University of Groningen

Heat Shock Factor 1 (HSF1-pSer326) Predicts Response to Bortezomib-Containing Chemotherapy in Pediatric AML

Hoff, Fieke W; van Dijk, Anneke D; Qiu, Yi Hua; Ruvolo, Peter P; Gerbing, Robert B; Leonti, Amanda R; Jenkins, Gaye N; Gamis, Alan S; Aplenc, Richard; Kolb, E Anders

Published in:
Blood

DOI:
[10.1182/blood.2020005208](https://doi.org/10.1182/blood.2020005208)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Final author's version (accepted by publisher, after peer review)

Publication date:
2021

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Hoff, F. W., van Dijk, A. D., Qiu, Y. H., Ruvolo, P. P., Gerbing, R. B., Leonti, A. R., Jenkins, G. N., Gamis, A. S., Aplenc, R., Kolb, E. A., Alonzo, T., Meshinchi, S., De Bont, E., Bruggeman, S. W., Kornblau, S. M., & Horton, T. M. (2021). Heat Shock Factor 1 (HSF1-pSer326) Predicts Response to Bortezomib-Containing Chemotherapy in Pediatric AML: A COG Study. *Blood*, 137(8), 1050-1060.
<https://doi.org/10.1182/blood.2020005208>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
editorial@hematology.org

Heat Shock Factor 1 (HSF1-pSer³²⁶) Predicts Response to Bortezomib-Containing Chemotherapy in Pediatric AML: A COG Study

Tracking no: BLD-2020-005208R1

Fieke Hoff (University Medical Center Groningen, Netherlands) Anneke van Dijk (University Medical Center Groningen, Netherlands) Yi Hua Qiu (M.D.Anderson Cancer Center, United States) Peter Ruvolo (University of Texas MD Anderson Cancer Center, United States) Robert Gerbing (Children's Oncology Group, United States) Amanda Leonti (Fred Hutchinson Cancer Research Center, United States) Gaye Jenkins (Baylor College of Medicine/Dan L. Duncan Cancer Center and Texas Children's Cancer Center, United States) Alan Gamis (Children's Mercy Hospital, United States) Richard Aplenc (CHOP/UPENN, United States) E. Anders Kolb (Nemours/Alfred I duPont Hospital for Children, United States) Todd Alonzo (University of Southern California, United States) Soheil Meshinchi (Fred Hutchinson Cancer Research Center, United States) Eveline De Bont (University Medical Center Groningen, Netherlands) Sophia Bruggeman (European Research Institute for the Biology of Ageing (ERIBA), Netherlands) Steven Kornblau (University of Texas M. D. Anderson Cancer Center, United States) Terzah Horton (Baylor College of Medicine/Dan L. Duncan Cancer Center and Texas Children's Cancer Center, United States)

Abstract:

Bortezomib (BTZ) was recently evaluated in a randomized Phase 3 clinical trial which compared standard chemotherapy (cytarabine, daunorubicin, etoposide; ADE) to standard therapy with BTZ (ADEB) for *de novo* pediatric acute myeloid leukemia. While the study concluded that BTZ did not improve outcome overall, we examined patient subgroups benefitting from BTZ-containing chemotherapy using proteomic analyses. The proteasome inhibitor BTZ disrupts protein homeostasis and activates cytoprotective heat shock responses. We measured total heat shock factor 1 (HSF1) and phosphorylated HSF1 (HSF1-pSer³²⁶) in leukemic cells from 483 pediatric patients using Reverse Phase Protein Arrays. HSF1-pSer³²⁶ phosphorylation was significantly lower in pediatric AML compared to CD34+ non-malignant cells. We identified a strong correlation between HSF1-pSer³²⁶ expression and BTZ sensitivity. BTZ significantly improved outcome of patients with low-HSF1-pSer³²⁶ with a 5-year event-free survival of 44% (ADE) vs. 67% for low-HSF1-pSer³²⁶ treated with ADEB ($P=0.019$). To determine the effect of HSF1 expression on BTZ potency *in vitro*, cell viability with HSF1 gene variants that mimicked phosphorylated (S326A) and non-phosphorylated (S326E) HSF1-pSer³²⁶ were examined. Those with increased HSF1 phosphorylation showed clear resistance to BTZ vs. those with wild type or reduced HSF1-phosphorylation. We hypothesize that HSF1-pSer³²⁶ expression could identify patients that benefit from BTZ-containing chemotherapy.

Conflict of interest: COI declared - see note

COI notes: TMH receives research funding from Takeda Pharmaceuticals.

Preprint server: No;

Author contributions and disclosures: F.W.H., E.S.J.M.d.B., S.W.M., S.M.K., T.M.H. designed and supervised the research, F.W.H., A.D.D., Y.Q., G.N.J. performed research, A.S.G., R.A., E.A.K., T.A.A., S.M.K., T.M.H. collected data, F.W.H., P.P.R., R.G., A.R.L., S.M., S.M.K., T.M.H. analyzed data, and F.W.H., S.M.K., T.M.H. wrote the paper.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Data will be provided in a data supplement available with the online version of this article. Data will also be deposited to "www.leukemiaatlas.org"

Clinical trial registration information (if any):

Heat Shock Factor 1 (HSF1-pSer³²⁶) Predicts Response to Bortezomib-Containing Chemotherapy in Pediatric AML: A COG Report

Running head: HSF1-pSer³²⁶ Predicts Bortezomib Response in AML

Fieke W. Hoff¹, Anneke D. van Dijk¹, Yihua Qiu², Peter P. Ruvolo^{2,3}, Robert B. Gerbing⁴, Amanda R. Leonti⁵, Gaye N. Jenkins⁶, Alan S. Gamis⁷, Richard Aplenc⁸, E. Anders Kolb⁹, Todd A. Alonzo^{4,10}, Soheil Meshinchi⁵, Eveline S. J. M. de Bont¹, Sophia W. M. Bruggeman¹¹, Steven M. Kornblau^{2,†}, Terzah M. Horton^{6,†}

¹Department of Pediatric Oncology/Hematology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; ²Department of Leukemia, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA; ³Section of Molecular Hematology, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA; ⁴COG Statistics and Data Center, Monrovia, CA, USA; ⁵Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA; ⁶Department of Pediatrics, Baylor College of Medicine/Dan L. Duncan Cancer Center and Texas Children's Cancer and Hematology Centers, Houston, TX, USA; ⁷Department of Hematology-Oncology, Children's Mercy Hospitals and Clinics, Kansas City, MO, USA; ⁸Division of Pediatric Oncology/Stem Cell Transplant, Children's Hospital of Philadelphia, Philadelphia, PA, USA; ⁹Nemours/Alfred I. Dupont Hospital for Children, Atlanta, GA, USA; ¹⁰Keck School of Medicine, University of Southern California, CA, USA; ¹¹European Research Institute for the Biology of Ageing (ERIBA), University Medical Center Groningen, University of Groningen, the Netherlands; [†]Co-senior author.

