

Age-Related Changes in miRNA Expression Influence GSTZ1 and Other Drug Metabolizing Enzymes

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Keywords: dichloroacetate, drug metabolism, GSTZ1, liver, miR-376c-3p, miRNA

Running Title: miRNA control of GSTZ1

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Number of text pages: 24

Number of tables: 4 (including 3 supplemental tables)

Number of figures: 7

Number of references: 31

Abstract words: 215

Introduction words: 543

Discussion words: 1015

Abbreviations: ADH, alcohol dehydrogenase; Ago, argonaute; ALDH, aldehyde dehydrogenase; AKR, aldo-keto reductase, AO, aldehyde oxidase; CDS, coding sequence; CYP, Cytochrome P450; DCA, dichloroacetate; eIF4e, eukaryotic translation initiation factor 4E; EPHX, epoxide hydrolase; FMO, flavin containing monooxygenase; GST, glutathione transferase; GSTZ1, glutathione transferase zeta 1; GW182, glycine-tryptophan protein of 182 kDa; MAO, monoamine oxidase; MT, methyl transferase; NAT, N-acetyltransferase; NQO, NAD(P)H dehydrogenase; PABP, poly-A-binding protein; Rb, retinoblastoma protein; RNA-induced silencing complex, RISC; SULF, sulfotransferase; UGT, UDP-glucuronosyltransferase; UTR, untranslated region

Abstract

Previous work has shown that hepatic levels of human glutathione transferase zeta 1 (GSTZ1) protein, involved in tyrosine catabolism and responsible for metabolism of the investigational drug dichloroacetate, increase in cytosol after birth before reaching a plateau around age seven. However, the mechanism regulating this change of expression is still unknown, and previous studies showed that *GSTZ1* mRNA levels did not correlate with GSTZ1 protein expression. In this study, we addressed the hypothesis that microRNAs could regulate expression of GSTZ1. We obtained liver samples from donors aged less than one year or older than thirteen years and isolated total RNA for use in a microarray to identify miRNAs that were downregulated in the livers of adults compared to children. From a total of 2,578 human miRNAs tested, 63 miRNAs were more than two-fold down-regulated in adults, of which miR-376c-3p was predicted to bind to the 3'-UTR of *GSTZ1* mRNA. There was an inverse correlation of miR-376c-3p and GSTZ1 protein expression in the liver samples. Using cell culture, we confirmed that miR-376c-3p could downregulate GSTZ1 protein expression. Our findings suggest that miR-376c-3p prevents production of GSTZ1 through inhibition of translation. These experiments further our understanding of GSTZ1 regulation. Furthermore, our array results provide a database resource for future studies on mechanisms regulating human hepatic developmental expression.

Significance Statement: Hepatic GSTZ1 is responsible for metabolism of the tyrosine catabolite maleylacetoacetate as well as the investigational drug dichloroacetate. Through examination of miRNA expression in liver from infants and adults and studies in cells, we showed that expression of GSTZ1 is controlled by miRNA. This finding has application to the dosing regimen of the drug dichloroacetate. The miRNA expression profiles are provided and will prove useful for future studies of drug-metabolizing enzymes in infants and adults.

Introduction

Glutathione transferase zeta 1 (GSTZ1), also known as maleylacetoacetate isomerase, is a primarily hepatic enzyme, present in cytosol and mitochondria, that is physiologically important in the tyrosine catabolism pathway (James et al., 2017). It is also the sole enzyme responsible for the metabolism of the experimental drug dichloroacetate (DCA), which has shown promise in the treatment of mitochondrial disorders, cancer, and pulmonary hypertension, among other diseases. While humans are born with little to no hepatic GSTZ1 expression, its levels undergo an increase shortly after birth before plateauing around age 7 years in cytosol and 21 years in mitochondria (Li et al., 2012; Zhong et al., 2018). This age-related expression is clinically relevant, as dosage with DCA must be appropriate for the extent of GSTZ1-mediated metabolism (reviewed in (James et al., 2017)). To date, the regulatory mechanisms controlling GSTZ1 expression have gone undiscovered.

Across the proteome, protein expression is regulated at multiple stages (reviewed in (Bina, 2013)). DNA and histone modifications determine the accessibility of genes. The expression levels, availability (i.e. the sequestration of E2F by retinoblastoma protein (Rb)), and subcellular localization of transcription factors influence the rate at which mRNA is transcribed from an accessible gene. Post-transcriptional modifications to newly formed mRNA determine the rate at which it becomes suitable for translation, and alternative splicing modulates the distinct fate of the pre-mRNA. Even after translation, post-translational modifications can have dramatic effects on the stability, and therefore observed expression levels, of proteins.

With the discovery of microRNAs (miRNAs) in the 1990s (Lee et al., 1993) and subsequent realization of their important role in gene regulatory mechanisms (Pasquinelli et al., 2000; Reinhart et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001), an additional layer of regulation has become apparent. The mechanism of action of miRNAs has traditionally been thought of as the regulation of mRNA stability. In this pathway, binding of miRNA to

complementary mRNA sequences leads to the recruitment of the RNA-induced silencing complex (RISC) and degradation of the mRNA (Gurtan and Sharp, 2013) . The subsequently lower mRNA levels result in lower protein expression.

