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Cloning of the 5′ Upstream Region of the Rat *p16* Gene and Its Role in Silencing

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Hypermethylation of the 5′ upstream region (5′ region) of the human *p16*$^{\mathrm{CDKN2A}}$ (*p16*) gene is known to cause silencing, which is involved in a wide range of human cancers. For the rat *p16* gene, its 5′ region has not been cloned, and it is uncertain whether surrogate use of exon 1α is adequate for analysis of *p16* silencing. In this study, we observed that methylation analysis of exon 1α gave false positive results in three samples of normal rat mammary epitheliums and in two of six primary mammary carcinomas. Therefore, we determined the nucleotide sequence of the 5′ region of the rat *p16* gene. To confirm that methylation status of the 5′ region is correlated with *p16* expression, the methylation status was analyzed by bisulfite sequencing and methylation-specific PCR in three samples of normal mammary glands, six samples of mammary carcinomas and four cell lines. The 5′ region was demethylated in all of the three normal and six carcinoma samples that fully expressed *p16*. On the other hand, the 5′ region was highly methylated in the 3Y1 cell line, which lacked *p16* expression, but without deletion. These results showed that the methylation status of the 5′ region was more closely correlated with *p16* expression than that of the exon 1α and analysis of the methylation status is useful in examining *p16* silencing in various rat tumors.

Key words: *p16* — Methylation — Rat — Silencing — CpG islands

Cyclin-dependent kinase inhibitor-2A (CDKN2A) is known to inhibit CDK4/6, and plays a critical role in cell cycle regulation.1) Inactivation of the *p16*$^{\mathrm{CDKN2A}}$ (*p16*) gene by homozygous deletion is one of the most common abnormalities in human cancers.2) The human *p16* gene is also inactivated by methylation of a CpG island (CGI) in its promoter region, and its silencing has been reported in a variety of cancers, such as those of the lung, brain, breast, colon, and bladder.2–4) Detailed analysis of the methylation pattern of the human *p16* gene showed that an approximately 250-bp region overlapping the transcription start site was capable of down-regulating the promoter activity, while *p16* expression could occur in the presence of heavy methylation in the coding region.5) It is generally considered that methylation of a CGI in the promoter region of a gene is responsible for transcriptional silencing, but methylation outside the promoter region does not block transcription.6, 7)

The rat is widely used for mechanistic research on human cancers, including molecular alterations and studies of carcinogens, promoters and preventive agents.8–10) The rat *p16* gene, like human *p16* gene,11) consists of three exons, exons 1α, 2 and 3.12) Exon 1α is specific for *p16*, and exons 2 and 3 are commonly used with the *p14* gene.11) The sequence of rat exon 1α has been determined,12) and methylation of exon 1α has been used for analysis of *p16* involvement in rat lung, liver and renal cancers.12–17) However, some inconsistencies have been noted, in that some carcinomas with full *p16* expression had completely methylated exon 1α.14) Expression analyses have been performed on limited fractions of samples.15–17)

In this study, we observed methylation of exon 1α in rat normal mammary epithelial cells and primary mammary carcinomas that had *p16* expression. This prompted us to clone the 5′ upstream region (5′ region) of the rat *p16* gene, whose methylation showed a better correlation with *p16* expression than did that of exon 1α.

MATERIALS AND METHODS

Samples and DNA and RNA extraction Rat fibroblast cell lines 3Y1 and BBR-2 were obtained from Japanese Cell Research Bank (Tokyo) and Stratagene (La Jolla, CA), respectively. Two rat mammary carcinoma cell lines, PhIP12-1 and PhIP7-4, were established in our laboratory.18) Primary mammary carcinomas were induced by oral administration of ten doses of 75 mg/kg 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-HCl to female 6-week-old (F344×SD)F_1 rats.19) Three samples of normal mammary epithelial cells were obtained from three non-treated female (F344×SD)F_1 rats, 56–69 weeks old, by the gland isolation technique.19) DNA was serially

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extracted with phenol and chloroform and ethanol precipitation,[20] and total RNA was isolated using ISOGEN (Nippon Gene, Tokyo).

