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Granzyme B and miR-378a Interaction in Acetaminophen Toxicity in Children

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Abstract

Background and Aim: Hepatic phase I drug-metabolizing enzymes CYP2E1, CYP1A2 and CYP3A4 catalyze the biotransformation of Acetaminophen (APAP) and are important in the mediation of toxicity. The potential role of other hepatic and non-hepatic Phase I enzymes in APAP toxicity has not been established.

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AUTHORS' CONTRIBUTIONS

Pritmohinder Gill conceived ideas for the study; Sandra McCullough, Pritmohinder Gill, Aaron Woodall, Mitchell R. McGill and Sara Frankowski designed and performed wet molecular biology experiments. Harsh Dweep, Sandra McCullough, Mitchell R. McGill and Sudeepa Bhattacharyya, analyzed the data. Pritmohinder Gill, Laura James, Gregory Kearns, Sandra McCullough, Mitchell R. McGill and Sudeepa Bhattacharyya wrote the manuscript. All authors reviewed and approved the manuscript for submission.

PATENT DISCLOSURE

Dr. James has a patent application pending for the measurement of APAP protein adducts in human blood samples.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The ethical approval was provided by the Institutional Review Board (IRB) University of Arkansas for Medical Sciences (UAMS), AR, USA.

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. All reported human were experimented in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsniki Declaration of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10ethics/10helsinki/).

CONSENT FOR PUBLICATION

Written informed consent was taken from all participants.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

CONFLICT OF INTEREST

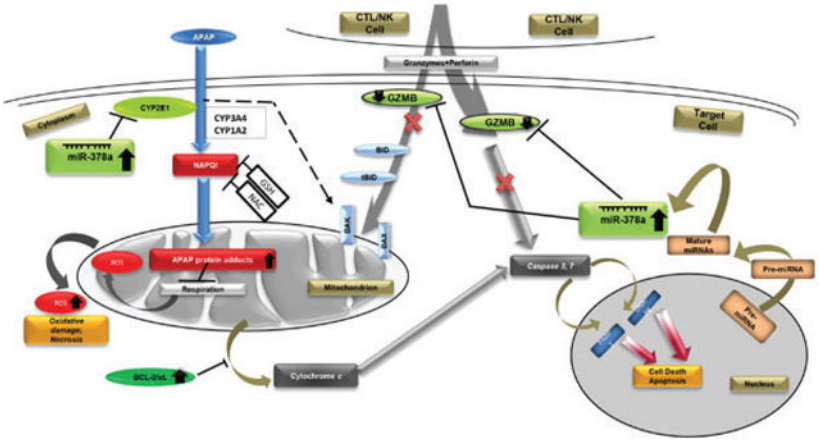
Dr. James is a partner in Acetaminophen Toxicity Diagnostics (ATD), LLC which is a recipient of a grant (DK79387) from the National Institute of Diabetes, Digestion, and Kidney Disease of the United States Department of Health and Human Services to develop a new diagnostic assay for acetaminophen toxicity.

Methods: PCR array containing 84 genes involved in phase I drug metabolism was examined in subgroups of hospitalized children for APAP overdose, categorized as no toxicity (ALT ≤ 45 IU/L, n=5) and moderate toxicity (ALT ≥ 500 IU/L, n=5).

Results: Significant downregulation was observed for ALDH6A1, CYP4F12 and GZMB in the no toxicity subgroup and ALDH1A1, CYP27A1 and GZMB in the moderate toxicity subgroup. qRT-PCR confirmed significant downregulation for ALDH1A1, CYP4F12, and GZMB. *In-silico* analysis identified GZMB 3'UTR to be a target of miR-378a-5p. Overexpression of miR-378a-5p reduced the luciferase activity of GZMB 3'UTR reporter plasmid reportedly by 50%. NK-92 cells transfected with the miR-378a-5p mimic extended the effect of APAP on GZMB protein expression compared to mimic controls. In addition, miR-378a-5p was significantly upregulated in blood samples of children with APAP overdose undergoing NAC treatment.

Conclusion: Overall, our study suggests the presence of a novel signaling pathway, whereby miR-378a-5p inhibits GZMB expression in children with APAP overdose.

Graphical Abstract



Keywords

Acetaminophen (APAP); APAP-Induced Liver Injury (AILI); Cytochrome P450; Granzyme B; miR-378a; biomarkers

1. INTRODUCTION

Acetaminophen (APAP; *N*-acetyl-*p*-aminophenol) is an analgesic and antipyretic drug that produces centrilobular hepatic necrosis at high doses [1–3]. Mechanisms for the development of APAP toxicity involve hepatic glutathione depletion and bioactivation *via* cytochromes P450 isoforms (CYP2E1, CYP1A2 and CYP3A4) to the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) [4]. The initiation of hepatocellular injury occurs when cellular detoxification pathways (*e.g.*, glucuronide and sulfate conjugation) are overwhelmed and NAPQI accumulates. MicroRNAs (MiR-NAs) are small non-protein-coding RNAs [5] that regulate gene expression by targeting the 3′ untranslated region (3′ UTR) of mRNA. They play a critical role in the regulation of cellular processes involving differentiation and proliferation [6] and can control innate and adaptive immune pathways

[7, 8] and could be important prognostic biomarkers as well as therapeutic targets in various disease conditions. In vertebrates, miRNA present highly managed regulation of gene expression as a single miRNA can target approximately hundreds to thousands of transcripts and a single transcript may have binding sites for multiple miRNAs [9].

A number of miRNAs have also been associated with APAP toxicity [10–12], but mechanisms by which dysregulated miRNAs cause hepatotoxicity have been lacking and several reports have identified liver-specific miR-122 as a potential biomarker of APAP toxicity in humans [13–17]. In addition, miRNAs have been linked to post-transcriptional gene regulation of CYP P450 phase I enzymes [18] and a few publications have investigated the relationships of specific miRNAs that regulate CYP P450 expression to APAP toxicity, characterized by alanine aminotransferase (ALT) elevation [19–21]. Our laboratory recently reported that miR-378a-5p was upregulated in the serum samples of APAP overdose subjects and that *in-vitro* overexpression of miR-378a-5p led to translational repression of CYP2E1 [20].

We herein looked at phase I drug metabolism enzymes gene expression profile in children with APAP overdose and identified GZMB as a relevant downstream target of miR-378a-5p. We show that miR-378a-5p upregulation modulates GZMB expression at the post-transcriptional level through direct interaction with the 3’ UTR of GZMB mRNA. These findings uncovered a novel putative molecular mechanism involving GZMB and miR-378a signaling in APAP overdose.

2. MATERIALS & METHODS

2.1. Chemicals

APAP, nuclease-free water and ethanol (molecular grade) were purchased from Sigma-Aldrich (St. Louis, MO). miRNA and RNA isolation, cDNA synthesis reagents were purchased from Qiagen (Valencia, CA). The miR-378a-5p mimic, miR-378a-5p inhibitor and AllStars Negative control siRNA were purchased from Qiagen (Valencia, CA). HiPerfect and Attractene transfection reagents were obtained from Qiagen (Carlsbad, CA).

2.2. Human Samples

Peripheral blood samples were collected as part of a previous study on APAP toxicity in children, approved by the University of Arkansas for Medical Sciences (UAMS) Institutional Review Board (IRB) [22]. Participants were enrolled by informed parental consent and by subject assent when appropriate (*i.e.*, age > 6 years), prior to the onset of study procedures and their ages ranged 6–9 years. The study included healthy controls (n=5, defined as no APAP exposure two weeks prior to sample collection) and children with APAP overdose that were hospitalized for treatment with N-Acetyl Cysteine (NAC), stratified by serum ALT values: no toxicity ≤ 45 IU/L (n=5); or moderate toxicity ≥ 500 IU/L (n=5). Blood samples (1–2.5 mL) were collected into PAXgene Blood RNA Tubes.

2.3. Clinical and Laboratory Data Collection

Demographic information included subject age, gender, weight, height, body surface area, past medical history, relevant history concerning recent APAP dosing and concomitant medications. The dose of the APAP ingestion, quantitation of APAP in peripheral blood, and time to N-Acetyl Cysteine (NAC) treatment were also recorded.

2.4. Assay for ALT and APAP Protein Adducts

Clinical laboratory results included APAP concentrations and ALT determinations performed in hospital clinical chemistry laboratories. APAP protein adducts, an indicator of the oxidative metabolism of APAP, were analyzed using a method previously published by our laboratory [23, 24]. Briefly, 100 μl of serum was analyzed using High-Performance Liquid Chromatography (HPLC) with an ESA CoulArray electrochemical detector. APAP cysteine was resolved on a 150 mm C18 column and detected using an ESA CoulArray electrochemical detector. Concentrations of adducts were determined relative to a standard curve of authentic APAP cysteine and reported as nmol/mL a-cys.

2.5. PAXgene Total RNA and miRNA Isolation

The PAXgene Blood miRNA system was used for the purification of total RNA (including miRNA) from peripheral blood samples collected in PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Switzerland). Paxgene extracted RNA was quantified using a spectrophotometer (NanoDrop™ 1000, Thermo Fisher Scientific, Wilmington, DE). Supplementary Methods details the cDNA synthesis and qRT-PCR conditions.

2.6. Phase I PCR Array

RT^2 Profiler™ PCR Arrays (PAHS-068Z, Qiagen) were used based on their high concordance with TaqMan PCR and high-density microarrays [25]. The PCR Array, which contains 84 genes involved in phase I human drug metabolism reactions involving oxidation, reduction, hydrolysis, cyclization and decyclization, was used with the SYBR®Green system (Qiagen). PCR array data analysis is outlined in the section dealing with Supplementary Methods.

2.7. PAXgene mRNA and miRNA Expression Analysis

Quantification of mRNA and miRNA expression was accomplished with assays and accompanying reagents from Qiagen on QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific). The relative abundance of significantly dysregulated target transcripts from Phase I PCR array was validated using RT^2 qPCR Assays and RT^2 SYBR Green Mastermixes. Gene expression assays used in this study were: ALDH1A1, ALDH6A1, CYP27A1, CYP4F12, GZMB, ACTB, hsa-miR-378a-5p and SNORD68. Refer to Supplementary Table S1 for a full list of qRT-PCR assays with their Entrez Gene ID, band size, reference position and Qiagen Catalog Number.

