was increased in peripheral blood during an increase in leukemia blast numbers (Fig. 3). CKB mRNA expression was higher in patient 1 and lower in patient 2, similar to the CK-BB activity. In contrast, none of the other 26 patients with hematologic malignancies showed CK-BB activity or expression of CKB mRNA (data not shown). In hematologic malignancies, it is likely that expression of CKB is a relatively rare occurrence.

We performed methylation analysis and compared promoter methylation and CKB expression. Representative results of BiPS and bisulfite sequencing are shown in Figures 4 and 5. Methylation pattern was decided primarily by BiPS and confirmed by DNA sequencing. Figure 5 shows that MKN7 is fully unmethylated and that patient 1 is fully methylated. In the present 2 patients, the analyzed region was mostly unmethylated, similar to the level of methylation observed in the other 26 patients with various hematologic disorders. The 2 patients showed a small amount of non-unmethylated band by BiPS, however, we could not affirm that the difference of methylation pattern resulted in regulation of CKB expression.

Four of 6 hematologic cancer cell lines, 7 of 11 solid cancer cell lines, and 1 of 6 brain tumor cell lines showed the methylated pattern. CKB mRNA expression level was qualitatively categorized into 5 grades by the band intensity electrophoresed on agarose gels. The hematologic cancer and braintumor cell lines with a relatively unmethylated CKB promoter showed higher CKB mRNA expression than that of cells with a methylated promoter. In solid tumor cell lines also, CKB mRNA was expressed at higher levels in cells with an unmethylated CKB promoter than in cells with a methylated promoter, but that this difference in expression was not significant (Table 3).

4. Discussion

Chen et al. [5] identified several molecular markers of MRD in B-lineage acute lymphoblastic leukemia (ALL). Seven genes including CKB were highly expressed in B-lineage ALL cells. Previously, CK-BB isoenzyme activity was occasionally observed in the sera

of patients with hematologic malignancies [3,4]. Our present study shows that expression of CKB mRNA and CK-B sometimes occurred in blastic transformation of the hematopoietic system, and these phenomena are possibly linked to a poor prognosis.

The 5' end of the CKB gene has an extremely high C+G content with a large number of CpG dinucleotides. Such CpG clusters are typically found in the promoters of housekeeping genes and are thought to be involved in the regulation of gene expression [8]. In neoplastic cells, some CpG islands become aberrantly hypermethylated or hypomethylated, which leads to transcriptional silencing or upregulation [9]. From these findings, we expected that abnormal methylation would result in abnormal expression of the CKB gene *in vivo*. We observed a relation between *CKB* mRNA expression and methylation of the CKB promoter; however, we could not confirm a direct effect of promoter methylation on CKB expression in hematologic malignancies. Expression of CKB is reported to be induced by estrogen responsive element, cAMP responsive element, myocyte enhancer factor-2, Sp1, and other enhancer sites located in the promoter region of CKB [10]. Repression of CKB by p53 mutation or loss has also been proposed [11]. Further studies to clarify the control of CKB expression are needed.

Acknowledgments

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Table 1. Patients with hematologic malignancies for this study

Disease	Patients n	CK activity (IU/l)	Leukemia Blast or lymphoma cell (%)
ALL	6	13 – 161	7 - 95
AML	8	36 - 1555	0 - 100
APL	2	39 - 83	0 - 69
CLL	1	143	0
CML	2	47 - 106	4 - 32
MDS	3	24 - 184	4 - 28
ML	4	43 - 70	0.5 – 42.5

AML, acute myeloid leukemia; CML, chronic myeloid leukemia; APL, acute promyelocytic leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphoblastic leukemia; MDS, myelodysplastic syndrome; ML, malignant lymphoma

Table 2. Primer sequences for RT-PCR and methylation analysis

Primer name	Primer sequence (5' to 3')
RT-PCR	
CKBmR-F2	GCAGCTCATCGACGACCACTTC
CKBmR-R1	GGGCACTGCAGGCAATAAGTTA
β-actin-F1	ACACTGTGCCCATCTACGAGG
β-actin-R1	AGGGGCCGGACTCGTCATACT
BiPS	
CKB-S-F1	GAATGAATGGGTTATAAATA
CKB-S-R2	ACCAAAATCAACGAAATCC

Table 3. Expression of *CKB* mRNA as determined by RT-PCR and *CKB* promoter methylation in cancer cell lines

Cell line group	Cell line	Origin of cell line	RT-PCR#	Methylation pattern
Hematologic cancer cell lines	HL-60	APL	+	Methylated
	NB4	APL	++	Unmethylated
	K562	CML	+++	Unmethylated
	Raji	Burkitt lymphoma	+	Methylated
	Daudi	Burkitt lymphoma	-	Methylated
	U937	Histiocytic lymphoma	+	Methylated
Solid tumor cell lines	MKN1	Gastric cancer	+++	Unmethylated
	MKN7	Gastric cancer	+/-	Methylated
	MKN28	Gastric cancer	+	Methylated/Unmethylated*
	MKN45	Gastric cancer	+++	Unmethylated
	MKN74	Gastric cancer	++	Methylated
	KATO III	Gastric cancer	++	Methylated
	NEDATE	Gastric cancer	+	Methylated
	PSN1	Pancreatic cancer	+	Methylated
	SW1116	Colorectal cancer	+++	Methylated
	C-1	Colorectal cancer	+	Unmethylated
	Lu65	Lung cancer	+++	Unmethylated
Brain tumor cell lines	Daoy	Medulloblastoma	+++	Unmethylated
	ONS-76	Medulloblastoma	+++	Unmethylated
	UW228	Medulloblastoma	++	Unmethylated
	TE671	Medulloblastoma	+	Unmethylated
	D283	Medulloblastoma	+/-	Methylated
	PFSK	Neuroepithelial tumor	+++	Unmethylated

CKB mRNA expression level was qualitatively categorized into 5 grades by the band intensity of RT-PCR products.

