

Research article

General Control Nonderepressible 2 and ATF4 Direct Liver Genes during Asparaginase Treatment in Mice

Rana Jaber Tarish

Department of Physiology, College of Veterinary Medicine, University of Al-Qadisiyah, Iraq

Corresponding Author Email: Rana.tarish@qu.edu.iq

(Received 24/8/2017, Accepted 25/12/2017)

Abstract

Asparaginase (ASNase) treatment results in synthesis of some factors such as ATF4. The eIF2-ATF4 pathway is essential for cell survival during amino acid starvation conditions. Activation of the AAR in liver requires the eIF2 kinase called general control nonderepressible 2 kinase (GCN2). To what extent activation of the GCN2-eIF2-AAR is mediated by ATF4 is unknown. Our objective and hypothesis are addressed in our aim to describe the liver response to ASNase in mice deleted for Atf4. RNA sequencing alongside complementary biochemical approaches were performed in the livers of mice treated with eight daily injections of ASNase or saline excipient. Cellular pathways examined in detail included the AAR. We discovered that global hepatic gene expression patterns in Atf4 knockout mice overlapped with Gcn2 knockout mice. Shared hepatic pathways or processes altered during ASNase included mTOR signaling, and xenobiotic metabolism. On the other hand, loss of Atf4 during ASNase uniquely altered gene expression signatures reflecting signaling via eIF2 and ER stress. This research provides insight into the importance of genetic background of patients in choosing ASNase as a treatment.

Keywords: ATF4 Direct Liver Genes, Asparaginase, Mice.

Introduction

One of the frequent causes of death in those under age 20 is acute lymphoblastic leukemia (ALL). Asparaginase (ASNase) is a drug that is used to treat ALL (1), and it is widely recommended as it improves remission induction rate (2). Nevertheless, ASNase has many deleterious side effects such as liver failure (3). ASNase works to decrease some amino acids causing amino acid deprivation. Our laboratory was the first to demonstrate that ASNase reduces liver protein synthesis by increasing phosphorylation of eukaryotic initiation translation factor 2 (eIF2) (4) via the general control nonderepressible 2 kinase (GCN2) (5). Phosphorylation of eIF2 by GCN2 dampens global protein synthesis rates while simultaneously promoting gene-specific translation of protein factors. This GCN2-eIF2-ATF4-driven adaptive mechanism is described, AAR (6). ATF4 is described in

many research articles as a master regulator of metabolism in response to many cellular stressors. It was found to play critical role in amino acid deficiency as a member of the GCN2-eIF2-ATF4 AAR pathway (6). The goal of this project is to determine whether the role of GCN2 is fully mediated by ATF4 or shared with other factors. This will help to better identify the causes of drug toxicity and perhaps reveal new treatment and prevention approaches.

Materials and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 443

Mice from Jackson Laboratories, Bar Harbor, ME were used in these experiments. All mice were individually housed in clear

plastic cages with corncob bedding and freely provided commercial diet. Protocols of animal use were according to Rutgers (IACUC). Animals were bred and maintained at the Rutgers Bartlett animal care facility.

Design.

Mice were administered once-daily intraperitoneally with ASNase (Deerfield, Illinois) after the start of the light cycle as previously detailed. The doses are based on our previous work as described in (4) and enzyme activity was determined prior to injection by the Nesslerization technique by detecting the level of ammonia as was described (4, 5).

Sample Collection.

Mice from all treatment groups were euthanized by decapitation ~8 h after the eighth daily injection. Trunk blood collected to obtain serum, and RNA isolation was performed on frozen samples that were stored at -80°C until analysis.

RNA-isolation.

Frozen liver samples were processed to obtain high quality RNA for RNA-Sequencing (RNA-Seq). Total RNA was extracted from frozen livers using NucleoSpin® RNA Kit (Macherey-Nagel, Newmann-Neander, Germany) followed by DNase treatment. The A260/280 and 260/230 absorbance ratios were identified using NanoDrop1000 (Thermo Fisher Scientific, Wilmington, DE).