Corresponding author: Terzah M. Horton, Department of Pediatrics, Baylor College of Medicine/Dan L. Duncan Cancer Center and Texas Children's Cancer and Pediatric Centers, 1102 Bates, Suite 750, Houston, TX 77030. Phone: 832-824-4269, Email: tmhorton@txch.org.

Key words: Integrated Stress Response; Reverse Phase Protein Array; RPPA; Proteomics; HSF1; Heat Shock Response

Financial support: TMH was funded by the NIH COG Grants U10 CA98543, U10 CA98413, U10 CA180886, 1U24-CA196173 and U10 CA180899, the St. Baldrick's Foundation, the R01-CA164024 and a grant from Takeda Pharmaceuticals. FWH was funded by the Junior Scientific Masterclass, Groningen, the Netherlands, the Van der Meer Boerema Foundation, and the Ter Meulen Grant of the Royal Netherlands Academy of Arts and Sciences.

Word count main text: 3946

Word count abstract: 200

Number of tables: 2

Number of figures: 6

Reference count: 46

Scientific category: Myeloid neoplasia

Key points

1. Low-HSF1-pSer³² is a favorable prognostic protein in patients treated with bortezomib-containing chemotherapy;
2. Addition of bortezomib to standard chemotherapy significantly improves outcome in low-HSF1-pSer³²⁶ pediatric AML patients.

Abstract

Bortezomib (BTZ) was recently evaluated in a randomized Phase 3 clinical trial which compared standard chemotherapy (cytarabine, daunorubicin, etoposide; ADE) to standard therapy with BTZ (ADEB) for *de novo* pediatric acute myeloid leukemia. While the study concluded that BTZ did not improve outcome overall, we examined patient subgroups benefitting from BTZ-containing chemotherapy using proteomic analyses. The proteasome inhibitor BTZ disrupts protein homeostasis and activates cytoprotective heat shock responses. We measured total heat shock factor 1 (HSF1) and phosphorylated HSF1 (HSF1-pSer³²⁶) in leukemic cells from 483 pediatric patients using Reverse Phase Protein Arrays. HSF1-pSer³²⁶ phosphorylation was significantly lower in pediatric AML compared to CD34+ non-malignant cells. We identified a strong correlation between HSF1-pSer³²⁶ expression and BTZ sensitivity. BTZ significantly improved outcome of patients with low-HSF1-pSer³²⁶ with a 5-year event-free survival of 44% (ADE) vs. 67% for low-HSF1-pSer³²⁶ treated with ADEB ($P=0.019$). To determine the effect of HSF1 expression on BTZ potency *in vitro*, cell viability with HSF1 gene variants that mimicked phosphorylated (S326A) and non-phosphorylated (S326E) HSF1-pSer³²⁶ were examined. Those with increased HSF1 phosphorylation showed clear resistance to BTZ vs. those with wild type or reduced HSF1-phosphorylation. We hypothesize that HSF1-pSer³²⁶ expression could identify patients that benefit from BTZ-containing chemotherapy.

Introduction

Despite significant improvements in long-term survival, pediatric acute myeloid leukemia (AML) still fare poorly when compared to pediatric acute lymphocytic leukemia (ALL)¹. Following induction therapy, approximately 90% of children achieve complete remission (CR), but 50% will eventually relapse². Curability after relapse is poor, with only one-third being cured³. This indicates that new therapeutic strategies are needed to further improve outcome. The proteasome inhibitor bortezomib (BTZ) is effective in multiple myeloma^{4,5} and mantle cell lymphoma⁶. Adult AML clinical trials have also demonstrated that BTZ can be safely combined with standard AML chemotherapy⁷⁻⁹. The Children's Oncology Group (COG) recently evaluated BTZ in a Phase 3 clinical trial randomizing *de novo* AML patients to cytarabine arabinoside (Ara-C)/daunorubicin/etoposide (ADE) with or without BTZ (ADEB). The trial was closed early, after inclusion of 950 of 1150 planned patients, when results suggested that BTZ addition did not improve overall survival (OS), event-free survival (EFS) or treatment-related mortality¹⁰. As part of a pre-planned analysis, we examined protein expression to determine if there were patients with specific proteomic patterns that benefitted from BTZ-containing chemotherapy.

BTZ is a proteasome inhibitor that specifically targets the $\beta 5$ -subunit of the 26S proteasome, disturbing cellular protein homeostasis. This results in a toxic buildup of misfolded proteins in the cytosol and proteomic stress that can result in cell death. With BTZ treatment, cells activate their adaptive mechanisms, including the endoplasmic reticulum-mediated unfolded protein response and the heat shock response (HSR). The HSR is a protective and pro-survival mechanism that maintains protein stability by inducing protein chaperones, allowing malignant cells to survive proteotoxic stress. The heat shock response is mediated by the

evolutionarily conserved heat shock factor 1 (HSF1). High levels of HSF1 correlate with unfavorable prognosis in solid cancers, including breast, lung, and colon cancer. However, no role for HSF1 has yet been ascertained in AML¹¹.

In response to a wide range of stresses, HSF1 is activated and translocates to the nucleus where it binds to heat shock-responsive DNA elements and upregulates genes encoding heat shock proteins¹². One of the important phosphorylation sites responsible for this activation is serine domain 326 (HSF1-pSer³²⁶), which is phosphorylated via the upstream kinases p38¹³, MEK¹⁴ and mTOR^{15,16}. Emerging evidence in multiple myeloma¹⁷ and non-small lung carcinoma¹⁸ demonstrates that heat shock proteins (HSP), including HSF1-pSer³²⁶, are upregulated following BTZ treatment and that knock down of HSF1 increases sensitivity to BTZ¹⁹⁻²¹. Others report that defects in the ubiquitin-proteasome system leads to activation of members of the heat shock family²². This suggests that HSF1 and BTZ act in opposing circumstances, which implies that activation of the HSR via activation of HSF1 could limit the efficacy of BTZ. We sought to determine if the level of HSF1-pSer³²⁶ modulated the effectiveness of BTZ-containing chemotherapy when used in pediatric AML treatment. Using Reverse Phase Protein Arrays (RPPA), we measured protein expression levels of HSF1-pSer³²⁶ in peripheral blood (PB) samples from 483 pediatric AML patients treated on the COG clinical trial AAML1031 (NCT01371981). We determined a significant and clinically relevant correlation between low HSF1-pSer³²⁶ expression and outcome in children with pediatric AML treated with BTZ-containing chemotherapy.