* In this cell a heterogeneous BiPS pattern was observed and indicated that there were both methylated and unmethylated DNA for the CKB promoter in the cell.

Legend for Figures

Fig. 1. Diagram of *CKB* promoter region sequences based on the published genomic sequences. CpG sites are indicated by vertical bars. The PCR primers designed for BiPS analysis are indicated by solid boxes. The PCR product is 299 bp long with 48 CpG sites and contains non-coding exon 1.

Fig. 2A. Relation between CK-BB activity and proportion of leukemia blasts in peripheral blood from patient 1 (A) and patient 2 (B). CK-BB activity was correlated with leukemia blast (%) (linear regression line: y=5.57x+250; correlation coefficient, 0.869 for patient 1, and Analyses of CK-BB activity, and CKB mRNA expression, and methylation of the CKB promoter.

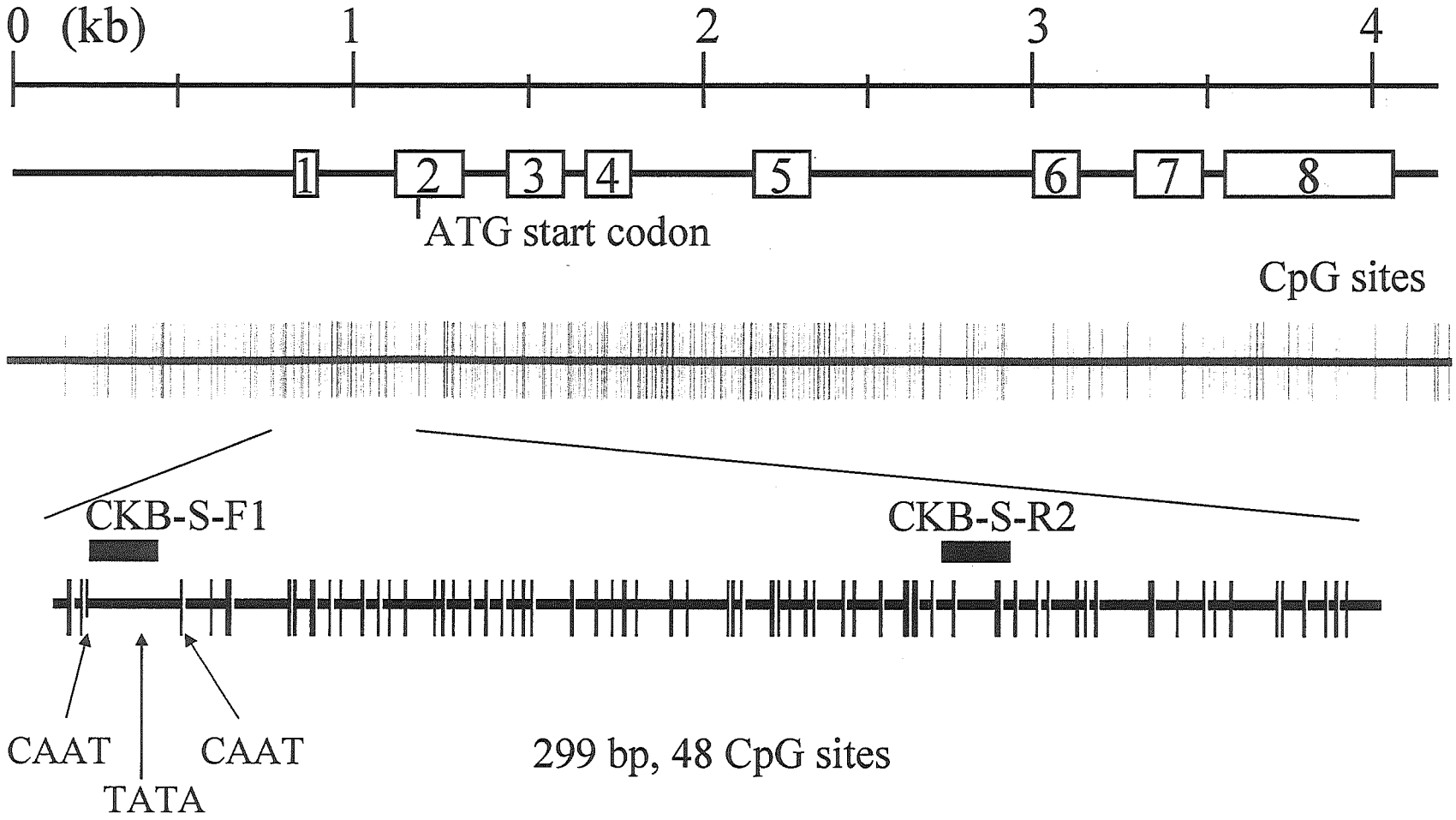
Fig. 3. RT-PCR analysis of CKB. Lanes 1 and 12, negative control (water); lanes 2, 5, and 6, Patient 1; lanes 3 and 4, other leukemic patients (negative result); lanes 7-11, Patient 2 (only a trace band was visible in lanes 7 and 8). Target length of the RT-PCR product is 651 base pairs (bp). Each lane of patients 1 and 2 show the results of RNA samples obtained at different time. CK-BB activities of the samples are 757, 824, 652 IU/l in lanes 2, 5, and 6, and 41, 32 13, 1, and 4 IU/l in lanes 7-11, respectively.