2.8. microRNA Target Predictions

The alignment interaction between miR-378a-5p and GZMB (EntrezID: 3002) was predicted using DIANA-microT-CDS [26], a component of 13 algorithms documented in the microRNA retrieval system of miRWalk2.0 [27, 28].

2.9. Functional Analysis of miR-378a-5p Interaction with 3'UTR of Human GZMB

The luciferase assay was used to assess whether miR-378a-5p targets the 3' UTR of GZMB mRNA. A pEZX-MT06-GZMB-LUC reporter plasmid containing 113 bp of human GZMB 3' UTR (HmiT008690-MT06) and negative control with no 3' UTR (pEZX-MT06) was purchased from GeneCopoeia™, Rockville, MD. HEK293T cells were plated 24 hours prior to transfection in 96-well plates (3×10^4 cells/well). pEZX-MT06-GZMB-LUC or pEZX-MT06 plasmids were co-transfected with or without miR-378a-5p mimic or inhibitor using Attractene transfection reagent (Qiagen). Cells were harvested 24 hours after transfection. Firefly and renilla luciferase activities were determined using the Luc-Pair™ Duo-Luciferase Assay kit 2.0 (GeneCopoeia™, Rockville, MD). Relative luciferase activities were calculated by normalization of firefly luciferase activity to renilla luciferase activity to correct for transfection efficiencies in each sample and compared to cells transfected with the negative control plasmid (pEZX-MT06) or GZMB-Luc plasmid (pEZX-MT06-3'UTR GZMB-Luc).

2.10. Cell Culture, APAP Treatment and RNA Extraction

HepaRG™ (Catalog Number HPRGC10, ThermoFisher Scientific, Carlsbad, CA), human primary pediatric hepatocytes (Product Number M00995-P, BioIVT, Baltimore, MD) and NK-92 cells (Catalog Number CRL-2407, ATCC, Manassas, VA) were purchased and cultured in vendor suggested media. The cells were plated in 6- or 24-well plate and placed in a humidified atmosphere containing 5% CO_2 at 37°C. After 24 hours, the cells were exposed to APAP (5 mM or 20 mM) for various time points. Total RNA was isolated from cells using the miRNeasy Mini Kit (Qiagen). Supplementary Methods section lists cDNA synthesis and qRT-PCR conditions.

All the experiments and methods were carried out in accordance with the approved guidelines as described in Bhattacharyya *et al.*, [22].

2.11. Transfection of miR-378a-5p Mimic in NK-92 Cells

miR-378a-5p mimics were used to overexpress miRNA in NK-92 cells. For transfection experiments, cells were plated in a 24-well plate with a seeding density of 2.5×10^5 per well and transfected with 5 nM mimic and ALLStar negative control siRNA using HiPerfect Transfection Reagent. After 24 hours, the transfection medium was replaced with regular medium and cells were treated with 20 mM APAP for 18 hours and harvested for protein analysis.

2.12. Western Blot Analysis of NK-92 Cells

NK-92 cells were washed with ice-cold PBS and lysed in RIPA buffer (Sigma-Aldrich) supplemented with a protease inhibitor cocktail tablet (Roche). Cell lysates were quantified using the BCA protein assay kit (ThermoFisher) and 75 ug of cell lysates were resolved

using Criterion™ 10% TGX™ (Tris-Glycine eXtended) precast PAGE gels (Bio-Rad.com) along with PageRuler™ protein ladder (ThermoFisher). The gels were transferred to a PVDF membrane immobilon-P (Sigma-Aldrich) and blocked in 5% (w/v) non-fat milk. The membrane was probed with Abs specific to GZMB (1:10,000, ab208586, Abcam) and β-actin (Sigma-Aldrich). After incubation with goat anti-rabbit IgG H & L (HRP) (1:20,000, ab6721, Abcam), signals were detected using SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher). Western blots were quantified using NIH Image J software (National Institutes of Health).

2.13. Statistical Analysis

All experimental data are presented as the means ± SEM Statistical comparisons between subgroups for toxicity (ALT), oxidative metabolism (APAP protein adducts), mRNA and miRNA were performed using one-way analysis of variance (ANOVA) followed by Tukey A post-hoc test. Differences between the two groups were examined using Student's *t*-test (2-tailed). The limit of significance accepted for all statistical analyses was $\alpha=0.05$.

3. RESULTS

3.1. Elevated Levels of ALT and APAP Protein Adducts in APAP Overdose Subjects

Serum samples from children with APAP overdose and controls were analyzed for ALT and APAP protein adducts (Table 1). Median levels of ALT and APAP protein adducts were elevated in the serum samples of APAP overdose subgroups as compared to healthy controls. Subjects in the no toxicity subgroup (ALT ≤ 45 IU/L, n=5) had elevated APAP levels and received treatment with N-Acetyl Cysteine (NAC) within 24 hours of the APAP ingestion. For the moderate toxicity subgroup (ALT ≥ 500 IU/L, n=5), the time of NAC relative to the APAP ingestion was beyond 24 hours (n=2), within 24 hours (n=1), and time of NAC treatment unavailable (n=2).

3.2. Identification of Differentially Expressed Genes between Subjects with no Toxicity and Moderate Toxicity

Volcano plot analysis was used to illustrate the regulation of 84 Phase 1 drug metabolism genes in subgroups of APAP overdose with no toxicity or moderate toxicity compared to control subjects. Only 3 genes were significantly downregulated in the no toxicity subgroup and included ALDH6A1 (−2 fold), GZMB (−2.8 fold) and CYP4F12 (−4 fold) ((Fig. 1A). Similar results were observed in samples from the moderate toxicity subgroup ((Fig. 1B), showing significant downregulation of ALDH1A1 (−2.4 fold) and GZMB (−2.6 fold), as well as CYP27A1 (−2.2 fold), but not for CYP4F12. Subsequently, qRT-PCR confirmed the downregulation of GZMB and CYP4F12 in no toxicity subgroup ((Fig. 2A) and GZMB and ALDH1A1 in medium toxicity subgroup ((Fig. 2B). The difference in no toxicity *vs.* moderate toxicity for GZMB was not significant (p=0.17).

3.2.1. miR-378a-5p Targets GZMB—Based on the PCR array analysis, GZMB was common in no toxicity and moderate toxicity subgroups and it is one of the esterases involved in hydrolysis reactions of phase I metabolism. We hypothesized that the observed suppression of GZMB expression in APAP toxicity may be due to miRNA-mediated gene

silencing. Alignment interaction obtained from DIANA-microT-CDS [26] indicated that miR-378a-5p has a putative binding site at the 3'UTR of GZMB ((Fig. 3A). GZMB 3′ UTR has an evolutionarily conserved miR-378a-5p binding seed site among different mammalian species including rat, mouse, dog, chimpanzee and human ((Fig. 3A).

To further examine possible miRNA-mRNA interactions, gain-of-function studies were performed using a miR-378a-5p mimic. Overexpression of miR-378a-5p reduced luciferase activity in a dose-dependent manner ((Fig. 3B). Transfection with 5nM mir-378a-5p mimics reduced the luciferase activity by ~50% in HEK293 cells co-transfected with a reporter plasmid containing the 3' UTR sequence of the human GZMB mRNA, while no change was observed in cells transfected with a negative control plasmid with no 3' UTR ((Fig. 3B). Moreover, upon co-transfection of miR-378 mimics and inhibitor restored the luciferase activity to that of controls. Collectively, these data demonstrate a specific inhibitory effect of miR-378a-5p on the 3' UTR of GZMB.

3.3. APAP Treatment of NK-92 Cells Downregulates GZMB Expression

We further hypothesized that APAP may have a direct effect on GZMB expression. In an attempt to identify cell type expressing GZMB, studies were undertaken with APAP treatment of HepaRG™, human primary pediatric hepatocytes and NK-92 cells. Only NK-92 cells expressed appreciable and detectable levels of GZMB and NK cells are known to express high levels of GZMB (GTEx Consortium) [29]. Light microscopic quantification with Trypan Blue method (Sigma) was used in estimating the proportion of viable NK-92 cells over 18-48 hrs period after APAP treatment. The proportion of NK cells live after APAP treatment of 5, 10 or 20 mM was approximately 92, 81 and 69 %, respectively (n=3, data not shown). Subsequent experiments with NK-92 cells were treated with 5 or 20 mM APAP and samples were harvested at 12 and 18 hours. As shown in (Fig. 4A), no significant difference in GZMB gene expression was observed with the 5 mM APAP exposure, but statistically, significant downregulation was observed for the 20 mM APAP exposure at 12 and 18 hours ($P \leq 0.05$). Moreover, at 18 h, GZMB protein expression was reduced by ~74% in NK-92 cells treated with APAP 20 mM compared to controls ((Fig. 4B and Supplementary Fig. S1). This effect was extended by the transfection of NK-92 cells with the miR-378a-5p mimic in APAP treated cells and GZMB protein expression was reduced by ~86% compared to mimic controls ((Fig. 4B and Supplementary Fig. S2). Positive control of 3T3 transfected with GZMB showing 28 KDa observed band size (Fig. S1). ALLStars negative control transfection did not impact GZMB expression (Fig. S2). We did not investigate the endogenous levels of miR-378a-5p in the NK cells, because NK cells do not encode miR-378a [30]. Altogether, the data demonstrate that APAP may directly reduce GZMB expression in NK cells, and this reduction can further enhance by the presence of miR-378a.

3.4. Elevated miR-378a Levels Decrease GZMB Levels in APAP Overdose Subjects

As noted above, gene expression levels of GZMB were reduced in APAP overdose sub-groups, and miR-378a-5p can cause that effect. To further assess the mechanism of miRNA-mRNA interaction, Paxgene extracted miRNA from patient peripheral blood was analyzed for the expression of miR-378a-5p ((Fig. 5). The expression of miR-378a in

the no toxicity subgroup was upregulated by 2.2-fold compared to controls ($p<0.05$), whereas miR-378a in the moderate toxicity subgroup was upregulated 3.8 fold compared to controls ($p<0.001$). The difference between no toxicity *vs.* moderate toxicity subgroup for miR-378a-5p approached significance ($p=0.05$). Of interest, elevations of miR-378a appeared to be minimally impacted by NAC treatment, as collectively, patients with APAP overdose, regardless of evidence of liver injury as quantified by ALT or APAP adduct values, had higher levels of miR-378a compared to controls. This data unequivocally establishes a key role for miR-378a-5p in regulating GZMB expression in APAP overdose.

