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Research Article

MicroRNAs and Their Predicted Target Messenger RNAs are Deregulated by Exposure to a Carcinogenic Dose of Comfrey in Rat Liver

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MicroRNAs (MiRNAs) are small noncoding RNAs that function as regulators of gene expression to control cell growth and differentiation. In this study, we analyzed miRNA and mRNA expression in the livers of rats treated with a carcinogenic dose of comfrey (*Symphytum officinale*) for 12 weeks. Groups of six rats were fed a normal diet or a diet containing 8% comfrey root. The animals were sacrificed 1 day after the last treatment and the livers were isolated for miRNA expression analysis using LC Sciences miRNA microarrays and for mRNA expression analysis using Affymetrix rat genome microarrays. MiRNA expression levels were significantly changed by comfrey treatment. The treated samples were separated clearly from the control samples in both principal component analysis (PCA) and hierarchical clustering analysis (HCA). Quantitative measurements of seven miRNAs using TaqMan real-time PCR were consistent with the microarray results in terms of fold-change and the direction of the change in expression. Forty-five miRNAs ($P < 0.01$) and 1,921 mRNAs ($q = 0$) were significantly changed by comfrey treatment. Using a target prediction algorithm, 434 differentially expressed genes (DEGs) were predicted to be targeted by the differentially expressed miRNAs (DEMs). The DEM-targeted DEGs were more likely to be involved in carcinogenesis than the DEGs that were not targeted by the DEMs. The nontargeted DEGs were enriched in noncancer-related biological processes. Our data suggest that comfrey may exert its carcinogenic effects by disturbing miRNA expression resulting in altered mRNA levels of the DEM-targeted genes that are functionally associated with carcinogenesis. Environ. Mol. Mutagen. 52:469–478, 2011.

Key words: microRNA; microRNA regulation; predicted mRNA; gene expression; genotoxic carcinogen

INTRODUCTION

Comfrey (*Symphytum officinale* L.) is a tall perennial plant that grows throughout the world. This plant has been used as a popular dietary supplement and herbal remedy and consumed as a green vegetable or tonic in many cultures for more than 2,000 years [Hirono et al., 1978; Buchman, 1979; Rode, 2002]. Comfrey has been used both internally and externally in different forms for the treatment of a variety of diseases, such as back pain, bone fractures, joint inflammation, wounds, gout, distortions, gastritis, gastroduodenal ulcers, lung congestion, and liver tumors [Roeder, 1995; Stickel and Seitz, 2000; Rode, 2002; Koll et al., 2004; Predel et al., 2005; Grube et al., 2007; Bleakley et al., 2008; Sakakura et al., 2008; Giannetti et al., 2010]. Comfrey, however, is hepatotoxic in livestock and humans, and carcinogenic in experimental animals [Hirono et al., 1978; Ridker and McDermont, 1989]. It induces hepatic veno-occlusive disease in humans [Ridker and McDermont. 1989] and hepatocellular adenomas and hemangioendothelial sarcomas of the liver in rats [Hirono et al., 1978].

Comfrey contains approximately nine pyrrolizidine alkaloids (PAs) and these have been implicated in its toxicity [Betz et al., 1994]. The major unsaturated PAs in comfrey are the monoesters lycopsamine and intermedine, their acetyl derivatives (7-acetyllycopsamine and 7-acetylintermedine), and symphytine [Stengl et al., 1982; Vollmer et al., 1987]. The unsaturated PAs are metabolically

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activated to toxic compounds in the liver by mixed function oxidases. This process involves oxidation of PAs to produce dehydropyrrolizidine (pyrrolic ester). Pyrrolic ester and its metabolites are very reactive and can bind to DNA and protein to generate DNA adducts, protein adducts, and DNA and protein cross-links [Vollmer et al., 1987; Fu et al., 2004; Chen et al., 2010]. Thus, the PAs are considered the primary components responsible for the genotoxicity and carcinogenicity of comfrey.

Previous studies have demonstrated that comfrey is a mutagen in bacteria and rat liver and lung. Acetone extracts from comfrey produced mutations in *Salmonella* without microsomal bioactivation using strains TA98 and TA100 [White et al., 1983]. The mutant frequencies in both rat liver and lung were increased by comfrey treatment, with a much higher mutant frequency in liver compared with that in lung [Mei et al., 2005; Mei and Chen, 2007], consistent with liver being the major tumor target tissue [Hirono et al., 1978]. Sequencing analysis of the comfrey-induced mutations showed that the PA mutational signatures included a high induction of G:C→T:A transversions and tandem base substitutions [Mei et al., 2005; Mei and Chen, 2007]. Therefore, these mutational data are consistent with the idea that the mutations induced by comfrey in rat liver and lung were due to the PAs in comfrey.

