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High levels of brain-type creatine kinase activity in human platelets and leukocytes: A genetic anomaly with autosomal dominant inheritance

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ABSTRACT

The ectopic expression in peripheral blood cells of the brain-type creatine kinase (CKB) is an autosomal dominant inherited anomaly named CKBE (MIM ID 123270). Here, we characterized the CK activity in serum, platelets (PLT) and leukocytes (WBC) of 22 probands (from 8 unrelated families) and 10 controls. CK activity was measured by standard UV-photometry. Expression of the *CKB* gene was analyzed by real-time PCR and Western blotting. DNA sequencing including bisulfite treatment was used for molecular analysis of the *CKB* gene. Serum CK levels were comparable between probands and controls. CKBE probands revealed significantly higher CK activity in PLT (3.7 ± 2.7 *versus* 179.2 ± 83.0 U/10^{12} PLT; $p<0.001$) and WBC (0.4 ± 0.3 *versus* 2.6 ± 2.1 U/10^9 WBC; $p=0.004$). Inhibitory anti-CKM antibodies did not affect CK activity indicating that the CK activity is generated exclusively by the CK-BB isoenzyme. CKB mRNA and protein levels were significantly higher in PLT and WBC from probands compared to controls. Re-sequencing of the entire *CKB* gene and methylation analysis of a CpG island revealed no alteration in CKBE probands. The genetic basis of CKBE remains unclear, however, we propose that a de-methylated *CKB* gene is inherited that leads to high CKB expression levels in myeloic precursor cells in the bone marrow.

Introduction

Creatine kinase (CK, ATP: creatine phosphotransferase, EC 2.7.3.2) is present in tissues with high energy turnover such as muscle and nervous tissues. Because of the dimeric structure of human CK, three different cytosolic isoenzymes CK-MM, CK-MB and CK-BB can be distinguished. In skeletal muscle, nearly 100% of the activity results from the MM isoenzyme, in heart muscle, 5–25% from the MB hybrid form and in brain and nerve tissue nearly 100% are of the BB isoenzyme. For diagnostic Monitoring of CK activity in serum or plasma is widely used in the diagnosis of myopathies and encephalopathies [1–3].

During a newborn screening program for Duchenne muscular dystrophy in the late 1970s [4] using a dry spot test of whole blood [5] some newborns with a positive test with respect to this screening program were found. However, the serum CK was in the normal range and no muscle disease present, that is, the tests were "false positive". To our surprise, we found in one family unusually high levels of CK-BB within the erythrocyte fraction and this anomaly was autosomal dominant transmitted [6]. In some families unusually high levels of this enzyme within platelets were identified. Meanwhile, in this screening program 501,000 newborns were tested and 132 were found "false positive" with respect to the screening program (unpublished data). This ectopic expression of CKB (CKBE, MIM ID 123270) in blood cells is an autosomal dominant inherited anomaly with an estimated frequency of about 1 in 5000 persons. The underlying CKBE mutation has been mapped to chromosome 14q32 where the *CKB* gene is located [7]. However, the molecular basis of this phenomenon remains unknown.

In this study, we re-investigated CK activity in serum, platelets and leukocytes from CKBE probands of eight unrelated families and in normal controls. We further characterized the isoenzyme using inhibitory antibodies against CKM. The CKB expression in platelets and leukocytes was determined at the mRNA and the protein level in probands and normal controls. The promoter, silencer and the coding region of the *CKB* gene were analyzed by re-sequencing. In addition, DNA methylation was determined by bisulfite sequencing.

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Subjects and methods

Subjects

Because of the need for rapid separation of the blood cells, only families living in Southern Germany were included. Therefore, it was achievable to start cell separation within 3 h of blood withdrawal. From eight unrelated families 22 probands with previously diagnosed high levels of CK activity were included. In addition, we analyzed a control group with three normal individuals from the families and seven unrelated controls (healthy volunteers). For blood collection standard systems (Sarstedt, Nümbrecht, Germany) were used to obtain serum, EDTA blood for automated cell counting (Cell-Dyn 3700; Abbott), and citrated whole blood for isolation of platelets and leukocytes. The baseline characteristics of all study individuals are summarized in Table 1.