Results

We found 545 genes were dependent (either directly or indirectly) on GCN2 while 408 genes required ATF4 relative to WT PBS-treated controls with 263 shared between *the groups* (B). Genes within the three categories indicated that many of the effected biological processes were shared; among them were stellate cell activation of hepatic fibrosis, complement cascades, calcium signaling, glycolysis and gluconeogenesis Figure (1-C). Several pathways such as epithelial nitric oxide synthase signaling, the coagulation system,

RNA-Seq Data differential gene expression.

Differences in gene expression were checked. Fastq files were aligned to the mouse genome (mm10) using TopHat (v2.1.0) and Bowtie (v1.1.2) (<http://ccb.jhu.edu/software.shtml>) and mapped reads were submitted to Cufflinks (v2.2.1) using the default settings. The assembled transcript files were quantified by Cuffdiff and then indexed and visualized using CummeRbund (v2.12.0).

Pathway Analyses.

Resulting gene lists from the Venn categories (A-G) were analyzed for biological relevance by Ingenuity Pathway Analysis (IPA). In addition, differentially expressed gene lists were evaluated for gene ontology and KEGG pathway enrichment analysis using DAVID (v6.7) (4). In both approaches, statistical significance of gene enrichment was $p < 0.05$.

Heat maps.

Heat maps Figures (1-C) and (1-D) show the level of significance of pathways across multiple gene sets. The $-\log_{10}$ of P values represent the statistical significance values were color-indexed (white for no significance to dark red for the highest significance).