Previous studies by our lab and others have failed to identify any transcriptional regulators or post-translational modifications that would be expected to modulate stability of GSTZ1 (Ammini et al., 2003; Zhong et al., 2018). Additionally, there was no correlation between GSTZ1 mRNA levels and protein expression (Langae et al., 2015). A possible explanation for this finding is related to a second function of miRNA. In addition to the canonical degradation pathway induced by miRNA binding, miRNAs also play an important role in gene regulation through the inhibition of translation (Gu and Kay, 2010; Dluzen and Lazarus, 2015). In this scenario, protein levels can be controlled without changing observed mRNA levels.

In the present study we tested the hypothesis that the differences in GSTZ1 enzyme expression that have been observed between young children and adults could be caused by differential expression of one or more miRNAs. We used a microarray to measure the expression of all known human miRNAs in liver from infants and adults and completed further studies on those that were downregulated in adults while also being predicted to bind to the GSTZ1 mRNA 3' untranslated region (UTR). The experimental flowchart is presented in Figure 1.

Materials and Methods

Human Liver Samples

Human liver samples were obtained under an exempt protocol that was approved by the University of Florida Institutional Review Board. Samples came from the University of Florida Clinical and Translational Science Institute Biorepository, the NIH Neurobiobank *via* the University of Maryland, and the Cooperative Human Tissue Network *via* Nationwide Children's

Hospital and the University of Alabama at Birmingham. Young samples were from fifteen donors of both sexes aged 1 – 303 days (mean 69.1 ± 93.1) and adult samples were from fifteen donors of both sexes aged 13 – 86 years (mean 53.2 ± 22.9).

Microarray Analysis

Total RNA was isolated from a subset of the human liver samples comprising ten young (range 1 to 303 days, mean 90.0 ± 108.1 days) and ten adult (range 32 to 86 years, mean 62.4 ± 18.5 years) livers using the miRNeasy mini kit from Qiagen (Valencia, CA) according to the manufacturer's instructions. A sample size of 10 in each age group would enable us to detect differences of 1.5-fold between groups with statistical power of 90% and $p < 0.05$. Previous studies showing 4-fold differences in expression of GSTZ1 protein between these age groups informed selection of this group size (Li et al., 2012; Zhong et al., 2018). The total RNA was delivered to the University of Florida Interdisciplinary Center for Biotechnology Research, where miRNA expression was determined using the GeneChip miRNA 4.0 array covering 2,578 human mature miRNAs from ThermoFisher Scientific (Carlsbad, CA). The .CEL files that we received from the core facility were then analyzed using the Transcriptome Analysis Console 4.0 software from ThermoFisher Scientific. Specific filter settings are described in more detail in subsequent sections. In all cases, a filter of $p \leq 0.05$ was applied.

RT-qPCR Analysis

Total RNA was isolated from all 30 human liver samples using the miRNeasy kit and then reverse transcribed using the miScript II RT kit. miScript primer assays for hsa-miR-376c-3p (MS00004046), hsa-miR-6738-5p (MS00046417), hsa-miR-23b-3p (MS00031647), and hsa-miR-152-3p (MS00003591) were then carried out in triplicate using Quantitect SYBR green on a QuantStudio 12K Flex Real-Time PCR System (ThermoFisher). All reagents were from Qiagen. ΔC_t values were calculated for miR-376c-3p and miR-6738-5p by subtracting the geometric

mean C_t value obtained from miR-23b-3p and miR-152-3p from the C_t value of the miRNA of interest (Lamba et al., 2014).

Generation of Stable Cell Lines

The 293T and MDA-MB-231 cell lines were grown in high glucose DMEM + 10% FBS and the HepG2 cell line was grown in low glucose DMEM + 10% FBS because high concentrations of glucose induce apoptosis in this cell line (Chandrasekaran et al., 2010). A lentiviral system was used to generate stable cell lines by transfecting 293T cells with the viral packaging plasmids PMD2.G and PsPax2 (Addgene, Cambridge, MA) along with the lentiviral vector pEZX-MR03 containing either a scrambled miRNA negative control or miR-376c-3p (Genecopoeia, Rockville, MD). The lentivirus that was produced was then used to transduce the MDA-MB-231 and HepG2 cell lines. All infected cell lines were then selected using puromycin from ThermoFisher Scientific.

Luciferase Assay

The PGL3 firefly luciferase plasmid was modified to contain nucleotides 1016 – 1282 of *hGSTZ1* mRNA, which is part of the 3' untranslated region (3'-UTR) (Fang et al., 2006). Forward (5'-gccgtgtaattctagaTTGAGGAGATGGGAGACTCG-3') and reverse (5'-gctcgaagcggccggccTGGAGTCACACACAACAAAAG-3') primers were amplified from genomic DNA obtained from the BCBL-1 human cell line. The amplicon was further amplified with Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) and inserted into the pGL3 plasmid that had been linearized by *Xho1* and *FseI* (New England Biolabs) using the Genart Seamless Cloning and Assembly kit (ThermoFisher Scientific).

293T cell lines stably expressing either a negative control scrambled miRNA or miR-376c-3p were plated at 130,000 cells per well of a 24-well plate. The next day, the cells were transfected using lipofectamine 2000 (ThermoFisher Scientific) to introduce the pGL3-GSTZ1

3'-UTR plasmid along with a PEF *Renilla* luciferase expressing plasmid as a transfection control. The following morning, the cells were refed, and then processed after an additional 24 hours had passed. Lysates were generated and analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). All transfections were done in quadruplicate and firefly luciferase expression was normalized to *Renilla* luciferase expression.

