Eur J Cancer Clin Oncol, Vol. 23, No. 3, pp. 331–337, 1987.
Printed in Great Britain

0277-5379/87$3.00+0.00
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Expression and Methylation of the Blym Gene in Human Tumor Cell Lines*

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Abstract-*We have examined Blym expression in 11 human tumor cell lines. Increased Blym expression was observed in one of three osteosarcoma cell lines relative to nontransformed human foreskin fibroblasts. In addition, enhanced Blym expression was observed in a melanoma cell line and in 2 of 6 squamous carcinoma cell lines relative to nontransformed, low passage human epithelial cells. We found no evidence of gene amplification or rearrangements of Blym sequences in any of the cell lines we have examined. We further analyzed the state of methylation of the Blym gene in several of the tumor cell lines by Msp I/Hpa 11 restriction endonuclease digestion. All cell lines examined had similar Msp I digestion patterns. However, the different tumor cell lines had different Hpa 11 digestion patterns. Therefore, our results indicate that the Blym gene is differentially expressed and methylated in human tumor cell lines.*

INTRODUCTION

THE HUMAN Blym gene (HuBlym-1) was originally isolated from the genomic DNA of a Burkitt's lymphoma cell line by DNA mediated gene transfer into NIH 3T3 cells [1]. The transforming activity of human Blym has been localized to a 0.95 Kb Eco R1 fragment [1]. HuBlym-1 which contains the 0.95 Kb Eco R1 human Blym gene [1, 2], will transform human foreskin fibroblasts to anchorage-independent growth [3]. DNA sequence analysis has revealed that Blym shows significant sequence homology to the amino terminal region of the iron binding protein, transferrin [2]. The Blym structural gene is transcribed into a 0.6 Kb messenger RNA which encodes a protein of 58 amino acids with a mol. wt of 7000 daltons [2, 4]. In humans, Blym has been mapped, by chromosomal *in situ* hybridization, to the short arm of chromosome 1 (1p32) [5]. The mechanism of activation of the Blym gene is unknown; a recent report indicates that the nucleotide sequence of the transforming gene is identical to that of the nontransforming Blym allele [4].

Little is known about the expression of Blym in normal and human tumor cell lines. In the present study, we have investigated Blym expression and the genomic organization of the Blym gene in several non-lymphoid human tumor cell lines.

Accepted 19 September 1986.
*This work was supported by NIH Grants CA 22704 and ES 00002.
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MATERIALS AND METHODS

Cells

The cell lines examined in these studies included: AG1522, low passage human foreskin fibroblasts; HN-2, HN-4, SCC-9, SCC-29, SCC-35, SCC-61, squamous cell carcinoma cell lines of the head and neck [6]; MG-63, U20S, and SAOS, osteosarcoma cell lines [7, 8]; U1, melanoma cell lines. Cells were grown in Eagle's minimal essential medium supplemented with 15% heat inactivated fetal calf serum at 37° C in a humidified atmosphere containing 5% CO_2. Early passage human foreskin epithelial cells were grown on 3T3 fibroblast feeder layers [9] and were the generous gift of Dr. R. Rice, Dept. of Toxicology, Harvard University.

RNA isolation

RNA was isolated from log phase cells. For RNA dot blot analysis, the method of White and Bancroft [10] was utilized. Cells were washed twice with ice cold Earle's balanced salt solution (EBSS), harvested from the dishes and resuspended in 10 ml of EBSS. An aliquot of this mixture was counted in a Coulter counter to determine cell number. The cells were pelleted by low speed centrifugation. Cell pellets (containing approx. 10^7 cells) were resuspended in 45 μl of ice cold TE [10 mM Tris, (pH7), 1 mM EDTA] and lysed by the addition of two 5 μl aliquots of 5% Nonidet P-40 (NP-40) and mixing on ice. The nuclei were pelleted by centrifugation at 10,000 ***g*** × 10 min.

The supernatant was transferred to a sterile tube containing 30 μl of 20 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate) and 20 μl of 37% formaldehyde, incubated at 60°C × 15 min and stored at −70° C until use. For dot blot analysis, RNA samples were serially diluted with 15 × SSC in a 96 well microtiter plate, and an equal aliquot from each dilution was spotted onto nitrocellulose using a Schleicher and Schuell 96 well manifold apparatus. The nitrocellulose filters were allowed to air dry and then baked in a vacuum oven at 80° C for 2 hr.