Fig. 4. BiPS analysis of the *CKB* promoter region. Lane 1, MKN7 cells (methylated); lanes 2 and 3, whole blood from Patient 1 (both unmethylated); lane 4, prepared leukocytes from Patient 1 (unmethylated + methylated); lane 5, prepared leukocytes from patient with ALL (unmethylated); lane 6, whole blood from Patient 2 (unmethylated); lane 7, prepared leukocytes from patient with AML (unmethylated); lane 8, NEDATE (methylated); lane 9, MKN1 cells (unmethylated); and lane 10, unmethylated control. Each lanes of patients 1 and 2 show the

results of RNA samples obtained at different time. Parenthesis indicate our decision for methylation pattern.

Fig. 5. Partial sequence of the *CKB* promoter after bisulfite treatment (MKN7 cells and patient 1). CpG sites are underlined. All of the CpG sites shown in this figure are methylated in MKN7 cell line and unmethylated in patient 1.

Figure(s)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ATG start codon
CpG sites
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CKB-S-R2
CAAT
TATA
CAAT
299 bp, 48 CpG sites

Figure(s)
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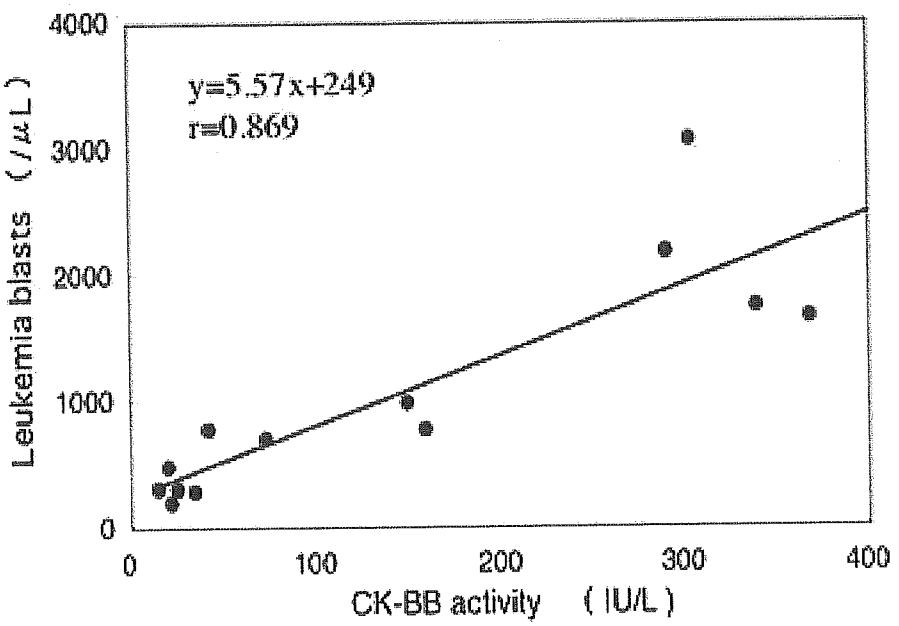

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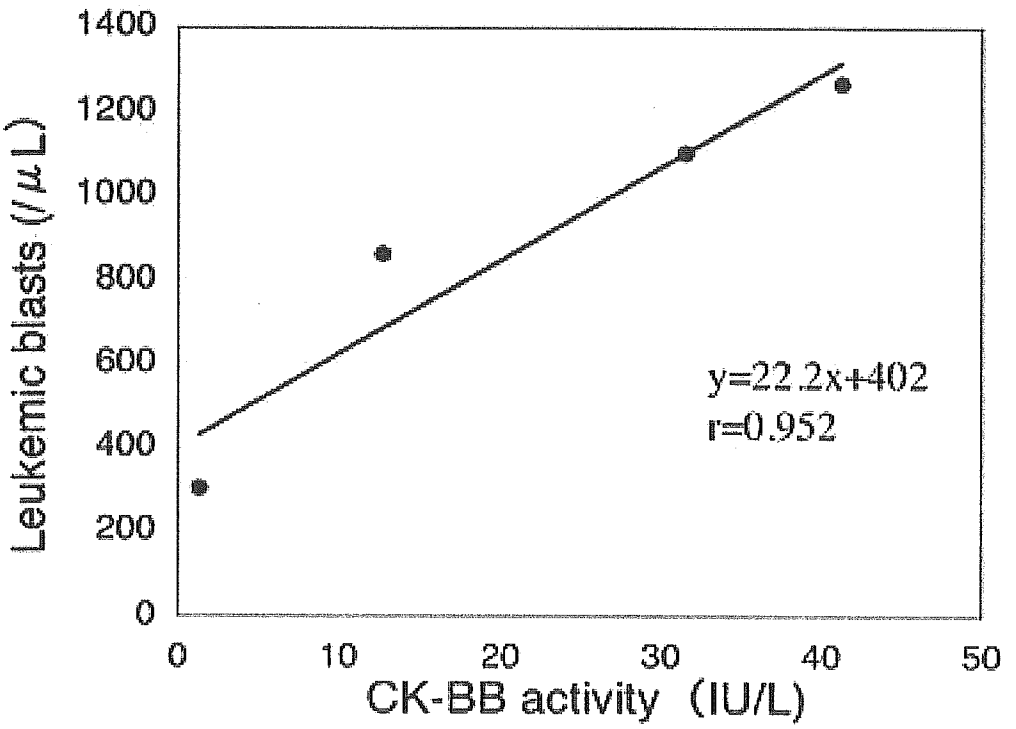