Immunoblot Analysis

Samples of liver, 100 mg, were homogenized in four volumes of 1.15% KCl/0.05M potassium phosphate buffer pH 7.4 and centrifuged at 600 x G to sediment nuclei and unbroken cells. Western blots and subsequent immunoblots were carried out as described previously (Li et al., 2011). The primary antibody was a custom polyclonal product produced by Cocalico Biologicals (Stevens, PA) and raised in rabbits against whole, purified recombinant human GSTZ1C. The peroxidase-linked secondary antibody was purchased from Sigma Aldrich (St. Louis, MO). Wells of the SDS-polyacrylamide gels were loaded with 30 µg of total protein from the 600xG supernatant of each sample. After transfer, the Western blot was normalized using total protein loading as determined by staining with Ponceau dye (ThermoFisher Scientific). Blots were quantified using a Chemidoc (Biorad, Hercules, CA) and ImageJ software from the National Institutes of Health.

Statistical Analysis

Student's t-tests and Pearson correlation coefficients were calculated using GraphPad Prism software (GraphPad Software, v6.07 San Diego, CA).

Results

miRNA Expression is Associated with Age

We previously showed that hepatic GSTZ1 expression is higher in adults than in children (Li et al., 2012; Zhong et al., 2018). In order to test our hypothesis that this change in expression is due to differential miRNA expression, we obtained liver samples from young (< 1 year old) and adult donors. Microarray analysis of total RNA from ten samples of each group, twenty samples total, yielded data showing the relative expression of 2,578 miRNAs in these age groups. In total, 378 miRNAs were differentially regulated ($p \leq 0.05$, Figure 2A), with 104 miRNAs showing at least a 2-fold change in expression (Figure 2B). Of these, 63 were downregulated, while 41 were upregulated in the adults. A list of these miRNAs is found in Supplemental Table 1. The values presented were calculated by the Transcriptome Analysis Console 4.0 software.

Identification of Candidate miRNAs Regulating GSTZ1

Since GSTZ1 protein expression is higher in adults, a miRNA regulating its expression would be expected to be lower in this same group. Therefore, we focused our attention on the 63 miRNAs that showed at least a 2-fold decrease in expression and had $p \leq 0.05$. Comparing this list to miRNAs predicted to bind to the GSTZ1 3'-UTR by the miRanda (John et al., 2004) or Targetscan (Lewis et al., 2005) algorithms, or that were found associated with GSTZ1 in the Tarbase 8.0 database (Karagkouni et al., 2018), yielded two candidate miRNAs: hsa-miR-376c-3p (previously known as miR-368) and hsa-miR-6738-5p. The list of downregulated miRNA, together with those from the miRanda, Targetscan, and Tarbase databases, are in Supplemental Table 2.

To verify the downregulation of these miRNAs in adults compared to children, we measured the expression of miR-376c-3p and miR-6738-5p using RT-qPCR in the livers that were used for the microarray analysis, plus five additional young samples and five additional adult samples providing 15 samples for each age group. Basic demographic information for all samples can be found in Supplemental Table 3. C_t values for these miRNAs were compared to

those obtained for miR-23b-3p and miR-152-3p, which have been shown to be suitable loading controls for analysis of miRNAs in the liver (Lamba et al., 2014). High ΔC_t values represent low miRNA expression, and ΔC_t values were significantly higher ($p < 0.005$, $n = 15$) in the adults, with mean values of 2.53 ± 2.56 and 5.23 ± 2.18 in the young and adult groups, respectively (Figure 3A). miR-6738-5p showed C_t values in the low- to mid- thirties, indicating very low expression (Figure 3B). Additionally, there was no difference seen between the age groups. We therefore determined that the differential expression seen for miR-6738-5p in the array was most likely a false-positive with an unknown cause. The predicted complementarity between the *GSTZ1* mRNA and miR-376c-3p is shown in Figure 3C, beginning with nucleotide 208 of the *GSTZ1* 3'-UTR.

miR-376c-3p Expression Correlates with GSTZ1 Protein Expression

While we have previously shown a large increase in GSTZ1 protein expression in adults compared to children, we sought to confirm that this difference was also present in the samples used in this study. Immunoblots to GSTZ1 were carried out on 600xG supernatants of homogenates of these livers. GSTZ1 expression was then quantified using densitometry and relative expression was calculated compared to a reference sample that was included on all blots. As expected, GSTZ1 protein expression was significantly higher in the adults ($p < 0.01$), with relative expressions of $62.4 \pm 35.2\%$ ($n = 14$) and $143 \pm 77.8\%$ ($n = 15$) in the young and adult groups, respectively (Figure 4A). Generation of a scatterplot with miR-376c-3p ΔC_t values and GSTZ1 protein expression in individual liver samples shows a direct correlation (Figure 4B). A Pearson correlation coefficient analysis yielded $r = 0.505$ and $p < 0.01$.

