Activation of the Unfolded Protein Response Is Associated with Favorable Prognosis in Acute Myeloid Leukemia

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Abstract

Purpose: The unfolded protein response is triggered by the accumulation of misfolded proteins within the endoplasmic reticulum. Previous studies suggest that the unfolded protein response is activated in some cancer cell lines and involved in tumor development. The role of the unfolded protein response during leukemogenesis is unknown thus far.

Experimental Design: Here, we assessed the induction of key effectors of the unfolded protein response in leukemic cells at diagnosis of 105 acute myeloid leukemia (AML) patients comprising all subtypes. We determined the formation of the spliced variant of the X-box–binding protein 1 (*XBP1*) mRNA, as well as expression levels of calreticulin, *GRP78*, and *CHOP* mRNA.

Results: The formation of the spliced variant of *XBP1s* was detectable in 16.2% (17 of 105) of AML patients. Consistent with activated unfolded protein response, this group also had significantly increased expression of calreticulin, *GRP78*, and *CHOP*. AML patients with activated unfolded protein response had lower WBC counts, lactate dehydrogenase levels, and more frequently, secondary AML. The incidence of *fms*-related tyrosine kinase 3 (*FLT3*) mutations was significantly lower in patients with activated unfolded protein response. In addition, an association was observed between activated unfolded protein response and deletion of chromosome 7. Finally, the clinical course of AML patients with activated unfolded protein response was more favorable with lower relapse rate ($P = 0.0182$) and better overall ($P = 0.041$) and disease-free survival ($P = 0.022$).

Conclusions: These results suggest that the unfolded protein response is activated in a considerable subset of AML patients. AML patients with activated unfolded protein response present specific clinical characteristics and a more favorable course of the disease.

The current paradigm on leukemogenesis indicates that leukemias are propagated by leukemic stem cells. The genomic events and pathways involved in the transformation of hematopoietic precursors into leukemic stem cells are increasingly clarified. Traditionally, research on the pathogenesis of acute myeloid leukemia (AML) has focused on the analysis of tumor suppressor genes or oncogenes, which regulate proliferation. Disruption of normal differentiation represents another hallmark of AML because leukemic blasts typically display a block in their normal differentiation process at a particular stage (1). Additional characteristics of leukemic stem cells may involve deregulation of cell death and self-renewal pathways.

Another feature of some cancer stem cells is the activation of the unfolded protein response, which is triggered by the accumulation of misfolded proteins in the endoplasmic reticulum, leading to endoplasmic reticulum stress (2–4). Two key events are noteworthy during the unfolded protein response. First, the global protein synthesis is reduced, which is achieved by a reduction of the protein load entering the endoplasmic reticulum, and second, specific endoplasmic reticulum chaperone molecules are activated, thereby markedly increasing the capacity to handle misfolded proteins (2, 3). If homeostasis cannot be achieved after activation of the unfolded protein response, cell death is triggered (4, 5). Initial key events of the unfolded protein response comprise the activation of the inositol-requiring protein-1 (*IRE1*), of the activating transcription factor 6 (*ATF6*), and of the protein kinase RNA–like endoplasmic reticulum kinase (6). In particular, activation of

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Translational Relevance

Here, we identified for the first time the activation of the unfolded protein response in a subset of patients with acute myeloid leukemia (AML) by assessing the spliced variant of the X-box–binding protein 1 (*XBP1*) mRNA, as well as the expression of calreticulin, *GRP78*, and *CHOP* mRNA. In particular, the PCR assay to detect the spliced variant of the *XBP1* mRNA has the potential to be applied in clinical practice. Remarkably, we identified AML patients with activated unfolded protein response to have a favorable course of their disease. In addition, an association was observed between activated unfolded protein response and deletion of chromosome 7 among AML patients with bad-risk karyotype abnormalities. Finally, cell lines with activated unfolded protein response are especially prone to undergo apoptosis after treatment with the proteasome inhibitor bortezomib. Thus, our studies might provide a rationale for the use of bortezomib in AML patients presenting with an activated unfolded protein response and/or deletion of chromosome 7 in the leukemic cells at diagnosis.

IRE1 induces the cleavage of the X-box–binding protein 1 (*XBP1*) mRNA, generating a spliced mRNA that encodes a potent novel transcriptional activator of downstream unfolded protein response target genes (7–10). Other prominent cellular mediators of the unfolded protein response besides *XBP1* are calreticulin, *CHOP*, and *GRP78* (2–4).