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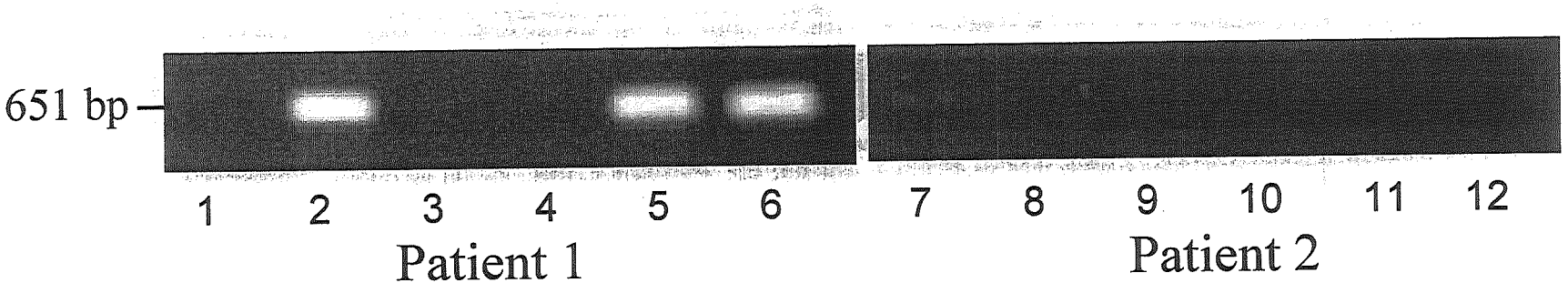

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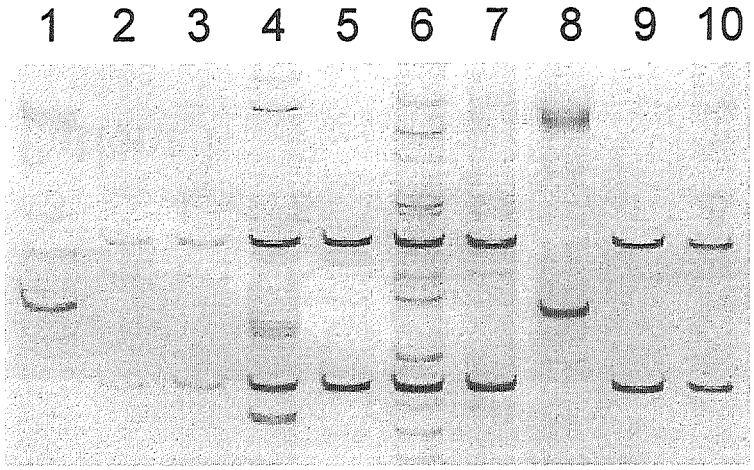
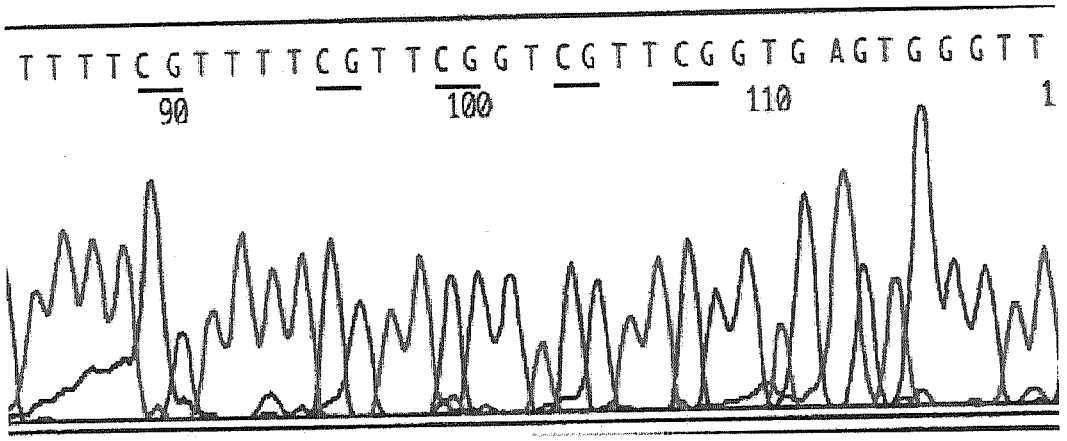
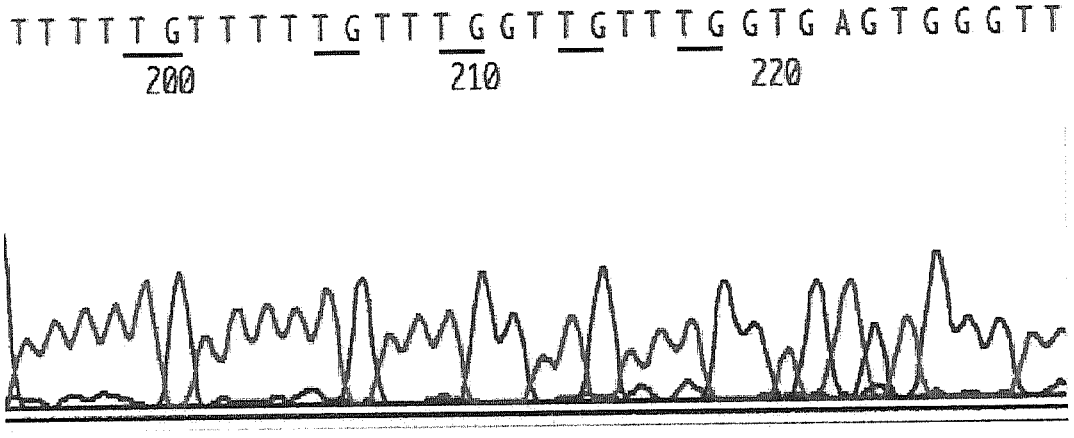