4. DISCUSSION

Acetaminophen overdose is associated with the development of acute hepatocellular injury evaluated in the clinical setting by elevations of serum alanine aminotransferase (ALT). As ALT elevations and other clinical indicators are relatively late stage biomarkers of liver injury, considerable interest has arisen for the identification of other, more sensitive and mechanism-related biomarkers of APAP-Induced Liver Injury (AILI). MiRNAs function as rheostats to finetune signaling pathways by controlling mRNA and protein output of target genes. MiRNAs have been shown to modulate signaling for phase I drug-metabolizing enzymes directly or indirectly through epigenetic mechanisms [18] and thus can play crucial roles in metabolism and in the development of AILI. Recent work showed elevation of miR-378a-5p in APAP overdose children [20] and adults [11]. However, the mechanistic significance of these elevations has not been previously examined. We recently found that miR-378a-5p repressed CYP2E1 [20] and in the current study, both invivo and in-vitro data demonstrate that miR-378a-5p regulates GZMB signaling in APAP toxicity. GZMB, known to regulate cell cycle and immune responses, belongs to the esterase family of enzymes important in hydrolysis reactions of phase I drug metabolism. Bioinformatics analysis identified the GZMB gene as a regulatory target of miR-378a-5p. This mRNA-miRNA interaction was experimentally verified by the gain-of-function luciferase reporter assay, showing that miR-378a-5p controls GZMB transcription (Fig. 3B). Moreover, NK-92 cells directly exposed to APAP had downregulation of GZMB expression at both mRNA and protein levels ((Fig. 4). Gain-of-function studies showed translational repression of GZMB expression in NK-92 cells transfected with the miR-378a mimic and then treated with APAP ((Fig. 4B). In addition, peripheral blood samples of APAP overdose subjects showed decreased expression of GZMB ((Fig. 2) and increased expression of miR-378a-5p ((Fig. 5). While the clinical data presented herein are correlative and consistent with other reports of miR-378a elevation in APAP toxicity [11, 20], the cell-based studies suggest a potential inhibitory effect of miR-378a-5p to down-regulate GZMB expression in APAP toxicity, possibly exacerbating a direct effect of APAP.

A proposed schematic model of APAP induced dysregulation of signaling pathways is illustrated in (Fig. 6). Hepatic glutathione (GSH) effectively detoxifies NAPQI [31] and with toxic APAP exposures, GSH stores are diminished and NAPQI covalently binds to cysteine residues on hepatic cellular proteins to form APAP protein adducts [1, 32, 33]. In addition, oxidative and nitrosative stress are major mechanisms in the development of hepatocellular necrosis [34, 35], as well as mitochondrial dysfunction and ATP depletion [36-39]. APAP toxicity is also associated with the early release and translocation of BAX

(BCL2-associated X protein) (Fig. 6) to mitochondria [38, 40–43], leading to mitochondrial swelling, and the rupture of the outer membrane with the passive release of intermembrane proteins [41].

The miRNA Tissue atlas (https://ccb-web.cs.uni-saarland.de/tissueatlas/), show a predominant source of miR-378a-5p to be muscle [44–46, 49]. NK cell cytotoxicity was shown to be activated by IFN-α and miR-378 was reported to directly target GZMB in NK cells [50]. GZMB play a critical role in antibody-dependent cellular cytotoxicity [51] and results in tumor target cell apoptosis. Other studies have shown that miR-378a-3p targets 3'UTR of GZMB in dengue fever [52], and acute myeloid leukemia [53]. Taken together, these studies provide a comprehensive understanding of miR-378 directly targeting 3' UTR of GZMB in NK cells. A recent investigation demonstrated miR-378a expression in CD14 monocytes, but its expression was not reported for CD56 cell surface receptors on Natural Killer (NK) cells [30]. Blood and immune cells are the predominant sources of GZMB [54, 55]. GZMB is a cytotoxic T-lymphocyte-associated serine esterase involved in cell death processes and influences innate and adaptive immune responses [47–49]. GZMB enters mitochondria [56] and triggers cell death both in a caspase-dependent and caspase-independent manner [57]. In mitochondria, GZMB induces cell death through oligomerization of BAX and BAK in the outer mitochondrial membrane mediated by BID (BH3 Interacting Domain Death Agonist) [58, 59]. Davis and colleagues [60] showed BCL-2 inhibition of GZMB-mediated cell death (Fig. 6). GZMB can also induce cell death in a ROS-dependent manner [61], independent of caspases and Mitochondrial Outer Membrane Permeabilization (MOMP). It can cleave and activate several important pro-inflammatory cytokines [62]. Our findings suggest that activation of BID may be blocked by downregulation of GZMB in natural killer cells, which is a direct target of miR-378a-5p (Fig. 6).

It is well-established that mitochondria are central in APAP hepatotoxicity [39], leading to programmed necrosis [70, 71] and ROS generation [36]. In addition, Bax is known to be important in APAP hepatotoxicity, as Bax-deficient mice have reduced liver injury [41]. Therefore, it is possible that GZMB could enter hepatocytes and accelerate liver injury by enhancing mitochondrial damage and Bax translocation, as described in (Fig. 6). Interestingly, depletion of NK cells with an intraperitoneal injection of mouse anti-NK1.1 antibody has no effect on APAP hepatotoxicity, despite NK cells being present in large numbers in the liver and likely activated after APAP overdose [72]. On the other hand, mice deficient in IL-13 have increased sensitivity to APAP hepatotoxicity associated with increased NK cells and NKT cells [69], indicating a role for NK cells in APAP-induced liver injury. It is possible that elevated circulating miR-378-5p normally suppresses GZMB expression in NK cells after APAP overdose and that prevents NK cell-mediated enhancement of the liver injury, which would explain why depleting NK cells does nothing but increasing them could still exacerbate the toxicity. Though it is clear that many crucial signaling pathways contribute to APAP hepatotoxicity and in principle reflect the complexity of regulatory cross-talk between pathways. Overall, our data highlights a novel molecular mechanism whereby miR-378a-5p upregulation in APAP toxicity may have a regulatory role in controlling GZMB expression.

CONCLUSION

In summary, although further studies are required to define the GZMB-miR-378 interaction, our clinical data potentially highlight a novel role of GZMB and miR-378a-5p in the development of APAP toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

[1]. Hinson JA, Roberts DW, James LP. Mechanisms of acetaminophen-induced liver necrosis. Handbook Exp Pharmacol 2010; 196: 369–405. 10.1007/978-3-642-00663-0_12

[2]. Lee WM. Acetaminophen and the U.S. Acute liver failure study group: lowering the risks of hepatic failure. Hepatology 2004; 40(1): 6–9. 10.1002/hep.20293 [PubMed: 15239078]

[3]. Ostapowicz G, Fontana RJ, Schiødt FV, et al. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. Ann Intern Med 2002; 137(12): 947–54. 10.7326/0003-4819-137-12-200212170-00007 [PubMed: 12484709]

[4]. Dahlin DC, Miwa GT, Lu AY, Nelson SD. *N*-acetyl-*p*-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proc Natl Acad Sci USA 1984; 81(5): 1327–31. 10.1073/pnas.81.5.1327 [PubMed: 6424115]

[5]. Ambros V. The functions of animal microRNAs. Nature 2004; 431(7006): 350–5. 10.1038/nature02871 [PubMed: 15372042]

[6]. Ivey KN, Srivastava D. MicroRNAs as regulators of differentiation and cell fate decisions. Cell Stem Cell 2010; 7(1): 36–41. 10.1016/j.stem.2010.06.012 [PubMed: 20621048]

[7]. Li Y, Shi X. MicroRNAs in the regulation of TLR and RIG-I pathways. Cell Mol Immunol 2013; 10(1): 65–71. 10.1038/cmi.2012.55 [PubMed: 23262976]

[8]. O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D. Physiological and pathological roles for microRNAs in the immune system. Nat Rev Immunol 2010; 10(2): 111–22. 10.1038/nri2708 [PubMed: 20098459]

[9]. Krek A, Grün D, Poy MN, et al. Combinatorial microRNA target predictions. Nat Genet 2005; 37(5): 495–500. 10.1038/ng1536 [PubMed: 15806104]

[10]. Vliegenthart AD, Shaffer JM, Clarke JI, et al. Comprehensive microRNA profiling in acetaminophen toxicity identifies novel circulating biomarkers for human liver and kidney injury. Sci Rep 2015; 5: 15501. 10.1038/srep15501 [PubMed: 26489516]

[11]. Ward J, Kanchagar C, Veksler-Lublinsky I, et al. Circulating microRNA profiles in human patients with acetaminophen hepatotoxicity or ischemic hepatitis. Proc Natl Acad Sci USA 2014; 111(33): 12169–74. 10.1073/pnas.1412608111 [PubMed: 25092309]

[12]. Yang X, Salminen WF, Shi Q, et al. Potential of extracellular microRNAs as biomarkers of acetaminophen toxicity in children. Toxicol Appl Pharmacol 2015; 284(2): 180–7. 10.1016/j.taap.2015.02.013 [PubMed: 25708609]

[13]. Antoine DJ, Dear JW, Lewis PS, et al. Mechanistic biomarkers provide early and sensitive detection of acetaminophen-induced acute liver injury at first presentation to hospital. Hepatology 2013; 58(2): 777–87. 10.1002/hep.26294 [PubMed: 23390034]

[14]. Dear JW, Antoine DJ, Starkey-Lewis P, Goldring CE, Park BK. Early detection of paracetamol toxicity using circulating liver microRNA and markers of cell necrosis. Br J Clin Pharmacol 2014; 77(5): 904–5. 10.1111/bcp.12214 [PubMed: 23879521]