For northern analysis, total cytoplasmic RNA was isolated as described [11]. Briefly, cells were washed twice with ice cold EBSS, scraped from the dishes and pelleted by low speed centrifugation. The cell pellets were resuspended in 5 ml lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.6), 0.5% NP-40, 100 units/ml RNAsin), and vortexed alternately on ice for 30 sec. The cell suspension was centrifuged at 5000 ***g*** for 20 min at 4° C. One-quarter vol. of 4x PK buffer [0.4M Tris (pH 7.5), 50 mM EDTA, 0.6 M NaCl, 4% sodium dodecyl sulfate (SDS)] [12] and proteinase K (final concentration 100 μg/ml) was added to the supernatant which was incubated for 30 min at 37° C. The RNA was extracted once with phenol, followed by two chloroform extractions and precipitated by the addition of 2 vol. of 95% ethanol. For northern analysis, 10 μg of cytoplasmic RNA was size fractionated on 1% agarose gels containing 2.2 M formaldehyde [13], and transferred to nitrocellulose in 20 × SSC [14]. After transfer, the filters were air dried and vacuum baked at 80° C for 2 hr.

Isolation of genomic DNA

Cells were washed twice with EBSS, scraped from the dishes, and pelleted by low speed centrifugation. The cell pellet was resuspended in 10 vol. of solution A [10 mM Tris (pH 8.0), 5 mM $MgCl_2$, 0.32 M sucrose, 1% Triton X-100] and centrifuged at 10,000 ***g*** × 10 min [15]. The pellet was resuspended in 10 vol. of solution A, centrifuged and the pellet was resuspended in 10 vol. of solution B [10 mM Tris (pH 8.0), 24 mM EDTA, 75 mM NaCl] [15]. Following the addition of SDS (final concentration 0.1%) and proteinase K (final concentration 100 μg/ml) the mixture was incubated at 37° C for 8–12 hr. The mixture was extracted once with phenol followed by three extractions with chloroform : isoamyl alcohol (24 : 1). After extraction, the DNA was precipitated by the addition of 2 vol. 95% ethanol. DNAs were digested with restriction enzymes in buffers recommended by the suppliers, and sized fractionated on 0.8% agarose gels. The DNA was transferred to nitrocellulose in 20 × SSC, the filters were air dried and baked at 80° C in a vacuum oven [14].

Hybridization of filters

Nitrocellulose filters were hybridized in 0.8 M NaCl, 0.1 M pipes, 100 μg/ml sonicated and denatured herring sperm DNA, 0.1% sarcosine, 2 × Denhardt's solution (1 × Denhardt's = 0.2% ficoll 400, 0.2% PVP 360, 0.2% BSA) containing 50% formamide and 10% dextran sulfate at 42° C [16] containing 10^6 dpm/ml of ^{32}P-labelled Blym probe (0.95 KB Eco Rl fragment from pHuBlym-1) [1] for 18–24 hr. Following hybridization, the filters were washed in 2 × SSC containing 0.1% sarcosine and 0.05% sodium pyrophosphate at 42° C for 30 min followed by a second series of washes in 0.2 × SSC containing 0.1% sarcosine and 0.05% sodium pyrophosphate at 60° C. The filters were air dried and autoradiographed with Kodak XAR 5 X-ray film and an intensifying screen at −70° C.

RESULTS

We examined 10 non-lymphoid human tumor cell lines for Blym expression by RNA dot blot and northern analysis. All of the cell lines examined expressed Blym. Those cells expressing the highest amounts of Blym message included SCC-35, SAOS and U1 lines (Figs. 1, 2). The Blym message migrates as a single band of about 0.5–0.6 Kb on agarose gels (Fig. 2). As a control, RNA from Raji Burkitt's lymphoma cells was also analyzed by northern analysis. These cells have been shown to express Blym [4]. The SAOS cell line produces a Blym transcript which comigrates with that obtained from Raji cells. SAOS and U1 cells express Blym at about the same level as the Raji cell line. Our sizing of the Blym messenger RNA is consistent with recently published data [4].