Figure(s)

MKN7



Patient 1



Promoter Hypermethylation in Cancer Silences *LDHB*, Eliminating Lactate Dehydrogenase Isoenzymes 1-4,

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Lactate dehydrogenase (LD; EC 1.1.1.27) isoenzymes are formed by the random combination of two different subunits encoded by two structurally distinct genes, *LDHA* and *LDHB* (*1*). Expression of mammalian *LDHA* and *LDHB* is regulated during development and is tissue specific (*2, 3*); therefore, alterations in the serum LD isoenzyme pattern serve as indicators of pathologic involvement and cancer development (*3*). In cancer patients, LD isoenzymes originate primarily from tumor tissues and partly from healthy tissues damaged by tumor expansion and invasion. Different phenotypes may originate from expression regulation by other regulatory genes and by the alteration of *LDHA* or *LDHB* caused by mutation; chromosomal deletion; duplication, or increase of copy number; and promoter methylation. The increase in LD1 correlates with the total copy number of the short arm of chromosome 12 in tumor cells (*4*). Recently, we found a high proportion of LD1 in a patient with retinoblastoma. The unique LD isoenzyme pattern was attributable to transcriptional silencing by promoter hypermethylation of *LDHA* (*5*).

In mammals, DNA methylation usually occurs at CpG dinucleotides, which are cytosines located 5′ of guanines. Methylation is known to play a role in regulating gene expression during cell development, X chromosome inactivation, genomic imprinting, and carcinogenesis (*6, 7*). In neoplastic cells, some CpG islands in the promoter region that are usually unmethylated become aberrantly methylated, and this leads to transcriptional silencing. Therefore, an epigenetic event is thought to be one mechanism for the inactivation of tumor suppressor genes (*8*).

Human *LDHB* has a CpG-rich region in its promoter that is similar to that of human *LDHA* and *LDHC* (*9*). We found that five cancer cell lines had only *LDHA* mRNA (*10*). Most gastrointestinal cancer patients had electrophoretically slow-moving isoenzymes and the LD-A subunit in their sera (*3*). We predicted that this unique pattern was derived partly from transcriptional silencing attributable to the aberrant promoter hypermethylation of *LDHB*. We have focused in this work on the aberrant methylation of the promoter region of *LDHB* in some cancer cell lines and gastrointestinal cancer tissues, with the intention of identifying the relationship between the LD isoenzyme pattern and aberrant promoter methylation.

The present study included 12 cancer cell lines, 20 patients with gastric cancer, and 25 patients with colorectal cancer. The cancer cell lines were the same as those used in our previous studies (*10, 11*). For the patients, both malignant and nonmalignant tissues were examined. They were obtained from the National Cancer Center Hospital and Hamamatsu University School of Medicine. Each patient consented to the experimental use of specimens and examination of the specimens for pathology. DNA was extracted from the cancer cell lines and the resected tissues by a method described previously (*10, 11*).

For methylation analysis, bisulfite-PCR single-strand DNA conformation polymorphism (BiPS) analyses were done (*12*). Briefly, bisulfite treatment was carried out and PCR-single-strand conformation polymorphism analysis was performed with 10% nondenaturing polyacrylamide gels and silver-staining detection (Daiichi Pure Chemicals). The primer sequences for amplification of *LDHB* were 5′-AGGGAGTGTGTATATTTGAGTT-3′ (sense) and 5′-TCAAACTTACCTATAAACCAAA-3′ (antisense). The promoter sequence of *LDHB* was taken from GenBank accession no. X13794 and is shown in Fig. 1A. The region selected for amplification contained exon 0 and has been related to the promoter activity (*13*). The PCR product was expected to contain 282 bp and 14 CpG sites. The PCR products were sequenced directly by the dideoxy-sequencing procedure with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and a PRISM 310 Genetic Analyzer (PE Applied Biosystems) according to the manufacturer's instructions (*12*). The amplified products were also cloned into a pDRIVE cloning vector (Qiagen) for sequencing.