[15]. Jetten MJ, Gaj S, Ruiz-Aracama A, et al. 'Omics analysis of low dose acetaminophen intake demonstrates novel response pathways in humans. Toxicol Appl Pharmacol 2012; 259(3): 320–8. 10.1016/j.taap.2012.01.009 [PubMed: 22285215]

[16]. Krauskopf J, de Kok TM, Schomaker SJ, et al. Serum microRNA signatures as "liquid biopsies" for interrogating hepatotoxic mechanisms and liver pathogenesis in human. PLoS One 2017; 12(5): e0177928. 10.1371/journal.pone.0177928

[17]. Starkey Lewis PJ, Merz M, Couttet P, et al. Serum microRNA biomarkers for drug-induced liver injury. Clin Pharmacol Ther 2012; 92(3): 291–3. 10.1038/clpt.2012.101 [PubMed: 22828715]

[18]. Yu AM, Tian Y, Tu MJ, Ho PY, Jilek JL. MicroRNA pharmacoepigenetics: posttranscriptional regulation mechanisms behind variable drug disposition and strategy to develop more effective therapy. Drug Metab Dispos 2016; 44(3): 308–19. 10.1124/dmd.115.067470 [PubMed: 26566807]

[19]. Chowdhary V, Teng KY, Thakral S, et al. miRNA-122 protects mice and human hepatocytes from acetaminophen toxicity by regulating cytochrome P450 family 1 subfamily A member 2 and family 2 subfamily E member 1 expression. Am J Pathol 2017; 187(12): 2758–74. 10.1016/j.ajpath.2017.08.026 [PubMed: 28963035]

[20]. Gill P, Bhattacharyya S, McCullough S, et al. MicroRNA regulation of CYP 1A2, CYP3A4 and CYP2E1 expression in acetaminophen toxicity. Sci Rep 2017; 7(1): 12331. 10.1038/s41598-017-11811-y [PubMed: 28951593]

[21]. Yu D, Wu L, Gill P, et al. Multiple microRNAs function as self-protective modules in acetaminophen-induced hepatotoxicity in humans. Arch Toxicol 2018; 92(2): 845–58. 10.1007/s00204-017-2090-y [PubMed: 29067470]

[22]. Bhattacharyya S, Yan K, Pence L, et al. Targeted liquid chromatography-mass spectrometry analysis of serum acylcarnitines in acetaminophen toxicity in children. Biomarkers Med 2014; 8(2): 147–59. 10.2217/bmm.13.150

[23]. Khandelwal N, James LP, Sanders C, Larson AM, Lee WM. Unrecognized acetaminophen toxicity as a cause of indeterminate acute liver failure. Hepatology 2011; 53(2): 567–76. 10.1002/hep.24060 [PubMed: 21274877]

[24]. Muldrew KL, James LP, Coop L, et al. Determination of acetaminophen-protein adducts in mouse liver and serum and human serum after hepatotoxic doses of acetaminophen using high-performance liquid chromatography with electrochemical detection. Drug Metab Dispos 2002; 30(4): 446–51. 10.1124/dmd.30.4.446 [PubMed: 11901099]

[25]. Arikawa E, Sun Y, Wang J, et al. Cross-platform comparison of SYBR Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. BMC Genomics 2008; 9: 328. 10.1186/1471-2164-9-328 [PubMed: 18620571]

[26]. Paraskevopoulou MD, Georgakilas G, Kostoulas N, et al. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic Acids Res 2013; 41: 169–73. 10.1093/nar/gkt393

[27]. Dweep H, Gretz N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. Nat Methods 2015; 12(8): 697. 10.1038/nmeth.3485 [PubMed: 26226356]

[28]. Dweep H, Sticht C, Pandey P, Gretz N. miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform 2011; 44(5): 839–47. 10.1016/j.jbi.2011.05.002 [PubMed: 21605702]

[29]. Battle A, Brown CD, Engelhardt BE, Montgomery SB. Genetic effects on gene expression across human tissues. Nature 2017; 550(7675): 204–13. 10.1038/nature24277 [PubMed: 29022597]

[30]. Leidinger P, Backes C, Meder B, Meese E, Keller A. The human miRNA repertoire of different blood compounds. BMC Genomics 2014; 15: 474. 10.1186/1471-2164-15-474 [PubMed: 24928098]

[31]. Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J Pharmacol Exp Ther 1973; 187(1): 211–7. [PubMed: 4746329]

[32]. Han D, Dara L, Win S, et al. Regulation of drug-induced liver injury by signal transduction pathways: critical role of mitochondria. Trends Pharmacol Sci 2013; 34(4): 243–53. 10.1016/j.tips.2013.01.009 [PubMed: 23453390]

[33]. James LP, Mayeux PR, Hinson JA. Acetaminophen-induced hepatotoxicity. Drug Metab Dispos 2003; 31(12): 1499–506. 10.1124/dmd.31.12.1499 [PubMed: 14625346]

[34]. Hinson JA, Pike SL, Pumford NR, Mayeux PR. Nitrotyrosine-protein adducts in hepatic centrilobular areas following toxic doses of acetaminophen in mice. Chem Res Toxicol 1998; 11(6): 604–7. 10.1021/tx9800349 [PubMed: 9625727]

[35]. James LP, Letzig L, Simpson PM, et al. Pharmacokinetics of acetaminophen-protein adducts in adults with acetaminophen overdose and acute liver failure. Drug Metab Dispos 2009; 37(8): 1779–84. 10.1124/dmd.108.026195 [PubMed: 19439490]

[36]. Du K, Ramachandran A, Jaeschke H. Oxidative stress during acetaminophen hepatotoxicity: sources, pathophysiological role and therapeutic potential. Redox Biol 2016; 10: 148–56. 10.1016/j.redox.2016.10.001 [PubMed: 27744120]

[37]. Gonçalves DF, de Carvalho NR, Leite MB, et al. Caffeine and acetaminophen association: effects on mitochondrial bioenergetics. Life Sci 2018; 193: 234–41. 10.1016/j.lfs.2017.10.039 [PubMed: 29107792]

[38]. Hanawa N, Shinohara M, Saberi B, Gaarde WA, Han D, Kaplowitz N. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. J Biol Chem 2008; 283(20): 13565–77. 10.1074/jbc.M708916200 [PubMed: 18337250]

[39]. Jaeschke H, McGill MR, Ramachandran A. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. Drug Metab Rev 2012; 44(1): 88–106. 10.3109/03602532.2011.602688 [PubMed: 22229890]

[40]. Adams ML, Pierce RH, Vail ME, et al. Enhanced acetaminophen hepatotoxicity in transgenic mice overexpressing BCL-2. Mol Pharmacol 2001; 60(5): 907–15. 10.1124/mol.60.5.907 [PubMed: 11641418]

[41]. Bajt ML, Farhood A, Lemasters JJ, Jaeschke H. Mitochondrial bax translocation accelerates DNA fragmentation and cell necrosis in a murine model of acetaminophen hepatotoxicity. J Pharmacol Exp Ther 2008; 324(1): 8–14. 10.1124/jpet.107.129445 [PubMed: 17906064]

[42]. El-Hassan H, Anwar K, Macanas-Pirard P, et al. Involvement of mitochondria in acetaminophen-induced apoptosis and hepatic injury: roles of cytochrome C, Bax, Bid, and caspases. Toxicol Appl Pharmacol 2003; 191(2): 118–29. 10.1016/S0041-008X(03)00240-0 [PubMed: 12946648]

[43]. Gunawan BK, Liu ZX, Han D, Hanawa N, Gaarde WA, Kaplowitz N. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. Gastroenterology 2006; 131(1): 165–78. 10.1053/j.gastro.2006.03.045 [PubMed: 16831600]

[44]. Ludwig N, Leidinger P, Becker K, et al. Distribution of miRNA expression across human tissues. Nucleic Acids Res 2016; 44(8): 3865–77. 10.1093/nar/gkw116 [PubMed: 26921406]

[45]. Carrer M, Liu N, Grueter CE, et al. Control of mitochondrial metabolism and systemic energy homeostasis by microRNAs 378 and 378*. Proc Natl Acad Sci USA 2012; 109(38): 15330–5. 10.1073/pnas.1207605109 [PubMed: 22949648]

[46]. Eichner LJ, Perry MC, Dufour CR, et al. miR-378(*) mediates metabolic shift in breast cancer cells *via* the PGC-1β/ERRγ transcriptional pathway. Cell Metab 2010; 12(4): 352–61. 10.1016/j.cmet.2010.09.002 [PubMed: 20889127]

[47]. Crome SQ, Lang PA, Lang KS, Ohashi PS. Natural killer cells regulate diverse T cell responses. Trends Immunol 2013; 34(7): 342–9. 10.1016/j.it.2013.03.002 [PubMed: 23601842]

[48]. Pallmer K, Oxenius A. Recognition and regulation of T Cells by NK Cells. Front Immunol 2016; 7: 251. 10.3389/fimmu.2016.00251 [PubMed: 27446081]

[49]. Krist B, Florczyk U, Pietraszek-Gremplewicz K, Józkowicz A, Dulak J. The role of miR-378a in metabolism, angiogenesis, and muscle biology. Int J Endocrinol 2015; 2015281756 10.1155/2015/281756

[50]. Wang P, Gu Y, Zhang Q, et al. Identification of resting and type I IFN-activated human NK cell miRNomes reveals microRNA-378 and microRNA-30e as negative regulators of NK cell cytotoxicity. J Immunol 2012; 189(1): 211–21. 10.4049/jimmunol.1200609 [PubMed: 22649192]

[51]. Elavazhagan S, Fatehchand K, Santhanam V, et al. Granzyme B expression is enhanced in human monocytes by TLR8 agonists and contributes to antibody-dependent cellular cytotoxicity. J Immunol 2015; 194(6): 2786–95. 10.4049/jimmunol.1402316 [PubMed: 25667415]

[52]. Liu S, Chen L, Zeng Y, et al. Suppressed expression of miR-378 targeting GZMB in NK cells is required to control dengue virus infection. Cell Mol Immunol 2016; 13(5): 700–8. 10.1038/cmi.2015.52 [PubMed: 26166761]