We analyzed the structure of the Blym gene in the genomic DNAs of several tumor cell lines on Southern blots. No evidence of amplification or rearrangement of Blym sequences was observed in any of these cells with the restriction enzymes Hind III (Fig. 3), Msp I (Fig. 4) or Eco R1 (data not shown). To examine the state of methylation of the Blym gene in these cells, genomic DNAs were analyzed by digestion with Hpa II, the isoschizomer of Msp I (Fig. 4). Hpa II and Msp I cut the recognition sequence CCGG [17, 18]. However, Hpa II digestion is inhibited if the internal cytosine residue in the recognition sequence is methylated (i.e. CC*GG), while Msp 1 digestion is not [18]. Msp 1 digestion produces a single band of about 4 Kb in all cell lines examined. In contrast, the Hpa II digestion patterns of genomic DNAs from the different tumor lines was variable. In AG1522, MG-63, and U20S cell lines at least

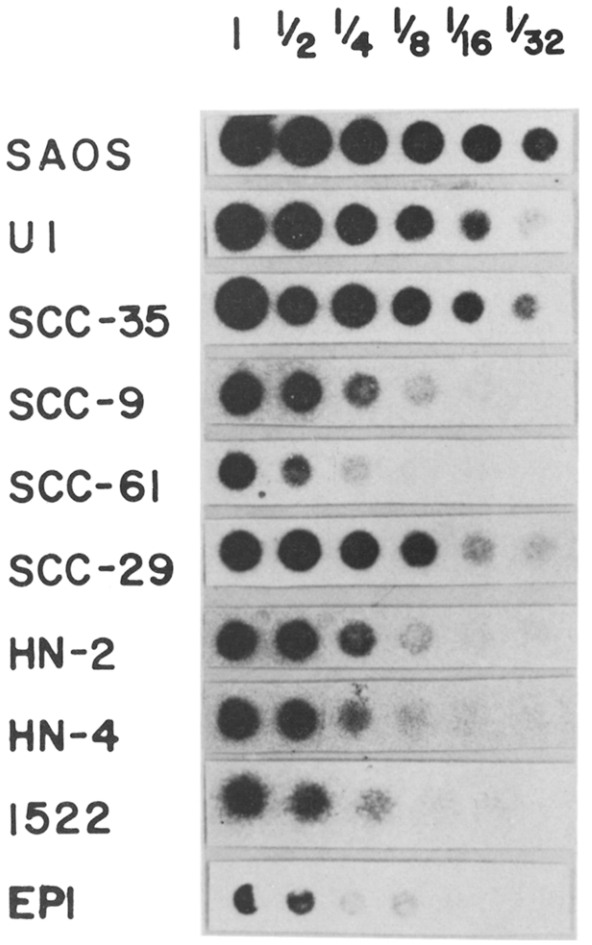

Fig. 1. RNA dot blots demonstrating Blym expression in several human tumor cell lines. HN, SCC: squamous cell carcinoma cell lines derived from head and neck tumors. EPI, epithelial cell RNA. Numbers along top: dilution of RNA samples spotted onto nitrocellulose. RNAs in this figure are normalized for cell number.