Promoter methylation of *LDHB* was detected in four gastric cancer cell lines and one pancreatic cancer cell line (Table 1; Fig. 1B). The five cancer cell lines, the *LDHB* promoters of which were methylated, had only the LD5 isoenzyme at the enzyme activity level and only the *LDHA* cDNA signal at the mRNA level (*10*). The methylation status of *LDHB* in the cell lines was in complete accord with the mRNA and activity expression results. One pancreatic cancer cell line, PSN1, yielded a partially methylated band by BiPS and sequence analysis (Fig. 1, B and D). This complicated BiPS pattern was reproduced by a repetitive BiPS analysis. The four gastric cancer cell lines yielded only a completely methylated BiPS band, and the other cancer cell lines yielded only an unmethylated BiPS band.

Promoter methylation of *LDHB* was observed in 3 of 20 gastric cancer tissues and in none of the corresponding healthy mucosa (Fig. 1C). None of 25 colorectal cancer tissues and corresponding healthy mucosa had promoter methylation in *LDHB*. Therefore, *LDHB* promoter was methylated in 5 of 12 cancer cell lines and in 3 of 45 cancers ($P = 0.007$, Fisher exact test).

The methylation pattern found by BiPS was heterogeneous in the three gastric cancer tissues. Sequence analysis of the PCR products after cloning revealed differences among the three gastric cancer tissues (Fig. 1D). GC-1, which had slight methylation in *LDHB*, was an early gastric cancer (3.5 × 3 cm; stage Ia) with severe intestinal

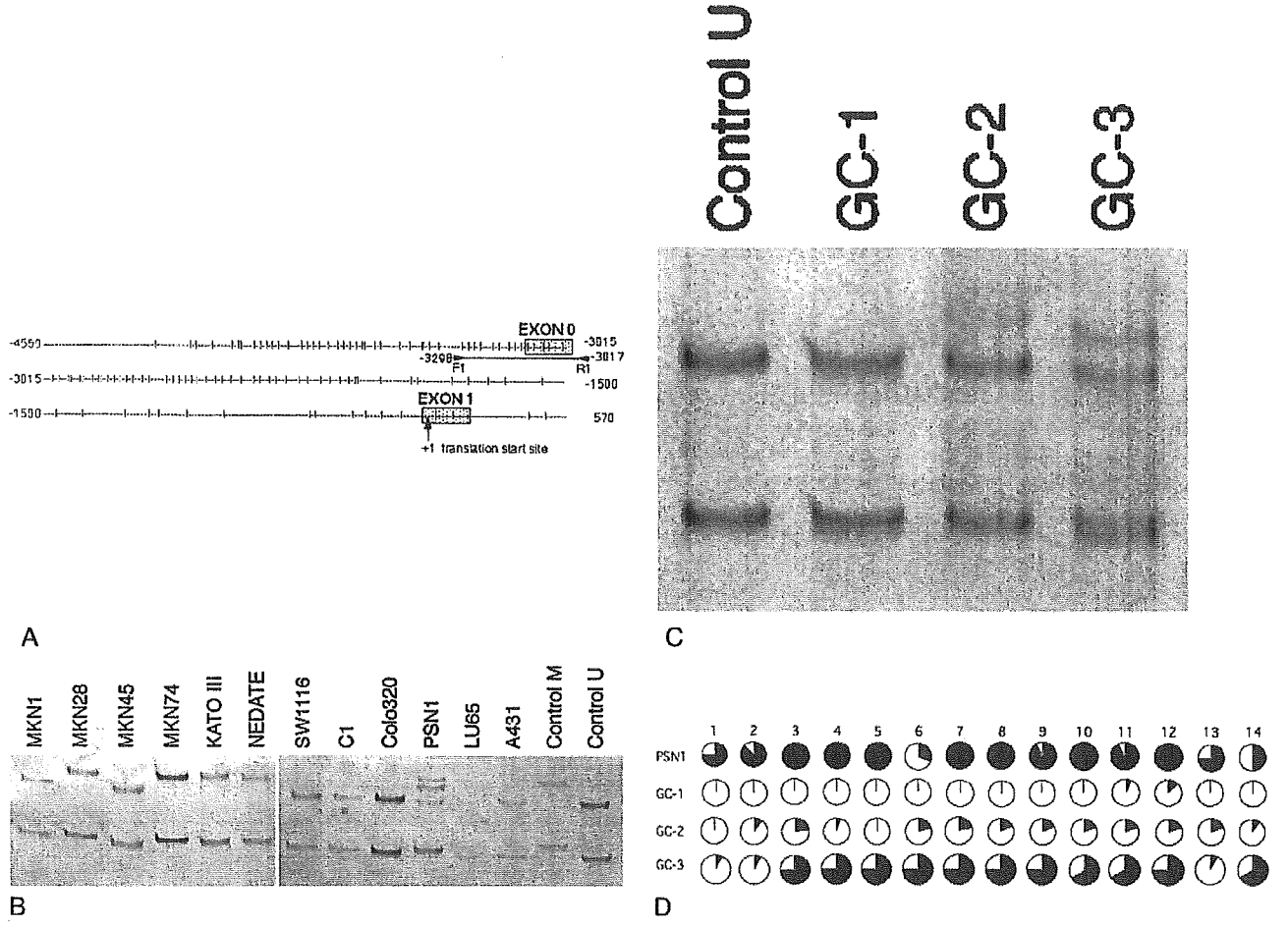

Fig. 1. Methylation analysis.