The role of the unfolded protein response during leukemogenesis has not been investigated thus far. However, we previously reported that the endoplasmic reticulum chaperone calreticulin is specifically induced in AML cells expressing the *AML1-MDS1-EVI1* protein (11), suggesting that the unfolded protein response can be activated in subtypes of AML patients. In this report, we screened a large collection of primary leukemic cells of all AML subtypes for the activation of the unfolded protein response. We found that activated unfolded protein response is observed in 16.2% of AML patients, as determined by the induction of the *XBP1* spliced variant and increased expression of *GRP78*, *CHOP*, and calreticulin. AML patients with activated unfolded protein response had specific clinical characteristics, and an association was observed between activated unfolded protein response and deletion of (parts of) chromosome 7. Remarkably, the clinical course of AML patients with activated unfolded protein response was more favorable with lower relapse rate and better survival. These results suggest that assessing AML cells for the presence of the spliced variant of *XBP1s* mRNA is a sensitive marker for activated unfolded protein response and that the unfolded protein response is activated in a considerable subgroup of AML patients.

Patients, Materials, and Methods

Patients. Source of malignant cells was Ficoll-separated mononucleated cells of bone marrow (82 patients) or peripheral blood (23 patients). All AML patients were consecutively diagnosed between 2005 and 2007 using standard morphology and immunophenotype markers at the Department of Oncology, University Hospital, Bern, Switzerland. Informed consent from all patients was obtained according to the Declaration of Helsinki, approved by decisions of the local ethics committee of Bern, Switzerland. Only patients fit for intensive treatment were included in this study, and they were all treated within the HOVON-SAKK 30/00 protocol for patients ≤60 y or the HOVON-SAKK 30/01 protocol for patients >60 y. Treatment for patients consisted of cytarabine and an anthracycline in cycle 1 and of cytarabine in cycle 2 (and amsacrin for patients ≤60 y). Patients ≤60 y in complete remission after cycle 2 were randomly assigned to a third cycle of chemotherapy with etoposide and mitoxantrone or high-dose chemotherapy with busulfan and cyclophosphamide, followed by autologous transplant. Allogenous transplant was done in patients <<55 y of age and with intermediate- or bad-risk cytogenetics.

Immunophenotyping and cytogenetic analysis. A panel of monoclonal antibodies against myeloid lineage associated antigens (including

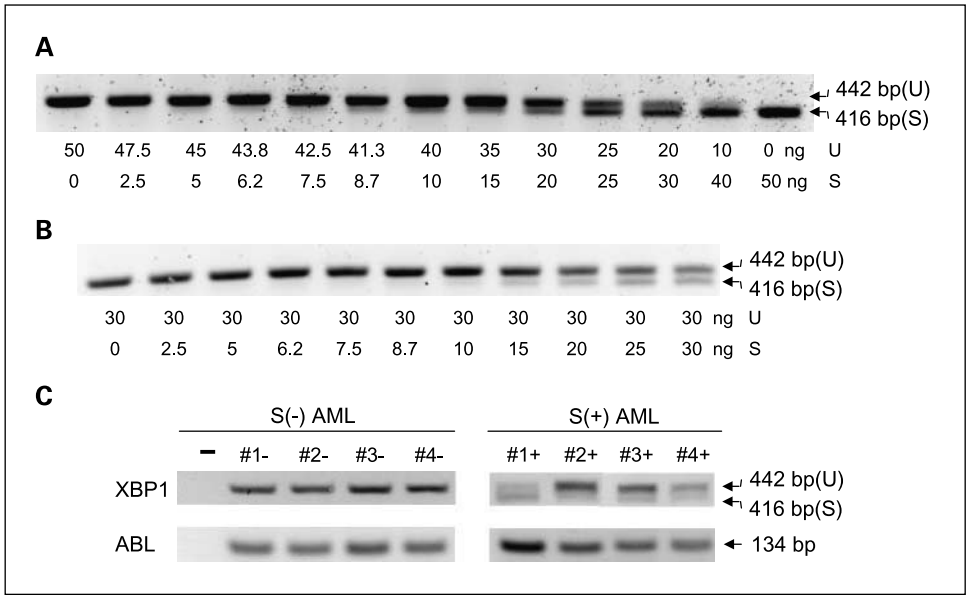

Fig. 1. A subgroup of AML patients is expressing *XBP1s*. *A*, the sensitivity of the PCR assay was determined using pcDNA3 plasmids encoding for human *XBP1* WT (U) or the spliced (S) form of *XBP1*. Top, illustrates a competitive PCR with decreasing amounts of plasmid encoding for *XBP1* WT and increasing amounts of plasmid encoding for the spliced form of *XBP1*. *B*, middle, a competitive PCR with constant amounts of *XBP1* WT plasmid combined with increasing amounts of the spliced form of *XBP1*. The ratio observed with 30 ng of *XBP1* WT plasmid and 20 ng of the spliced form of *XBP1* in (*A*) and (*B*) was determined to separate AML patients with induced versus uninduced XBP1s mRNA accordingly. *C*, whereas only the unspliced form of *XBP-1* mRNA is detectable in S- AML patients (*left, bottom*), both the spliced and unspliced form are observed in S+ AML patients (*right, bottom*), as assessed by semiquantitative reverse transcription-PCR. The expression of the *ABL1* gene is presented as a control.