Isolation of blood cells

Platelets were isolated and leukocyte-depleted according to a protocol published earlier [8]. Briefly, platelet-rich plasma (PRP) was obtained from citrated blood after centrifugation for 20 min at 150 *g*. PRP was leukocyte-depleted with the use of Purecell PL leukocyte removal filters (Pall Medical GmbH; Bad Kreuznach, Germany). Leukocytes (WBC) were obtained from the residual citrated blood after lysis of the red blood cells. Cell numbers of the platelet (PLT) and the WBC fraction were determined using automated cell counting.

Creatine kinase assays

CK activity was measured in an optical test using a standard test system (CK; Roche Diagnostics, Mannheim, Germany). A similar test for determination of the CK-MB isoenzyme included a mixture of four monoclonal antibodies (mouse) against human CKM with an inhibiting capacity of more than 99% (CK-MB; Roche). In both tests the reduction of NADP was followed at 340 nm in a spectral photometer (Hitachi Life Science; Mannheim, Germany).

Relative CKB mRNA quantification by real-time PCR

Total platelet RNA was extracted as described before [9]. From leukocytes total RNA was extracted using a commercial kit (RNeasy Mini Kit; Qiagen, Hilden, Germany). First strand cDNA synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR for detection of the house keeping genes *GAPDH* and *YWHAZ* as well as the *CKB* gene was performed on a LightCycler 480 system (Roche) using universal probes according to standard protocols. C_p values were used for normalization and relative quantification of mRNA levels according to geNorm algorithms [10].

Relative CKB protein quantification by Western blotting

Total protein extracts from platelets and WBCs of probands and controls were processed for Western blotting using standard procedures. The CKB protein was detected by a goat polyclonal antibody (clone N-20; Santa Cruz Biotechnology, Inc., Heidelberg, Germany). For relative quantification the same blots were stripped and re-hybridized using a monoclonal anti-actin antibody (clone C-11; Santa Cruz Biotechnology). Images from ECL detection of protein bands were taken by a 12-bit CCD camera and further processed using image analysis software (LabWorks 4.5; UVP Ltd., Upland CA, USA).

DNA sequencing of the CKB gene

For promoter and exon re-sequencing genomic DNA was prepared from EDTA-anticoagulated blood samples using a common salting-out method. PCR primers were developed with the help of the primer3 software [11]. Due to high GC content PCR amplification of the promoter region and exon 1 was achieved using specialized kits (GC-rich PCR system, Roche). PCR fragments were sequenced directly after purification in both directions by applying the same amplifying primers or additional primers (Table 2) and the dideoxy chain termination method (DYEnamic ET Kit, GE Healthcare, Munich, Germany) on a MegaBACE500 sequencer. For bisulfite sequencing genomic DNA was isolated from EDTA-anticoagulated blood samples using a commercial kit (QIAamp DNA Blood Mini Kit; Qiagen). Bisulfite conversion was done using EpiTect Bisulfite Kit (Qiagen). The CpG island was amplified and sequenced using primers given in Table 2.

Statistical evaluation

The statistical comparison of blood and CK activity values as well as *CKB* mRNA and protein expression levels was performed using the SPSS statistical software (SPSS v 12.0; SPSS, Munich, Germany). P-values <0.05 represented significant differences.

Results

Inheritance of the CKBE phenotype in eight unrelated families

The CKBE phenotype could be confirmed by a positive test CK activity in blood cells and normal CK activity in the serum in 22 probands from eight unrelated families. All pedigrees indicate an autosomal dominant mode of inheritance (Fig. 1). Three of the probands

Table 1
Baseline characteristics and CK activity of study individuals.