RT-PCR Five micrograms of total RNA was used for first-strand cDNA synthesis with Superscript II reverse transcriptase (Invitrogen, Leek, Netherlands), and 1 μl of a 50-μl reaction mixture was used as a template for PCR. The primer sequences used for amplification of the rat *p16* gene are listed in Table I. The rat glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene was used as an internal control gene with the following primers; *Gapdh*-A, 5′-TGGTGAAGGTCGGTGTGAAC-3′, and *Gapdh*-B, 5′-AGGGGTCGTTGATGGCAACA-3′ (annealing temperature: 55°C). For each gene, multiple cycles of PCR were tested. The cycle at which a sample having the highest expression reached an amplification plateau was determined, and a cycle number smaller than this was adopted for the analysis.

Southern blot analysis Genomic DNA was digested with *Bam*HI restriction enzyme, and electrophoresed in 0.9% agarose gel. After capillary blotting onto a filter, it was hybridized with a probe that had been labeled with $[\alpha\text{-}^{32}\text{P}]$dCTP using a Megaprime DNA labeling system (Amersham Pharmacia Biotech, Uppsala, Sweden). The filter was washed and exposed to Kodak XAR film. The probe for the 5′ region covered nt. −988 to 12 (translation start site=1) and that for exon 2 covered codons 63 to 156. A control probe was prepared from the *Atrn* gene, covering codons 534 to 1054.[21]

Methylation-specific PCR (MSP) and bisulfite sequencing Bisulfite treatment of genomic DNA was performed essentially as previously described.[22] Genomic DNA (500 ng) was digested with *Bam*HI restriction enzyme. The DNA was denatured in 0.3 *N* NaOH, then 2.9 *M* sodium bisulfite (Sigma, St. Louis, MO) and 0.5 m*M* hydroquinone (Sigma) was added and the DNA sample underwent 15 cycles of 30-s denaturation at 95°C and 15 min incubation at 50°C. The sample was then desalted using the Wizard DNA cleanup system (Promega, Madison, WI), and desulfonated by treatment with 0.3 *N* NaOH at room temperature for 5 min. After ethanol precipitation with ammonium acetate, DNA was dissolved in TE buffer.

MSP[23] was performed using the bisulfite-treated DNA and primers listed in Table I. The minimum number of PCR cycles was determined by observing the amplification of positive controls. As a positive control for primers for unmethylated DNA, the normal mammary epithelial cells of rat 16-2, which has full *p16* expression, was used. DNA having all CpG sites methylated by *Sss*I-methylase was used as a positive control for primers for methylated DNA.

For bisulfite sequencing, PCR was performed using the primers in Table I, and PCR products were cloned into pGEM-T Easy Vector (Promega). More than 10 clones

Table I. List of Primers

	Name	5′ position (nt.)	Sequence (5′ → 3′)	Annealing temperature (°C)
RT-PCR	Rp16-a	100	AACACTTTCGGTCGTACCC	61
	Rp16-b	198	GTCCTCGCAGTTCGAATC	
Bisulfite sequencing	p16-bis-A1	−478	GTTTGTTGGGAGGAGGAGAGATT	55
	p16-bis-A2	−201	AAACACCTCTAAAAACACTACTACCC	
	p16-bis-B1	−233	GTGGGGTGGGTAGTAGTGTT	58
	p16-bis-B2	26	ACTAATCTATCTACAAAAAACTCCAT	
MSP	BS1 (U)[a]	−30	GTGAATTTGAGGAGAGTGATTTG	60
	BS2 (U)[a]	99	CAAAACATTTAATAAAACCCCAA	
	BSM1 (M)[a]	−27	AATTCGAGGAGAGCGATTCG	60
	BSM2 (M)[a]	96	AACGTTTAATAAAACCCCGA	
	A1 (U)	−468	AGGAGGAGAGATTTTGATTTT	57
	A2 (U)	−368	AAATACTAAACTCCTTTCAAACA	
	A3 (M)	−469	GAGGAGGAGAGATTTCGATTTC	60
	A4 (M)	−367	AAAATACTAAACTCCTTTCGAACG	

a) Published in ref. 12).

were cycle-sequenced with the primers used for the initial PCR. Complete conversion of cytosines not flanked by guanine was confirmed. Unbiased amplification of methylated and unmethylated sequences was confirmed by sequencing an equal mixture of DNA derived from normal mammary epithelial cells of rat 16-2 and DNA treated with *Sss*I-methylase; the yields from these DNAs were approximately equal.