CD9, CD11b, CD13, CD14, CD15, CD33, glycophorin, and myeloperoxidase), as well as lymphoid lineage–associated antigens (including CD2, CD3, CD7, CD10, CD19, CD22, CD79, and lineage nonspecific antigens, including HLA-DR, TdT, CD34, CD45, and CD56), was used to analyze the leukemic cells. The cutoff for a positive result of a particular marker was set at >20%.

All cytogenetic analyses were done at a single reference institution, at the University Hospital of Lausanne, Switzerland. Metaphase chromosomes were banded by conventional banding technique and karyotyped according to the International System for Human Cytogenetic Nomenclature. A karyotype was considered normal if at least 20 metaphases remained without evidence of a clonal abnormality.

***Reverse transcription-PCR and assay to detect the spliced variant of* XBP1 *mRNA* (XBP1s).** RNA was extracted using the RNA-Easy kit (Qiagen). In a 25-μL reaction, 1 μg of total RNA was incubated with 200 U M-MLV Reverse Transcriptase (Promega Corp.), 2.8 μg random primers (Roche Diagnostics), 1.12 U rRNasin (Promega Corp.), 13 nmol deoxynucleotide triphosphates (SIGMA), and 1× M-MLV RT reaction buffer (Promega Corp.) for 1 h at 42°C, followed by 5 min at 95°C. Quantitative reverse transcription-PCR was carried out on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using Taqman Universal PCR Master Mix (Roche). For *GRP78*, calreticulin, and *CHOP*, assay-on-demand gene expression probes (Applied Biosystems) were applied.

For *ABL* detection, the primers had the following sequences: sense, 5′-TGGAGATAACACTCTAAGCATAACTAAAGGT-3′; antisense, 5′-GATGTAGTTGCTGGGACCCA-3′; and probe, 5′-FAM-CCATTTTTGGTTTGGGCTTCACACCAT-TAMRA-3′. The relative quantitation was indicated by Ct values, determined based on duplicate reactions for each target and internal control gene. Duplicate Ct values were averaged and subtracted to obtain ΔCt [ΔCt = Ct (target gene) - Ct (control gene)]. The relative expression level was determined as $2^{-\Delta\Delta Ct}$, wherein ΔΔCt is ΔCt (target sample) – ΔCt (reference sample). For the reference sample, ΔΔCt = 0 and $2^{-0} = 1$. For the other samples, evaluation of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the reference sample (12).

To assess the induction of the *XBP1s* variant by PCR, 100 ng of cDNA were amplified applying one cycle of 94°C for 2 min, 37 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min, and a final extension for 5 min at 72°C. The following primers were used for *XBP1*: sense, 5′-GGGAATGAAGTGAGGCCAG-3′, and antisense, 5′-CAATACCGCCAGAATCCATG-3′. The primers correspond to nucleotides 412 to 431 and 834 to 853 of the *XBP1* cDNA, generating two PCR products of 442 (*XBP1u*) and 416 (*XBP1s*) bps, respectively (10). The samples were subsequently analyzed by gel electrophoresis. We confirmed in five endoplasmatic reticulum stress (ERS)(+) patients by sequencing of purified PCR products the bands to be in fact wild-type (WT) and spliced XBP1, respectively.

Statistical analysis. Overall survival was defined as the time from diagnosis to death. Disease-free survival was defined as the time from achievement of complete remission to first appearance of progression/relapse or death from any cause, and event-free survival was defined as the time from diagnosis to first appearance of progression/relapse or death from any cause. Patients alive without progression/relapse by the time of analysis were censored at the time of their last follow-up. overall survival, disease-free survival, and event-free survival were estimated with 95% confidence intervals. Survival curves were generated using the Kaplan-Meier method and were compared with the log-rank χ^2 test, with $P \leq 0.05$ defining significance. Complete remission was defined as patients having <5% blasts in their bone marrow assessed between days 18 and 20 after the onset of chemotherapy and having achieved peripheral hematopoietic recovery, defined as neutrophils >1.5 G/L and platelets >100 G/L, with >3 d after the last transfusion of blood products.

The Cox regression proportional hazard model (stepwise regression) was used in the multivariable analysis to explore the independent effect of the variables [activated unfolded protein response, WBC count, age, mutation of nucleophosmin (NPM1), and mutation of *fms*-related

