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CREB has been described as critical for leukemia progress. We investigated CREB expression in ALL and AML pediatric patients. CREB protein was significantly high (p<0.001) at diagnosis but not during remission. This study underlines the role of CREB in leukemia and suggests new insights into the transformation process.

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Acute leukemia accounts for about one third of childhood cancers.[1] Recent advances in functional genomics has highlighted the need to investigate complex gene expression patterns to discover novel signaling and disease-related pathways.[2] CREB is a transcription factor that regulates gene expression principally through activation of the cyclic AMP (cAMP)-dependent cell signal transduction pathways by binding to the cAMP response element (CRE) region at the promoters.[3] CREB is phosphorylated in Serine 133 principally by protein kinase A, and this modification enhances its transactivation potential.[4] It has been reported to be over expressed in adult myeloid leukemia and to play a role in myeloid transformation and leukemia progression.[5,6]

We investigated the expression of CREB in childhood acute leukemia. We analyzed a total of 86 acute lymphoid (ALL), 40 acute myeloid (AML) leukemia patients (Table 1) and a series of leukemia cell lines to reveal the potential role of this signaling pathway in leukemogenesis. CREB expression was also studied in samples collected from 86 ALL and 21 AML patients during remission to establish a correlation with the disease stage. As control group we used bone marrow (BM) samples from 19 patients affected by other non-neoplastic hematologic disorders (diagnoses: neutropenia, n=4; thrombocytopenia, n=9; anemia, n=3; lymphoma, without BM infiltration n=3) and from 9 healthy donors (7 pediatric and 2 adult samples collected in the process of transplants).

Western blot (WB) analysis showed CREB protein overexpression in all leukemia cell lines but not in sorted normal hematopoietic stem cells (Figure 1A). WB analysis showed CREB overexpressed in 29 out of the 31 (94%) patients with ALL (20/23 with B-lineage ALL; and 7/8 with T-ALL) and in 13 out of 17 (76%) with AML. In addition, CREB resulted in the active form (pCREB) at diagnosis. On the contrary, CREB was not detected in any remission samples or in the control group. Interestingly, CREB expression in samples collected from diagnosis to treatment completion showed a clear correlation with the disease stage (Figure 1B). To define CREB protein level, a competitive ELISA assay was performed on 16 ALL and 7 AML patients previously analyzed by WB as well as in 55 new ALL and 23 AML patients collected during the study. Average CREB concentration was 151.5 ng/mL in ALL at diagnosis (n=71), and 72 ng/mL in AML at diagnosis (n=30). The distribution of CREB level was significantly lower in ALL and AML remission samples (4.47 ng/mL; n=80) than in the control group (7.28 ng/ml in 9 healthy bone marrow; 3.15 ng/ml in 19 non-neoplastic disorders samples; p<0,001). Overall, the quantification of pCREB level in the same samples showed that CREB was almost completely in its active form (p<0.001). Considering the cut-off value of 26 ng/mL from ELISA results, we found CREB protein overexpressed in 60/71(84%) of ALL and in 20/30 (66%) of AML. This value was used in paired samples of ALL or AML patients at diagnosis and during therapy course until relapse. It confirmed the correlation between CREB expression level and disease activity (Figure 1C). Values under this cut-off were undetectable by WB.

By analyzing CREB transcriptional activity using a gel shift assay, we found that CREB bound CRE region in samples at diagnosis while in remissions and controls its expression was insufficient to induce the CREB/CRE shift (Figure 1D). Overall, these results agree with Molstad *et al.*'s suggestion that CREB controls the transcription machinery in leukemic tissue, upregulating target genes.[7] We then focused on the cAMP early inducible repressor (ICER) expression which represses CREB activity by binding to the same promoter genes. A lower ICER expression was found at diagnosis. This significantly increased in remission samples and in the control group. On the contrary, Cyclin A1, one of CREB target genes, was upregulated at diagnosis and downregulated in remission samples and in the control group (p<0.03) (Figure 1E). These findings suggest a role for ICER in the modulation of leukemic gene expression.[8,9] The induction of Cyclin-A1 in patients suggests additional

Table 1. Main biologic and clinical features of childhood acute leukemia patients enrolled.