Cloning and sequencing of the 5′ region of rat *p16* A rat BAC library (RPCI-32 segment 1, CHORI BACPAC Resources, Oakland, CA) was screened by hybridization with a probe derived from exon 2 of the rat *p16* gene. Positive BAC clones were purchased from BACPAC Resources. A BAC clone, 63F13, was directly sequenced by cycle-sequencing using the BigDye Terminator Ready Reaction Mix (PE Applied Biosystems, Foster City, CA) and primers serially synthesized based on the sequence obtained. The final sequence was confirmed by PCR of genomic DNA and direct sequencing of the product. Homology search and motif search were performed using GENETYX-MAC software.

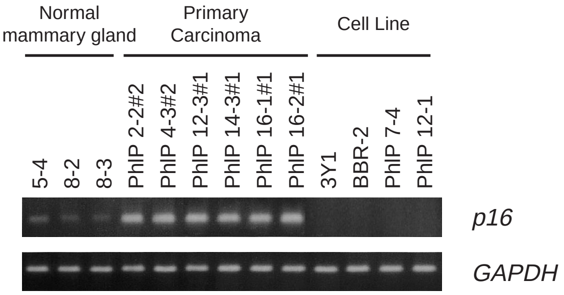

Fig. 1. *p16* expression in three samples of normal mammary epithelial cells, six mammary carcinomas and four cell lines. The three normal samples expressed *p16* at similar levels and all of the six mammary carcinomas had elevated expression levels. In contrast, the four cell lines did not have *p16* expression.

RESULTS

***p16* expression by RT-PCR** Expression of *p16* was analyzed in three samples of normal mammary epithelial cells, six primary mammary carcinomas and four rat cell lines (Fig. 1). *p16* was found to be expressed at similar levels in the three samples of normal mammary ducts, and its expression was elevated in the six primary carcinomas. However, *p16* expression was completely lost in the four cell lines, and could not be detected even after five additional PCR cycles.

Homozygous deletion of *p16* in three cell lines To identify the reason for the complete loss of *p16* expression in the four cell lines, homozygous deletion of the *p16* gene

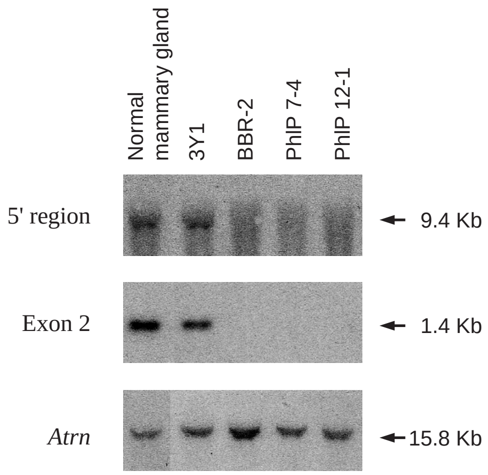

Fig. 2. Southern blot analysis of the *p16* gene. Genomic DNA of a control sample of normal mammary epithelial cells and the four cell lines were analyzed. Hybridization with probes from the 5′ region and exon 2 showed that BBR-2, PhIP7-4 and PhIP12-1 had homozygous deletions of the *p16* gene. A probe for the *Atrn* gene was used as a control.

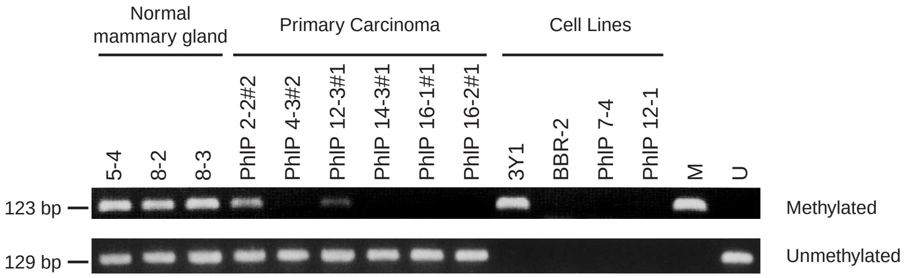

Fig. 3. MSP analysis of the three samples of normal mammary epithelial cells, six mammary carcinomas and four cell lines. 3Y1 was shown to have only methylated DNA. The three samples of normal mammary epithelial cells and two of the six mammary carcinomas displayed bands using specific primer for methylated DNA. These samples were considered to have both methylated DNA and unmethylated DNA.

