h. After that, the cells were washed with PBS twice and changed into medium of RPMI 1640 supplemented with 10% fetal calf serum. The cells were harvested 24 h later. For continuous transfection, cells were transfected with antisense oligo or control oligo every day and were split every second day.

3. Results

3.1. Methylation status of PRA and PRB before and after 2-Aza-CdR treatment

By MSP without 5-Aza-CdR treatment, methylation bands were observed whereas unmethylated bands did not appear in either one of the three cell lines used (Fig. 1A and B). After 5-

Table 1
PCR primer sequences and PCR conditions for PR and DNMT1

Primer	Sequence	Pre-PCR incubation	Denature	Annealing	Extension	Cycle	Final incubation
PRA-Wf	5′-ACGGGCTACTCTTCCCTCG-3′	94 °C, 5 min	94 °C, 30 s	63 °C, 30 s	72 °C, 30 s	40	72 °C, 8 min
PRA-Wr	5′-TGGAATATGCGCCCTCCACG-3′						
PRA-Uf	5′-ATGGGTTATTTTTTTTTTG-3′	94 °C, 5 min	94 °C, 30 s	55 °C, 30 s	72 °C, 30 s	40	72 °C, 8 min
PRA-Ur	5′-TAAAATATACACCCTCCACA-3′						
PRA-Mf	5′-ACGGGTTATTTTTTTTTCG-3′	95 °C, 5 min	94 °C, 30 s	55 °C, 30 s	72 °C, 30 s	40	72 °C, 8 min
PRA-Mr	5′-TAAAATATACGCCCTCCACG-3′						
PRB-Wf	5′-TGACTGTCGCCCGCAGTACG-3′	95 °C, 5 min	94 °C, 30 s	63 °C, 30 s	72 °C, 30 s	40	72 °C, 8 min
PRB-Wr	5′-CGGCAATTTAGTGACACGCG-3′						
PRB-Uf	5′-TGATTGTTGTTTGTAGTATG-3′	95 °C, 5 min	94 °C, 30 s	55 °C, 30 s	72 °C, 30 s	40	72 °C, 8 min
PRB-Ur	5′-CAACAATTTAATAACACACA-3′						
PRB-Mf	5′-TGATTGTCGTTCGTAGTACG-3′	95 °C, 5 min	94 °C, 30 s	55 °C, 30 s	72 °C, 30 s	40	72 °C, 8 min
PRB-Mr	5′-CGACAATTTAATAACACGCG-3′						
RT-PCR AB-f	5′-AGCCGGTCCGGGTGCAAG-3′	94 °C, 2 min	94 °C, 30 s	62 °C, 30 s	72 °C, 30 s	35	72 °C, 8 min
RT-PCR AB-r	5′-CCACCCAGAGCCCGAGGG-3′						
RT-PCR B-f	5′-ACTGAGCTGAAGGCAAAGGGT-3′	94 °C, 2 min	94 °C, 30 s	62 °C, 30 s	72 °C, 30 s	35	72 °C, 8 min
RT-PCR B-r	5′-GTCCTGTCCCTGGCAGGGC-3′						
RT-PCR DNMT1-f	5′-GTGGCAGTGGACGGAGCAAG-3′	94 °C, 2 min	94 °C, 30 s	62 °C, 30 s	72 °C, 30 s	35	72 °C, 8 min
RT-PCR DNMT1-r	5′-AACCAGTGGGCGTGAAACAT-3′						
β-Actin-f	5′-AAGGCCAACCGCGAGAAGAT-3′	94 °C, 2 min	94 °C, 30 s	62 °C, 30 s	72 °C, 30 s	35	72 °C, 8 min
β-Actin-r	5′-TCGGTGAGGATCTTCATGAG-3′						