	ALL			AML		
	CREB +	*CREB -*		*CREB +*	*CREB -*	
WB analysis*	29/31	2/31		13/17	4/17	
ELISA analysis*	60/71	11/71		20/30	10/30	
CREB protein expression§	73/86 (84%)	13/86	p<0.001	32/40 (80%)	8/40	p<0.001
Sex	42 M 31F	10M 3F	p=0.06	17M 15 F	6M 2F	p=0.4
Age	7>10 yrs 66<10 yrs	3>10 yrs 10<10 yrs	p=0.2	4>10 yrs 28<10 yrs	1>10 yrs 7<10 yrs	p=1
Immunophenotype and morphology						
	61 B-lineage (47 CALL; 22 PreB)	8 B-lineage (7 CALL; 3 PreB)		9 M1 1 M2 3 M3 3 M4 8 M5 2 M7 6 Unc	1 M1 2 M2 2 M3 1 M4 2 Unc	
	12 T-lineage (9 Early-T; 8 T mature)	5 T-lineage (3 Early-T)	p=0.2			
Cytogenetics and molecular genetics						
t(9;22) *BCR-ABL*	3	–	p=1	–	–	
t(12;21)*TEL-AML1*	14	1	p=0.5	–	–	
t(1;19) *E2A-PBX1*	4	–	p=1	–	–	
t(4,11) *MLL-AF4*	3	–	p=1	–	–	
t(8;21) *AML1-ETO*	–	–		3	1	p=1
t(15;17) *PML-RARA*	–	–		1	1	p=0.4
inv(16) *CBFB-MYH11*	–	–		2	–	p=1
t(11;19) *MLL-ENL*	–	–		2	–	p=1
Hyperdiploid	11	3	p=0.2	1	–	p=1

**For 16 ALL samples and 7 AML samples analyzed by western blot, material was sufficient to also perform ELISA quantification ([8]16/16 ALL were positive for both analyses; 1/7 for AML patients). CREB+ defines CREB protein overexpression found by WB or by ELISA (>26 ng/mL). ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; M: male; F: female; yrs: years. Unc: Unclassified.*