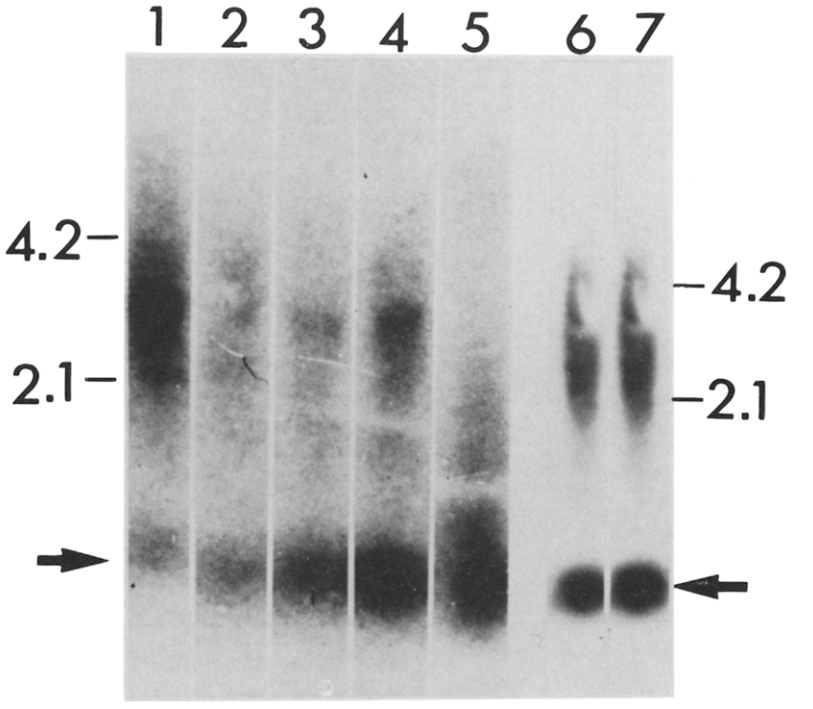

Fig. 2. Northern analysis of cytoplasmic RNAs from human tumor cell lines. Lane 1, AG1522 RNA; 2, MG-63 RNA; 3, U20S RNA; 4, SAOS RNA; 5, U1 RNA; 6, SAOS RNA; 7, Raji RNA. Ten micrograms of total cytoplasmic RNA was loaded in each lane. Numbers on right and left, mol. wt markers in Kb. Raji cell RNA was the generous gift of C. Pik and G. Cooper, Harvard University. The arrow designates the Blym transcript.

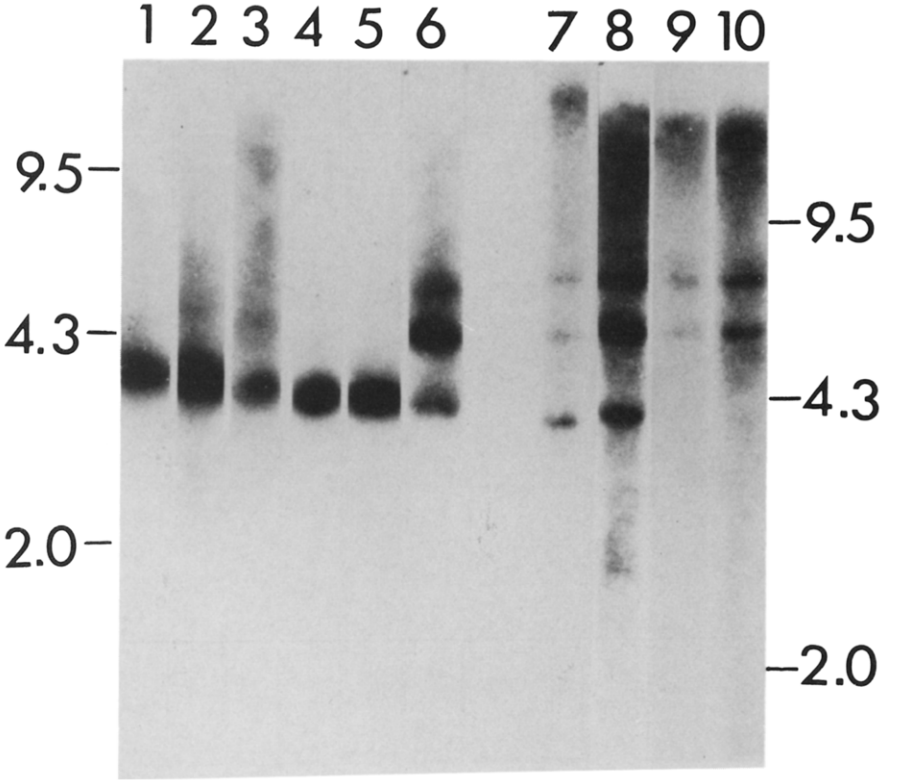

Fig. 4. Southern analysis of genomic DNAs from human tumor cell lines digested with Hpa II *and Msp* I. *Lanes* 1–5, *samples digested with MSP* 1; *Lanes* 6–10, *samples digested with Hpa* II. *Lanes* 1,6: *AG*1522 *cells*; *Lanes* 2,7: *MG*-63 *cells*; *Lanes* 3,8: *U*20*S cells*; *Lanes* 4,9: *SAOS cells*; *Lanes* 5, 10: *U*1 *cells*. *Numbers on right and left, mol. wt markers in Kb.*