Parameter	Probands (n = 22)	Controls (n = 10)	p-value*
Age, mean ± SD (range)	35.8 ± 20.3	45.7 ± 15.5	0.183
Male, % (n)	68% (15)	30% (3)	0.062
Blood values			
RBC, mean ± SD [M/µl]	5.4 ± 0.7**	4.7 ± 0.2	0.005
HGB, mean ± SD [g/dl]	14.7 ± 2.0	14.2 ± 0.6	0.468
PLT, mean ± SD [K/µl]	282.5 ± 71.3	279.4 ± 34.9	0.896
WBC, mean ± SD [K/µl]	6.9 ± 1.3	6.1 ± 1.1	0.117
Creatine kinase activity			
Serum CK, mean ± SD [U/L]	55.3 ± 33.6	36.2 ± 28.5	0.130
PLT CK, mean ± SD [$U/10^{12}$ PLT]	179.2 ± 83.0	3.7 ± 2.7	<0.001
WBC CK, mean ± SD [$U/10^{9}$ WBC]	2.56 ± 2.09	0.35 ± 0.27	0.004

* Comparison of the two groups by *t*-test or Fisher's exact test (for gender); significant differences showed p-values<0.05.
** Significantly higher RBC counts in probands were due to one Turkish family (four individuals) with thalassemia minor.

Table 2
Primers for re-sequencing and bisulfite sequencing of the *CKB* gene.

Region	Forward (5′–3′)	Reverse (5′–3′)
Silencer	ACAGATGAGGGTCCCTGATG	ACTCGAGGACGCTTGGGTTC
Promoter	TGCGGGCACCTTGAGGAAG	CTTTGCACGCAGCGCCCCTAGCC
Exon 1	CCAATGGAATGAATGGGCTA	CAGCTTCAGTGCGTTGTG
Exon 2	CTGAGTGGTACGCGGGAG	TGCAGGTTGTCGGGGTTG
Exon 3–4	CTTCACGCTGGACGACG	GGGCGACGTAAACAAAAGC
Exon 5	ACTGGACGCCCGCAGAT	GAGAGGGAAAGCGGAGGTAG
Exon 6–8	TGGGAAGTTCCTTTGGTCTG	GGCACCACTCAGACGCA
Additional sequencing primers		
Silencer	CCAGCTCAAAGCCAGCAATG	
Exon 2		GGGACGGGGACAGTGAC
Exon 3	GCCACCCGTACATCATG	TGCAGGTTGTCGGGGTTG
Exon 7	CCTGTAGGGGTTTCAGGCA	CTGGGGAGACAGCAAGTCA
Bisulfite sequencing		
CpG	ATCCAAGGACCTGAGGTTTCGG	GGGACGCGGCCAAGGTCAGCG