There was overlap in GSTZ1 protein expression in samples from infants and adults. The GSTZ1 protein expression in the highest expressing young samples were similar to the lowest expressing adult samples. Comparison of ΔC_t values measuring miR-376c-3p expression in these samples showed no difference between age groups (Figure 4C).

miR-376c-3p Inhibits Protein Expression

To show that miR-376c-3p can directly inhibit GSTZ1 protein expression, we utilized cell culture models. HepG2 (hepatocellular carcinoma) and MDA-MB-231 (breast cancer) cell lines, both of which express a small amount of GSTZ1 endogenously, were engineered to express either a scrambled negative control miRNA (miR-CTRL) or miR-376c-3p. Immunoblots of lysates obtained from these cell lines showed that the HepG2 cells expressing exogenous miR-376c-3p had approximately 90% as much GSTZ1 protein as did the cells expressing miR-CTRL (Figure 5A, n = 3). The MDA-MB-231 cell line with exogenous miR-376c-3p showed a greater decrease, to below 60% of the cell line expressing miR-CTRL. The changes in both the HepG2 and MDA-MB-231 cell lines were significant, $p < 0.05$.

We also showed that miR-376c-3p could inhibit the expression of firefly luciferase that had been modified such that its mRNA contained a portion of the 3'-UTR from *GSTZ1*. 293T cell lines expressing either miR-CTRL or miR-376-3p were transfected with the firefly luciferase along with a second plasmid containing *Renilla* luciferase as a transfection control. The cell line expressing miR-376c-3p had roughly 75% the level of firefly luciferase expression when compared to the cell line expressing miR-CTRL (Figure 5B, n = 4). A diagram showing the construction of the firefly luciferase reporter plasmid is shown in Figure 5C.

Several Drug Metabolizing Enzymes are Targeted by miRNAs Differentially Regulated with Age

A number of drug metabolizing enzymes are differentially regulated with age (Hines, 2008), but the mechanisms controlling such changes are currently not fully understood. In order to use our data more fully, we sought to identify which of these enzymes might be controlled by miRNAs. To do so, we utilized the results of our miRNA array to identify all miRNAs that were significantly up- or downregulated between the age groups ($p < 0.05$, fold change > 2). This

yielded 65 miRNAs that were expressed to a lesser degree in the adult group and 39 that showed higher expression in the adults.

We made a list of all predicted target mRNAs for these miRNAs using the TargetScan and miRDB (Wang, 2016) prediction algorithms. This procedure allowed us to search this list for families of drug metabolizing enzymes that are potentially targeted by miRNAs that are differentially regulated with age. Figure 6A shows families of enzymes and the total number of differentially regulated miRNAs that were predicted to bind their target mRNA. Figures 6B and 6C show how many of that total number were upregulated and how many were downregulated.

We then identified individual genes that had a differential of at least five between the targeting miRNAs that were up- and downregulated (i.e., 6 upregulated and 1 downregulated or 2 upregulated and 7 downregulated) (Table 1), as we considered these genes most likely to be influenced by the changes in miRNA expression. Interestingly, this list of twenty-eight genes contains six whose expression is known to be higher in adults than in children and showed a downregulation in miRNAs predicted to target them, giving a potential mechanism for the expression changes. These were CYP2B6 (Croom et al., 2009), GSTM (Strange et al., 1989), FMO3 (Dolphin et al., 1996) and UGTs1A1, 1A6 and 1A9 (Bhatt et al., 2019).

Discussion

The hepatic expression of many drug metabolizing enzymes changes with age, but the mechanism by which these changes occur is often unknown, reviewed in (Hines, 2008). GSTZ1, the sole enzyme responsible for the metabolism of the investigational drug DCA, falls into this category. While its increase in expression over the early years of life has been well characterized (Li et al., 2012; Zhong et al., 2018), efforts to identify mechanisms controlling its expression had been unsuccessful.

In this study we have demonstrated for the first time that an endogenous process is capable of modulating levels of GSTZ1 protein. Using an array containing all known human mature miRNAs, we identified 63 miRNAs that are downregulated in liver samples from adults compared to very young children. By comparing this list to those miRNAs predicted to bind to the 3'-UTR of *GSTZ1* mRNA, we identified two potential miRNA regulators of GSTZ1.

RT-qPCR confirmed that miR-376c-3p expression decreased with age, and a significant correlation was found between miR-376c-3p and GSTZ1 protein expression in our samples. Cell culture experiments showed that introduction of miR-376c-3p caused a decrease in GSTZ1 expression. These data indicate that declining miR-376c-3p expression during the human maturation process likely results in increasing GSTZ1 protein levels. These experiments were carried out using HepG2, MDA-MB-231, and 293T cells. We believe that these are appropriate models since the cell culture experiments were intended as proof-of-principle experiments. The cells provided a vehicle for miRNA to bind either endogenous *GSTZ1* mRNA or exogenous luciferase mRNA. Use of primary hepatocytes, or intact liver, would potentially provide more contextual data, but would also introduce experimental complications that would make these experiments impractical.

In addition to GSTZ1's role in the metabolism of DCA, it is also a critical enzyme in the tyrosine-catabolism pathway, where it is known as maleylacetoacetate isomerase. While it is possible that increasing GSTZ1 expression is an evolutionary response to altered tyrosine consumption with age, it is also possible that the increase is simply a bystander effect. Since each miRNA regulates many mRNAs, if miR-376c-3p expression changes with age in order to change the expression of another gene, it would also affect the expression of GSTZ1. There is experimental evidence of miR-376c-3p interacting with 325 other mRNAs (Paraskevopoulou et al., 2016), therefore, elucidating the primary target(s) will require substantial future work.