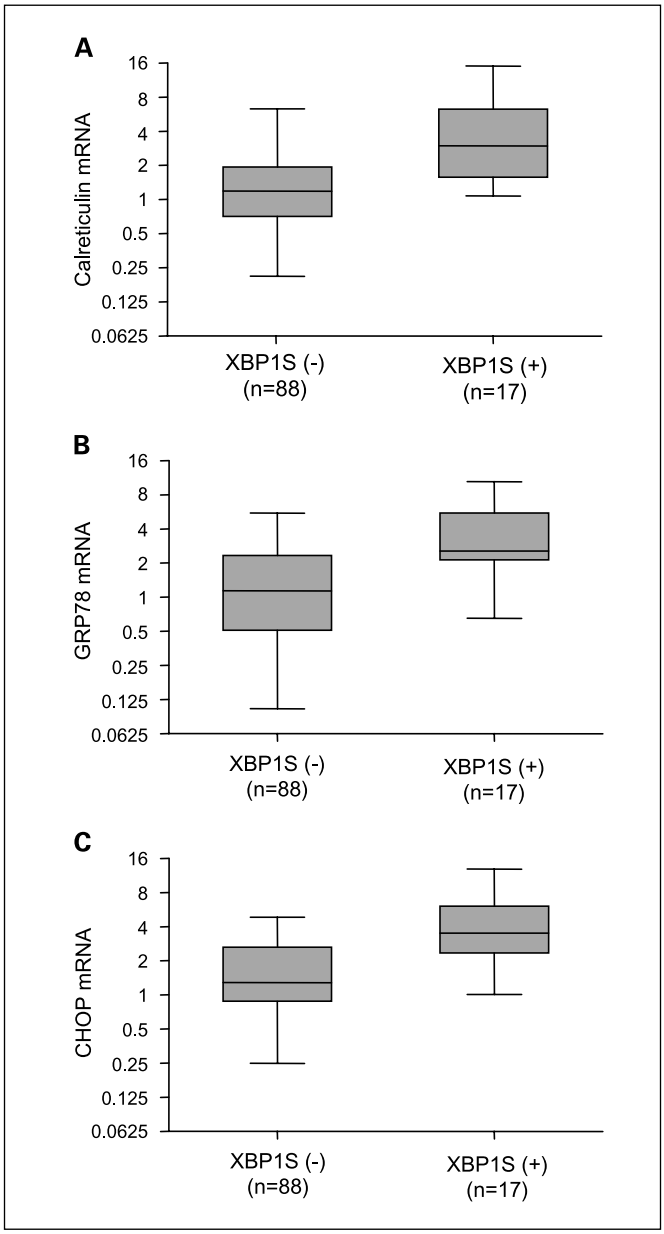

Fig. 2. Expression levels of the unfolded protein response target genes calreticulin, *GRP78*, and *CHOP* are increased in *XBP1s(+)* AML patients. AML patients expressing *XBP1s(+)* or patients lacking expression of the spliced form of *XBP1* mRNA [*XBP1s(-)*] are analyzed for calreticulin, *CHOP*, and *GRP78* mRNA expression. *N*-fold expression levels are depicted in log 2 scale for each gene. Boxes, interquartile ranges; horizontal bars, medians; thin bars, the 5th and 95th percentiles. *A, XBP1s(+)* AML patients expressed higher calreticulin levels compared with patients lacking expression of *XBP1s* (mean ± SD, 4.12 ± 2.6 versus 1.72 ± 1.8; $P = 0.0016$). *B, GRP78* mRNA levels were increased in *XBP1s(+)* AML patients compared with patients negative for *XBP1s* (mean ± SD, 3.54 ± 2.2 versus 1.94 ± 2.0; $P = 0.0220$). *C, CHOP* mRNA levels were increased in *XBP1s(+)* AML patients compared with patients negative for *XBP1s* (mean ± SD, 3.72 ± 1.8 versus 1.88 ± 2.2; $P = 0.0088$).

Table 1. Clinical characteristics of AML with and without activated unfolded protein response

		All (n = 105)	ERS(+) (n = 17)	ERS(-) (n = 88)	P
Sex		42 f/63 m	6 f/11 m	37 f/51 m	
Median age at diagnosis* (range)		61 (20-81)	60.5 (32-79)	61 (20-81)	0.6302[†]
Median WBC, $\times 10^9$/L (range)		19.85 (0.6-360)	4.3 (0.6-43)	27.05 (0.8-360)	0.0059
Median blasts in blood, % (range)		68 (0-99)	24.5 (0-78)	76 (0-99)	0.0079
Median LDH, units/L[‡]		798	495	892	0.0156
Extramedullary manifestations, n		21	3	16	0.5882
De novo AML, %		93.3	82.3	96.2	
secondary AML, %		6.7	17.7	3.8	0.0310
MDS, n		6	3	3	
Therapy related, n		0	0	0	
Consolidation in CR1[§]					
Chemotherapy, n		39	10	29	
Autologous transplant, n		14	1	13	
Allogenous transplant, n		17	3	14	
FAB classification	M0	9	2	7	
	M1	26	4	22	
	M2	27	4	23	
	M3	9	3	6	
	M4	20	3	17	
	M5	11	0	11	
	M6	2	1	1	
	M7	1	0	1	

Abbreviations: FAB, French American British; LDH, lactate dehydrogenase; MDS, myelodysplastic syndrome; f, female; m, male; CR1, first complete remission.
*Fifty-one of the 105 patients were >60 y at diagnosis.
[†]P values were calculated with the Mann-Whitney test; significance was assumed when $P < 0.05$.
[‡]LDH normal, <480 units/L.
[§]Seventy of 105 patients achieved a first complete remission after two cycles of induction and thus underwent consolidation therapy.

tyrosine kinase 3 (FLT3)] on overall survival and disease-free survival. Variables were retained in the model if they showed a statistically significant predictive value ($P \leq 0.05$). For all statistical analysis, SPSS software (version 12.0; SPSS) was used.