Methods

Pediatric AML patient samples

PB samples were obtained from 483 *de novo* pediatric AML patients that participated in the COG AAML1031 Phase 3 clinical trial, and CD34+ bone marrow (BM) samples obtained from 30 healthy donors; 20 pediatric CD34+ samples and 10 adults CD34+ samples. Samples from adults were used included to allow future comparison to adult AML samples which were published previously²³. Patient samples from the AAML1031 were acquired during routine diagnostic assessments prior to (i.e. at time of diagnosis), 10 hours after and 24 hours after the start of chemotherapy. Samples were collected between July 2011 and February 2017. Written informed consent was obtained in accordance with *Declaration of Helsinki*. The associated demographics are described in **Table 1**.

Outcome data was available for 410 of the patients enrolled on the AAML1031 study. One hundred and sixty-four patients received standard ADE induction therapy, 210 patients received ADE in combination with BTZ (ADEB), and 36, with known FLT3-ITD mutations, received ADE + sorafenib (ADES). As we are particularly interested in the additional effect of BTZ, and as the patients treated with ADE and ADES had no statistically significant difference in outcome, patient treated with ADE and ADES were combined for proteomic analysis ($N=200$). Three hundred forty-eight (85%) patients achieved CR by the end of course 2, 31 (7.6%) patients were refractory or died (failed therapy), 156 (45%) patients relapsed after remission, and 286 were still alive at the end of their follow-up (70%). Patients had mutation data available for CEBP α , NPM1 and FLT3-ITD.

Cell lines

HEK 293T cells were purchased from the DSMZ (Braunschweig, Germany) and were cultured in DMEM containing 10% fetal calf serum (GIBCO), 1% penicillin and streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO₂.

RPPA methodology

The methodology and validation of the RPPA technique are fully described elsewhere²⁴⁻²⁷. Briefly, fresh samples were isolated by centrifugation using lymphocyte separation solution (Sigma) and enriched for leukemic cells by CD3/CD19 depletion (Miltenyi Biotech, Cologne, Germany). Protein preparations were normalized to a concentration of 1x10⁷ cells/mL and printed in five serial dilutions onto slides along with normalization and expression controls. Samples were printed on two slides per antibody, each containing 1152 samples (i.e., patient samples, controls). Given the high inter- and intraarray reproducibility and high staining precision of RPPA, no replicate samples were used²⁷. Slides were probed with 300 antibodies listed in **Table S1**, including a primary validated antibody against HSF1 phosphorylated at serine 326 (Abcam, Cambridge, UK,) and HSF1-total (Cell signaling, Danvers, MA). Stained slides were analyzed using Microvigen® Software (version 3.0, Vigen Tech, Carlisle, MA). *SuperCurve* algorithms were used to generate a single value from the five serial dilutions²⁸. Loading controls²⁹ and topographical normalization³⁰ procedures were performed to account for protein concentration and background staining variations on each array. Replicates-Based Normalization³¹ was used to align samples from two different slides. Overlapping samples (n=98) between the two slides had an average Pearson's correlation coefficient of 0.88 (SD: +/-0.07). Of the 300 antibodies, two antibodies were excluded due to a poor Pearson's correlation coefficient (CDX2, PSMB9). Based on significant differences in protein expression of paired samples that were collected in different preservative tubes (i.e., heparin vs. Cell Save), three antibodies were excluded (H2AX-pSer¹⁴⁰, MYH9-pSer¹⁹⁴³,

PRKAA1_2-pTyr¹⁷²), leaving 293 antibodies in addition to total and HSF1-pSer³²⁶ for analysis. For each protein, the median expression levels of the 30 normal BM CD34+ samples were subtracted from the expression in the patient samples to obtain relative protein expression levels.

Quality control

To exclude low-quality samples, we removed samples that showed a greater number of proteins with extreme levels of expression, using a cut-off value of + 3SD mean protein expression. Twenty-two (1.4%) of the 1524 samples did not meet the QC criteria and were excluded.³²

Gene expression profiling

Ribodepleted RNA-sequencing data was generated as described elsewhere³³. Expression data was available for 390/483 RPPA-patients and for 1,063 eligible patients enrolled in the clinical trial. HSF1 gene expression was compared to 21,927 other genes.

Western blotting

Western blot analysis was performed as previously described³⁴. Antibodies were used targeting HSF1-total (Cell signaling; Catalog #4353; 1:1,000), HSF1-pSer³²⁶ (Abcam; Catalog #ab76076; 1:10,000), and Actin beta (Santa Cruz Biotechnology; Catalog #Sc-47778; 1:2,000).

HSF1 and HSF1 mutants' construction and overexpression

pIRES-puro3 expression vectors were used containing an empty vector, RNA sequence targeting HSF1 S326A or HSF1 S326E mutants. The full method is described elsewhere³⁵.

Cell viability and chemicals

Quantification of leukemia cell viability was carried out using Water Soluble Tetrazolium-1 cell proliferation assays as described previously³⁴. Cells were seeded at a density of 20,000 cells per 100 μ l/well in a 96-well plate format and treated with appropriate inhibitor concentrations. Compounds: bortezomib (Bio-Connect), etoposide (Accord).

Statistical analysis

Outcome data for each patient was calculated from June 30, 2019, with a median follow-up period of 4.4 years (range 0.3-7.5 years). Estimates of OS and EFS were calculated using the Kaplan–Meier method. OS and EFS were defined as time from study entry until death or until relapse, secondary malignancy or death, respectively. Relapse risk (RR) was calculated using methods of competing events and was defined as the time from the end of two courses of induction (for patients in CR) to relapse, where deaths without a relapse were considered competing events. The significance of predictor variables was tested with the log-rank statistic for OS, EFS and with Gray’s statistic for RR. Cox proportional hazard models were used to estimate hazard ratios for univariable and multivariable analyses of OS and EFS. Competing risk regression models were used for analyses of RR. Outcome by treatment arm was based on intention-to treat analysis.

Relative HSF1-pSer³²⁶ expression was initially examined for continuous vs. step-wise correlations with outcome (**Figure S1**). HSF1-pSer³²⁶ protein expression negatively correlated with outcome in a step-wise fashion in ADEB-treated patients. Based on this observation, patients were divided by relative HSF1-pSer³²⁶ expression into thirds. Proportions between the three clusters (low, medium, and high HSF1-pSer³²⁶) and categorical

clinical variables were compared using Pearson's Chi-square test. The medians of the continuous variables were compared using the Kruskal-Wallis test. Pearson's correlation analyses were performed to investigate correlation between HSF1-pSer³²⁶ protein expression levels and the other 293 proteins assessed on the RPPA and between gene expression data of HSF1 and 21,927 other genes. *P*-values were adjusted using FDR correction. All statistical analyses were performed in R (Version 0.99.484 –2009-2015 RStudio, Inc.) or SAS (version 9.4).