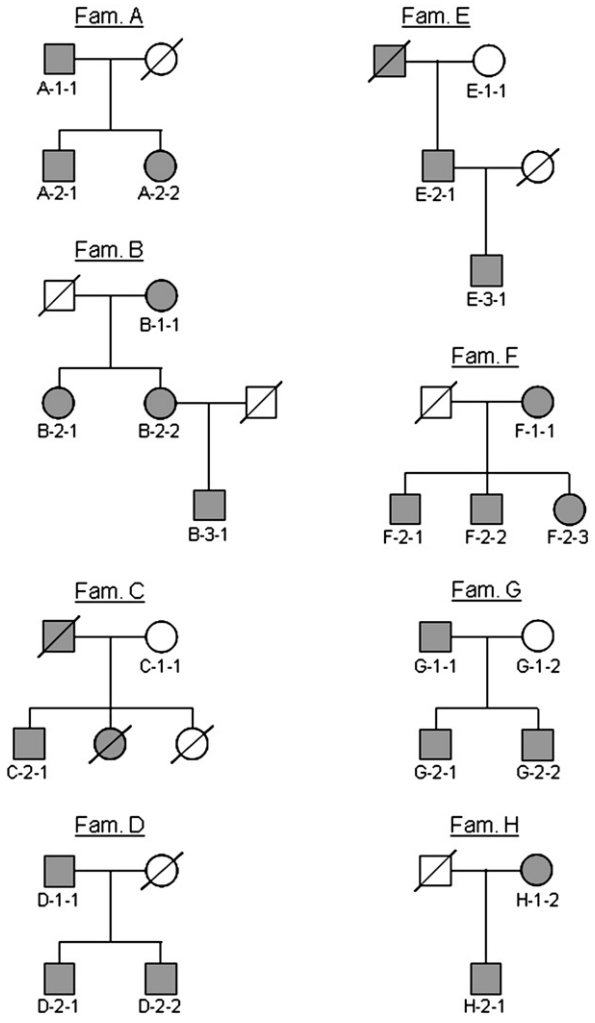

Fig. 1. Pedigrees of the eight CKBE families included in this study. Gray symbols denote individuals with CKBE phenotype; open symbols denote unaffected individuals. Crossed-out symbols indicate individuals that have not been re-investigated in this study, however, diagnosis of the CKBE phenotype was obtained in former investigations. For the present study each individual was given a sample code (*e.g.* A-1-1 from family A).

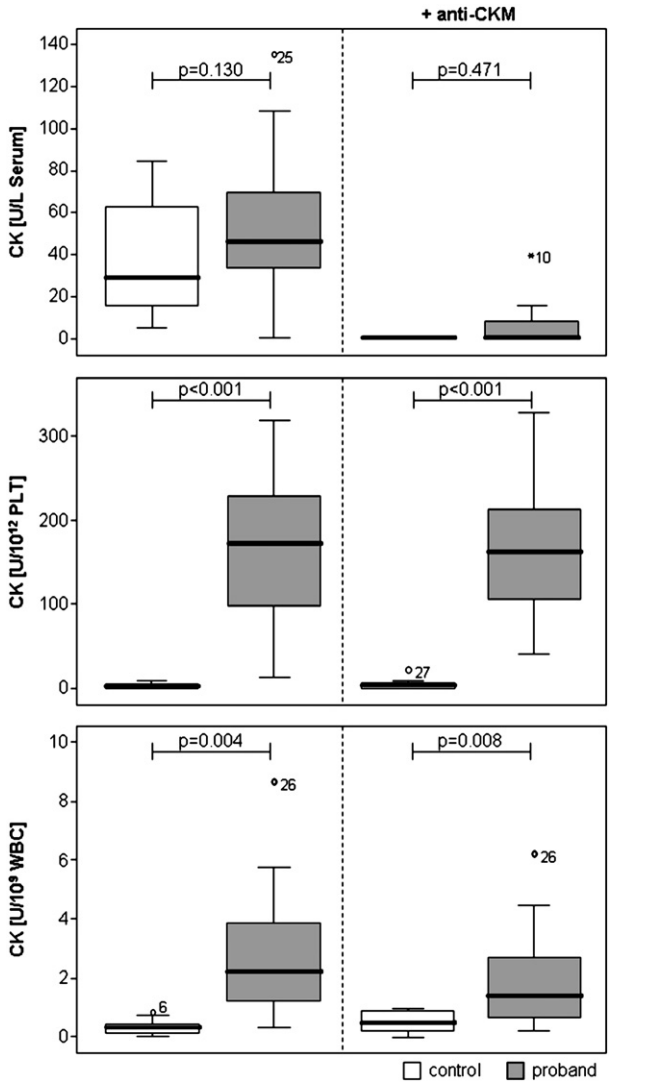

Fig. 2. Box plots showing total CK activity (left panel) in serum and lysates of platelets (PLT) and leukocytes (WBC) of controls (n = 10; white boxes) and CKBE probands (n = 22; gray boxes). Boxes show the 25th to 75th percentile with the median indicated by the line inside each box. Whiskers represent the 1.5 interquartile range (IQR) and individuals with values outside the 1.5 IQR range are marked by symbols (° or *) and the corresponding sample number. The addition of monoclonal anti-CKM antibodies (right panel) significantly inhibited serum CK activity but did not affect CK activity in PLT or WBC.

relatives had normal CK values which also confirmed the results from previous screening tests. Thirty years after the initial investigation the former newborns are adults and all of them are in good health. They did not show any signs of muscular dystrophy, platelet dysfunction or any other diseases. All probands showed normal blood cell values (Table 1) with one exception: in the Turkish family (Family F, Fig. 1) all four probands exhibited red cell data indicating thalassemia minor.