Due to the toxicity of comfrey, its use has been restricted and limited to external use in many countries [Snider, 1991; Integrated Laboratory Systems, 1997; FDA, 2001; Koll et al., 2004]. Although its popularity has declined due to the restrictions, comfrey is still available commercially in several forms. In many parts of the world, there are presently no restrictions on its use. It is still available in many countries or through Internet ordering. Thus, the toxicity of comfrey requires further investigation, especially for the mechanism(s) underlying its carcinogenicity and potential biomarkers of exposure.

MicroRNAs (miRNAs) are a small class of nucleic acids (approximately 20–24 bases) that post-transcriptionally regulate gene expression [Ambros, 2003]. They are single-stranded RNA molecules that are not translated into proteins. Each miRNA molecule is partially complementary to one or more mRNA molecules, and functions to down-regulate gene expression by inhibiting protein translation or destabilizing target transcripts [Bartel, 2004]. Since these early discoveries, many studies have confirmed that the expression of miRNAs in different types of species is regulated developmentally and spatially, and is involved in differentiation and proliferation of cells [Shivdasani, 2006]. Other various functions of miRNAs have also been found, ranging from control of leaf and flower development in plants [Aukerman and Sakai, 2003] and neuronal patterning in nematodes [Johnston and Hobert, 2003] to acting as tumor suppressor genes and oncogenes [Lu et al., 2005; Cummins and Velculescu, 2006; Volinia et al., 2006; Mott, 2009].

Recently, a number of studies have been reported on the involvement of miRNAs in the regulation of cancer initiation, development, and metastasis [Croce, 2009; Stallings, 2009; Ventura and Jacks, 2009]. In cancer, miRNAs are often dysregulated with their expression patterns being correlated with clinically relevant tumor characteristics [Lu et al., 2005; Volinia et al., 2006; Shi and Guo, 2009]. MiRNAs have been shown to be directly involved in cancer initiation and progression [Hagan and Croce, 2007; Lynam-Lennon et al., 2009]. Studies on the relationship between miRNAs and carcinogen exposure have also been reported [Chen, 2010]. These studies indicate that alterations in miRNA expression play an important role in carcinogenesis.

The objective of this study was to evaluate the role of miRNAs in the carcinogenesis of comfrey. In previous studies, we found that comfrey treatment of rats resulted in expression changes of a large number of genes in liver using multiple microarray platforms [Guo et al., 2006] and the functions of those differentially expressed genes (DEGs) were involved in PA metabolism, injury of endothelial cells, and liver injury and abnormalities, including liver fibrosis and cancer development [Mei et al., 2006; Guo et al., 2007]. Because miRNAs regulate gene expression, alteration of gene expression could be related to miRNA up- or down-regulation. We hypothesize that comfrey treatment induces miRNA deregulation and the differentially expressed miRNAs (DEMs) are functionally associated with comfrey toxicity and carcinogenicity. To confirm our hypothesis, we measured the relative levels of miRNA expression in livers of rats treated with comfrey and performed functional analysis of the target and non-target DEGs of the DEMs.

MATERIALS AND METHODS

Animal Treatment

The animal treatment has been described previously [Mei et al., 2006]. Briefly, Big Blue transgenic rats were purchased from Taconic Laboratories (Germantown, NY). The treatment schedule of comfrey was based on a previous study that evaluated its carcinogenicity [Hirono et al., 1978] and indicated such treatment would induce hepatocellular adenomas and liver hemangioendothelial sarcoma in the comfrey-treated rats. Comfrey roots were obtained from Camas Prairie Products (Trout Lake, WA). PAs in the comfrey roots were determined by mass spectral analysis of an extract and included symphytine, 7-acetyllycopsamine, and 7-acetylintermedine as major components in near equal amounts; intermedine and lycopsamine were present in a relatively smaller quantity [Mei et al., 2005]. The comfrey roots were ground and then blended with basal diet (NIH-31 pellets, Purina Mills International, Brentwood, MO) in a Hobart Mixer to make an 8% comfrey root diet. Groups of six 6-week-old male Big Blue rats were fed either a basal diet or the comfrey diet for 12 weeks. The animals were sacrificed one day after the last treatment. The diet containing 8% comfrey root induced a statistically significant increase in DNA mutation frequency and an altered DNA mutation spectra in the liver of these rats [Mei et al., 2006]. The recommendations set forth by our Institutional Animal Care and Use Committee for the handling, maintenance, treatment, and sacrifice of the animals were followed. The livers were isolated, frozen quickly by liquid nitrogen, and stored at -80°C.