(*A*), diagram of the *LDHB* promoter region sequence obtained from published genomic sequences [GenBank accession no. X13794 and Ref. *(18)*]. CpG sites are indicated by *vertical bars*, and the 5′ noncoding exon 0 and protein coding exon 1 are indicated by *shaded boxes*. The PCR primers designed for the bisulfite-PCR methods are indicated by the *paired arrowheads* with their positions relative to the adenine residue at the translation start codon. (*B and C*), results of bisulfite treatment and PCR-single-strand conformation polymorphism analysis of the *LDHB* promoter in 12 cancer cell lines (*B*) and 3 gastric cancer tissues (*C*) that had methylated DNA. *U*, unmethylated; *M*, methylated. (*D*), methylation pattern for the PSN1 cancer cell line and three gastric cancer tissues. *Each column* represents a tumor, and *each row* represents CpG sites. The proportion of methylated DNA is indicated in *black*.

metaplasia located at the proximal portion. GC-2, which showed mild methylation, was an invasive cancer (stage IIIa) with histologic heterogeneity. GC-3, which had heavy methylation, was an invasive cancer (stage II) with histologic heterogeneity.

LDHB promoter was methylated in 5 of 12 cancer cell lines, but in only 3 (7%) of 45 clinical cancer tissues. Therefore, methylation of the promoter is a relatively uncommon mechanism for the frequent increase of cathodal LD isoenzymes in gastric and colorectal cancer patients. The high proportion of cell lines with methylation of the promoter might be attributable to our selection of a small series of cell lines, but the higher proportion in cell lines than in cancer samples from patients might also reflect the biological differences found in the recent report *(14)*.

The presence of partially methylated DNA in the PSN1 cancer cell line is similar to that of *hMLH1* in the colorectal cancer cell line C-1 *(12)*. Partial methylation may be caused by the heterogeneous methylation status in the cultured PSN1 cells. The reason *LDHB* mRNA is not expressed in PSN1 could be that the unmethylated allele in the *LDHB* promoter has a mutation, a large deletion in a downstream coding region, or a methylation in other regions not examined in this study.

Interestingly, the present study showed that the methylation of the *LDHB* promoter was a characteristic for the cancers and not a germ-line abnormality of the patients. It means that promoter methylation of *LDHB* is an epigenetic abnormality but not a genomic alteration.

The three gastric cancer patients with promoter methylation of *LDHB* did not share any common specific similarities in clinical and pathologic findings except for widely expanded cancer. This issue should be further investigated in the future. The heterogeneous BiPS pattern in the three gastric cancer tissues might be attributable to clonal differentiation with different methylation patterns and histologic heterogeneity. This issue, how-

Table 1. Association of LD isoenzyme activity, mRNA expression, and methylation status.

		mRNA[a]		Enzyme activity					Methylation	
Cell line	Origin	*LDHA* (%)	*LDHB* (%)	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	*LDHA*	*LDHB*
MKN1	GC[b]	58.9	41.1	2.5	18.1	40.7	31.8	6.9	–	–
MKN28	GC	100.0	0	0.0	0.0	0.0	0.0	100.0	–	+
MKN45	GC	34.5	65.5	23.3	42.5	31.4	2.8	0.0	–	–
MKN74	GC	100.0	0	0.0	0.0	0.0	0.0	100.0	–	+
KATO III	GC	100.0	0	0.0	0.0	0.0	0.0	100.0	–	+
NEDATE	GC	100.0	0	0.0	0.0	0.0	0.0	100.0	–	+
SW1116	CRC	40.0	60.0	15.8	39.8	36.6	7.9	0.0	–	–
C-1	CRC	34.8	65.2	13.6	40.0	40.8	5.6	0.0	–	–
Colo320	CRC	ND	ND	ND	ND	ND	ND	ND	–	–
PSN1	PC	100.0	0	0.0	0.0	0.0	0.0	100.0	–	+
LU65	LC	ND	ND	ND	ND	ND	ND	ND	–	–
A431	EDC	ND	ND	ND	ND	ND	ND	ND	–	–

[a] mRNA *LDHA* (%) and *LDHB* (%) were obtained from our previous report *(6)*.

[b] GC, gastric cancer; CRC, colorectal cancer; ND, not determined; PC, pancreatic cancer; LC, lung cancer; EDC, epidermoid cancer.

ever, should also be elucidated by a large-scale clinicopathologic study.

Patients with many malignancies combined with increased serum LD activity have a poor prognosis *(15)*. Therefore, additional studies of the regulation of expression of LD and the release of LD from the cancers in patients are warranted.

LDHA expression can be induced by estrogen *(16)*, cyclic AMP *(17)*, hypoxia *(18)*, and c-*Myc* *(19)*. Induction is caused presumably by the actions of these agents on the *LDHA* promoter *(20)*. Accordingly, serum LD3, LD4, and LD5 are frequently increased in patients with malignant diseases and reflect increased expression of *LDHA* by neoplastic cells. We found that the other important reason for the increase in the amounts of electrophoretically slow-moving LD isoenzymes in cancer patients is transcriptional silencing of *LDHB* expression because of aberrant methylation in the promoter region of *LDHB*. The frequency of *LDHB* methylation is not high, but it should be noted that enzyme abnormalities in tumors occasionally originate from aberrant methylation.