CK activity in serum and blood cells

The CK activity was measured in platelets, leukocytes and serum of 10 controls (seven unrelated controls and three normal controls from three of the families) and 22 probands (Table 1 and Fig. 2). Serum levels of CK activity were comparable between CKBE probands and controls (mean ± SD: 55.3 ± 33.6 *versus* 36.2 ± 28.5 U/L; $p = 0.130$). In platelets we found a more than 50-fold higher CK activity in the probands compared to controls (179.2 ± 83.0 *versus* 3.7 ± 2.7 U/10^{12} platelets; $p < 0.001$). In leukocytes the difference was less prominent (approximately 7-fold higher in probands), however, still significant (2.6 ± 2.1 *versus* 0.4 ± 0.3 U/10^9 leukocytes; $p = 0.004$). In order to further characterize the structure of the active enzyme in the different blood fractions we used a standard assay that included inhibitory antibodies against CKM and compared the values with the total CK activity. As expected, in serum samples of both probands and controls the anti-CKM antibodies significantly inhibited CK activity indicating the CK-MB isoenzyme (Fig. 2). In platelet and leukocyte samples of probands no significant difference of CK activity could be observed after anti-CKM incubation. Thus, the CK activity in platelets and leukocytes of both CKBE proband and controls resulted exclusively from the CK-BB isoenzyme.

CKB expression levels in platelets and leukocytes

We determined relative mRNA levels of the *CKB* gene compared to mRNA levels of two reference genes (*GAPDH* and *YWHAZ*) in platelet RNA from of 16 probands and 8 controls. Probands revealed significantly higher mRNA levels compared to controls ($p = 0.005$) and the relative *CKB* mRNA levels strongly correlated ($r = 0.578$; $p = 0.003$) with the CK activity in platelets (Fig. 3). Very similar results could be obtained at the protein level by using Western blotting. The relative CKB protein expression level was significantly higher in platelets of probands compared to controls ($p = 0.001$)