Results

Heterogeneous protein expression of HSF1-pSer³²⁶ in pediatric AML samples

Relative protein expression levels of HSF1-total and HSF1-pSer³²⁶ were analyzed using RPPA in myeloblast cells from 483 *de novo* pediatric AML patients and 20 pediatric and 10 adult CD34+ samples from healthy donors. Samples were collected at time of diagnosis prior to chemotherapy. HSF1-total was significantly lower in pediatric AML compared to CD34+ BM cells from healthy donors ($P=7.6E-09$) (**Figure 1A**). Likewise, expression of HSF1-pSer³²⁶ was statistically lower in pediatric AML compared to nonmalignant CD34+ cells ($P=2.2E-12$) (**Figure 1B**). To assess the correlation between expression of HSF1-total and HSF1-pSer³²⁶, Pearson's correlation analysis was performed. This resulted in a modest correlation of 0.34 ($P=8.4 E-15$); most variation in HSF1 phosphorylation intensities was observed in the patients that expressed higher levels of HSF1-total (**Figure 1C**).

Low-HSF1-pSer³²⁶ protein expression is prognostically favorable in patients receiving BTZ.

Among all patients (ADE and ADEB combined) HSF1-total expression was in general not predictive for OS, EFS and CR duration. However, patients with the highest 3rd HSF1-total protein expression experienced inferior OS when compared to the lowest 2/3rd ($P=0.032$), and a trend toward a higher EFS ($P=0.078$) and RR ($P=0.035$) (**Figure S2A-C**). Analysis of HSF1-pSer³²⁶ expression also showed no significant differences in outcome associated with increasing-HSF1-pSer³²⁶ expression levels (OS: $P=0.140$; EFS: $P=0.203$; RR: $P=0.375$) (**Figure S2D-F**).

However, a very different picture emerged when the effect of HSF1-pSer³²⁶ level was assessed separately within the ADE or ADEB treatment groups (**Figure 2**). For those treated with ADE alone the relative HSF1-pSer³²⁶ protein expression had no effect on OS, EFS or RR (OS: $P=0.698$; EFS: $P=0.572$; RR: $P=0.535$). In contrast, for those receiving BTZ-containing chemotherapy (**Figure 2D-F**), the level of HSF1-pSer³²⁶ affected all three outcome measures; patients with middle or high HSF-pSer³²⁶ expression had stepwise decreases in OS and EFS associated with a corresponding increase in the relapse rate compared to those with low-HSF1-pSer³²⁶ (OS: $P=0.011$, EFS: $P=0.002$, RR: $P=0.006$). The 5-year OS was 83% in the low-HSF1-pSer³²⁶ patients, versus 73% and 60% for the middle- and high-HSF1-pSer³²⁶ patients, respectively. The 5-year EFS decreased from 67% in low HSF1-pSer³²⁶ to 38% for high-HSF1-pSer³²⁶, and RR increased from 27% to 54%. This suggests that low-HSF1-pSer³²⁶ correlates with more favorable outcome in ADEB-treated pediatric AML patients. Although a similar trend was observed for HSF1-total, the effect was less strong compared to HSF1-pSer³²⁶ (**Figure S3**).

BTZ with ADE is beneficial in low-HSF1-pSer³²⁶, but not in high-HSF1-pSer³²⁶ patients

To evaluate whether the addition of BTZ to ADE therapy improved outcome compared to ADE alone, clinical outcome for low- and high-HSF1-pSer³²⁶ was compared between ADE and ADEB. Notably, the addition of BTZ significantly improved EFS and decreased the RR for those with low HSF1-pSer³²⁶ (5yr-OS, 68% vs. 83%, $P=0.056$; 5yr-EFS, 44% vs. 67%, $P=0.019$; 5-yr RR; 46% vs. 27%, $P=0.049$) (**Figure 3A-C**). However, in high-HSF1-pSer³²⁶ patients, the trend is reversed; EFS decreased from 55% to 38% in the patients with BTZ ($P=0.035$) and RR increased from 33% to 54% ($P=0.024$) (**Figure 3D-F**). Therefore, the addition of BTZ improved the outcome of those with low-HSF1-pSer³²⁶, but as levels of HSF1-pSer³²⁶ rise this beneficial effect is negated.

HSF1-pSer³²⁶ expression is independent of most clinical and patient characteristics

Because we observed a strong correlation with HSF1-pSer³²⁶ levels and outcome in the ADEB treated patients, we evaluated for associations between HSF1-pSer³²⁶ levels and various demographic and clinical features (**Table 1**). Among these, five showed differences between the cohorts. CEBP α was most frequently mutated in patients with low-HSF1-pSer³²⁶ (16.2% vs. 9.5% overall, $P=0.01$); however, the good-risk translocation (8;21) was unexpectedly enriched in the high-HSF1-pSer³²⁶ patients (23.4% vs. 15.9% overall, $P=0.01$). Median peripheral blast percentage (52.5% vs. 62%, $P=0.014$), white blood count ($34.6 \times 10^3/\mu\text{L}$ vs 55.4, $P=0.015$) and absolute number of peripheral blasts (14.59 vs 27.9, $P=0.004$) were all higher in the high-HSF1-pSer³²⁶ patients compared to the low- and middle-HSF1-pSer³²⁶ patients (**Table S2**).

HSF1-pSer³²⁶ as an independent predictor of survival in ADEB treated patients

Next, univariable and multivariable analysis were performed to determine if HSF1-pSer³²⁶ was an independent prognostic marker for OS, EFS or RR in ADEB treated patients. Independent predictors from univariate analysis were HSF1-pSer³²⁶ expression and the AAML1031 risk group stratification (**Table S3**). In multivariable analysis both HSF1-pSer³²⁶ and AAML1031 risk remained an independent prognostic variable for OS and EFS, while HSF1-pSer³²⁶ was the only independent prognostic variable for RR (**Table 2**). For patients treated without BTZ, HSF1-pSer³²⁶ was not found to be an independent prognostic marker.

HSF1-pSer³²⁶ sensitivity to BTZ *in vitro*

To determine if HSF1 phosphorylation affected BTZ potency *in vitro*, HSF1 variants³⁵ were expressed in 293T cells. One HSF1 variant mimicked the non-phosphorylated-HSF1 state by

replacing the phosphorylatable serine 326 (S326) with an unmodifiable (inert) alanine (S326A). The second mimicked the phosphorylated HSF1-pSer³²⁶ state by substituting glutamic acid (S326E), which simulates active pSer³²⁶. Cell viability assays were performed to assess the effect of HSF1 phosphorylation ON/OFF state on cell survival. Cells with overexpressed HSF1 S326E (increased/ON-state HSF1-pSer³²⁶ expression) were more resistant to BTZ, whereas cells that expressed HSF1 S326A (decreased/OFF-state HSF1-pSer³²⁶ expression) were more sensitive to BTZ compared to empty vector (**Figure 4A**). These two mutations of HSF1-pSer³²⁶ site had no effect on etoposide potency, suggesting that the serine 326 phosphorylation site is crucial for BTZ sensitivity (**Figure 4B**).

