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Abstract: Background: We encountered two patients with elevated levels of the creatine kinase (CK)-BB isoenzyme in their sera. Here we examined the relation among CK-BB activity, expression of CKB mRNA in peripheral blood, and hypermethylation of the CKB.
Methods: The two patients and other 26 patients with hematologic malignancies, and some cancer cell lines were subjected to measurement of serum CK activity, CK isoenzyme analysis, CKB mRNA expression analysis by RT-PCR, and methylation analysis of the CKB promoter region.
Results: CK-BB activity and proportion of leukemia blasts were correlated in the two patients. CKB mRNA was elevated in peripheral blood during an increase in blast numbers. In contrast, none of the other 26 patients showed CK-BB activity or expression of CKB mRNA. In all of the patients with hematologic

disorders, the analyzed region of CKB promoter was mostly unmethylated. However, some of cancer cell lines showed the methylated pattern. CKB mRNA was expressed at higher levels in cells with an unmethylated CKB promoter than in cells with a methylated promoter.
Conclusions: Expression of CKB mRNA and CK-B sometimes occurred in blastic transformation of the hematopoietic system. A relation between CKB mRNA expression and methylation of the CKB promoter was suggested.

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Increased creatine kinase BB activity and CKB mRNA expression in patients with hematologic disorders: relation to methylation status of the *CKB* promoter

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Abstract

Background: We encountered 2 patients with increased activities of the creatine kinase (CK)-BB isoenzyme in their sera. Here we examined the relation among CK-BB activity, expression of CKB mRNA in peripheral blood, and hypermethylation of the CKB. *Methods:* The 2 patients and other 26 patients with hematologic malignancies, and some cancer cell lines were subjected to measurement of serum CK activity, CK isoenzyme analysis, CKB mRNA expression analysis by RT-PCR, and methylation analysis of the *CKB* promoter region. *Results:* CK-BB activity and proportion of leukemia blasts were correlated in the 2 patients. CKB mRNA was increased in peripheral blood during an increase in leukemia blast numbers. In contrast, none of the other 26 patients showed CK-BB activity or expression of *CKB* mRNA. In all of the patients with hematologic disorders, the analyzed region of *CKB* promoter was mostly unmethylated. However, some of cancer cell lines showed the methylated pattern. CKB mRNA was expressed at higher levels in cells with an unmethylated CKB promoter than in cells with a methylated promoter. Conclusions: Expression of CKB mRNA and CK-B sometimes occurred in blastic transformation of the hematopoietic system. A relation between CKB mRNA expression and methylation of the CKB promoter was suggested.

Key words: creatine kinase; creatine kinase BB isoenzyme; *CKB* gene; methylation

1. Introduction

Creatine kinase (CK; EC 2.7.3.2) is involved in the maintenance of ATP at sites of cellular work [1]. Three cytoplasmic isoenzymes of CK have been identified in humans. These isoenzymes are dimeric molecules with 2 subunits designated M and B and are encoded by distinct genes [2]. Each CK isoenzyme has a distinct intracellular or tissue-specific pattern of the localization. The CK-BB isoenzyme is reported to be increased in patients with various malignancies, primarily solid tumors [3]. However, the CK-BB isoenzyme has also been detected in patients with various hematologic disorders [4]. Recently, we encountered 2 patients with increased activities of the CK-BB isoenzyme in their sera. Both suffered from hematological disorders. Chen et al. [5] recently reported that the CK-B subunit is a marker for monitoring minimal residual disease (MRD) in acute lymphoblastic leukemia. However, there are no reports of the correlation of CK-BB activity in serum with peripheral white blood cell counts or of expression of CKB mRNA in leukemic cells. There are also no reports that CKB mRNA is useful for monitoring of MRD in myeloid leukemia. Therefore, we examined CK-BB activity and expression of CKB mRNA in peripheral blood from patients with several types of hematologic disorders. We also examined the effect of hypermethylation of the *CKB* gene on expression of *CKB*.