Results

XBP1s *is expressed in a subset of AML patients.* One of the initiating events during activation of the unfolded protein response is the cleavage of the *XBP1* mRNA, generating *XBP1s* (10, 13). Thereby, activated *IRE1* removes 26 nucleotides from the *XBP1* mRNA. Because of this frame shift deletion, a novel potent transcription factor is encoded at a molecular weight of 54 kDa instead of 34 kDa.

To screen AML patients for activation of the unfolded protein response, we analyzed mRNA from mononucleated bone marrow samples of 105 patients of all subtypes. Using PCR primers spanning the critical 26 nucleotides of the *XBP1*

Table 2. Molecular and karyotype abnormalities of AML with and without activated unfolded protein response

	All (n = 105)	ERS(+) (n = 17)	ERS(-) (n = 88)	P
FLT3-ITD, n (%)	28 (26.7)	1 (5.9)	27 (30.7)	0.0411
NPM1 mutation, n (%)	51 (48.6)	4 (23.5)	47 (53.4)	0.0322
CEBPA mutation, n (%)	8 (7.6)	1 (5.9)	7 (7.9)	0.4548
Good risk*, n (%)	18 (17.1)	4 (23.5)	14 (15.9)	
Intermediate risk[†], n (%)	57 (54.3)	6 (35.3)	51 (60.0)	
Bad risk[‡], n (%)	30 (28.6)	7 (41.2)	23 (26.1)	

*Good-risk patients comprised t(15;17), t(8;21), and inv(16) with n = 9, n = 4, and n = 5 patients, respectively. Three ERS(+) patients in this group had t(15;17), and one patient had t(8;21).
[†]Intermediate-risk patients showed a normal karyotype (n = 54), +8 (n = 2), and -Y (n = 1). The ERS(+) patients in this group all had a normal karyotype.
[‡]Bad-risk patients had complex (more than two numerical and/or structural non–core binding factor) anomalies (n = 23); -7 (n = 2); -7;t(2;11) (n = 1); inv(3) (n = 2); +11 (n = 1); and del(6q);del(7q) (n = 1). ERS(+) patients in this group comprised complex (all also involving -7 or -7q) anomalies (n = 4); -7 (n = 2); and -7;t(2;11) (n = 1). Results of the karyotype analysis and molecular diagnostics were available from all patients.

Table 3. Clinical outcome of AML patients with and without activated unfolded protein response

	All (*n* = 105)	ERS(+) (*n* = 17)	ERS(-) (*n* = 88)	*P*
CR1 achieved, % (*n*)	70.6 (81)	76.2 (13)	69.7 (68)	0.5724
Death in CR1, *n*	4	1	3	
Relapse of patients with CR1, % (*n*)	60.5 (49/81)	28.5 (4/13)	66.2 (45/68)	0.0182
OS at 2 y, %	23.5	39.5	19.1	
DFS at 2 y, %	18.2	34.3	13.8	
EFS at 2 y, %	19.4	34.3	16.5	
Patients disease-free				
At follow-up, *n* (%)	35 (33.3)	8 (47.1)	27 (30.7)	
Median, mo	27	28.5	26.7	
Range, mo	4-119	4-119	10-112	

Abbreviations: OS, overall survival; DFS, disease-free survival; EFS, event-free survival.

cDNA, we assessed the expression of the spliced and unspliced forms of *XBP1* mRNA. The sensitivity of the assay was determined with pcDNA3 (Invitrogen, Carlsbad, CA) plasmids encoding for human *XBP1* WT or the spliced form of *XBP1*. Figure 1A illustrates a competitive PCR using decreasing amounts of *XBP1* WT and increasing amounts of the spliced form of *XBP1*. In addition, Fig. 1B presents a competitive PCR with constant amounts of *XBP1* WT plasmid combined with increasing amounts of the spliced form of *XBP1*. The ratio observed with 30 ng of *XBP1* WT plasmid and 20 ng of the spliced form of *XBP1* was determined to separate AML patients with induced versus uninduced XBP1s mRNA. Representative examples from AML patients are depicted in Fig. 1C. We found that the *XBP1s* mRNA variant was present in 17 (16.2%) of 105 patients, whereas it was not detectable in mononuclear cells from bone marrow samples of 10 healthy individuals (data not shown). The group of AML patients with induced *XBP1s* mRNA is designated as S+ and patients with undetectable *XBP1s* mRNA as S-. The percentage of S+ patients was not different in bone marrow (17%) and peripheral blood samples (13%).