[53]. Bhise NS, Chauhan L, Shin M, et al. MicroRNA-mRNA pairs associated with outcome in AML: from *in vitro* cell-based studies to AML patients. Front Pharmacol 2016; 6: 324. 10.3389/fphar.2015.00324 [PubMed: 26858643]

[54]. Consortium GT. The Genotype-Tissue Expression (GTEx) project. Nat Genet 2013; 45(6): 580–5. 10.1038/ng.2653 [PubMed: 23715323]

[55]. Wilhelm M, Schlegl J, Hahne H, et al. Mass-spectrometry-based draft of the human proteome. Nature 2014; 509(7502): 582–7. 10.1038/nature13319 [PubMed: 24870543]

[56]. Chiusolo V, Jacquemin G, Yonca Bassoy E, et al. Granzyme B enters the mitochondria in a Sam50-, Tim22- and mtHsp70-dependent manner to induce apoptosis. Cell Death Differ 2017; 24(4): 747–58. 10.1038/cdd.2017.3 [PubMed: 28338658]

[57]. Trapani JA. Immunity, granzymes and cell killing. In: eLS John Wiley and Sons, Ltd. 2001. 10.1002/9780470015902

[58]. Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. Immunol Rev 2010; 235(1): 105–16. 10.1111/j.0105-2896.2010.00908.x [PubMed: 20536558]

[59]. Cullen SP, Brunet M, Martin SJ. Granzymes in cancer and immunity. Cell Death Differ 2010; 17(4): 616–23. 10.1038/cdd.2009.206 [PubMed: 20075940]

[60]. Davis JE, Sutton VR, Smyth MJ, Trapani JA. Dependence of granzyme B-mediated cell death on a pathway regulated by Bcl-2 or its viral homolog, BHRF1. Cell Death Differ 2000; 7(10): 973–83. 10.1038/sj.cdd.4400725 [PubMed: 11279544]

[61]. Jacquemin G, Margiotta D, Kasahara A, et al. Granzyme B-induced mitochondrial ROS are required for apoptosis. Cell Death Differ 2015; 22(5): 862–74. 10.1038/cdd.2014.180 [PubMed: 25361078]

[62]. Wensink AC, Hack CE, Bovenschen N. Granzymes regulate pro inflammatory cytokine responses. J Immunol 2015; 194(2): 491–7. 10.4049/jimmunol.1401214 [PubMed: 25556251]

[63]. Ray SD, Jena N. A hepatotoxic dose of acetaminophen modulates expression of BCL-2, BCL-X(L), and BCL-X(S) during apoptotic and necrotic death of mouse liver cells *in vivo*. Arch Toxicol 2000; 73(10–11): 594–606. 10.1007/s002040050013 [PubMed: 10663392]

[64]. Qiu Y, Benet LZ, Burlingame AL. Identification of the hepatic protein targets of reactive metabolites of acetaminophen *in vivo* in mice using two-dimensional gel electrophoresis and mass spectrometry. J Biol Chem 1998; 273(28): 17940–53. 10.1074/jbc.273.28.17940 [PubMed: 9651401]

[65]. Donnelly PJ, Walker RM, Racz WJ. Inhibition of mitochondrial respiration *in vivo* is an early event in acetaminophen-induced hepatotoxicity. Arch Toxicol 1994; 68(2): 110–8. 10.1007/s002040050043 [PubMed: 8179480]

[66]. Boulares AH, Zoltoski AJ, Stoica BA, Cuvillier O, Smulson ME. Acetaminophen induces a caspase-dependent and Bcl-XL sensitive apoptosis in human hepatoma cells and lymphocytes. Pharmacol Toxicol 2002; 90(1): 38–50. 10.1034/j.1600-0773.2002.900108.x [PubMed: 12005112]

[67]. Krenkel O, Mossanen JC, Tacke F. Immune mechanisms in acetaminophen-induced acute liver failure. Hepatobiliary Surg Nutr 2014; 3(6): 331–43. 10.3978/j.issn.2304-3881.2014.11.01 [PubMed: 25568858]

[68]. Liu ZX, Govindarajan S, Kaplowitz N. Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity. Gastroenterology 2004; 127(6): 1760–74. 10.1053/j.gastro.2004.08.053 [PubMed: 15578514]

[69]. Yee SB, Bourdi M, Masson MJ, Pohl LR. Hepatoprotective role of endogenous interleukin-13 in a murine model of acetaminophen-induced liver disease. Chem Res Toxicol 2007; 20(5): 734–44. 10.1021/tx600349f [PubMed: 17439248]

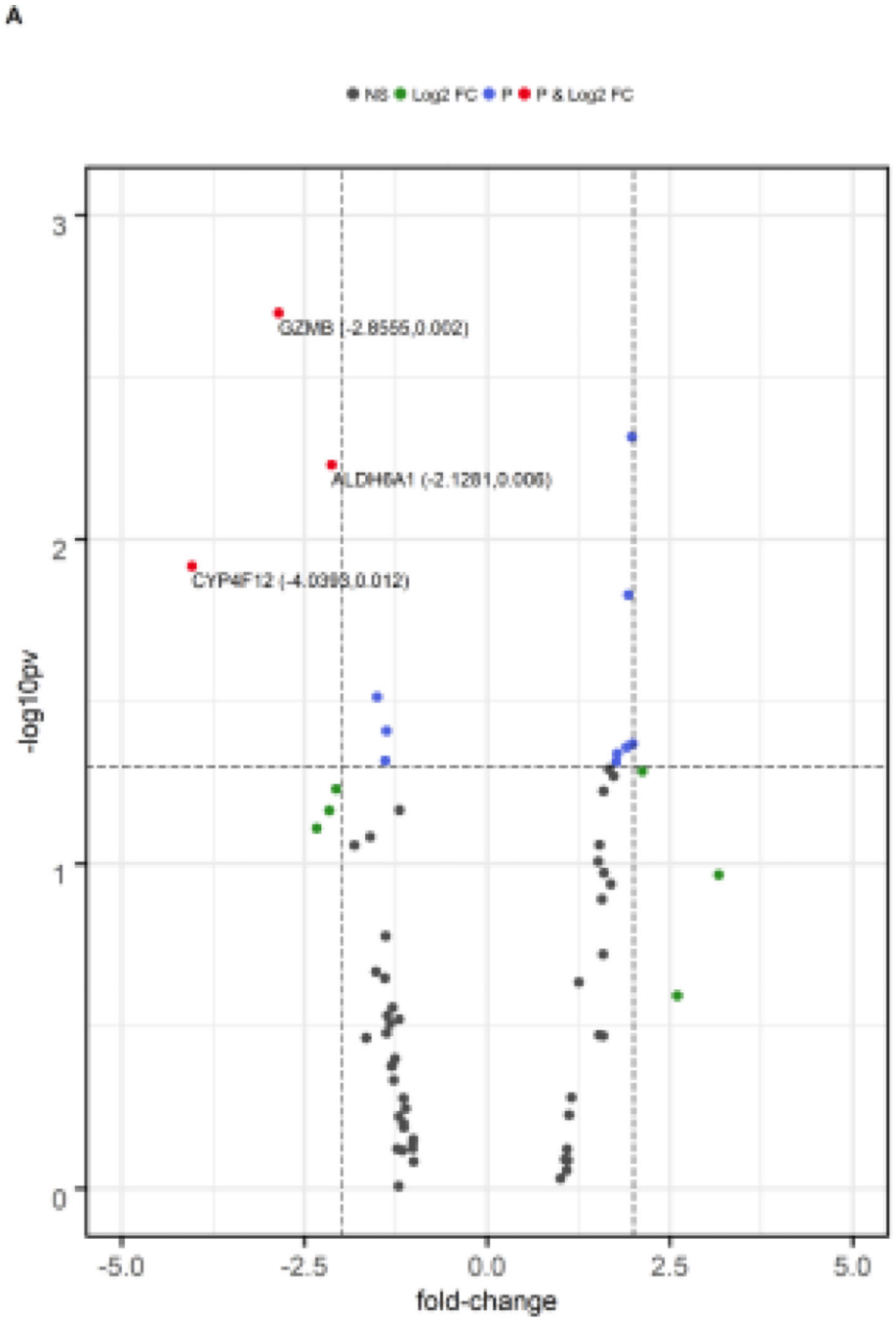
[70]. Jaeschke H, Duan L, Akakpo JY, Farhood A, Ramachandran A. The role of apoptosis in acetaminophen hepatotoxicity. Food Chem Toxicol 2018; 118: 709–18. 10.1016/j.fct.2018.06.025 [PubMed: 29920288]

[71]. Ramachandran A, McGill MR, Xie Y, Ni HM, Ding WX, Jaeschke H. Receptor interacting protein kinase 3 is a critical early mediator of acetaminophen-induced hepatocyte necrosis in mice. Hepatology 2013; 58(6): 2099–108. 10.1002/hep.26547 [PubMed: 23744808]

[72]. Masson MJ, Carpenter LD, Graf ML, Pohl LR. Pathogenic role of natural killer T and natural killer cells in acetaminophen-induced liver injury in mice is dependent on the presence of dimethyl sulfoxide. Hepatology 2008; 48(3): 889–97. 10.1002/hep.22400 [PubMed: 18712839]

[73]. Ida H, Utz PJ, Anderson P, Eguchi K. Granzyme B and Natural Killer (NK) cell death. Mod Rheumatol 2005; 15(5): 315–22. 10.3109/s10165-005-0426-6 [PubMed: 17029086]

[74]. Rousalova I, Krepela E. Granzyme B-induced apoptosis in cancer cells and its regulation. Int J Oncol 2010; 37(6): 1361–78. [PubMed: 21042704]