Just as each miRNA regulates many mRNAs, many mRNAs are bound and controlled by multiple miRNAs. While we showed that miR-376c-3p decreases GSTZ1 protein expression, the decreases seen in our cell culture studies were not as large as the changes seen with age in human liver. We identified miR-376c-3p due to its large expression change, but it is possible that other miRNAs, showing less than a two-fold decrease with age, work in concert to regulate GSTZ1. Figure 7A shows the 3'-UTR binding locations of five miRNAs that were shown by our array to be downregulated ($p < 0.05$), regardless of the magnitude of the decrease. Due to the relatively small decreases in expression seen for the other miRNAs, there are likely other unknown factors that will need to be identified in future studies.

While the 3'-UTR of *GSTZ1* has not been sequenced in a large number of samples, it is important to note that the predicted binding between all miRNAs discussed in this paper and the 3' UTR are based off reference sequences. It is unknown if polymorphisms exist that would influence miRNA binding, but it would be expected that changes in the mRNA sequence at the miRNA binding site could increase or decrease the effects of miRNA expression of *GSTZ1* protein expression. This could result in large variations in the population and is an interesting area for follow-up studies.

Previous experiments have noted that there is a lack of correlation between *GSTZ1* mRNA and its protein. Therefore, miR-376c-3p likely does not regulate *GSTZ1* through the canonical mRNA destruction pathway. In recent years it has become apparent that miRNAs function through multiple mechanisms, including the inhibition of translation (Gu and Kay, 2010). Upon binding to the 3'-UTR, the argonaute (Ago) and glycine-tryptophan protein of 182 kDa (GW182) protein complex is recruited. These large proteins may act to sterically block the circularization of mRNA that results from the binding of eukaryotic translation initiation factor 4E (eIF4e) to the poly-A binding protein (PABP). These proteins may also prevent the binding of the ribosomal subunits or block them from progressing the full length of the coding sequence.

The results of our array may be useful for other studies examining changes in hepatic protein expression with age. The data are freely available from the Gene Expression Omnibus database (accession: GSE132763). Mining these data, we identified drug metabolizing enzymes whose mRNA are predicted to be bound by miRNA that showed altered regulation with age. Flavin containing monooxygenases (FMO), which show a very well documented, though poorly understood, switch in predominant expression from FMO1 to FMO3 during development (Dolphin et al., 1996; Yeung et al., 2000) may be candidates for further study. As shown in Table 1, FMO1 showed upregulation with age of more miRNAs targeting it than downregulating it, and FMO3 showed the opposite. Other genes whose targeting miRNA showed large changes consistent with known mRNA or protein levels were several members of the UDP-glucuronosyltransferase (UGT) family, where miRNAs including miR-376c-3p have previously been implicated (Papageorgiou and Court, 2017), and GSTs in the A and M families (Strange et al., 1989). For CYP3A4 and SULT2A1, however, the observed miRNA differential was not in accord with the known higher expression of these enzymes in adults compared to infants (Dubaisi et al., 2019).

It is possible that miRNAs influence the expression of many drug metabolizing enzymes and we have only begun to identify these cases. To our knowledge, this study is the first to report that expression of a member of the GST superfamily, GSTZ1, is regulated at least in part by a miRNA. Our data can be used in future studies examining not only the regulation of GSTZ1 and other drug metabolizing enzymes in human liver, but also any protein that is differentially regulated with age.

Acknowledgements

Tissue samples were obtained from the University of Florida Clinical and Translational Science Institute (NIH National Center for Advancing Translational Sciences award number UL1TR001427), the NCI Cooperative Human Tissue Network (CHTN), and the NIH NeuroBioBank. HepG2, MDA-MB-231, and 293T cells were a gift from Brian Law. We also thank Jacquelyn Serfecz for her help in obtaining the luciferase measurements.

Authorship Contributions

Participated in research design: Jahn, Gay, Weaver, Stacpoole, James

Conducted experiments: Jahn, Gay, Weaver

Contributed new reagents or analytic tools: Renne, Langae

Performed data analysis: Jahn, Weaver, James

Wrote or contributed to the writing of the manuscript: Jahn, Weaver, James, Langae

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Footnote

This work was funded by grants from the US Public Health Service, National Institutes of Health, National Institute of General Medical Sciences [Grant RO1 GM 099871].

Figure Legends

Figure 1. Flowchart showing the experimental design to identify and confirm miRNAs that regulate GSTZ1 protein expression.

Figure 2. miRNA microarray results comparing expression in liver from young and adult populations (A) miRNAs that showed statistically significant expression differences between the age groups ($p \leq 0.05$) Each dot represents one miRNA. A miRNA expressed equally in both populations would fall directly on the $y = x$ line. (B) miRNAs that showed a statistically significant change with a fold change of ≥ 2 or ≤ -2 . The circled area contains 63 miRNAs that represented miRNAs of potential interest in the regulation of GSTZ1.

Figure 3. qPCR confirmation of array results for (A) miR-376c-5p and (B) miR-6738-5p. Total RNA from young and adult livers was analyzed with RT-qPCR to identify expression levels of the indicated miRNA. Statistical differences were determined by Student's t-test, $n = 15$ in each group.

Figure 4. Correlation of GSTZ1 protein expression with miR-376c-3p (A) Relative expression of GSTZ1 protein, as determined by densitometry of Western blots, in livers from young and adult populations. Protein levels are expressed as a percentage compared to a single sample that was used as a reference on all blots. ** indicates a significant difference between groups, $p < 0.01$ as determined by Student's t-test, $n = 14$ in young and $n = 15$ in adult (B) Scatterplot showing a direct correlation between GSTZ1 protein expression and miR-376c-3p ΔC_t (and therefore an inverse correlation with miR-376c-3p expression). Each point represents one liver sample. (C) Comparison of miR-376c-3p ΔC_t values from the three young samples with the highest expression of GSTZ1 and three low-expressing adults revealed no difference.