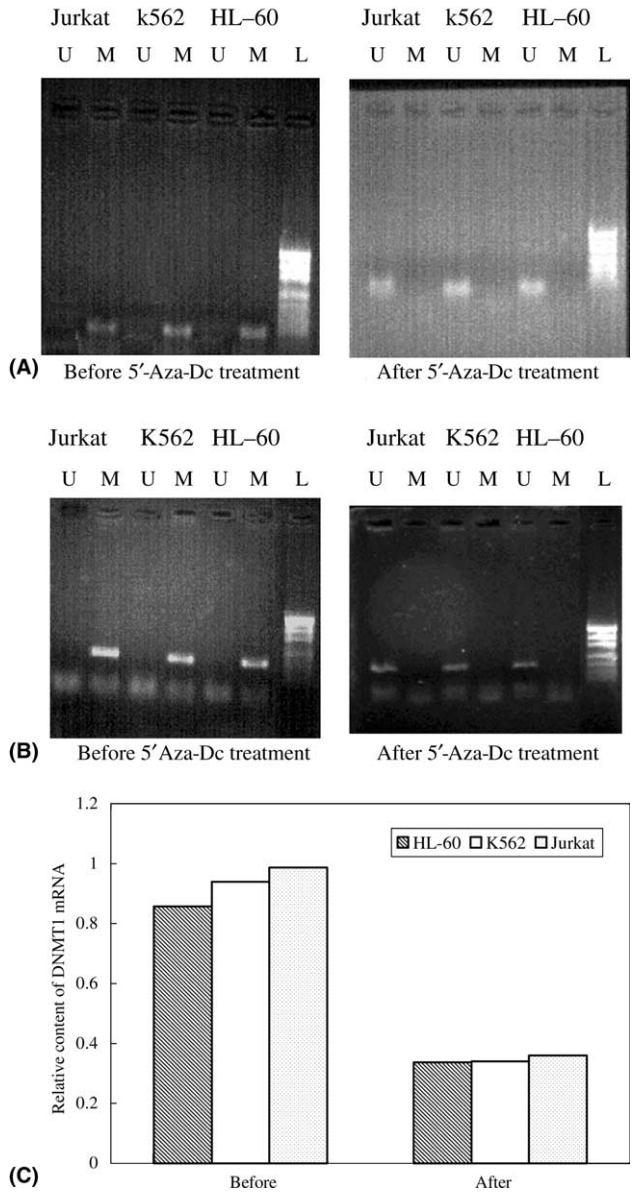

Fig. 1. (A) Methylation status of PRA before and after treatment with demethylating reagent 5′-Aza-CdR. U, unmethylated bands; M, methylated bands; L, marker. (B) Methylation status of PRB before and after treatment with demethylating reagent 5′-Aza-CdR. U, unmethylated bands; M, methylated bands; L, DNA marker. (C) Changes of *DNMT1* mRNA before and after 5-Aza CdR treatment.

Aza-CdR treatment, all cell lines had only unmethylated bands of PRA and PRB by MSP (Fig. 1A and B).

3.2. The mRNA expression of PRAB and PRB before and after 2-Aza-CdR treatment

Before 2-Aza-CdR treatment, no PRAB or PRB expression was found by RT-PCR (Fig. 2). Treatment of cell lines with 2-Aza-CdR restored PRAB and PRB expression in all cancer cell lines (Fig. 2).

3.3. The mRNA expression of DNMT1 before and after 2-Aza-CdR treatment

Treatment of cell lines with 2-Aza-CdR lowered the mRNA expression of DNMT1 about two times compared with mRNA

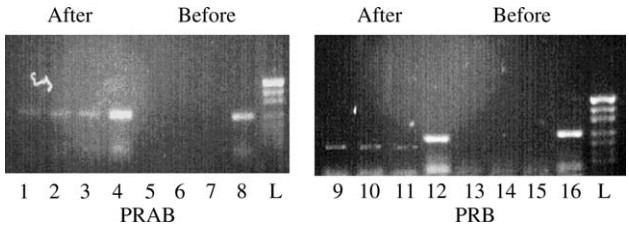

Fig. 2. The mRNA expression of PRAB, PRB after (1–4; 9–12) and before (5–8; 13–16) 5′-Aza-CdR treatment. L – DNA marker; 1,5,9, 13– Jurkat; 2,6,10,14 – K562; 3,7,11,15 – HL-60; 4,8,12,16 – β-actin.

Table 2
Changes of DNMT1 mRNA before and after 5-Aza CdR treatment ($\bar{x} \pm$ S.D.)

	HL-60	K562	Jurkat
Before	0.857 ± 0.025	0.940 ± 0.026	0.987 ± 0.021
After	0.337 ± 0.025	0.340 ± 0.026	0.360 ± 0.026
	$P < 0.01$	$P < 0.01$	$P < 0.01$

expression of DNMT1 before 2-Aza-CdR treatment (Fig. 1C, Table 2).