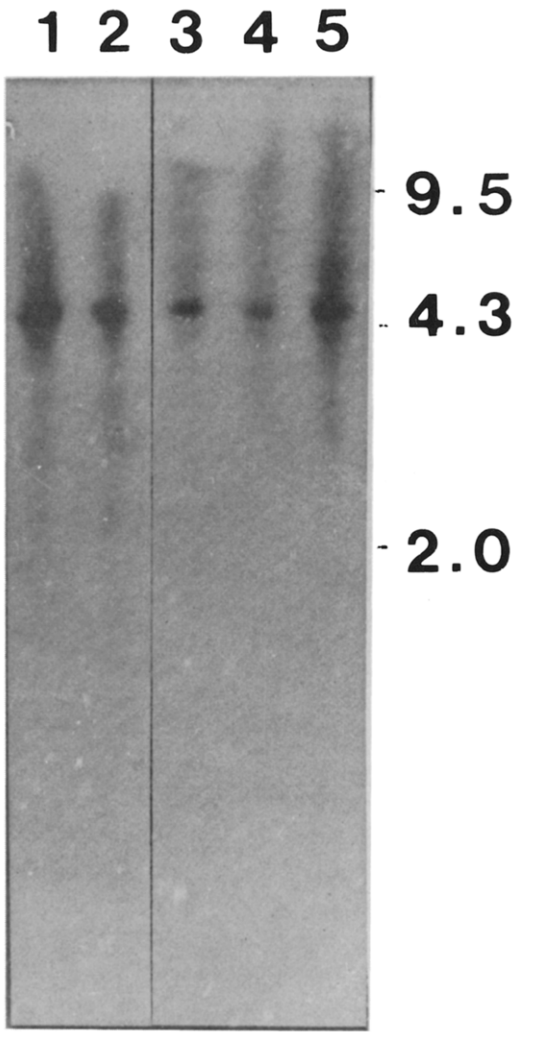

Fig. 3. Southern analysis of Genomic DNAs from human tumor cell lines digested with Hind III. *Lane* 1, *AG*1522 *cells*; *Lane* 2, *MG*-63 *cells*; *Lane* 3, *U*20*S cells*; *Lane* 4, *SAOS cells*; *Lane* 5, *U*1 *cells*. *Numbers on right mol. wt markers in Kb.*

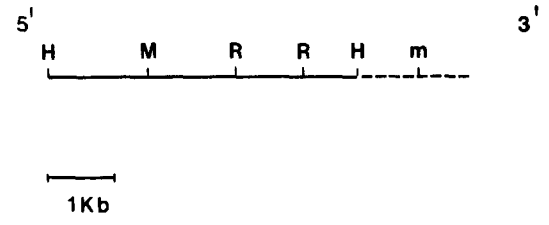

Fig. 5. Restriction map of the 4.6 Kb *Hind* III *fragment cloned in plasmid pH* 5 *which contains the structural human Blym gene. The solid line represents the* 4.6 Kb *fragment cloned in pH* 5, *in which we have mapped one Msp* I *site* (M), *while the dashed line represents non-mapped flanking sequences not present in clone pH* 5. *The Blym gene is delineated by the two Eco R*I *sites. A second Msp* I *site* (m) *can be placed* 3' *to the Blym gene based on our genomic blots (only one band is observed following digestion of genomic DNAs with Msp* I). *R, Eco R*I *sites*; H, *Hind* III *sites*; *M*,m, *Msp* I *sites. Msp* I *recognition sites (i.e. CCGG) are potential sites of DNA methylation.*

three bands were observed on Hpa II blots while in the SAOS and U1 cell lines two bands were observed.

Since we observed different hybridization patterns on the Hpa II blots (Fig. 4), we determined the location of the MSP I cleavage site(s) in the vicinity of the structural Blym gene. We constructed a restriction map of clone pH 5 which consists of a 4.6 Kb Hind III fragment containing the structural human Blym gene (0.95 Kb Eco R1 fragment), and cloned in plasmid pBR 322 (this plasmid was the generous gift of A. Diamond and G. Cooper, Harvard Unversity). One Msp I site was mapped about 1 Kb 5' to the structural Blym gene (Fig. 5). Since only one band is observed in Msp I digests of genomic DNAs, a second Msp II can be placed 4 Kb from the first site.