Figure 5. Regulation of GSTZ1 protein expression by miR-376c-3p (A) GSTZ1 protein expression in the indicated cell lines stably expressing transgenic miR-376c-3p. Values are

expressed as a percentage of the GSTZ1 protein in a sister cell line expressing a negative control miRNA (miR-CTRL) derived from the same parental cells. N = 3 for each cell line. (B) Luciferase activity in 293T cells expressing miR-CTRL or miR-376c-3p stably and luciferase containing the 3'-UTR of *GSTZ1* transiently. miR-CTRL was defined as 100% expression. N = 4 replicates for each cell line. (C) Scheme showing the construction of the luciferase plasmid. Into the pGL3 plasmid was inserted the luciferase coding sequence followed immediately by a portion of the *GSTZ1* 3'-UTR containing the predicted binding region for miR-376c-3p (highlighted). In all panels, values represent the mean of replicate measurements, error bars represent the standard deviation, * indicates $p < 0.05$, and ** indicates $p < 0.01$, as determined by Student's T-test

Figure 6. miRNAs targeting several drug metabolizing enzymes are differentially expressed in young and adult populations (A) The total number of miRNAs that were differentially regulated ($p < 0.05$, fold change ≥ 2 or ≤ -2) that were predicted to bind to any member of the indicated enzyme family. CYP, cytochrome P450; SULT, sulfotransferase; UGT, UDP glucuronosyltransferase; GST, glutathione transferase; NAT, N-acetyltransferase; MT, methyl transferase; NQO, NAD(P)H quinone oxidoreductase; FMO, flavin-containing monooxygenase; MAO, monoamine oxidase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; AO, aldehyde oxidase; AKR, aldo-keto reductase; EPHX, epoxide hydrolase (B) and (C) show the number of miRNAs from the total that are up- or downregulated, respectively.

Figure 7. Binding of miRNAs to the *GSTZ1* 3'-UTR. 3'-UTR binding location of all miRNAs that showed a statistically significant downregulation ($p < 0.05$) in the adult population, regardless of fold change. The fold change (i.e. -1.14x) for each, along with its exact binding site (i.e. n. 47-69) are given for each.

Table 1. Gene candidates for miRNA regulation. These genes showed a large differential (≥ 5 , ≤ -5) in miRNAs predicted to target their mRNA and that are up- or down-regulated in adult populations vs. young. This differential was calculated by subtracting the number of downregulated miRNA from the upregulated miRNA. A positive number would predict downregulated protein in adults while a negative number would predict upregulated protein.

Gene	miRNA Differential	Gene	miRNA Differential
CYP2B6	-7	CYP3A4	5
CYP2S1	-5	CYP3A5	8
CYP2U1	-5	CYP51A1	5
CYP4F12	-5	GSTM4	10
CYP4F22	-6	GSTO1	6
FMO3	-5	SULT2A1	5
GSTA2	-5		
GSTM5	-5		
GSTO2	-9		
GSTT2	-5		
mGST1	-6		
UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7	-5		
UGT1A5, UGT1A8, UGT1A9, UGT1A10	-6		