Effect of chemotherapy on HSF1-pSer³²⁶ expression is dependent on the relative abundance of HSF1-pSer³²⁶

HSF1 is a stress protein and it has previously been shown that HSF1-pSer³²⁶ increases with BTZ treatment in multiple myeloma^{21,41}. To compare HSF1-pSer³²⁶ protein expression following chemotherapy in patients with high-HSF1-pSer³²⁶ vs. low-HSF1-pSer³²⁶, we compared expression levels in pretreatment PB samples to those collected at 10 and 24 hours after the start of chemotherapy. We observed that among patients with lower pretreatment expression of HSF1-pSer³²⁶ (**Figure 5A**), expression levels increased following treatment (median + 0.7 log2), but did not reach the pretreatment levels observed in high HSF1-pSer³²⁶ patients. Those with high pretreatment HSF1-pSer³²⁶ (**Figure 5B**), had no change in the level of HSF1-pSer³²⁶ over time. These patterns were unaffected by which treatment arm was received (ADE vs. ADEB). (**Figure S4**).

HSF1-pSer³²⁶ strongly correlated with translation and ribosomal proteins

The pediatric AML RPPA array identified 90 of the other 294 analyzed proteins that were significantly correlated with HSF1-pSer³²⁶ ($r \geq 0.25$ or $r \leq -0.25$, $P < 0.05$ / $292 < 0.00017$);

59 were negatively correlated and 31 (including HSF1-total) were positively correlated (**Figure 6/ Table S4**). Of the positively correlated proteins, 24 of 31 (77%) were proteins that had undergone post-translational modifications, versus only 5/59 (8%) of the negatively correlated proteins, suggesting that the predominant method of HSF1-pSer³²⁶ effect is through pathway activation, rather than by altering total protein expression. Proteins that were significantly correlated with HSF1-pSer³²⁶ included proteins involved in protein translation and ribosomal processes (e.g. EIF4EBP1-pTyr³⁷⁻⁴⁶, EIF4E, RPS6-pSer²³⁵⁻²³⁶ etc., **Figure 6A** shown in red), as well as phospho-MAP kinase (known to be responsible for phosphorylation of HSF1) phospho-SMAD2, phospho-GSK3, phospho-MDM2 and phospho-AKT.

To identify if similar pathways could be identified computationally, STRING-network analysis was performed using the proteins that were correlated with HSF1-pSer³²⁶. This resulted in a protein: protein enrichment p -value $< 1.0^{-16}$. STRING analysis revealed that HSF1-pSer³²⁶ associations were enriched for proteins associated with protein binding, translation regulation, RNA binding, ribonucleoprotein complex binding, translation factor activity, RNA binding and heat shock protein binding (**Table S5**).

HSF1-pSer³²⁶ protein relationships to HSF1 transcript expression

To compare HSF1 transcript levels with HSF1-total protein abundance, we correlated HSF1 mRNA levels from 390 pediatric AML patients with HSF1 transcripts to HSF1-total and HSF1-pSer³²⁶ RPPA protein data. This resulted in a Pearson's correlation coefficient between mRNA and protein profiles of 0.18 for HSF1-total ($P=0.00028$), corresponding to previous studies that showed that the correlation between high-throughput genomic and proteome analyses is quite low and varies between 0.17 and 0.40 (**Figure S5A**)³⁶⁻⁴⁰. As expected,

correlation between HSF1 mRNA and HSF1-pSer³²⁶ protein levels was even less marked ($R = 0.079$, $P=0.12$) (**Figure S5B**) as GEP cannot assess post translational modifications (PTM).

Although, HSF1-total protein expression by RPPA and HSF1 gene expression by RNA-seq did not correlate, we next wanted to determine if proteins correlated with HSF1-pSer³²⁶ also tracked with HSF1 gene expression. Correlations between HSF1 and 21,927 genes were examined in a large cohort of 1,063 pediatric AML patients enrolled on the AAML1031 clinical trial (including 390 of our 1,063 cases). This resulted in 228 significantly correlated genes with a correlation coefficient ≥ 0.45 (**Table S6**). Among the 90 proteins with expression correlated to HSF1-pSer³²⁶, gene expression was available for 85 genes (10/90 proteins were not measured by GEP and for 4 the antibody measured 2 or 3 isoforms. E.g., for protein GSK3A_B-pSer²¹⁻⁹, we had gene expression data for both GSK3A and GSK3B available). Among the 31/90 positively correlated RPPA proteins, only AKT1, AKT2, MAPK3, MDM2 and EIF4EBP1 had a correlation coefficient ≥ 0.35 with GEP. Among the 59 negatively correlated proteins, genes CASP9, MUC1 and TUBA1A had the strongest correlation (≥ 0.35) with HSF1-pSer³²⁶.

Discussion

This paper shows, for the first time, that response to BTZ-containing chemotherapy in pediatric AML is associated with HSF1-pSer³²⁶ phosphorylation. Using 483 samples obtained from the AAML1031 Phase 3 clinical trial in pediatric AML, we measured protein abundance for HSF1-total and HSF1-pSer³²⁶ in samples that were collected at time of diagnosis (pre-treatment). We observed a heterogeneous expression of HSF1-pSer³²⁶, while expression of HSF1-total was much more homogeneously expressed. The greatest variation in expression was observed in patients that expressed higher levels of HSF1-total, making intuitive sense as a given amount of HSF1-total is needed to be expressed before it can be phosphorylated. When we examined patient outcomes based on treatment arm, we observed that, in patients treated with BTZ-containing chemotherapy (ADEB), a significant amount of stratification was present, with outcome improving for those with lower HSF1-pSer³²⁶, but with worse survival for those with high HSF1-pSer³²⁶ expression. In patients treated with ADE alone, no stratification in outcome based on HSF1-pSer³²⁶ expression was observed. For those patients with low-HSF-pSer³²⁶, the addition of BTZ to standard chemotherapy significantly improved outcome, while EFS and RR significantly worsened in high-HSF1-pSer³²⁶ patients after BTZ addition. These results were supported *in vitro* where BTZ sensitivity was altered using gene constructs creating obligate phosphorylated and non-phosphorylatable forms of HSF1. Results showed that cells with constitutively active HSF1 were much less sensitive to BTZ than cells transfected with non-phosphorylated HSF1. These data imply that HSP activity (phosphorylation), and by extension the resulting downstream consequences, contribute to the regulation of BTZ [17].