3.4. The changes of mRNA expression of DNMT1 after antisense DNMT1 oligo treatment

We used an antisense DNMT1 oligo (MG88) and a control oligo (MG208) to abrogate DNMT1. The results showed that DNMT1 was significantly downregulated in the three cell lines after transfection. Fig. 3 shows the DNMT1 mRNA expression in HL-60. The similar result is obtained in K562 and Jurkat.

3.5. The changes of methylation status of PRA and PRB after antisense DNMT1 oligo treatment

By MSP after antisense DNMT1 oligo MG88 treatment, both methylation bands and unmethylated bands were observed in all three cell lines, whereas in the control (MG208 treatment) only methylated bands appeared (Fig. 4).

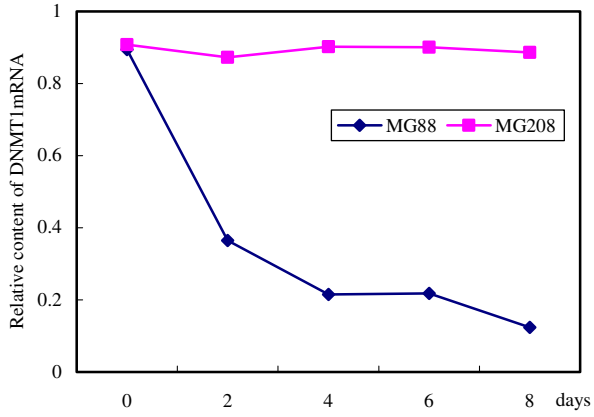

Fig. 3. The changes of mRNA expression of DNMT1 after antisense DNMT1 oligo treatment. MG88, antisense DNMT1 oligo; MG208, control oligo.

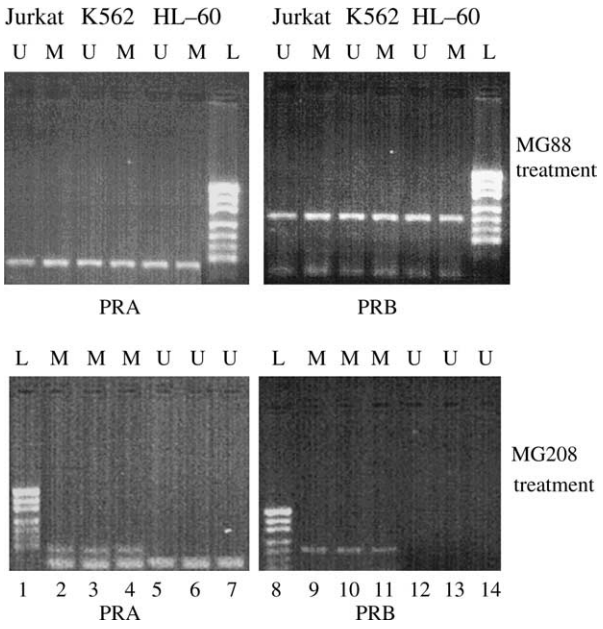

Fig. 4. Methylation status of PRA and PRB after 4 days DNMT1 oligo treatment. MG88 – DNMT1 antisense oligo; MG208 – control oligo; U – unmethylated bands; M – methylated bands; L – DNA marker; 2,5,9,12 – Jurkat; 3,6,10,13 – K562; 4,7,11,14 – HL-60.

3.6. *The relationship of PRB mRNA expressions and the MG88 treatment*

The PRB mRNA was increased in a time-dependent manner in the antisense oligo MG88 treatment group within 8 days in three cancer cell lines (Fig. 5).

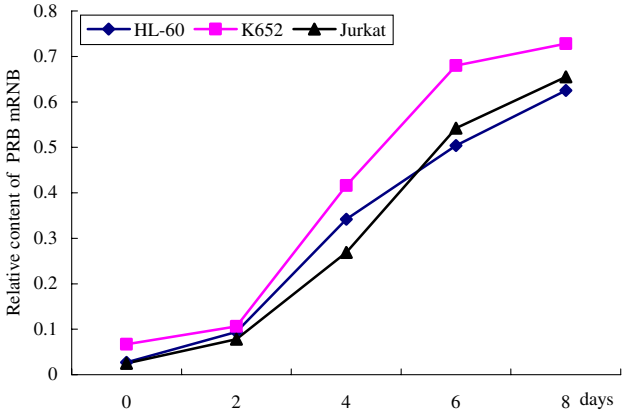

Fig. 5. Relation between PRB mRNA expressions and the time of MG88 treatment.