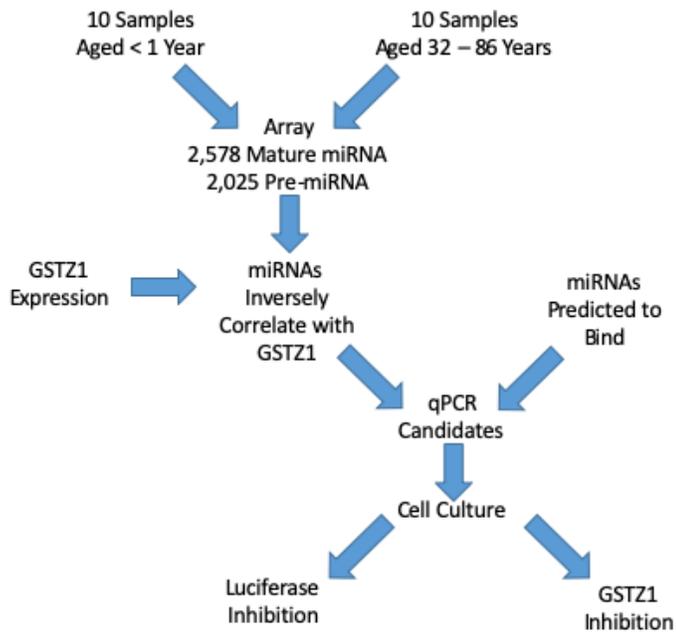


Figure 1

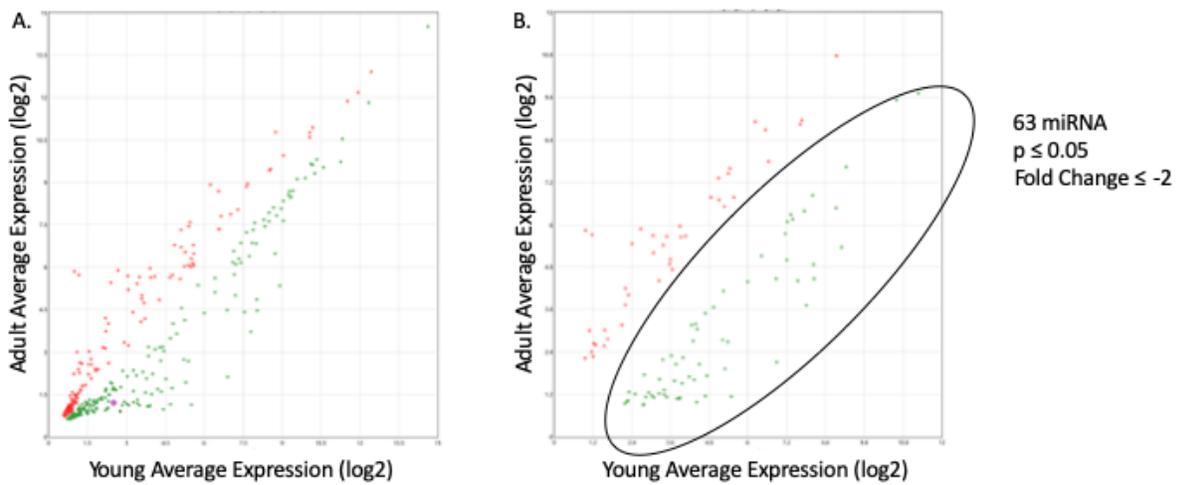


Figure 2

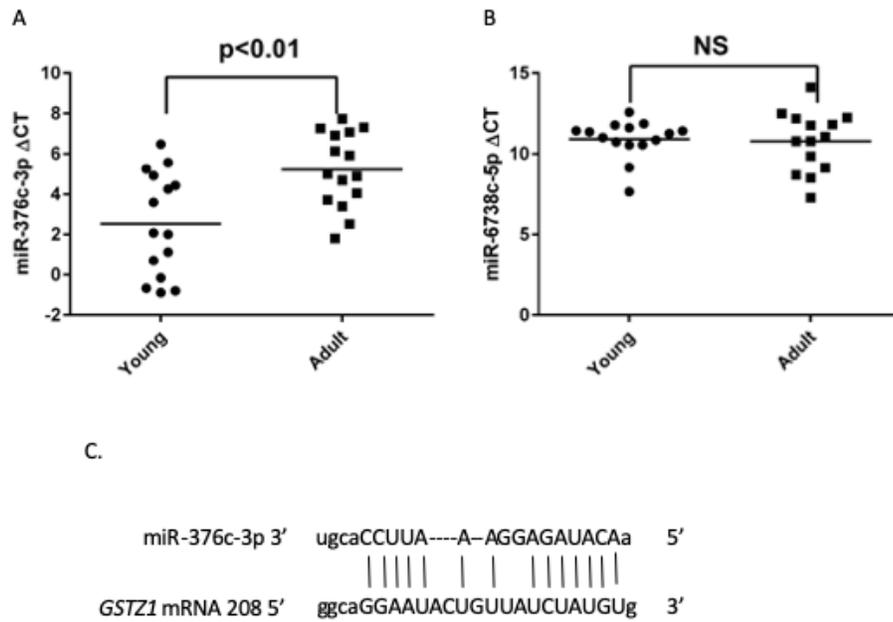


Figure 3

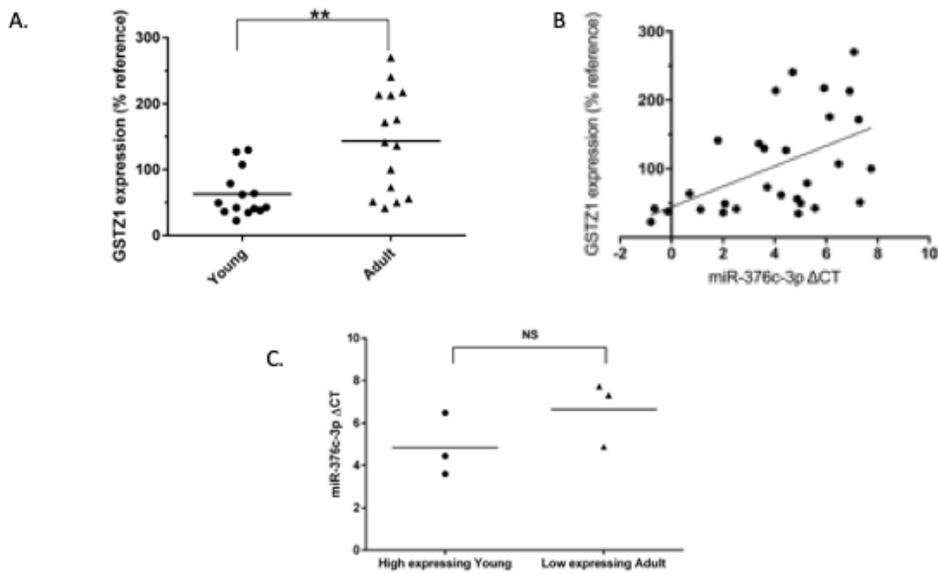