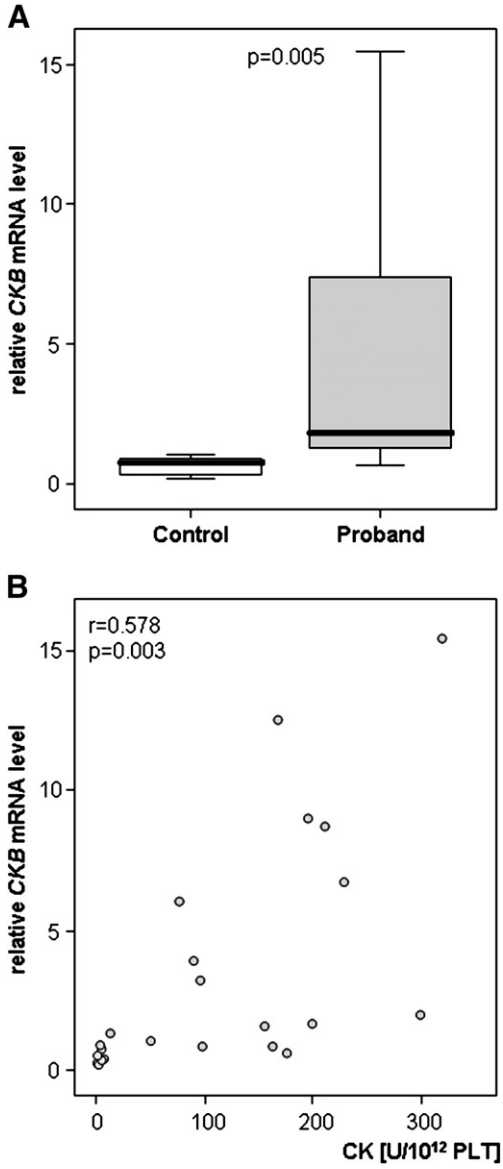

Fig. 3. Levels of *CKB* mRNA in platelets of probands (n = 16) and controls (n = 8) determined by real-time PCR. For relative quantification the *CKB* mRNA levels were compared to house keeping genes (*GAPDH* and *YWHAZ*) using geNorm normalization. (A) Probands showed significantly higher *CKB* mRNA levels compared to controls. (B) The *CKB* mRNA levels in all tested individuals significantly correlated with CK activity in platelets.

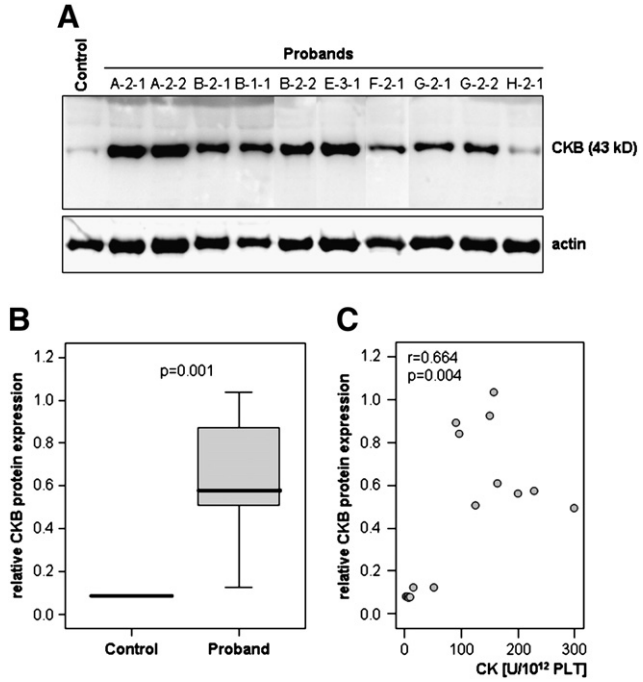

Fig. 4. Levels of CKB protein in platelets (PLT) of probands (n = 10) and controls (n = 5) determined by Western blotting (A). The polyclonal anti-CKB antibody specifically detected a protein band of expected size (43 kDa). (B) For relative quantification the intensities of the CKB and actin protein bands on the same blot were compared using image analysis software. Probands showed significantly higher CKB protein levels compared to controls. (C) The CKB protein levels in all tested individuals significantly correlated with CK activity in platelets.

and showed a strong correlation to the CK activity in platelets (r = 0.664; p = 0.004) (Fig. 4). In addition, the relative protein expression significantly correlated with relative CKB mRNA levels (r = 0.751; p = 0.001; data not shown). Western blotting experiments were also performed on protein extracts from WBC (Fig. 5). CKBE probands showed significantly higher CKB protein levels (p = 0.001) also in WBC. We found a moderate correlation of CKB protein expression and CK activity in WBC that did not reach statistical significance (r = 0.449; p = 0.166).

Molecular analysis of the CKB gene

Direct sequencing of the promoter and the coding region of the *CKB* gene was done in all probands and controls. We found no functional mutation. Two synonymous SNPs in exon 4 and exon 8 (rs1136165, Arg152Arg; rs1803283, Glu364Glu) were detected, which were in strong linkage. Genotyping for both SNPs of all