A

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-1435      aactaacccagcttgttggtgcagctatattcccacagtttagaggctgaagcag
-1380 ggcaacttggggtataatataaggtgagacttcacacatatacgctctgtctctgtctct
-1320 ctctctctctctctcacacacacacacacacacacacacacacacacacacacacagt
-1260 cagttctctgtctctctagttttgtgtccacagcatatgattggcattctcaccacacac
-1200 atatgtaaagtcaaaatagctgtggccatataaagaatatggggagagacaattattcag
-1140 cacctacagaaaataacgccaggtctttactcctggcactcgggaggctcggaaagcagc
-1080 aggcagagctcctccgagagtttaggttgagttccaggagagggagaaagggccgctgcc
-1020 ttttcggtgaggactctttttagtctacccgtttgtctagtaattttgcccaaagcgtg
 -960 ttcttcctgcctcacaagattgcaataagttttaaatgaacaatttaaacctgtgccatt
 -900 gggtgtgcagtagttctcaacatttatgtgcagcatatcaactcatttaaactcacaacg
 -840 ggtccataaaatagaaatactttataagcagattaccctccaaagacttcactcgctcgc
 -780 tttttgaaatttgtgtacagaatcctagcactgctaccgcaacatcagaaatatttctga
 -720 aaattcctcgccaaggttcgggtttcatacttggtatagtaccctccaaaataagttgtt
 -660 tgtttgagctagggttgttgggatctcaccttggccaggatgtagctctttcttctgaat
 -600 aaaagagaacgcgcctttccttttttttttttttttttttttttttttgctaagaagt
 -540 taaatatcttaagcttcaatttggcgatgaaaactccctccttcgttttcctaaaacctg
 -480 gggtttgctgggaggaggagagatttcgatttcgagcgggtagagaatggactagttcac
 -420 tttctcagatgtgctcctctatggtgtccccgcccgaaaggagcccagtacctcaagggg
 -360 tggtcaggcttgcgggaaagggtgtcagcgtgggtagcaggtgggggcagttcgatcctt
 -300 cagcgaagcttcaactcctagcactccacaggaacccctcacctcctctgcagtccgacc
 -240 ccgggggtggggtgggcagcagtgttttcagaggtgttcaattcctgctttattcaggg
 -180 cacatagcgccagctaaagcgggctgtggaaccaggtcaggagcaaagcgtggtttgccc
 -120 cccccccccgtcccccctcagaggaaggaaggagggccccactggtcacaccactggcga
  -60 ctgggcgggcactgCATCTCCGAGAGGAAGGCGAACTCGAGGAGAGCGATCCGGAGCAGC
    1 ATGGAGTCCTCTGCAGATAGACTAGCCAGGGCAGCGGCCCTGGGCCGTGAGCACGAGGTG
   61 CGGGCACTGCTGGAAGCCGGGGCTTCACCAAACGCCCCGAACACTTTCGGTCGTACCCCG
  121 ATACAG
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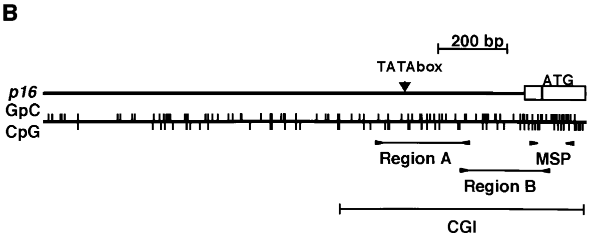

Fig. 4. Cloning of the 5′ region of the rat *p16* gene. A. Nucleotide sequence of the rat *p16* gene as far as 1435-bp upstream of the translation initiation site. CpG sites are shown in bold face, and a putative TATA box is boxed. The translation initiation codon is shown by an underline, and the nucleotides in reported exon 1α are shown in capital letters. B. Schematic representation of the GpC and CpG sites. The density of CpG sites gradually decreased with increasing separation from the initiation codon. The locations of the two regions used for bisulfite sequencing (region A and B) and a pair of primers for MSP of exon 1α are shown.

was analyzed by Southern blot analysis (Fig. 2). When a probe for exon 2 of the rat *p16* gene was used, hybridization signals were absent for three cell lines, BBR-2, PhIP7-4 and PhIP12-1, while clear signals were present for 3Y1 and for the normal sample of mammary epithelial cells. When a probe for the 5′ upstream region, which we had cloned (see the following sections), was used, hybridization signals were again absent for BBR-2, PhIP7-4 and PhIP12-1. All the samples gave similar hybridization signals when a control probe was used. These results showed that BBR-2, PhIP7-4 and PhIP12-1 had homozygous deletion of the *p16* gene.