Figure 4

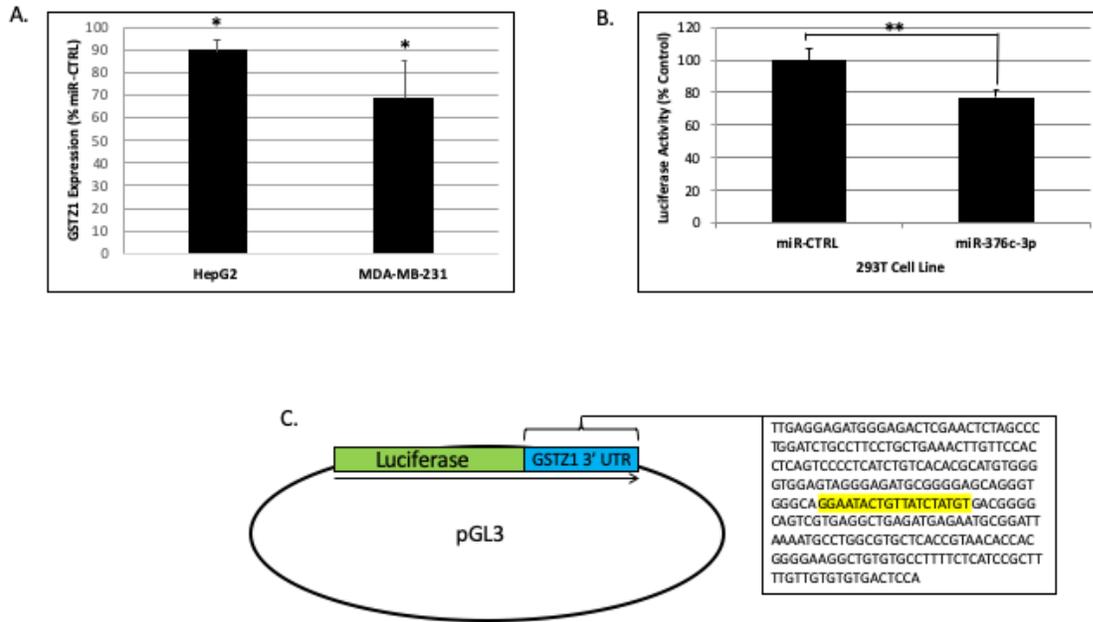


Figure 5

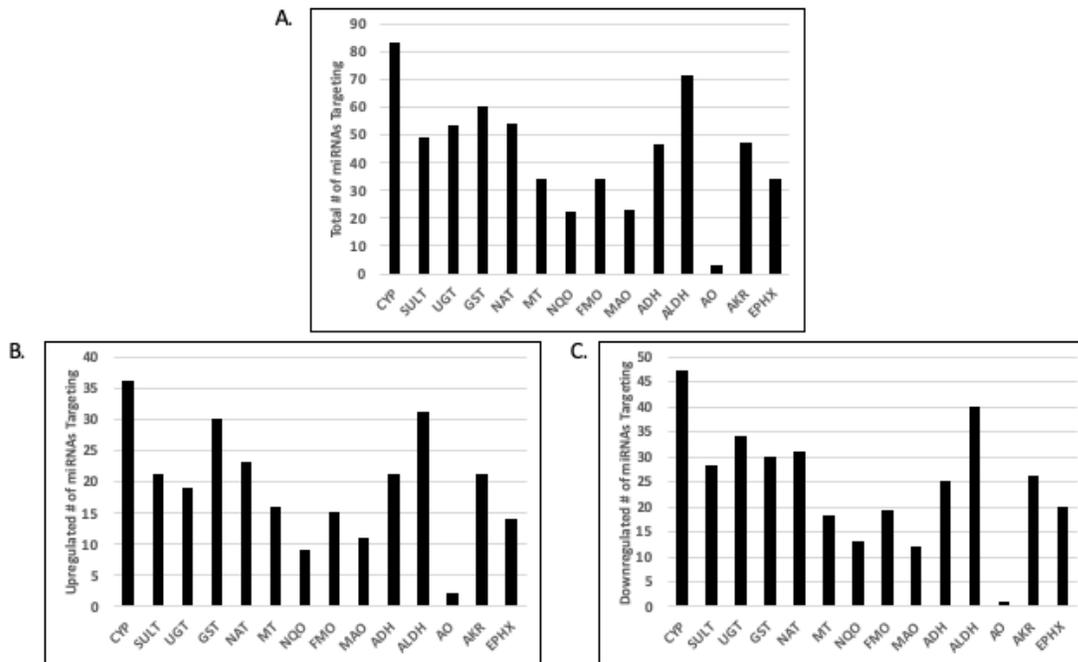


Figure 6

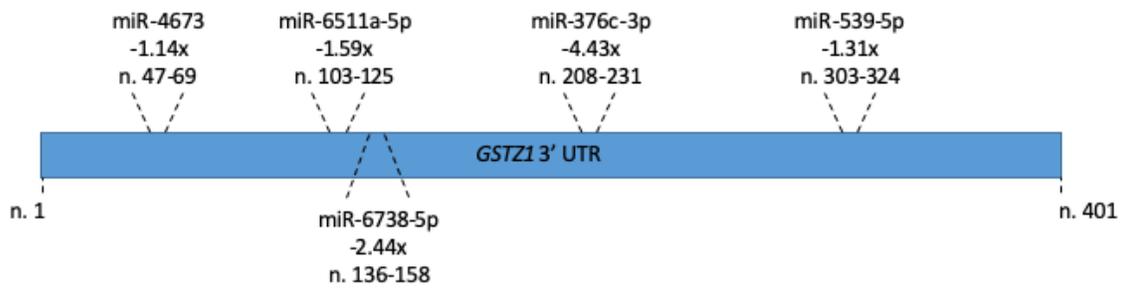


Figure 7