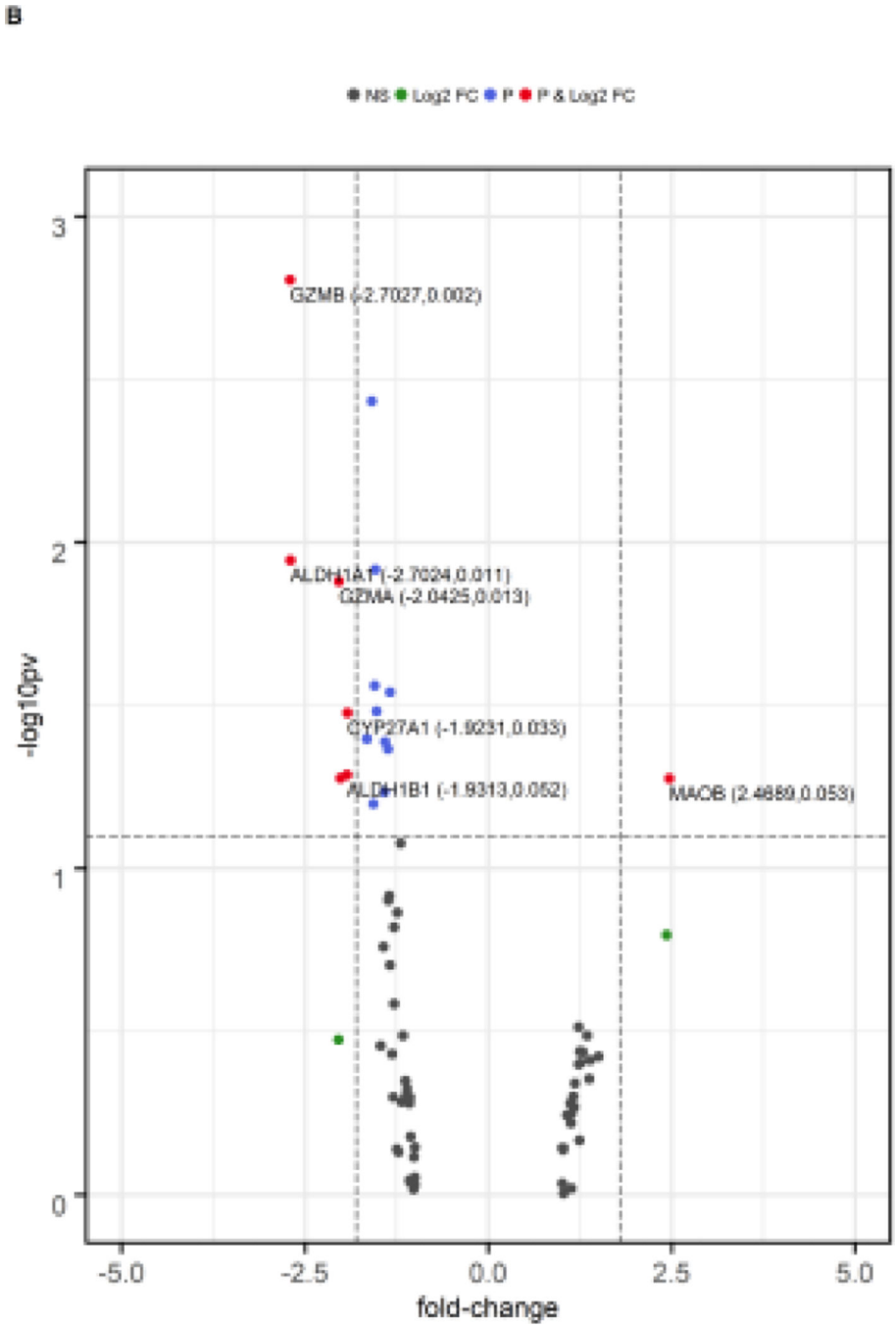


Fig. (1).

Phase I enzymes gene expression in PAXgene isolated total RNA from APAP overdose subjects. (**A**) With no toxicity subgroup (ALT ≤ 45 IU/L). Volcano plot of drug metabolism phase I PCR array. No toxicity APAP overdose subgroup (n=5) compared to controls (n=5). (**B**) With moderate toxicity subgroup (ALT ≥ 500 IU/L). Volcano plot of drug metabolism phase I PCR array. Moderate toxicity APAP overdose subgroup (n=5) compared to controls (n=5). Volcano plot analyses used significant genes that separated the two subgroups (control *vs* no toxicity or control *vs* moderate toxicity) and combined the Fold Change

(FC) and the *p* values from the *t*-tests. P value <0.05; fold cut off 2. To determine significant genes (red color dots), the p-value cut-off was set to 0.05 and the expression threshold was 2-fold (FC). The green color depicts genes that meet only FC criterion, whereas, the blue color shows candidates qualifying with only p-value cutoff, and the grey color represents non-significant genes.

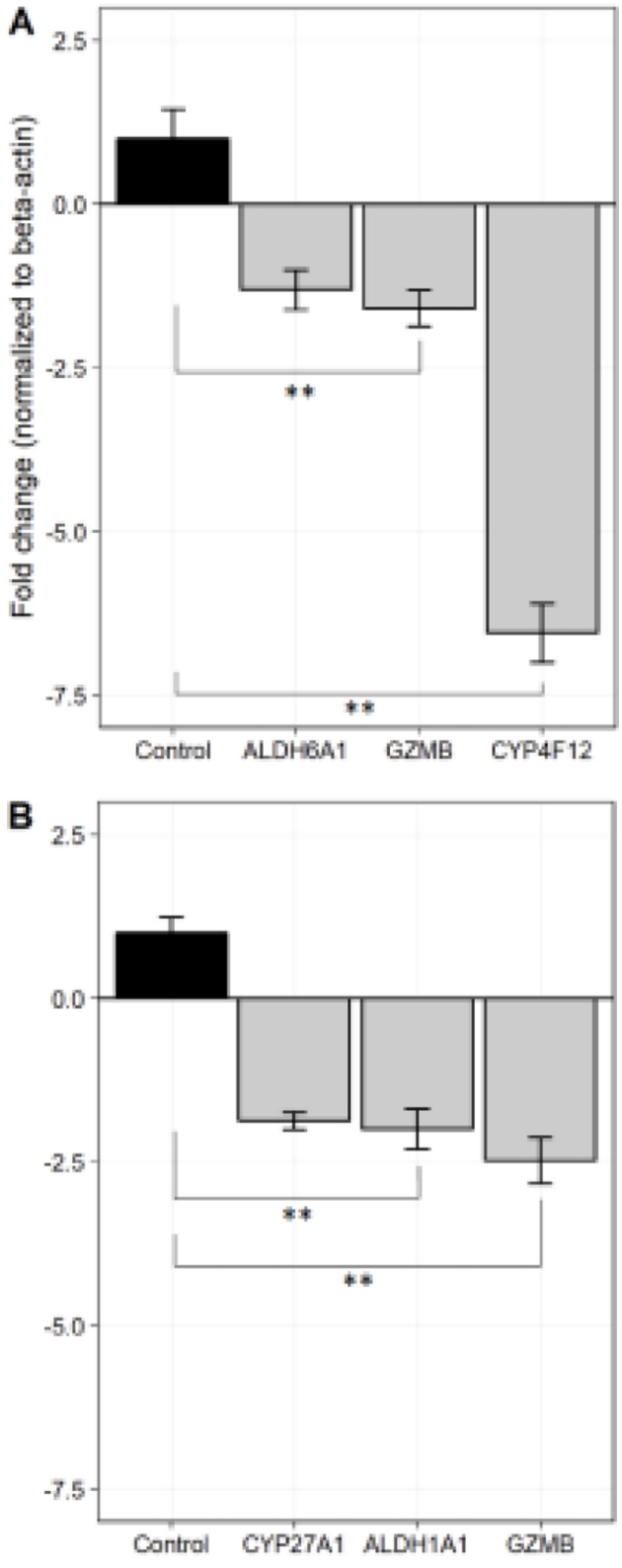


Fig. (2).

qRT-PCR validation of significantly dysregulated genes in PAXgene isolated total RNA from APAP overdose subjects. (**A**) CYP4F12, ALDH6A1 and GZMB in no toxicity subgroup (n=5) compared to controls (n=5). (**B**) CYP27A1, ALDH1A1 and GZMB in moderate toxicity (n=5) compared to controls (n=5). Data are means ± SEM, n = 5. *P ≤ 0.05; **P ≤ 0.01 versus controls. Supplementary Fig. (S3) shows specificity of PCR product using melt curve analysis for GZMB and β-actin.