Expression of key mediators of the unfolded protein response is induced in AML patients with activated unfolded protein response (S+). Expression of the spliced form of *XBP1* mRNA is considered a sensitive and early marker for activation of the unfolded protein response. To validate our result above, we determined mRNA expression levels of additional unfolded protein response–associated chaperones such as calreticulin, *GRP78*, and *CHOP* in S+ and S- AML patients (Fig. 2). The Ct value of the target gene was normalized (ΔCt) to the Ct value of the *ABL* gene of the sample. The mean ΔCt value of all patients diagnosed with French American British classification–subtype M0 served as reference (1-fold).

In the group of S+ patients, median expression levels increased for calreticulin (2.6-fold), for *GRP78* (2.3-fold), and for *CHOP* (2.5-fold) as compared with S- patients. *P* values were <0.05 for all three genes tested. The increased expression observed for these unfolded protein response target genes in S+ patients supports the concept that the unfolded protein response is indeed activated in this subset of AML patients.

Clinical characteristics of AML patients with activated unfolded protein response. AML patients with activated unfolded protein response (S+) differed in several aspects from S- patients, as summarized in Table 1. Patients with activated unfolded protein response had significantly lower WBC counts and lower percentage of blasts in the peripheral blood at diagnosis. In addition, lactate dehydrogenase levels were lower in S+ patients. Secondary AML evolving from precedent myelodysplastic syndrome were more frequently observed among S+ patients (17.7% versus 3.8%). No differences were detected for age at diagnosis, gender distribution, and extramedullary manifestations at diagnosis. In addition, no clear preferences for any French American British classification subtypes could be attributed to S+ patients, even if no S+ patients were identified in the M5 subgroup. Despite analysis of an extensive panel of immunophenotype markers, no consistent pattern characteristic for S+ patients could be identified.

Molecular and karyotype abnormalities of AML patients with activated unfolded protein response. All patients in this study were analyzed by conventional cytogenetics at a single reference laboratory, as well as for mutations in the CCAAT/enhancer-binding protein á gene (*CEBPA*), for internal tandem duplications of the *fms*-like tyrosine kinase 3 (*FLT3-ITD*), and for mutations in *NPM1* (assessed by PCR and subsequent sequencing). The results are summarized in Table 2. In our panel of AML patients, *CEBPA* mutations seemed to be balanced among S+ and S- patients. However, *FLT3-ITD* was only rarely observed among S+ patients (5.9% versus 30.7%). In addition, *NPM1* mutations were identified to a lesser extent among S+ patients (23.5% versus 53.4%).

The karyotype analysis was normal in 54 (51.4%) of the 105 patients in this study. In contrast, only 35.2% (6/17) of S+ patients had a normal karyotype. Because *NPM1* and *FLT3* mutations are predominantly observed among normal karyotype AML patients, this may partly explain the low representation of these mutations observed among S+ patients. A rather high percentage (23.5%) of patients with good-risk abnormalities were seen among S+ patients. This was based on three patients with t(15;17) and one patient with t(8;21). In addition, S+ patients also had more frequently bad-risk karyotype abnormalities (41.1%) than S- patients (26.1%). Remarkably, the karyotype abnormalities of all S+ patients with cytogenetic bad-risk features involved aberrations of chromosome 7. In detail, the seven patients had complex anomalies involving −7 in two patients, complex anomalies involving del7q in two patients, monosomy 7 alone in two patients, and −7;t (2;11) in one patient.