Methylation status of a CGI in exon 1α Methylation of a CGI in exon 1α is known to be important for rat *p16* silencing,[12] and the methylation status of the region was analyzed by MSP. 3Y1 gave a PCR product only with primers for methylated DNA (Fig. 3). The other three cell lines gave no PCR products using primers for methylated DNA or for unmethylated DNA, which was concordant with the *p16* homozygous deletion in the three cell lines. Surprisingly, this region was methylated to various degrees even in the three normal samples and in two of the six mammary carcinomas (Fig. 3) that had elevated *p16* expression.

Cloning of the 5′ region Considering that methylation of a CGI in the promoter region is generally well correlated with transcriptional repression,[6, 7] the nucleotide sequence in the 5′ region of the rat *p16* gene was determined (Fig. 4A; GenBank accession number, AB081658). Motif search predicted a TATA box between nt. −401 and −396 and a transcription start site 10-bp downstream of the box. A CGI was predicted to start from the 5′ region and extend into exon 1α (Fig. 4B), the CpG score and G+C content between nt. −591 and 120 being 0.63% and 57%, respectively, and meeting the criterion for CGIs proposed by Gardiner-Garden and Frommer.[24] The sequence had 74.2% homology with the mouse and 43.7% homology with the human 5′ region.

Methylation status of the 5′ region Methylation status of the 5′ region was determined by bisulfite sequencing of two regions (regions A and B; between nt. −478 and 23) that contained 22 CpG sites (Fig. 5A). CpG sites in the 5′ region were found to be demethylated in the three samples of normal mammary epithelial cells with normal *p16* expression and in the six mammary carcinomas with elevated *p16* expression. In contrast, these CpG sites were methylated in the 3Y1 cell line that lacked *p16* expression.

The methylation status of the 5′ region was in good accordance with *p16* expression. Since a convenient method to analyze the methylation status of the region was expected to be useful, we developed MSP primers for the 5′ region (A1 and A2 for unmethylated DNA; A3 and A4 for methylated DNA in Table I). The primers for the methylated sequence gave PCR products only for 3Y1, and never gave products in the three normal mammary epithelial cells and six primary carcinomas. The primers for the unmethylated sequence gave PCR products in the three samples of normal mammary ducts and six mammary carcinomas (Fig. 5B).

DISCUSSION

The 5′ region of the rat *p16* gene, which was newly identified in this study, was demethylated in normal mammary epithelial cells and mammary carcinomas that had *p16* expression, and was methylated in a cell line that had