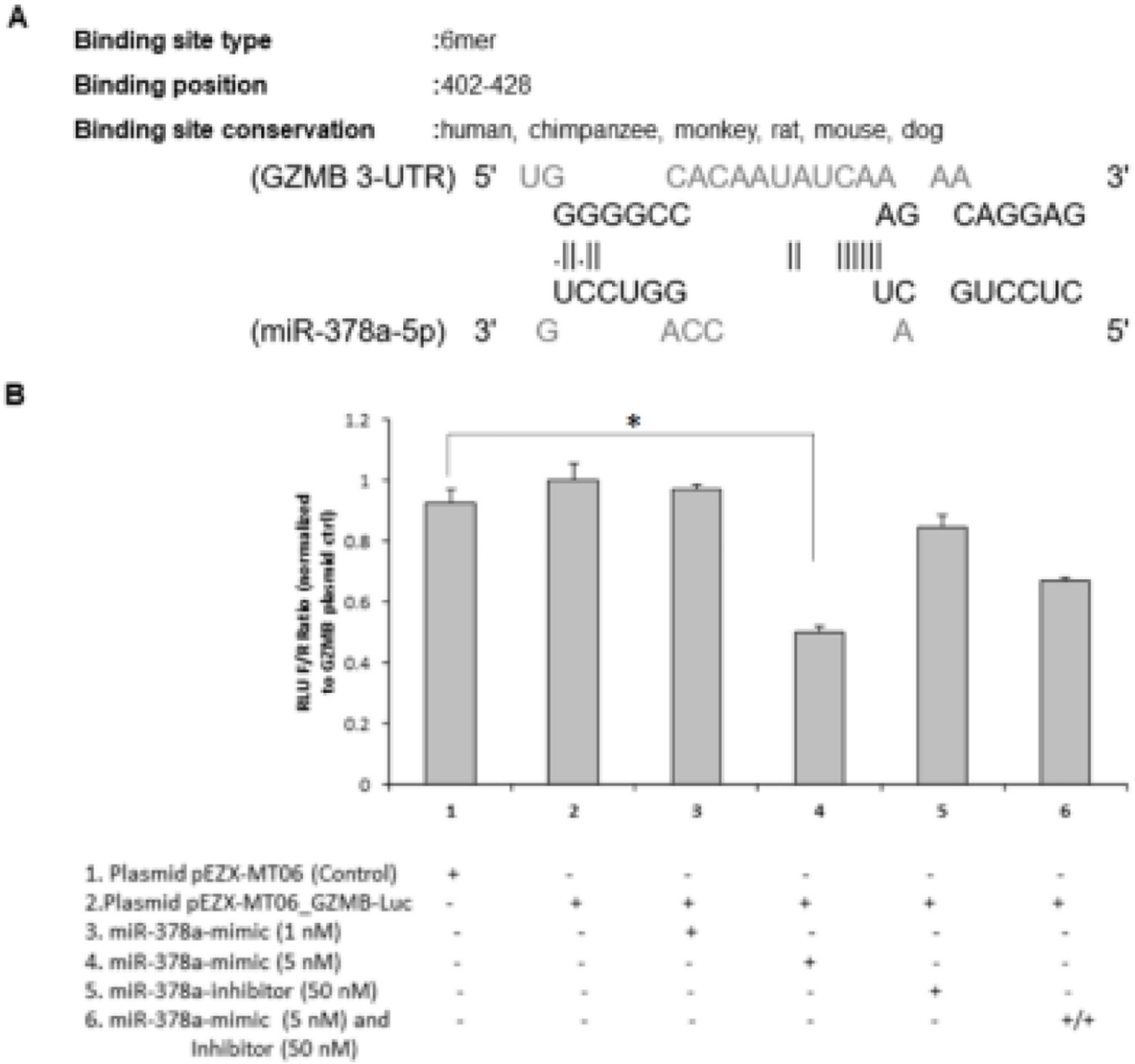
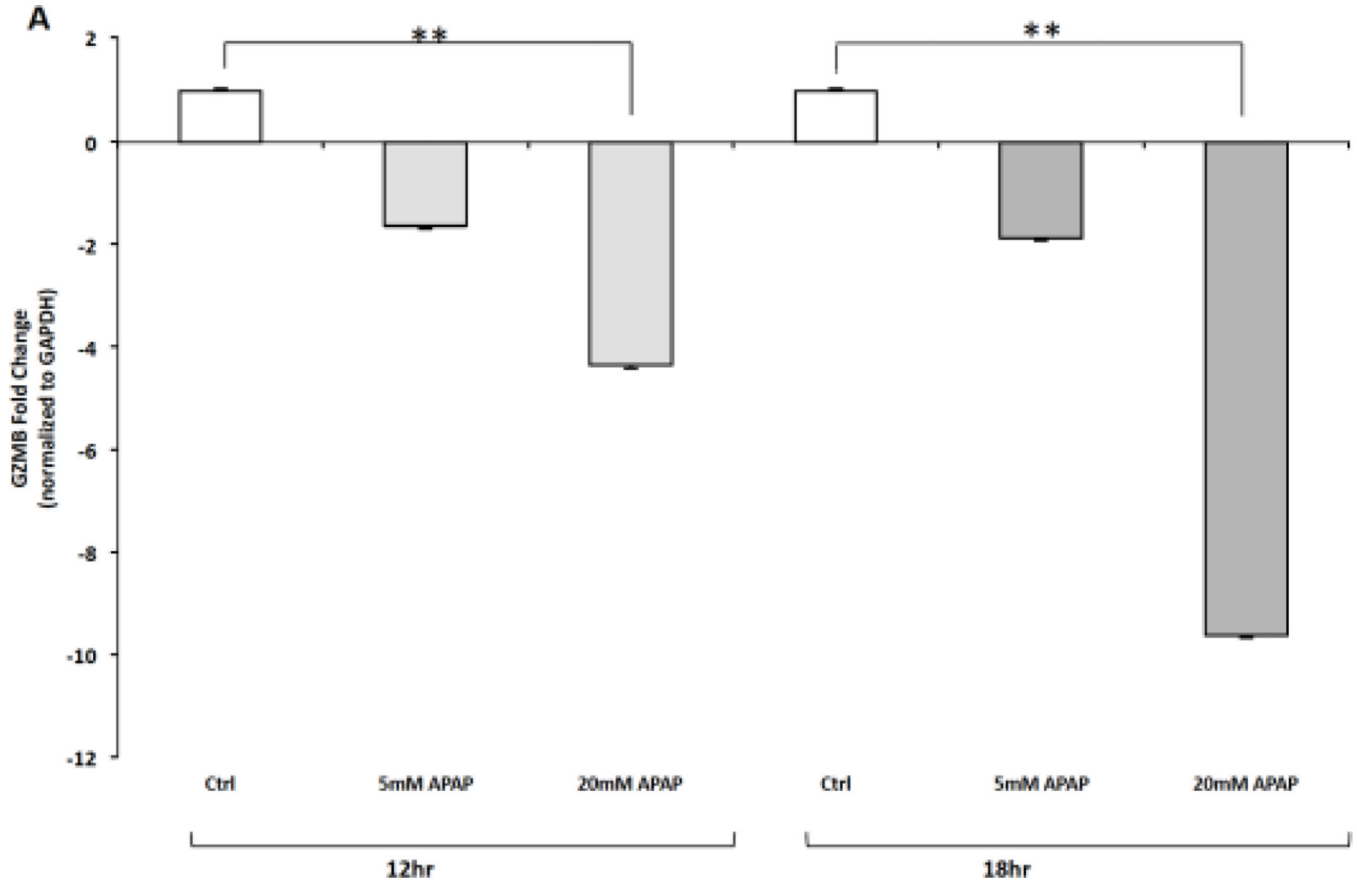


Fig. (3).

GZMB is a direct target of miR-378a-5p (**A**) Evolutionary conservation of the miR-378a-5p seed site in mammalian GZMB 3′ UTRs. Human, chimpanze, monkey, dog, and mouse show alignment with the seed region of miR-378a-5p. Base pairing comparison between miR-378a-5p and GZMB 3′UTR putative target site is shown according to the DIANA-microT-CDS algorithm. (**B**) MiR-378a-5p directed repression of renilla luciferase reporter genes bearing 3' UTR of GZMB : HEK 293FT cells were co-transfected with 200 ng of either pEZX-MT06 or pEZX-MT06-GZMB-LUC vector (GeneCopoeia) and miR-378a-5p mimic (1 nM, 5 nM) or inhibitor (50 nM) using Attractene transfection reagent (Qiagen). Experiments were repeated 3 independent times. Firefly luciferase activity which was

determined 24 h after the transfection and was normalized with respective Renilla luciferase activity. RLU=Relative Luciferase Units. Data are means ± SEM, n = 2. ANOVA with Tukey's post hoc test *P =0.029 *versus* controls.



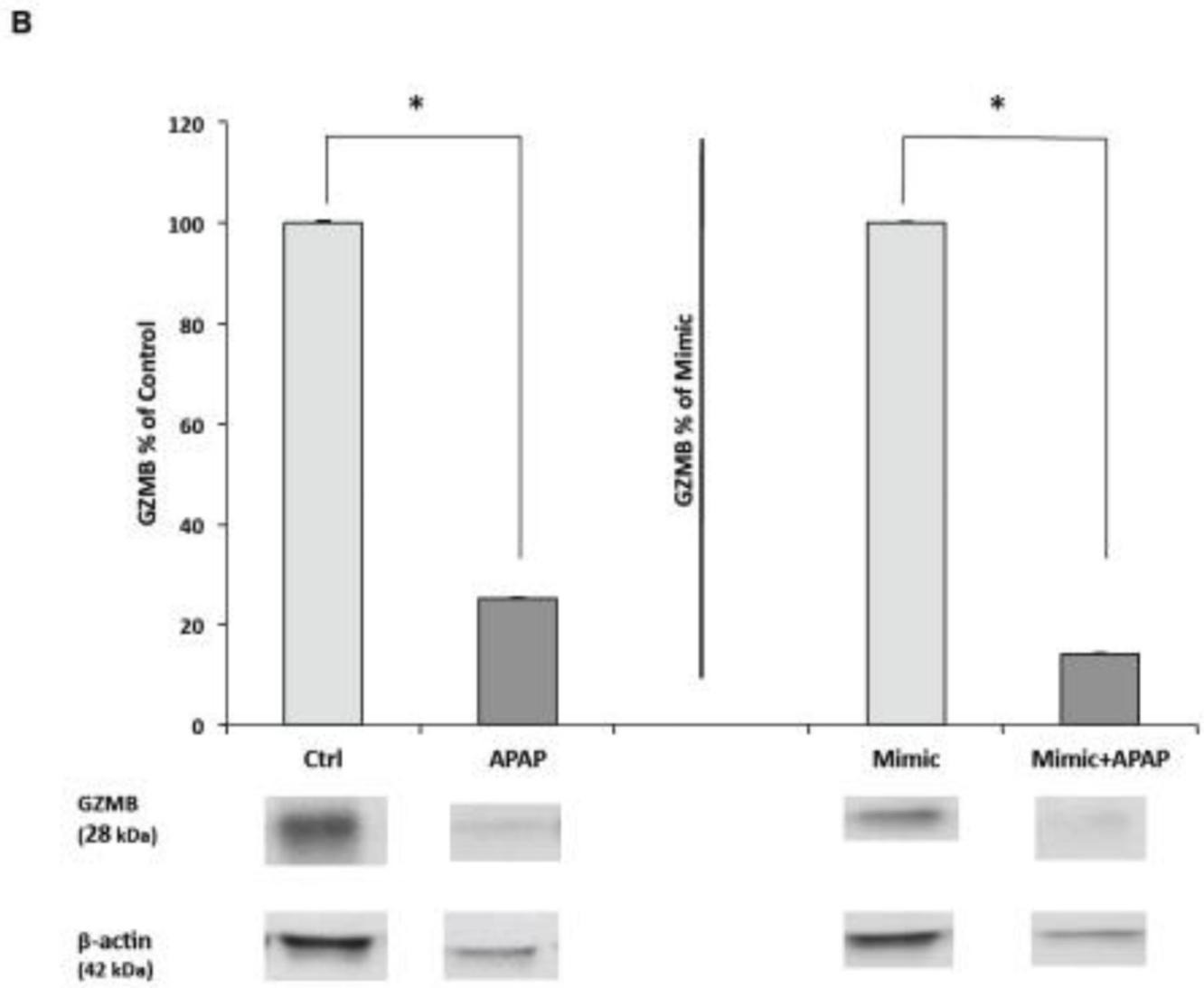


Fig. (4).