We hypothesize that patients with low-HSF1-pSer³²⁶ are more sensitive to BTZ, as reduced activity of HSF1 would be less likely to protect the cells from the pro-apoptotic effects of BTZ- containing chemotherapy. In addition, the observation the cells with high baseline HSF1-pSer³²⁶ expression were not able to further upregulate their phosphorylation, suggests that their stress response was already fully activated, thereby acting as a potential BTZ resistance mechanism. High-HSF1-pSer³²⁶ could reflect high pre-treatment cell stress. These leukemia cells would be used to high levels of proteotoxic stress and would be less sensitive to increased cell stress caused by bortezomib. Further experiments are required to test these hypotheses.

Although survival rates of pediatric AML have been greatly improved over the last decades, the curability remains low with an OS of approximately 65-70%, indicating that the need for better therapy to improve outcome is still needed. While the AAML1031 study recently concluded that BTZ should not be used in children with *de novo* AML in combination with standard chemotherapy¹⁰, because it failed to improve 3-year EFS or OS, we wanted to identify a subgroup of patients that did benefit from the addition of BTZ, to improve outcome. The crucial observation of our study is that the addition of BTZ improved the survival of a significant percentage of patients, and that there is an adverse effect associated with the addition of BTZ to a second subset of patients. Lacking the means to identify one subset from the other, the overall conclusion that BTZ addition did not improve clinical outcome missed this finding, creating a type II or false negative error. The BTZ sensitive cohort had an increase in the EFS from 44% to 67% (+23%) at 5-years. If this was achieved in the one third of patients with the BTZ sensitive HSF1-pSer³²⁶ profile then eight additional patients per 100 diagnosed with AML would be alive and event-free after 5-years, a significant improvement in the cure rate. Given the decrease in survival with BTZ addition in

high-HSF1-pSer³²⁶ patients, this again emphasizes the need for clear predictive cut off points, when utilizing HSF1-pSer³²⁶ in the process of therapy selection. We would envision this being done with a more direct assay than RPPA, such as an ELISA or other protein-based method that is quick and has good sensitivity and specificity. Antibodies are currently available for assembling such as assay.

As we are in an era where there are increasingly more targeted therapies and where genetics define smaller and smaller subsets of patients, it becomes more important to match the right patient to the right drug(s). To choose therapies most beneficial for each patient, an ELISA assay could be used to identify patients most likely to benefit from BTZ-containing chemotherapy. This assay would quickly quantify HSF1-pSer³²⁶ protein expression and could identify which patients might benefit from additional BTZ therapy. For those patients that do not fall into this category, the development of a (phosphorylation) HSF1 inhibitor should be continued^{41,42} to effectively suppress the function of HSF1 and in turn sensitize high-HSF1-pSer³²⁶ patients to BTZ addition. Although, this first requires testing, it could expand the cohort of patients benefitting from BTZ. Potentially, these inhibitors could also be used to augment sensitivity of low-HSF1-pSer³²⁶ patients who are already sensitive. Despite testing in several Phase I clinical trials, however, HSF1 inhibitors have been difficult to use in the clinic. While showing potent anticancer effects, *in vitro*, their lack of specificity often result in off-target effects. Utilization of a useful inhibitor would therefore require further *in vitro* and *in vivo* studies. Testing in combination with other compounds should be considered, as that may increase effectiveness and limit toxicity.

In summary, decreased expression of HSF1-pSer³²⁶ correlated with increased response to BTZ-containing chemotherapy in pediatric AML and suggests that the HSF1-pSer³²⁶ abundance may be important in BTZ-containing chemotherapy selection.

Acknowledgements

We thank Yoshihiko Hirohashi (Sapporo Medical University School of Medicine, Japan) for the mammalian expression plasmids encoding the HSF1 mutations.

Author contributions

F.W.H., E.S.J.M.d.B., S.W.M., S.M.K., T.M.H. designed and supervised the research, F.W.H., A.D.D., Y.Q., G.N.J. performed research, A.S.G., R.A., E.A.K., T.A.A., S.M.K., T.M.H. collected data, F.W.H., P.P.R., R.G., A.R.L., S.M., S.M.K., T.M.H. analyzed data, and F.W.H., S.M.K., T.M.H. wrote the paper.

Disclosure of Conflict of Interest

TMH receives research funding from Takeda Pharmaceuticals.

References

1. Pui C, Carroll WL, Meshinchi S, Arceci RJ. Biology, Risk Stratification, and Therapy of Pediatric Acute Leukemias: An Update. *J Clin Oncol*. 2011;29(5):551-565.
2. Estey EH. Acute myeloid leukemia: 2016 Update on risk-stratification and management. *Am J Hematol*. 2016;91(8):824-846.
3. Zwaan CM, Kolb EA, Reinhardt D, et al. Collaborative Efforts Driving Progress in Pediatric Acute Myeloid Leukemia. *J Clin Oncol*. 2015;33(27):2949-U40.
4. Moreau P, Richardson PG, Cavo M, et al. Proteasome inhibitors in multiple myeloma: 10 years later. *Blood*. 2012;120(5):947-959.
5. Popat R, Oakervee HE, Hallam S, et al. Bortezomib, doxorubicin and dexamethasone (PAD) front-line treatment of multiple myeloma: updated results after long-term follow-up. *Br J Haematol*. 2008;141(4):512-516.
6. Hambley B, Caimi PF, William BM. Bortezomib for the treatment of mantle cell lymphoma: an update. *Ther Adv Hematol*. 2016;7(4):196-208.
7. Attar EC, De Angelo DJ, Sirulnik A, et al. Addition of Bortezomib (Velcade) to AML Induction Chemotherapy Is Well Tolerated and Results in a High Complete Remission Rate [Abstract]. *Blood*. 2005;2782(106).
8. Attar EC, Johnson JL, Amrein PC, et al. Bortezomib Added to Daunorubicin and Cytarabine During Induction Therapy and to Intermediate-Dose Cytarabine for Consolidation in Patients with Previously Untreated Acute Myeloid Leukemia Age 60 to 75 Years: CALGB (Alliance) Study 10502. *J Clin Oncol*. 2013;31(7):923-929.
9. Attar EC, DeAngelo DJ, Supko JG, et al. Phase I and Pharmacokinetic Study of Bortezomib in Combination with Idarubicin and Cytarabine in Patients with Acute Myelogenous Leukemia. *Clin Cancer Res*. 2008;14(5):1446-1454.
10. Aplenc R, Meshinchi S, Sung L, et al. Bortezomib with standard chemotherapy for children with acute myeloid leukemia does not improve treatment outcomes: a report from the Children's Oncology Group. *Haematologica*. 2020.
11. Mendillo M, Santagata S, Koeva M, et al. HSF1 Drives a Transcriptional Program Distinct from Heat Shock to Support Highly Malignant Human Cancers. *Cell*. 2012;150(3):549-562.
12. Dai C, Whitesell L, Rogers AB, Lindquist S. Heat Shock Factor 1 Is a Powerful Multifaceted Modifier of Carcinogenesis. *Cell*. 2007;130(6):1005-1018.
13. Dayalan Naidu S, Sutherland C, Zhang Y, et al. Heat Shock Factor 1 Is a Substrate for p38 Mitogen-Activated Protein Kinases. *Mol Cell Biol*. 2016;36(18):2403-2417.