3.7. *Methylation frequencies of the PRA and PRA genes in primary leukemia samples*

To determine normal patterns of PR methylation in hematopoietic cells, we studied bone marrow DNA from 10 healthy individuals. MSP results showed that normal bone marrow cells are unmethylated in the PR double promoters CpG islands. Having established that normal hematopoietic cells are unmethylated at the PR CpG island, we next determined the methylation state of this gene in 44 human leukemia samples. Table 3 is the summary of PR methylation in leukemia samples and normal controls.

4. Discussion

The research of promoter methylation was mainly focused on mono-promoter gene before. But the gene regulation of multiple promoters is more complex than that of mono-promoter. Research of methylation of PR with two promoters was mainly focused on steroid-related neoplasms [1,18,21] before. This experiment was designed to disclose the methylation status of PR double promoters in leukemia and clarify the significance of PR promoter methylation in silencing PR expression in leukemia. The correlations of PR methylation and PR expression together with the DNMT1 was further studied, which provided theory basis for the relation between the epigenetic and the formation of neoplasm and new direction for the therapy of neoplasm.

Sasaki et al. [18] investigated the methylation status of PRA and PRB in UEC cell lines using MSP. They discovered that PRB is methylated in all human UEC cells, whereas PRA is unmethylated. We investigated the methylation status of PRA and PRB in the gastric cancer cell line, colorectal cancer cell line, lung cancer cell line, pancreas cancer cell line and epidermoid cancer cell line [22]. We discovered that PRA and PRB are both methylated in all the cancer cell lines investigated.

In this study, we studied the methylation status of PRA and PRB in leukemia cell lines using MSP. The results showed that CpG islands of PRA and PRB promoters were hypermethylated without unmethylation bands in three leukemia cell lines Jurkat, K562 and HL-60. In addition, the mRNA expression of PRAB and PRB was inactivated in the three leukemia cell lines researched.

To understand whether PR mRNA expression is inactivated by methylation, we treated cells with 5-Aza-CdR. The treatment of 5-Aza-CdR restored PRB and PRAB expression in all cell lines researched. The results showed clearly that there is a close relationship between the inactivation of PR gene and abnormal methylation of PR in leukemia cells.

Table 3
Summary of PR methylation in leukemia

	Methylated		Unmethylated	
	PRA	PRB	PRA	PRB
ALL	63.6% (7/11)	54.5% (6/11)	45.5% (5/11)	45.5% (5/11)
CLL	62.5% (5/8)	75.0% (6/8)	37.5% (3/8)	25.0% (2/8)
AML	66.7% (10/15)	60.0% (9/15)	40.0% (6/15)	53.3% (8/15)
CML	70.0% (7/10)	60.0% (6/10)	30.0% (3/10)	50.0% (5/10)
Normal	0% (0/10)	0% (0/10)	100% (10/10)	100% (10/10)

To further understand the molecular mechanism of hypermethylation of PR gene in leukemia cell, we used antisense methyltransferase oligo (MG88) to study the influence of DNMT1 mRNA expression to PR methylation. PR methylation is in accordance with an increase of DNMT1 mRNA in this study. After the MSP analysis, we demonstrated the evidence of DNA methylation in leukemia cell lines. We also found that a high level of DNMT was related to low PR mRNA expression and methylation of the promoter site. The antisense methyltransferase oligo in reactivating this gene further identified the role of DNMT1 in PR gene silencing. Kanai et al. [23] reported that overexpression of DNMT1 mRNA was significantly associated with CpG island methylator phenotype in 23% of colorectal cancers and 31% of stomach cancers. However, some other studies have failed to demonstrate a correlation between the level of DNMT1 expression in cancer cells and the state of methylation of the tumor suppressors [24,25]. Our study supports the hypothesis that PR gene silencing through DNA methylation may be mediated by an increase of DNMT in leukemia cancer cell lines.

Melki et al. [26] developed a standardized competitive RT-PCR assay to measure the level of DNMT transcripts in patients with acute leukemia. They observed a 4.4-fold mean increase in the level of DNMT mRNA compared with normal bone marrow. Their results support the hypothesis that an increase in DNMT activity is associated with malignant hematological diseases and may constitute a key step in carcinogenesis.