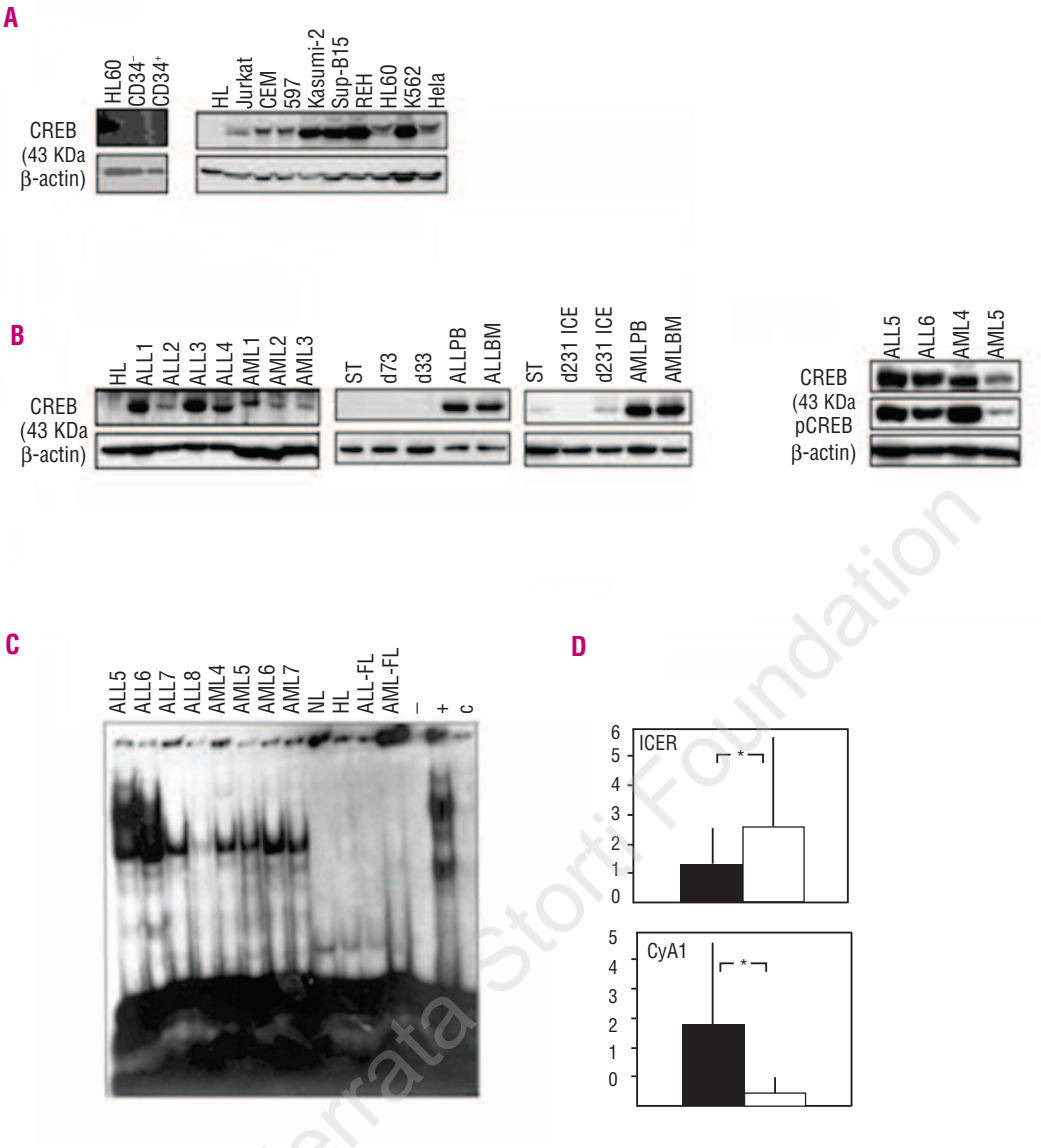

Figure 1. A. Western blot analysis of CREB protein expression in normal hematopoietic stem cell sorted from a healthy pediatric bone marrow (CD34$^+$, CD34$^-$) and the HL60 promyelocytic cell line (left) and Western blot (right) of a series of leukemic cell lines (JURKAT,CEM, 697, KASUMI-2, Sup-B15, REH, HL60, K562 and Hela) and a healthy bone marrow (HL). **B)** Western blot analysis of CREB in representative patients at diagnosis affected by lymphoid acute leukemia (ALL: 1-4) and myeloid leukemia (AML: 1-3) and HL (left). Western blots of specimens collected during the therapy course of the current AIEOP-ALL and AML trials in an ALL (center) and AML (right) patients. ST, stop therapy; d, day; PB, peripheral blood at diagnosis; BM, bone marrow at diagnosis. Western blot analysis of total CREB and pCREB and β-actin in ALL (ALL: 5-6) and AML (AML: 4-5) patients at diagnosis after stripping and reprobing the same membrane. Blots were probed using CREB rabbit polyclonal or PhosphoCREB (6 µg/mL) rabbit polyclonal (Upstate, Charlottesville, VA, USA), stripped, and reprobed with β-actin (3 µg/mL) (Sigma-Aldrich St. Louis, MO, USA). **C)** Gel shift assay in representative lymphoid (ALL: 1-4) and myeloid (AML: 5-8) patients at diagnosis of leukemia, non leukemia sample (NL), a healthy donor (HL), and in a sample in complete remission collected during the follow up (FL) of one representative ALL and AML patient. (-) negative control without cell lysates; (+) positive control from HeLa nuclear extracts and (c) competitive control are included in the analyses. **D)** Histogram describes ICER and Cyclin A1 expression by quantitative real time PCR analysis of ALL and AML patients at diagnosis (black bar) and of the remission samples and the control group (white bar). *Defines that data are statistically significant ($p<0.03$, Student's t-test). All samples were analyzed in triplicate. Error bars denote the standard deviation of each sample measured in triplicate.

CREB target genes that could be induced by its signaling pathway. The precise mechanism of CREB protein overexpression is not known but we focused on some aspects. Our results revealed that the upregulated CREB protein expression is not strictly correlated to mRNA expression.[10] Furthermore, the possible involvement of cAMP, one of the most important CREB phosphorylation activators, is not supported since we found comparable cAMP levels in both leukemia cells at diagnosis and in controls. Furthermore, the systematic sequencing of the CREB KIX domain in our study set up previously unrevealed genetic mutations of the sequence that could support inappropriate CREB phosphorylation or recruitment of co-factors (*data not shown*).

In conclusion, our results revealed a strong association between an aberrant CREB expression in the majority of cases of acute childhood leukemia, ALL and AML. This sug-

gests that CREB could be considered a candidate *hit* in leukemogenesis. We believe that the cAMP/CREB/ICER[9,10] pathway may have a critical role in leukemia gene expression and in the pathogenesis of childhood acute leukemia.

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