14. Tang Z, Dai S, Dai C, et al. MEK Guards Proteome Stability and Inhibits Tumor-Suppressive Amyloidogenesis via HSF1. *Cell*. 2015;160(4):729-744.
15. Chou S, Prince T, Gong J, Calderwood SK. mTOR Is Essential for the Proteotoxic Stress Response, HSF1 Activation and Heat Shock Protein Synthesis. *PLoS One*. 2012;7(6):e39679.
16. Guettouche T, Boellmann F, Lane WS, Voellmy R. Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC Biochem*. 2005;6(1):4.
17. Shah SP, Nooka AK, Jaye DL, Bahlis NJ, Lonial S, Boise LH. Bortezomib-induced heat shock response protects multiple myeloma cells and is activated by heat shock factor 1 serine 326 phosphorylation. *Oncotarget*. 2016;7(37):59727-59741.
18. Sojka DR, Gogler-Pigłowska A, Vydra N, et al. Functional redundancy of HSPA1, HSPA2 and other HSPA proteins in non-small cell lung carcinoma (NSCLC); an implication for NSCLC treatment. *Sci Rep*. 2019;9(1):1-15.
19. Kawazoe Y, Nakai A, Tanabe M, Nagata K. Proteasome inhibition leads to the activation of all members of the heat-shock-factor family. *Eur J Biochem*. 1998;255(2):356-362.
20. Heimberger T, Andrulis M, Riedel S, et al. The heat shock transcription factor 1 as a potential new therapeutic target in multiple myeloma. *Br J Haematol*. 2013;160(4):465-476.
21. Shah SP, Lonial S, Boise LH. When Cancer Fights Back: Multiple Myeloma, Proteasome Inhibition, and the Heat-Shock Response. *Mol Cancer Res*. 2015;13(8):1163-1173.
22. Barna J, Csermely P, Vellai T. Roles of heat shock factor 1 beyond the heat shock response. *Cell Mol Life Sci*. 2018;75(16):2897-2916.
23. Hu CW, Qiu Y, Ligerde A, et al. A quantitative analysis of heterogeneities and hallmarks in acute myelogenous leukaemia. *Nat Biomed Eng*. 2019.
24. Kornblau SM and Coombes KR. Use of reverse phase protein microarrays to study protein expression in leukemia: technical and methodological lessons learned. *Methods Mol Biol*. 2011;785:141.
25. Kornblau SM, Singh N, Qiu Y, Chen W, Zhang N, Coombes KR. Highly Phosphorylated FOXO3A Is an Adverse Prognostic Factor in Acute Myeloid Leukemia. *Clin Cancer Res*. 2010;16(6):1865-1874.
26. Kornblau SM, Tibes R, Qiu YH, et al. Functional proteomic profiling of AML predicts response and survival. *Blood*. 2009;113(1):154-164.
27. Tibes R, Qiu Y, Lu Y, et al. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. *Mol Cancer Ther*. 2006;5(10):2512-2521.
28. Hu J, He X, Baggerly KA, Coombes KR, Hennessy BTJ, Mills GB. Non-parametric quantification of protein lysate arrays. *Bioinformatics*. 2007;23(15):1986-1994.

29. Neeley ES, Kornblau SM, Coombes KR, Baggerly KA. Variable slope normalization of reverse phase protein arrays. *Bioinformatics*. 2009;25(11):1384-1389.
30. Neeley ES, Baggerly KA, Kornblau SM. Surface Adjustment of Reverse Phase Protein Arrays using Positive Control Spots. *Cancer Inform*. 2012;2012(11):77-86.
31. Akbani R, Ng PKS, Werner HMJ, et al. A pan-cancer proteomic perspective on The Cancer Genome Atlas. *Nat Commun*. 2014;5(1):3887.
32. Horton TM, Hoff FW, van Dijk AD, et al. The effects of sample handling on proteomics assessed by reverse phase protein arrays: functional proteomic profiling in leukemia. 2020. *Submitted*.
33. Smith JL, Ries RE, Hylkema T, et al. Comprehensive Transcriptome Profiling of Cryptic *CBFA2T3-GLIS2* Fusion-Positive AML Defines Novel Therapeutic Options: A COG and TARGET Pediatric AML Study. *Clin Can Res*. 2020;26(3):726-737.
34. Kampen KR, ter Elst A, Mahmud H, et al. Insights in dynamic kinome reprogramming as a consequence of MEK inhibition in MLL-rearranged AML. *Leukemia*. 2014;28(3):589-599.
35. Yasuda K, Hirohashi Y, Mariya T, et al. Phosphorylation of HSF1 at serine 326 residue is related to the maintenance of gynecologic cancer stem cells through expression of HSP27. *Oncotarget*. 2017;8(19):31540-31553.
36. Genshaft AS, Li S, Gallant CJ, et al. Multiplexed, targeted profiling of single-cell proteomes and transcriptomes in a single reaction. *Genome Biol*. 2016;17(1):188.
37. Vogel C and Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet*. 2012;13(4):227-232.
38. Payne SH. The utility of protein and mRNA correlation. *Trends Biochem Sci*. 2015;40(1):1-3.
39. Mun D, Bhin J, Kim J, et al. Proteogenomic characterization of human early-onset gastric cancer. *Cancer Cell*. 2019;35(1):111-124.e10.
40. Zhang B, Wang J, Wang X, et al. Proteogenomic characterization of human colon and rectal cancer. *Nature*. 2014;513(7518):382-387.
41. Shah SP, Nooka AK, Lonial S, Boise LH. TG02 inhibits proteasome inhibitor-induced HSF1 serine 326 phosphorylation and heat shock response in multiple myeloma. *Blood adv*. 2017;1(21):1848-1853.
42. Santagata S, Mendillo ML, Tang Y, et al. Tight coordination of protein translation and HSF1 activation supports the anabolic malignant state. *Science*. 2013;341(6143):250.