To further understand the role of DNMT1 in PR gene silencing in leukemia, we used an antisense methyltransferase oligo to test the hypothesis that a high level of DNMT is the main cause of the hypermethylation in the CpG island and contributes to the loss of PR gene mRNA expression. Although DNMT inhibitor 5-Aza-CdR can produce similar results and is widely used to study the reexpression of genes silenced by promoter methylation [27], this inhibitor is mutagenic and may cause DNA damage. To exert their biological effect, 5-Aza-CdR must be incorporated into the DNA where they covalently trap the bulky 190 kDa DNMT onto the DNA [28], so that they may alter gene expression by mechanisms unrelated to the inhibition of DNMT [29]. Antisense oligos have been proven to be an effective approach in many areas of cancer research and have been shown to act through an Rnase H-dependent cleavage of the target mRNA and then turnover of previously synthesized protein, and lead to a reduction in target protein [27]. MG88 is termed as second-generation antisense molecules because it contains both phosphorothioate backbone modifications as well as a 2′-*O*-methyl modification to the ribose on the four 3′ and 5′ terminal nucleotides. The combination of these chemistries increases stability and potency of the inhibitors, thus allowing very low concentrations to be used experimentally, thus minimizing non-specific effects [27]. In our study, we compared the effect of antisense DNA DNMT1 oligo MG88 and a control oligo with six mismatch bases. We found that DNMT mRNA can be reduced to a very low level on the second day after antisense oligo transfection. PR mRNA level was upregulated in all three cell lines. This suggested that DNMT plays an important role in PR gene silencing and promoter CpG methylation. A previous study also demonstrated that blocking DNMT1 by antisense oligo could activate the p16 gene through demethylation [30]. This study suggests that antisense DNMT1 can reactivate the PR gene in human cancer cell lines.

Cancer cell lines exhibit a higher degree of methylation than normal tissues [31]. Because cell lines are at the end stage of carcinogenesis and they may not truly represent each cancer in tissue. Therefore, we also performed methylation analysis using primary leukemia samples. The results are summarized in Table 3. The methylation frequencies of the PRA gene are 63.6% in ALL, 62.5% in CLL, 66.7% in AML, 70% in CML and 0% in normal control. The methylation frequencies of the PRB gene are 54.5% in ALL, 75% in CLL, 60% in AML, 60% in CML and 0% in normal control. These data confirm extensive methylation of the PRA and PRB CpG islands in these leukemias. This aberrant methylation might be an important step in the evolution of these tumors.

CpG islands are found in other members of the hormone receptor superfamily including androgen, progesterone, and ER genes [32–34]. Issa et al. [13] discovered that the ER CpG island is abnormally methylated in most hematopoietic neoplasms. This study observed that PR CpG island is also abnormally methylated in leukemia neoplasms. These results altogether supported the hypothesis that the hormone receptor superfamily might be involved in normal hematopoiesis.

In summary, this study indicated that reduced PR mRNA expression in leukemia cell lines was in part related to DNA methylation and a high level of DNMT. Antisense methyltransferase oligo can abrogate DNMT1 and increase PR mRNA expression. We conclude that DNA methylation plays an important role in PR gene silencing in leukemia.

References

[1] Lapidus, R.G., Ferguson, A.T., Ottaviano, Y.L., Parl, F.F., Smith, H.S., Weitzman, S.A., Baylin, S.B., Issa, J.P. and Davidson, N.E. (1996) Clin. Cancer Res. 2, 805–810.
[2] Graham, J.D. and Clarke, C.L. (1997) Endocr. Rev. 18, 502–519.
[3] Peterson, C.M. (2000) J. Soc. Gynecol. Invest. 7, S3-7.
[4] Horwitz, K.B. and McGuire, W.L. (1975) Steroids 25, 497–505.
[5] MacLusky, N.J. and McEwen, B.S. (1980) Brain Res. 189, 262–268.
[6] Perrot-Applanat, M., Cohen-Solal, K., Milgrom, E. and Finet, M. (1995) Circulation 92, 2975–2983.
[7] Pearce, P.T., Khalid, B.A.K. and Funder, J.W. (1983) Endocrinology, 1287–1291.
[8] Clarke, C.L. and Sutherland, R.L. (1990) Endocr. Rev. 11, 266–302.
[9] Fuqua, S.A., Hill, S.M., Chamness, G.C., Benedix, M.G., Greene, G.L., O'Malley, B.W. and McGuire, W.L. (1991) J. Natl. Cancer I 83, 1157–1160.
[10] Fuqua, S.A., Allred, D.C., Elledge, R.M., Krieg, S.L., Benedix, M.G., Nawaz, Z., O'Malley, B.W., Greene, G.L. and McGuire, W.L. (1993) Breast Cancer Res. Treat. 26, 191–202.
[11] Bird, A.P. (1986) Nature 321, 209–213.
[12] Antequera, F., Boyes, J. and Bird, A.P. (1990) Cell 62, 503–514.
[13] Issa, J.P., Zehnbauer, B.A., Civin, C.I., Collector, M.I., Sharkis, S.J., Davidson, N.E., Kaufmann, S.H. and Baylin, S.B. (1996) Cancer Res. 56, 973–977.
[14] Felgner, J., Heidorn, K., Korbacher, D., Frahm, S.O. and Parwar, R. (1999) Leukemia 13, 530–534.
[15] Meakawa, M., Sugano, K., Kashiwabara, H., Ushiama, M., Fujita, S., Yoshimori, M. and Kakizoe, T. (1999) Biochem. Biophys. Res. Commun. 262, 671–676.