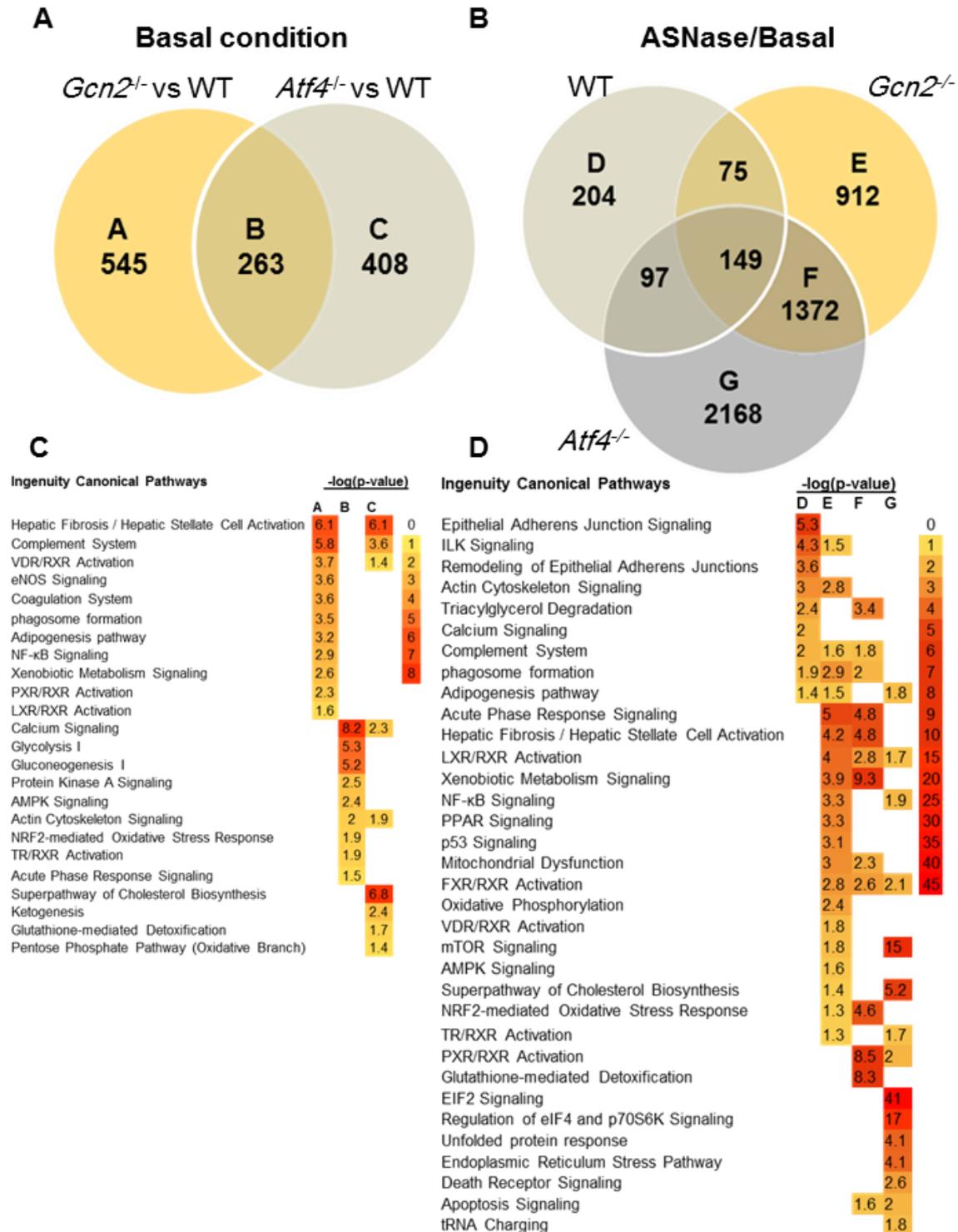
Statistics

R using *cummeRbund* was employed to do statistics for this study with $FDR < 0.1$.

phagosome formation, and adipogenesis were predominantly associated with GCN2 whereas ATF4 was exclusively associated with processes including cholesterol biosynthesis, ketogenesis and the oxidative branch of the pentose phosphate pathway. We also examined the effect of ASNase on hepatic gene expression by Venn analysis in Figure (1-B). In WT mice, ASNase affected 525 genes, of these, 61% changed independent of GCN2 and ATF4 (category D). ASNase altered 2,508 genes in *Gcn2*^{-/-} mice whereas ASNase altered 3,786 genes in

Atf4^{-/-} mice. Analyses were performed on these specified gene categories to explore biological processes affected Figure (1-D). These analyses identified signaling via

peroxisome proliferator-activated receptor, p53, and AMPK and oxidative phosphorylation.



Discussion

In this study, we show that absence of Gcn2 or Atf4 genes alters the liver transcriptome basally and during ASNase treatment. The study shows for the first time the difference in gene expression of liver genes in case of ASNase treatment in mice. The results are of high value since they help

to figure the response of the liver to such treatment in intact and mutants at the level of gene expression. There are a lot of discoveries that might light hidden pathways that may play significant roles in improving the ASNase treatment and the ALL remission simultaneously.

References

- 1-Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med.* (2006); Jan 12; 354:166-78.
- 2-Landau H, Lamanna N. Clinical manifestations and treatment of newly diagnosed acute lymphoblastic leukemia in adults. *Curr Hematol Malig Rep.* (2006); Sep; 1(3):171-9. doi: 10.1007/s11899-996-0005-8.
- 3-Appel IM, Hop WC, van Kessel-Bakvis C, Stigter R, Pieters R. L-ASNase and the effect of age on coagulation and fibrinolysis in childhood acute lymphoblastic leukemia. *Thromb Haemost.* (2008); Aug; 100:330-7.
- 4-Reinert RB, Oberle LM, Wek SA, Bunpo P, Wang XP, Mileva I, Goodwin LO, Aldrich CJ, Durden DL, McNurlan MA, Wek RC, Anthony TG. Role of glutamine depletion in directing tissue-specific nutrient stress responses to L-ASNase. *J Biol Chem.* (2006); Oct 20; 281(42):31222-33. Epub 2006 Aug 24.
- 5-Bunpo P, Dudley A, Cundiff JK, Cavener DR, Wek RC, Anthony TG. GCN2 protein kinase is required to activate amino acid deprivation responses in mice treated with the anti-cancer agent L-ASNase. *J Biol Chem.* (2009); Nov 20; 284(47):32742-9
- 6-Kilberg MS, Shan J, Su N. ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends Endocrinol Metab.* (2009); Nov; 20(9):436-43.