Tables

Table 1. Patient characteristics

		All cases	Low-HSF1-pSer ³²⁶	Middle-HSF1-pSer ³²⁶	High-HSF1-pSer ³²⁶	<i>P</i>
Number (n = 410)	Total	100%	31.7%	34.9%	33.4%	
Age (years old)	< 1	12.2%	13.9%	14.0%	8.8%	0.32
	2 - 10	33.4%	36.2%	28.0%	36.5%	0.23
	> 11	54.4%	50.0%	58.0%	54.7%	0.41
Gender	Female	49.0%	52.3%	49.7%	45.3%	0.51
Race[†]	American Indian or Alaska Native	1.1%	0.9%	2.3%	0%	0.20
	Asian	4.7%	4.3%	4.7%	5.0%	0.96
	Black	11.8%	6.8%	13.2%	15.1%	0.12
	White	82.2%	88.0%	79.8%	79.0%	0.13
	Multiple races	0.3%	0%	0%	0.8%	0.35
Ethnicity^{††}	Hispanic	17.1%	13.6%	19.0%	18.5%	0.45
	Not Hispanic	82.9%	86.4%	81.0%	81.5%	
Cytogenetics	t(8;21)	15.9%	10.0%	14.0%	23.4%	0.01
	inv(16)	13.9%	16.2%	16.8%	8.8%	0.10
	Normal karyotype	27.8%	33.8%	22.4%	27.7%	0.11
	t(9;11)(p22;q23)/11q23	17.6%	15.4%	17.5%	19.7%	0.65
	-5, -7, or +8	7.8%	9.2%	7.0%	7.3%	0.76
	Other	17.1%	15.4%	22.4%	13.1%	0.10
NPM1	Mutant	11.2%	10.0%	11.9%	11.7%	0.87
CEBPα	Mutant	9.5%	16.2%	7.0%	5.8%	0.01
FLT3-ITD	Mutant	22.4%	20.8%	19.6%	27.0%	0.28
High allelic ratio FTL3-ITD (≥ 0.4)	Yes	14.6%	13.1%	14.0%	16.8%	0.67
Risk stratification^{†††}	High	29.8%	31.0%	25.9%	32.6%	0.45
Complete remission	Yes	84.9%	86.2%	82.5%	86.1%	0.62

[†] N=45 patients have unknown race and are excluded from the results;

^{††} N=18 patients have unknown ethnicity and are excluded from the results;

^{†††} AAML1031 protocol risk group definition:

- Low risk: inv(16)/t(16;16) or t(8;21), or NPM or CEBPα mutation;
- High risk: FLT3/ITD+ with high allelic ratio ≥ 0.4 , or monosomy 5/del5q or 7, without lowrisk features;
- Risk status unknown for 10/410

*NA/ unknown values not considered in *P*-value calculations and are excluded from the results;

Table 2. Multivariable analysis in ADEB treated patients for relapse risk, overall survival and event-free survival.

		OS from study entry				EFS from study entry			RR from end of course 2			
		n	HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>	n	HR	95% CI	<i>P</i>
HSF1- pSer³²⁶	Low	52	1			1			48	1		
	Middle	83	2.10	0.89 – 4.95	0.090	1.92	1.07 – 3.43	0.028	70	1.60	0.84 – 3.04	0.156
	High	69	3.58	1.56 – 8.22	0.003	2.94	1.65-5.24	< 0.001	60	2.76	1.45 - 5.29	0.002
Risk group	Low	155	1			1			146	1		
	High	49	2.66	1.55-4.58	<0.001	2.18	1.42 – 3.34	< 0.001	32	1.48	0.82 – 2.66	0.194

Figure legends

Figure 1. RPPA protein expression levels for HSF1-total and HSF1-pSer³²⁶. RPPA protein expression levels for the 483 *de novo* pediatric AML patients and 30 CD34+ BM samples, for **A)** HSF1-total, and **B)** HSF1-pSer³²⁶. **C)** Correlation between HSF1-total and HSF1-pSer³²⁶ protein expression. HSF1-total was divided into 6 equally sized groups. Boxplots represent the dispersion of HSF1-pSer³²⁶ expression. Lower HSF1-total samples express lower levels of HSF1-pSer³²⁶, while higher HSF1-total levels are associated with higher levels of HSF1-pSer³²⁶ and show more variation in HSF1-pSer³²⁶ phosphorylation.

Figure 2. Kaplan-Meier survival curves for overall survival, event-free survival and relapse risk. **A)** Overall survival, **B)** event-free survival and **C)** relapse risk for patients treated without the addition of BTZ (left). **D)** Overall survival, **E)** event-free survival and **F)** relapse risk for patients treated with the addition of BTZ to ADE (right). Dashed lines (- - -) indicate low-HSF1-pSer³²⁶ patients, dotted (....) lines represent middle-HSF1-pSer³²⁶ and solid lines (—) the high-HSF1-pSer³²⁶.

Figure 3. Kaplan-Meier survival curves for low-HSF1-pSer³²⁶ and high-HSF1-pSer³²⁶ treated with ADE vs. ADEB. **A)** Overall survival, **B)** event-free survival and **C)** relapse risk for low-HSF1-pSer³²⁶ patients (left). **D)** Overall survival, **E)** event-free survival and **F)** relapse risk for high-HSF1-pSer³²⁶ patients (right). Dotted (....) lines indicate treatment with ADE alone, solid (—) lines represent the ADEB treated patients.

Figure 4. Reproduction HSF1-pSer³²⁶ sensitivity to BTZ in 293T cells. To assess the effect of high and low HSF1-pSer³²⁶ expression on **A)** BTZ sensitivity and **B)** etoposide

sensitivity, 293T cells were transfected with two HSF1-pSer³²⁶ mutants that mimicked high (solid line) and low (dashed line) HSF1-pSer³²⁶ patients, in addition to an empty vector as control (dotted line). **C)** Effect of mutation on HSF1-pSer³²⁶ expression was confirmed by Western blot.

Figure 5. Change in HSF1-pSer³²⁶ protein expression following chemotherapy. This figure shows the change in HSF1-pSer³²⁶ expression following chemotherapy over time. Low-HSF1-pSer³²⁶ patients (pretreatment) are shown in light gray. High-HSF1-pSer³²⁶ patients (pretreatment) are shown in dark gray.

Figure 6. Significantly correlated proteins with HSF1-pSer³²⁶. **A)** List of proteins significantly correlated with HSF1-pSer³²⁶ protein expression. Antibodies indicated with a red “+” are directed against post-translationally modified sites. Proteins shown in red are involved in initiation or elongation of protein translation or in ribosomal processes. Total HSF1 is highlighted in yellow. **B and C)** STRING network analysis of the positively and negatively correlated proteins.

Figure 1

A

B

C

Figure 2

Figure 3

Figure 4

A

B

C

Figure 5

A

C