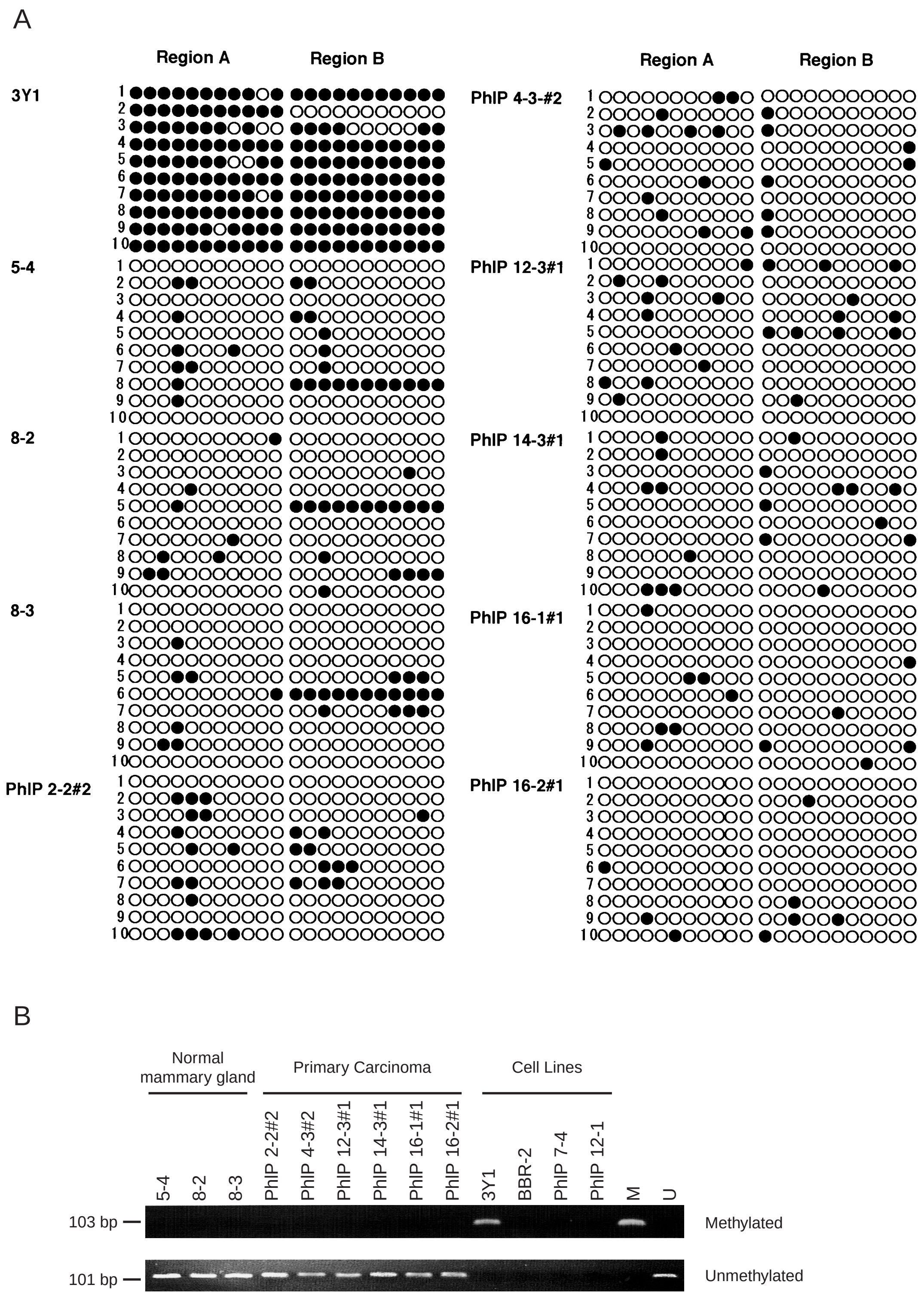

Fig. 5. Methylation analysis of the 5′ region. A. Results of bisulfite sequencing. For each sample, ten clones for both regions were sequenced. Methylated CpG sites are shown by closed circles, and unmethylated CpG sites are shown by open circles. B. MSP of region A. Only the 3Y1 cell line, which did not have *p16* expression, had methylated DNA in this region. This set of MSP primers is considered useful to analyze silencing of the *p16* gene.

lost *p16* expression. In contrast, exon 1α, which has been widely used to examine silencing of the rat *p16* gene, was partially methylated even in the normal mammary epithelial cells and mammary carcinomas that had *p16* expression. Generally, a CGI that is important for transcriptional regulation of a gene is located around its transcriptional start site,[6, 7] and regions outside the core CGI could be methylated by various factors, such as aging.[25, 26] In the case of the rat *p16* gene, a putative TATA box and a transcriptional initiation site were found 390-bp upstream of the translation initiation site. In the human *p16* gene, the essential region for *p16* expression is known to be located 50- to 300-bp upstream of the translation initiation site.[5] These findings indicate that the transcription status of the rat *p16* gene can be predicted more precisely by the methylation status of the 5′ region than by that of exon 1α.

Any sample, a primary sample or cell line, might consist of heterogeneous subpopulations regarding *p16* methylation and expression. If heterogeneous subpopulations are hypothesized, the exon 1α region could still play a critical role in rat *p16* silencing. However, it is unlikely that as many as three samples prepared from histologically normal mammary epithelial cells of untreated rats had two subpopulations with different biological properties; one having methylated exon 1α and thus silenced *p16* and one having unmethylated exon 1α and thus expressing *p16*. It is more likely that exon 1α does not play a critical role in *p16* silencing, as exon 1α does not in humans, and so could be methylated in a subpopulation without any biological consequence.

p16 expression was found to be lost in 3Y1 and BBR-2, which are considered to be normal fibroblasts.[19, 27] Inactivation of *p16* is known to be advantageous in immortalization,[28, 29] and it is not surprising to find *p16* inactivation in "normal" cell lines. In addition, 3Y1 is known to have a *p53* mutation.[27] In contrast to cell lines, elevation of *p16* expression was observed in primary mammary carcinomas. It is reported that *p16* expression is also elevated in human breast cancers,[30] and the elevation is considered to be one of the responses to accelerated cell cycles in breast cancers.

Analysis of the methylation status of the 5′ region, for example by using the MSP primers designed here, is expected to have a wide range of application for analysis of *p16* silencing in various rat tumors.

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