[16] Liu, Z.J. and Maekawa, M. (2003) Anal. Biochem. 317, 259–265.
[17] Maekawa, M., Sugano, K., Ushiama, M., Fukayama, N., Nomoto, K., Kashiwabara, H., Fujita, S. and Kakizoe, T. (2001) Clin. Chem. Lab. Med. 39, 121–128.
[18] Sasaki, M., Dharia, A., Oh, B.R., Tanaka, Y., Fujimoto, S. and Dahiya, R. (2001) Cancer Res. 61, 97–102.
[19] Chen, C.L., Liu, S.S., Ip, S.M., Wong, L.C., Ng, T.Y. and Ngan, N.Y. (2003) Eur. J. Cancer 39, 517–523.
[20] Fournel, M., Sapieha, P., Beaulieu, N., Besterman, J.M. and MacLeod, A.R. (1999) J. Biol. Chem. 274, 24250–24256.
[21] Srinivasan, G., Campbell, E. and Bashirelahi, N. (1995) Microsc. Res. Tech. 30, 293–304.
[22] Liu, Z.J., Maekawa, M., Horii, T. and Morita, M. (2003) Life Sci. 73, 1963–1972.
[23] Kanai, Y., Ushijima, S., Kondo, Y., Nakanishi, Y. and Hirohashi, S. (2001) Int. J. Cancer 91, 205–212.
[24] Jurgens, B., Schmitz-Drager, B.J. and Schulz, W.A. (1996) Cancer Res. 56, 5698–5703.
[25] Eads, C.A., Danenberg, K.D., Kawakami, K., Saltz, L.B., Danenberg, P.V. and Laird, P.W. (1999) Cancer Res. 59, 2302–2306.
[26] Melki, J.R., Warnecke, P., Vincent, P.C. and Clark, S.J. (1998) Leukemia 12, 311–316.
[27] Jones, P.A. and Taylor, S.M. (1980) Cell 20, 85–93.
[28] Juttermann, R., Li, E. and Jaenisch, R. (1994) Proc. Natl. Acad. Sci. USA 91, 11797–11801.
[29] Jackson-Grusby, L., Laird, P.W., Magge, S.N., Moeller, B.J. and Jaenisch, R. (1997) Proc. Natl. Acad. Sci. USA 94, 4681–4685.
[30] Chuang, L.S., Ian, H.I., Koh, T.W., Ng, H.H., Xu, G. and Li, B.F. (1997) Science 277, 1996–2000.
[31] Smiraglia, D.J., Rush, L.J., Fruhwald, M.C., Dai, Z., Held, W.A., Costello, J.F., Lang, J.C., Eng, C., Li, B., Wright, F.A., Caligiuri, M.A. and Plass, C. (2001) Hum. Mol. Genet. 10, 1413–1419.
[32] Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H. and Chambon, P. (1990) EMBO J. 9, 1603–1614.
[33] Ottaviano, Y.L., Issa, J.P., Parl, F.F., Smith, H.S., Baylin, S.B. and Davidson, N.E. (1994) Cancer Res. 54, 2552–2555.
[34] Issa, J.P., Baylin, S.B. and Belinsky, S.A. (1996) Cancer Res. 56, 3655–3658.