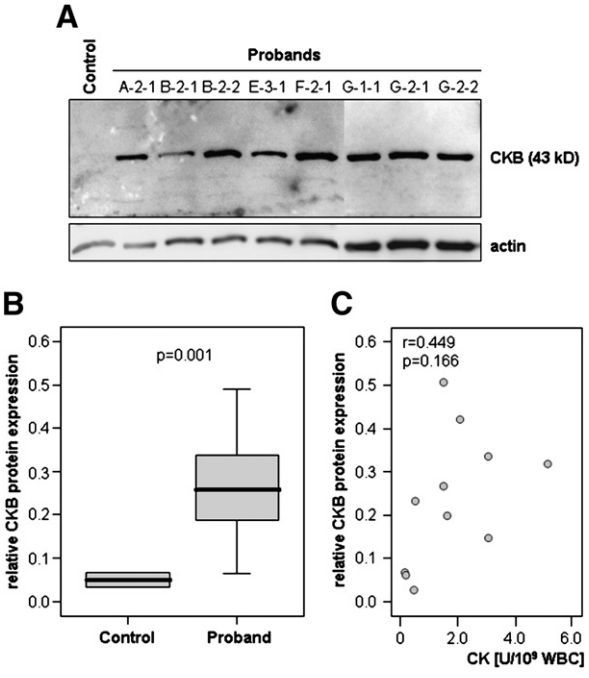

Fig. 5. Levels of CKB protein in leukocytes (WBC) of probands (n = 8) and controls (n = 4) determined by Western blotting (A). The polyclonal anti-CKB antibody specifically detected a protein band of expected size (43 kDa). (B) For relative quantification the intensities of the CKB and actin protein bands on the same blot were compared using image analysis software. Probands showed significantly higher CKB protein levels compared to controls. (C) The CKB protein levels in all tested individuals tended to correlate with CK activity in WBC without reaching statistical significance.

probands and controls showed a similar distribution of the allele frequencies (data not shown). In addition, we sequenced the entire *CKB* silencer region described by Ritchie et al. [12] at position −390 to −1150 upstream of the transcription start site (TSS) in all CKBE probands and controls. An INDEL (GGGGG/GGGG–) polymorphism in a polyG stretch ($G_{4/5}$-T-G_4-C-G_5) at position −426 was identified in probands as well as in controls. No other DNA sequence variation could be found.

For DNA methylation analysis we first analyzed the entire *CKB* sequence for the localization of possible CpG islands using the EMBOSS CpGPlot tool (www.ebi.ac.uk/Tools/emboss/cpgplot/). A CpG island was predicted with 205 bp in length including 32 CpG sites in the promoter and exon 1 region (Fig. 6). Bisulfite sequencing of the CpG island indicated complete methylation in leukocyte DNA samples from probands and controls (data not shown).

Discussion

The unusually high CK activity in platelets and leukocytes of probands from eight families is genetically transmitted in an autosomal dominant way. The activity seems exclusively generated by the CK-BB isoenzyme because the anti-CKM antibody did not affect CK activity in platelets and leukocytes. In an earlier study from 1978 we described the presence of CK-BB in erythrocytes of three probands from a family of Italian origin and we could not detect any CK activity in the platelets of normal controls using a test system based on creatine and ATP as substrates [6]. In the present study with the commercially available test system creatine phosphate and ADP were used as substrates. We found low CK activity in platelets and leukocytes of normal controls and clearly showed high CK levels in CKBE probands. A possible interference of adenylate kinase activity in these cells is excluded by inhibiting the activity with diadenosine pentaphosphate and AMP. In addition, we performed pre-run measurements before addition of the substrate creatine phosphate and could not observe a change in absorption. Furthermore, the CK activity could not be inhibited by anti-CKM antibodies.

In cells with high and fluctuating energy demands, such as muscle cells, a high activity of CK is responsible for the so called phosphocreatine shuttle. It consists of CK in cytosol and in mitochondria and performs fast energy transfer between mitochondria and peripheral energy consumers [13]. The CK activity in normal platelets and leukocytes as measured in the present study, even at low levels, lets us suppose that in these blood cells the phosphocreatine shuttle is of functional relevance. Previous reports of normal blood cells confirmed the presence of CK activity in granulocytes, astrocytes, monocytes and at low level in platelets, but not in erythrocytes and lymphocytes [14,15]. The CK-BB isoenzyme was also found in serum, platelets and erythrocytes from patients with a variety of hematologic disorders, mostly myeloproliferative diseases [16,17]. The probands of our study (except the Turkish family with thalassemia minor) had no clinical signs of bleeding or thrombosis or any other hematological disfunction. Thus, the substantial increase of CK activity in platelets and leukocytes had no negative effect on the number or function of the blood cells. However, *in vitro* function tests have not been done.