2. Materials and methods

2.1. Patients and subjects

Routine laboratory examination identified high serum CK activity (1632 IU/l) in a 64-y-old man (Patient 1). The reference interval was from 55 to 204 IU/l for men. Isoenzyme analysis by electrophoresis showed a very high proportion (55%) of the CK-BB band. This patient had been observed for 5 years due to chronic anemia and was diagnosed with myelodysplastic syndrome (MDS). At the time of the recent visit to our hospital he complained of high fever (39°C) and showed hyperplasia of erythroid cells in the bone marrow and

increased leukemia blasts (28%) in peripheral blood. He was diagnosed with acute myeloid leukemia.

Patient 2 was a 60-y-old man with thromboangitis obliterans (TAO) and hepatocellular carcinoma. During treatment for TAO, leukocytosis and thrombocytosis were observed, and he was diagnosed with chronic myeloid leukemia (CML). Recently he was admitted for acute lymphoblastic transformation of CML. Total CK activity and the proportion of CK-BB in his serum were 113 IU/l and 36.5%, respectively, and leukemia blast counts (21100/μl) were 6% of white blood cell counts.

Twenty-six other patients with hematologic malignancies (8 with acute myeloid leukemia, 2 with CML, 2 with acute promyelocytic leukemia, 6 with acute lymphoblastic leukemia, 1 with chronic lymphoblastic leukemia, 3 with MDS, and 4 with malignant lymphoma) (Table 1) were subjected to measurement of serum CK activity, CK isoenzyme analysis, CKB mRNA expression analysis by RT-PCR, and analysis of methylation of the *CKB* promoter region. Six hematologic cancer cell lines (HL-60, NB4, K562, Raji, Daudi, U937), 11 solid tumor cell lines (MKN1, MKN7, MKN28, MKN45, MKN74, KATO III, NEDATE, PSN1, SW1116, C-1, Lu65), and 6 brain tumor cell lines (Daoy, ONS-76, UW228, TE671, D283, PFSK) were also subjected to *CKB* mRNA expression analysis by RT-PCR and methylation analysis of the *CKB* promoter region. The procedures in this study were in accordance with the Helsinki Declaration of 1975 and the subsequent 1996 amendments.

2.2. Activity measurement and isoenzyme analysis

Serum CK activity was measured by enzymatic assay with a Hitachi 7350 analyzer (Hitachi High Technologies). Assay conditions were based on the method recommended by the Japanese Society of Clinical Chemistry [6]. CK isoenzymes in serum were separated electrophoretically with Titan III supporting media (Helena Laboratory). The reference interval of CK-BB is 0.1 to 2.6%.

2.3. RNA preparation and RT-PCR

Total RNA was extracted from peripheral whole blood with Isogene (Nippon Gene). Reverse transcription (20-μl reaction mixture) was carried out with approximately 0.5 to 1.0 μg total RNA as template with ReverTraAce-α kit (TOYOBO). One-twentieth of the resulting cDNA was amplified with primers specific for *CKB* and β-actin (Table 2). PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining and UV transillumination. Stained bands were analyzed densitometrically with Cool Saver imaging software (ATTO Corp.).

2.4. DNA preparation and methylation analysis

Genomic DNA was extracted from peripheral whole blood with a DNA Extractor WB Kit (sodium iodide method) (Nippon Gene). For methylation analysis, we used sodium bisulfite treatment and PCR-single-strand DNA conformation polymorphism (SSCP) (BiPS) [7] and DNA sequencing analysis. Sequences of primers for the BiPS analysis are shown in Supplemental Table 2. The promoter sequence of CKB was taken from GenBank X15334 and is shown in Figure 1. The region selected for amplification contained non-coding exon 1 and is reported to be related to promoter activity [8]. The PCR product was expected to contain 299 base pairs and 48 CpG sites. BiPS analysis was performed with 12% non-denaturing polyacrylamide gels and silver-staining detection (Daiichi Pure Chemicals). DNA sequencing analysis was performed with BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and a PRISM 310 Genetic Analyzer (Applied Biosystems).

3. Results

On the basis of the timecourse of the laboratory data for patients 1 and 2, CK-BB activity and proportion of leukemia blasts in peripheral blood were correlated (correlation coefficients for patients 1 and 2 were 0.869 and 0.952, respectively) (Fig. 2A, B). CKB mRNA

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.

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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.