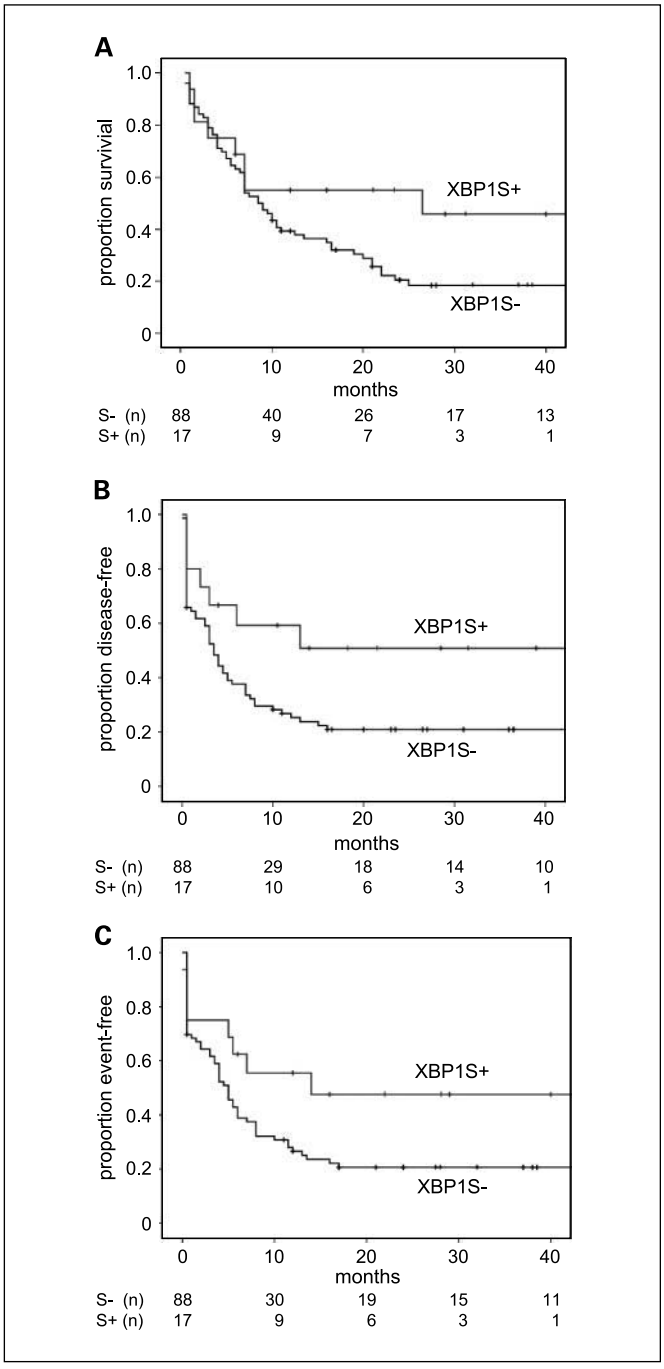

Fig. 3. Activated unfolded protein response is associated with favorable outcome in AML patients. Overall survival ($P = 0.041$; *A*), disease-free survival ($P = 0.022$; *B*), and event-free survival ($P = 0.018$; C) were determined for AML patients expressing *XBP1s(+*; $n = 17$) or patients lacking expression of the spliced form of *XBP1* mRNA [*XBP1s(-)*; $n = 88$]. Patients who received allogeneic stem cell transplant were censored at the time of transplant.

In summary, we observed S+ patients across all risk groups. They were seen less frequently among normal karyotype AML compared with S- patients. *FLT3-ITD* seemed to be rare among S+ patients. Finally, there was some evidence for an association between deletion of chromosome 7 and the activation of the unfolded protein response in AML patients. However, larger series are needed to clarify these observations.

Activation of the unfolded protein response is associated with favorable course of the disease in AML patients. To assess the prognostic significance of the activation of the unfolded protein response, we correlated activation of the unfolded protein response with clinical outcome data. Because S+ patients were observed in all three risk categories and because of small numbers in each risk group, the prognostic significance of activated unfolded protein response is assessed for S+ patients as one group and compared with S- patients.

The results depicted in Table 3 indicate that the response to induction chemotherapy was not significantly different. A complete remission was achieved in 76.2% of S+ patients compared with 69.7% of S- patients. Remarkably, however, the relapse rate was considerably lower (28.5%) for S+ patients than for S- patients (66.2%), with a *P* value of 0.0182. This translated into a significantly better overall survival at 2 years of 39.5% for S+ patients compared with 19.1 months for S- patients; and disease-free survival at 2 years was 34.3% (S+) and 13.8% (S-). The survival curves are presented in Fig. 3. These data indicate that activation of the unfolded protein response is associated with good response to induction chemotherapy and a favorable course of the disease.

Finally, we assessed the significance of the activation of the unfolded protein response in malignant cells of AML patients as an independent prognostic marker. In a multivariable analysis identifying *FLT3-ITD*, *NPM1* mutation, age at diagnosis, and peripheral leukocytes at diagnosis as independent factors, the activation of the unfolded protein response turned out to be of additional independent significance to indicate favorable overall and disease-free survival as presented in detail in Table 4.

Discussion

Current risk assessment in patients with AML is based on karyotype abnormalities and genomic mutations or deregulated expression of particular genes. Increasingly, treatment of patients with AML is directed by such markers if timely available. Here, we found that activation of the unfolded protein response in leukemic cells at diagnosis is associated with particular clinical characteristics and a more favorable course of the disease than in AML patients lacking activation of the unfolded protein response.

In this report, we determined the activation of the unfolded protein response in a large collection of AML patients involving all subtypes. Our analysis is based on the detection of the spliced variant of the *XBP1* mRNA, as well as the assessment of expression levels of early mediators of the unfolded protein response such as calreticulin, *GRP78*, and *CHOP*. We found a strong correlation between these effectors of the unfolded protein response. Of all AML patients, 16.2% were identified to have activated unfolded protein response in leukemic cells obtained at diagnosis. This is the first comprehensive report indicating that the unfolded protein response is activated in a subgroup of AML patients.