DISCUSSION

In this report we have demonstrated that Blym is expressed in human tumor cell lines derived from both carcinomas and sarcomas. The levels of Blym messenger RNA produced by the different tumor lines varied widely, but several cell lines expressed higher levels of Blym compared with nontransformed human fibroblast and epithelial cells. Of all the nonlymphoid tumor lines we examined the SAOS, U1, and SCC 35 cell lines had the highest levels of Blym message.

We found no evidence of polymorphisms, amplification or rearrangement of the Blym structural gene in any of the cell lines we examined. However, our data do demonstrate that different tumor cell lines have variable DNA methylation patterns in the vicinity of the Blym gene based on the Hpa II digestion patterns. Since Hpa II cutting is inhibited if the internal cytosine in the four base recognition sequence is methylated (i.e. CC*GG) [18], the loss of the smallest (4 Kb) Hpa II fragment in the SAOS and U1 cell lines suggests that at least one of the two Msp I sites which flank the structural Blym gene is methylated in all of the Blym alleles in these cells. The absence of the 4 Kb band in the Hpa II digests of genomic DNA from SAOS and U1 cells cannot be accounted for by a polymorphism because the same Msp 1 digestion pattern is observed with all of the cell lines.

Studies in several systems have demonstrated that DNA methylation can play an important role in gene expression [19, 20]. 5-Azacytidine, an inhibitor of cytosine methylation, has been shown to induce differentiation [19, 21] and oncogenically transform C3H/10T½ mouse embryo fibroblast cells [19, 22]. This compound will also activate hypoxanthine phosphoribosyltransferase (HPRT) and other genes on an inactive human X chromosome [20]. The active HPRT gene is hypomethylated in the 5' region relative to the inactive allele [23]. A different result has been obtained in studies examining the expression of type 1 collagen genes [24]. The DNA around the start site of transcription of the collagen gene is unmethylated in expressing or nonexpressing cells [24]. However, DNA in the central and 5' regions of the gene is methylated to about the same extent in nonproducing and collagen producing cells [24]. Therefore, these results show that collagen genes can be methylated and still be actively transcribed.

An interesting finding of our studies was that the U1 and SAOS, cell lines which both express somewhat higher levels of Blym compared with nontransformed AG1522 cells, showed increased methylation as evidenced by the loss of the 4 Kb band present in the Hpa II digests of the other cell lines examined. However, the cell lines we examined which contained the 4 Kb band in the Hpa II digests also expressed Blym. At the present time we do not know the relationship between Blym expression and DNA methylation.

If the Blym gene played a specific role in the development of a specific tumor type, then one might predict that consistent patterns of Blym expression or methylation would be observed in cell lines derived from a specific tumor type. However, we did not observe consistent patterns of Blym expression or methylation within each group of tumor cell lines derived from the same tumor type. Of the six squamous cell carcinoma cell lines we examined, SCC-35 and SCC-29 expressed higher levels of Blym compared with SCC-9, SCC-61, HN-2 and HN-4. Similar results were obtained with the three osteosarcoma cell lines we examined. The SAOS cell line expressed higher levels of Blym and presented a different Hpa II digestion pattern compared with the MG-63 or U20S cell lines. Therefore, these results suggest that Blym expression and methylation patterns are not tumor type specific.

The transforming activity of human Blym has been reported to be in the 0.95 Kb Eco R1 fragment which contains the structural gene [1, 2]. The mechanism of activation of this gene is unknown; no difference in the sequence of the transforming and nontransforming Blym genes has been observed [4]. However, our mapping of the Msp 1 sites suggests that DNA methylation of these sites is not directly involved in Blym activation, as these restriction sites are about 1 Kb upstream and downstream from the Blym gene and presumably would not necessarily cotransfect with the 0.95 Kb Eco R1 fragment, containing the structural Blym gene.

The role Blym plays in normal cellular physiology as well as malignant transformation is unknown. Our results demonstrate that Blym is expressed at higher levels in some human tumor cell lines relative to non-transformed, control cells. Further, our results with Hpa II/Msp 1 restriction endonuclease digestion suggest that DNA methylation patterns vary between different tumor cell lines.

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