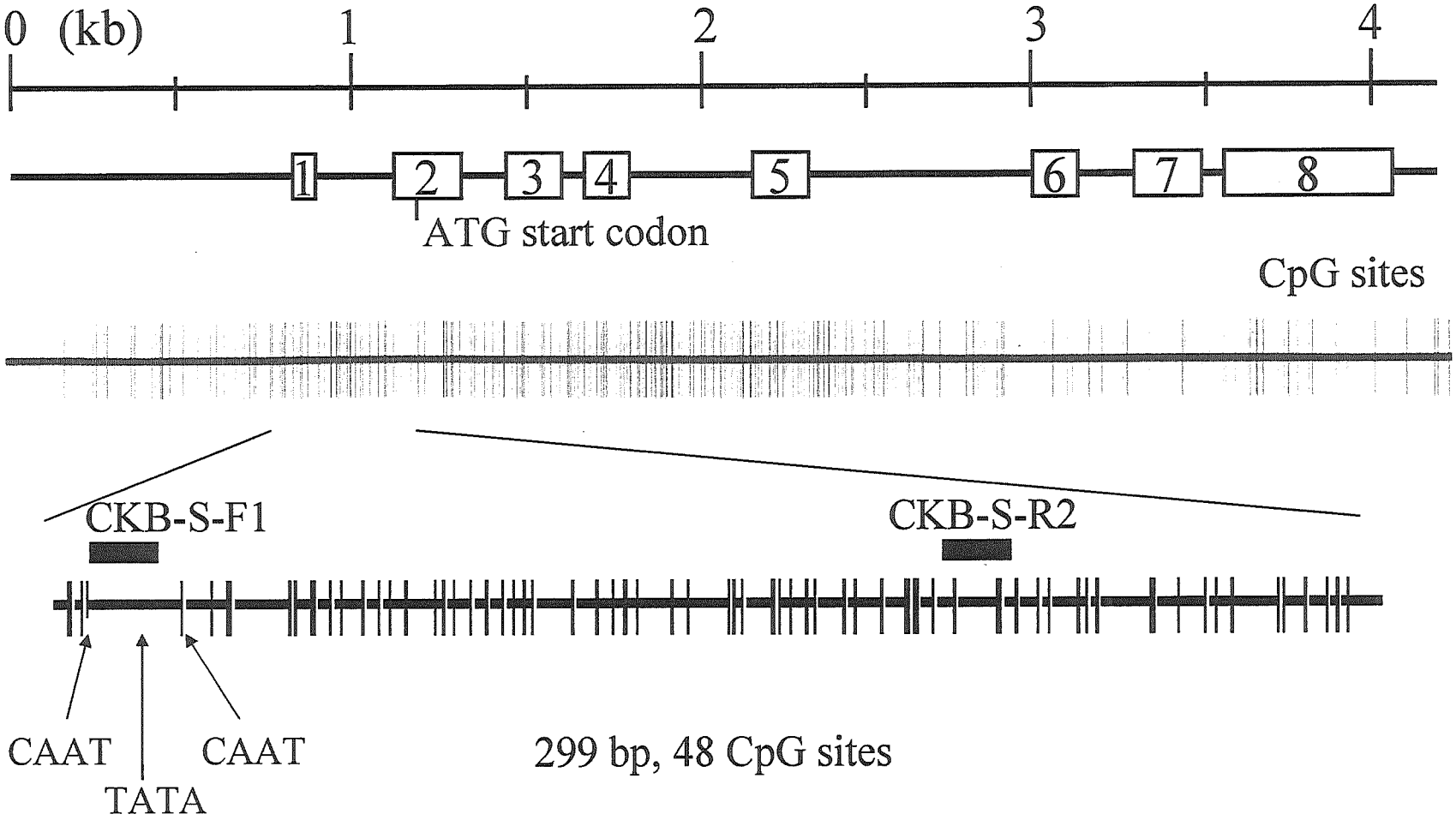
Fig. 3. RT-PCR analysis of CKB. Lanes 1 and 12, negative control (water); lanes 2, 5, and 6, Patient 1; lanes 3 and 4, other leukemic patients (negative result); lanes 7-11, Patient 2 (only a trace band was visible in lanes 7 and 8). Target length of the RT-PCR product is 651 base pairs (bp). Each lane 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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1
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ATG start codon
CpG sites
CKB-S-F1
CKB-S-R2
CAAT
TATA
CAAT
299 bp, 48 CpG sites

Figure(s)
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