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Pilot study of arbitrarily primed PCR-single stranded DNA conformation polymorphism analysis for screening genetic polymorphisms related to specific phenotypes

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Abstract

Background: To investigate relationships between phenotypes and genotypes is not simple. We propose a phenotype-to-genotype screening strategy and pooled DNA system. As a pilot study of this strategy, we used arbitrarily primed polymerase chain reaction (AP-PCR) in combination with single-stranded DNA conformation polymorphism (SSCP) to screen for genetic polymorphisms associated with longevity.
Methods: Study subjects were separated into 3 age groups, individuals aged >100 years, 90–99 years and 60–69 years. Genomic DNAs were prepared from each individual, pooled to represent the 5 study groups, and then the pooled genomic DNAs were subjected to AP-PCR-SSCP analysis.
Results: We found 1 SNP more frequently in senior citizens with longevity. The genotype frequency of the 82133G>A polymorphism of human chromosome 3 clone RP11-61K12 (AC011199) differed significantly (P=0.0189, Fisher's exact test) between older subjects (>90 years) and younger subjects (<70 years). It is noteworthy that the strategy we describe herein was useful for identifying an SNP that showed statistically significant differences in its distribution across the subject groups.
Conclusions: The pooled DNA strategy and quantitative genotype discrimination can also be applied to screening for the relationship between phenotype and genotype more effectively.

Keywords: Arbitrarily primed PCR; Genetic polymorphism; Phenotype; Single stranded DNA conformation polymorphism

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1. Introduction

Associations between phenotypes and single nucleotide polymorphisms (SNPs) or other polymorphisms, including insertions and deletions, have been shown by exhaustive screening of polymorphisms by sequencing and by etiological studies of genotype–phenotype correlations. This genotype-to-phenotype approach is useful; however, exhaustive screening is expensive and labor intensive. Relationships between phenotypes and genotypes have also been investigated by use of candidate genes for the phenotype. However, much effort cannot always lead to a good result [1]. Here we propose the reverse process, a phenotype-to-genotype screening strategy and pooled DNA system. In essence, we screened for common genotypes in pooled DNAs of individuals with a common phenotype. As a pilot study of this strategy, we used arbitrarily primed polymerase chain reaction (AP-PCR) [2] in combination with single-stranded DNA conformation polymorphism (SSCP). This technique takes advantage of the random nature of AP-PCR and the quantitative and resolution abilities of SSCP [3,4]. We used this method to screen for genetic polymorphisms associated with longevity.

2. Materials and methods

2.1. Subjects

Study subjects were separated into 3 groups, individuals aged >100 years (n=9), 90–99 years (3 sub-groups, n=8 each group), and 60–69 years (n=9). As a control group, we included 27 volunteers <30 years recruited from among medical students.

2.2. AP-PCR-SSCP

Genomic DNAs were prepared from each individual and then pooled to represent the 3 age groups described above. The pooled genomic DNAs were subjected to AP-PCR-SSCP analysis. Sequences of the primers used are shown in Table 1. Amplification conditions for AP-PCR were 94 °C for 3 min followed by 5 cycles of 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min, 35 cycles at 94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1 min, and a final cycle of 72 °C for 7 min. The amplified products were analyzed by SSCP with 6–10% polyacrylamide gel electrophoresis and silver staining detection (Daiichi Pure Chemicals, Tokyo, Japan) [4]. SSCP bands with different visual densities or mobilities were excised from the gel and cloned into pGEM-T (Qiagen, Tokyo, Japan) for sequencing (PE Applied Biosystems, Tokyo, Japan). Nucleotide sequences were searched with BLAST [5]. Gene-specific primers were synthesized and used for genotyping of individual genomic DNAs in 3 age groups and a control group of volunteers by PCR-SSCP and/or DNA sequencing.

Table 1
Primer sequences for the present study

Primer name	Primer sequence (5′ to 3′)
LDAcDF1	ACCGCCCGACGTGCATTCCC
LDAcDF2	CGCCCGACGTGCATTCCCGA
LDHA-m3	GTAATTATCATGGCTGGGACAT
Ae6F	TGAGGTGATCAAACTCAAAGGC
Ae6R	CTTAATCATGGTGGAAACTGGG
BAT26F	TGACTACTTTTGACTTCAGCC
BAT26R	AACCATTCAACATTTTTAACCC
MS3(A)F2	CCAGCTATCTTCTGTGCATC
UF1KAB1	CGAATCGCATGGCCTTG
EU1KAB1	TTCTCAGGCTCCCTCTCC
p16RNA1	CCCGCTTTCGTAGTTTTCAT
p16RNA2	TTATTTGAGCTTTGGTTCTG
D16S521L	GGAGCGAGACTCCGTCTAAA
D9S287L	GAGGATGCTCCTCACGC
NCC-COMM	AGGAATTCTTTTCTCTTNCAG

2.3. Statistical analysis

Pearson's χ^2 test and Fisher's exact test were used to compare allele and genotype frequencies between study groups. To analyze allele frequencies between the four age categories, the Kruskal–Wallis non-parametric test was used. Statistical significance was accepted at $P<0.05$.

3. Results and discussion

From a polyacrylamide gel of AP-PCR-SSCP (Fig. 1), we found 1 suspected band with different densities. By nucleotide sequence, it was originated from the 82133G>A polymorphism of human