The accumulation of unfolded or misfolded proteins in the lumen of the endoplasmic reticulum induces the activation of the *IRE1* (ref. 6). Activation of *IRE1* induces the cleavage of the *XBP1* mRNA, generating a spliced mRNA, which encodes a potent novel transcriptional activator of downstream unfolded protein response target genes (7–10, 13). This spliced

Table 4. Multivariable analysis for overall survival and disease-free survival to assess the prognostic significance of the activated unfolded protein response in AML patients

	Overall survival		Disease-free survival	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
UPR*	0.58 (0.33-0.73)	0.006†	0.61 (0.32-0.72)	0.012†
WBC‡	1.44 (1.01-1.88)	0.029†	1.39 (0.99-1.84)	0.038†
Age§	1.18 (1.04-1.28)	0.011†	1.16 (1.02-1.26)	0.024†
NPM1‖	0.53 (0.33-0.70)	<0.001†	0.50 (0.22-0.77)	<0.001†
FLT3 WT¶	0.62 (0.25-0.79)	0.010†	0.64 (0.24-0.80)	0.018†

Abbreviations: 95% CI, 95% confidence interval; UPR, unfolded protein response.
*Activated unfolded protein response status was compared to nonactivated unfolded protein response status.
†$P < 0.05$ was considered significant.
‡WBC >20 × 10^9/L were compared with <20 × 10^9/L.
§Age >60 y was compared with age <60 y.
‖*NPM1* mutation status was compared with *NPM1* WT status.
¶*FLT3* WT status was compared with *FLT3-ITD* status.

mRNA can rapidly be detected by semiquantitative PCR, allowing the identification of activated unfolded protein response. We found that the detection of *XBP1s* represents a sensitive marker for activated unfolded protein response, with possible implications for its use in clinical practice.

Besides splicing of the *XBP1* mRNA, we also observed that AML patients with activated unfolded protein response have increased levels of calreticulin, *GRP78*, and *CHOP*. Induction of these endoplasmic reticulum chaperones is crucial for cell survival by re-establishing correct folding of proteins, preventing their aggregation and ultimately protecting cancer cells against endoplasmic reticulum stress–induced apoptosis (14, 15). *GRP78* expression was reported to be induced in a variety of human solid tumors (16–23), whereas its expression in hematologic malignancies has not been investigated thus far. Here, we observed that malignant cells from AML patients with activated unfolded protein response expressed higher *GRP78* and *CHOP* levels.

The conditions activating the unfolded protein response in leukemic cells remain to be elucidated. There is some evidence that chromosome rearrangements may also lead to induction of endoplasmic reticulum stress. Acute promyelocytic leukemia is characterized by the expression of the fusion gene product of the promyelocytic leukemia gene (*PML*) and the retinoic acid receptor á (RARá). It was suggested that PML-RARá induces the accumulation of the nuclear hormone receptor corepressor (*N-CoR*) in the endoplasmic reticulum, leading to induction of endoplasmic reticulum stress and the processing of *ATF6* (24). The unfolded protein response–induced apoptosis in acute promyelocytic leukemia cells is circumvented through selective expression of a glycoprotease, which is processing misfolded *N-CoR* protein (25). Interestingly, we found activated unfolded protein response in a considerable percentage of AML-M3 patients in our study.

We identified calreticulin to be increased in the group of AML patients with activated unfolded protein response. Besides its role in proper folding of glycoproteins in the endoplasmic reticulum and its importance for maintaining intracellular Ca2+-homeostasis (26), calreticulin was previously identified as a potent RNA-binding protein, thereby blocking the translation of specific mRNAs such as *CEBPA* and *CEBPB* (27). Our group has reported that increased calreticulin expression in certain subtypes of AML is associated with activated binding of calreticulin to *CEBPA* mRNA and consecutive suppression of CEBPA protein (11). The concept of unfolded protein response triggered induction of calreticulin leading to suppression of CEBPA protein in AML patients provides a rationale for endoplasmic reticulum stress being involved in leukemogenesis.

Misfolded proteins are usually transported from the endoplasmic reticulum to the cytoplasm and targeted to proteasomal degradation. Accumulation of proteins in the cytoplasm inhibits the transport of unfolded proteins from the endoplasmic reticulum, thereby increasing the amount of misfolded proteins in the endoplasmic reticulum lumen, inevitably inducing the unfolded protein response and, ultimately, apoptosis of the cell. Our findings of activated unfolded protein response in malignant cells of AML patients might provide a rationale for novel treatment strategies in AML. Cell lines with activated unfolded protein response are especially prone to undergo apoptosis after treatment with the proteasome inhibitor bortezomib, and this concept has become a standard treatment for multiple myeloma patients (28). In line with this, high expression of calreticulin in patients with light chain amyloidosis predicts responsiveness to treatment with the alkylating agent melphalan (29). Moreover, toxicity of melphalan seems to be increased *in vitro* in cells pretreated with endoplasmic reticulum stress–inducing agents (30). These reports suggest increased responsiveness to chemotherapy in malignant cells with activated unfolded protein response. In particular, this study might provide a rationale for the use of bortezomib together with standard regimens in AML patients presenting with an activated unfolded protein response in the leukemic cells at diagnosis. Based on our study, patients with monosomy 7 or del7q might be promising candidates to profit from such a treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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