Effect of APAP on GZMB gene expression and protein levels in NK-92 cells. (**A**) Effect of APAP treatment (5 and 20 mM) on the GZMB gene expression in human NK-92 cells at 12 and 18 h. (**B**) GZMB protein levels in NK-92 cells treated with 20 mM APAP for 18 h (left panel). GZMB protein levels in NK-92 cells transfected with miR-378a mimic (5 nM) and treated with 20 mM APAP for 18 h (right panel). Data are means ± SEM of 3 independent experiments in each category $*P \leq 0.05$. GZMB was normalized to β-actin for each group.

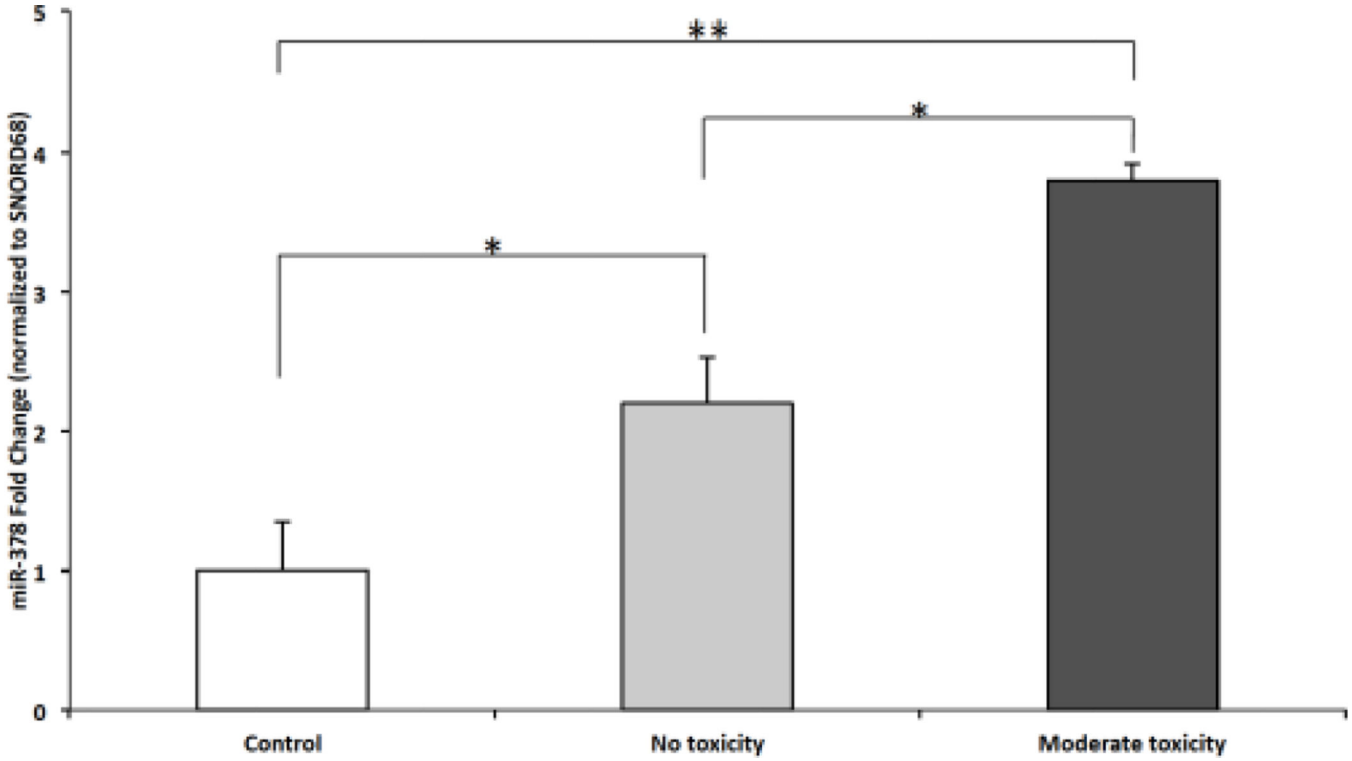


Fig. (5).

Expression of miR-378a-5p in PAXgene isolated micro**RNA. Controls (n=5)**; APAP overdose samples were no toxicity subgroup (n=5) and moderate toxicity subgroup (n=5), significance level *P ≤ 0.05. ** P<0.001. Supplementary Fig. S3 shows specificity of PCR product using melt curve analysis for miR-378a-5p and SNORD68.

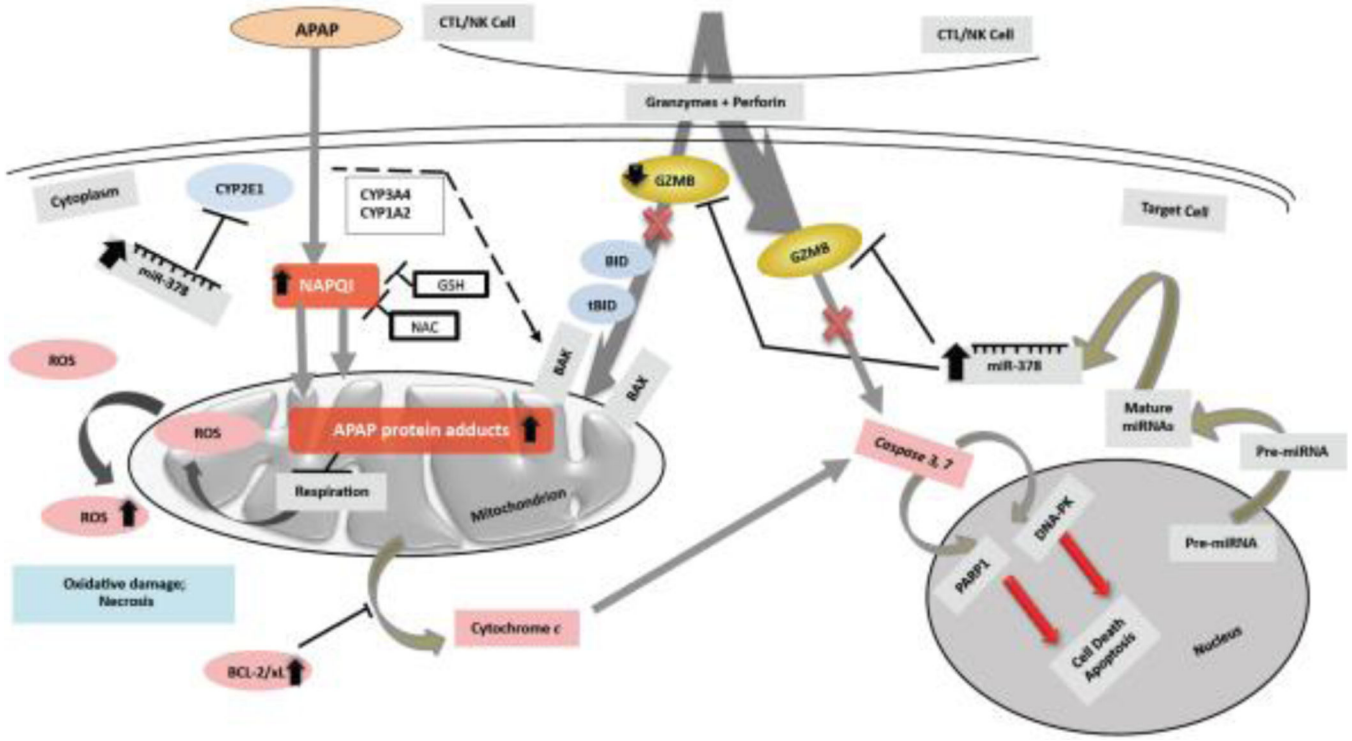


Fig. (6).

Model for APAP-induced dysregulation of signaling pathways regulating GZMB and miR-378a-5p. APAP overdose suppresses the expression of GZMB. miR-378a-5p which has putative binding site in the 3'UTR of GZMB directly regulate its expression. Downregulation of GZMB directly blocks the activation of BID and Caspase pathway, which are important in cell death. GZMB has several substrates and most prominent ones are BID, ICAD, Mcl-1, PARP1, and procaspases (2, 3, 7, 8, 9 and 10). BCL2= BCL2, Apoptosis regulator; BCL-xL= B-cell lymphoma-extra-large; BID= BH3 Interacting Domain Death Agonist; tBID= trucated BH3 Interacting Domain Death Agonist; BAX=BCL2-associated X protein; BAK= BCL2 Antagonist/Killer 1; DNA-PK= DNA-dependent protein kinase; GSH= Glutathione; GZMB= Granzyme B; miRNA= microRNA; miR-378a= miR-378a-5p; PARP1=Poly(ADP-Ribose) Polymerase 1; NAC= N-acetyl cysteine; NAPQI= N-acetyl-p-benzoquinone imine; ROS= Reactive oxygen species [32, 43–47, 57–59, 63–70, 73, 74].

Table 1.

Clinical parameters for subject with toxie APAP exposure (no or moderate toxicity subgroup).

Sample	N	ALT (IU/L) Median (Range)	Adducts (nmoles/ml) Median (Range)
Control	5	15 (7, 20)	0.006 (0.00, 0.01)
APAP Overdose (no toxicity)	5	35 (25, 45)	0.6821 (0.440, 0.777) *
APAP Overdose (moderate toxicity)	5	1409 (697, 2071) ***	0.936 (0.84, 1.244) **

ALT and adduct levels shown as median and range values. One-way analysis of variance (ANOVA) with Tukey's post hoc test.

*, ** and *** indicate p=0.015, p=0.00015 and p= 0.0002, respectively. For each no toxicity and moderate toxicity subgroup: ALT (n=5) and adducts (n=4). ALT= alanine aminotransferase; Adducts= APAP protein adducts.