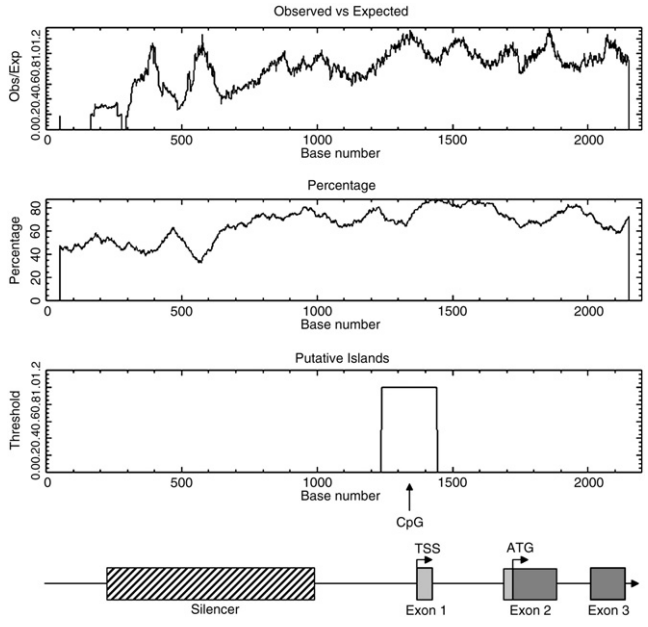

Fig. 6. Result of *in-silico* analysis of the CKB gene sequence using the EMBOSS CpGPlot tool to locate CpG islands. A CpG island was identified downstream of the silencer region including the transcription start site (TSS) and the entire exon 1. The start site of the coding region (ATG) is located in exon 2.

It could be speculated that the markedly elevated enzyme activity, especially in platelets, results in an acceleration of the phosphocreatine shuttle followed by a more rapid regeneration of ATP. Kuiper et al. [18] demonstrated that in macrophages – under the condition of phagocytosis – cytosolic CKB is mobilized and co-accumulates with F-actin in nascent phagosomes to provide access to ATP. The occurrence of CKB in blood cells under malignant conditions of hematopoesis may be explained as a positive reaction against the malignancy. Thus, the CKBE phenotype could possibly result in a protection against malignant transformation and other stress conditions of the cells.

The profiling of gene transcripts in normal human platelets by microarray hybridization analysis revealed the presence of *CKB* but not *CKM* mRNA [9]. In the present study we could confirm *CKB* gene expression in platelets and leukocytes by using real-time PCR. *CKM* gene transcripts could not be detected (data not shown). The relative *CKB* mRNA levels were significantly higher in CKBE probands indicating that the *CKB* gene is upregulated at the transcriptional level. However, the molecular basis of the upregulation remains unknown. Neither the coding region nor the promoter or silencer region of the *CKB* gene showed DNA sequence alteration in our samples. Altered methylation patterns of the *CKB* promoter were described in patients with hematologic disorders and increased CK-BB activity [17]. Unfortunately, the authors described a DNA region that we could not localize in the human *CKB* gene. In our investigation using DNA from leukocytes, methylation analysis of the CpG island did not show differences between CKBE probands and controls. We speculate that the CKBE phenotype is due to de-methylated *CKB* gene in myeloic precursor cells such as megakaryoblasts and myeloblasts in the bone marrow. During cell differentiation (including megakaryopoiesis) the *CKB* gene is then silenced by methylation. The increased levels of mRNA, protein and enzymatic activity could persist in the cells of the blood. The hypothesis of transmission of a de-methylated *CKB* gene fits with the autosomal dominant inheritance of the CKBE phenotype.

Conflict of interest

The authors declare no conflict of interest.

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