Messenger RNA (mRNA) Data Source and Analysis

The mRNA expression data were obtained through GEO (series accession number: GSE5350). The mRNA data were generated from the same

tissue samples as those used for the miRNA analysis in this study and published previously as a part of the MicroArray Quality Control (MAQC) project [Guo et al., 2006]. In our previous study, gene expression was analyzed via four microarray platforms (Affymetrix, Agilent, Applied Biosystems, and GE Healthcare) and high concordance was obtained between these different microarray platforms [Guo et al., 2006; Li et al., 2009b]. The data generated by the Affymetrix Rat Genome 230 2.0 microarray (Santa Clara, CA) were used in this study. The gene expression data were quantile-normalized and then the normalized data were analyzed using the Significant Analysis of Microarray (SAM) method [Tusher et al., 2001]. The DEGs were determined according to the q value ($q = 0$).

MiRNA Isolation

Total RNA containing miRNA was isolated from about 60 mg of liver tissue suspended in RNA-Later ICE (Ambion Inc., Austin, TX) using mirVana® miRNA isolation kit (Ambion). RNA concentrations were determined using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the qualities were checked on an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA) using an RNA6000 Nano LabChip (Agilent). The ratio of OD260/OD280 was >1.8 for all these RNA samples.

Microarray Analysis of miRNA Expression

The miRHumanMouseRat 12.0 microRNA microarray was obtained from LC Sciences (Houston, TX) and it included 1,292 unique probes that were complementary to all mature miRNAs of human (856), mouse (617), and rat (349) in miRBase release 12.0. To perform the miRNA array experiment, total RNA (2–5 μg) was first size fractionated using a YM-100 Microcon centrifugal filter (Millipore). The small RNAs (<300 nt) were then 3′-extended with a poly(A) tail using poly(A) polymerase. Thereafter, an oligonucleotide tag was ligated to the poly(A) tail for later staining with fluorescent dye Cy3. Hybridization was performed overnight on a μParaflo® microfluidic chip using a micro-circulation pump (Atactic Technologies, Houston, TX). Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD). Raw intensities were $\log_2$ transformed and normalized with cyclic LOWESS (locally weighted regression). MiRNAs with at least one of the 12 intensities (six control samples and six comfrey-treated samples) larger than the intensity threshold of 32 were considered detectable. In total, 215 rat miRNAs were found to be detectable on the microarray. t-Tests were performed to compare the detectable miRNAs in the samples from the control and comfrey-treated groups. MiRNAs with a P value <0.01 were considered significantly changed and named DEMs hereafter.

Real-Time PCR Confirmation of miRNA Expression

Seven miRNAs, including four up-regulated miRNAs (rno-miR-34a, rno-miR-200b, rno-miR-214, and rno-miR-182) and three down-regulated miRNAs (rno-miR-203, rno-miR-92a, and rno-miR-125b-5p), were selected for TaqMan real-time PCR confirmation. The reaction materials were purchased from Applied Biosystems (Forster City, CA). Reverse transcriptase (RT) reactions contained 84 ng total RNA, 50 nM stem-loop RT primer, 1× RT buffer, 0.25 mM each of dNTPs, 3.33 U/μl MultiScribe reverse transcriptase and 0.25 U/μl RNase inhibitor in 10 μl volume. The reactions were incubated in an Applied Biosystems 9700 Thermocycler in a 96-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. Real-time PCR was performed using a standard TaqMan® PCR kit protocol on an Applied Biosystems 7500 Fast Real-Time PCR System. The 10 μl of PCR reaction included 0.56 μl RT product, 1× TaqMan® Universal PCR Master Mix, 0.2 μM TaqMan® probe, 1.5 μM forward primer, and 0.7 μM reverse primer. The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The threshold cycle (C_T) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold 0.05.

Classification of miRNA Expression Profile

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were applied for clustering samples based on gene expression profiles. The normalized intensities of 215 detectable miRNAs were used to conduct the analyses within ArrayTrack™, an FDA-developed software package for managing, analyzing, and interpreting microarray gene expression data [Tong et al., 2003; Fang et al., 2009]. PCA uses analysis of the principal sources of variance in data and displays this information graphically either two-dimensionally or three-dimensionally [Wang and Gehan, 2005]. PCA were conducted using the autoscaled method. The normalized data were $\log_2$ transformed before analysis. The eigen values and percentage of variability from the first three principal components were identified and used for clustering the samples. For HCA, the distance matrix was calculated using the Euclidean method and the dendrogram was linked with average algorithm.

Prediction of miRNAs' Target Genes

The predicted target genes of DEMs were generated via TargetScan, an online tool for prediction of miRNA target genes (available at: http://www.targetscan.org/). TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8-mer or 7-mer sites in mRNAs that match the seed region of each miRNA [Lewis et al., 2005; Grimson et al., 2007]. TargetScan has been widely used and was demonstrated to be more accurate than other prediction software [Baek et al., 2008].

Functional Analysis of the DEMs

The functions relevant to the DEMs were determined by the predicted target genes of the DEMs that were also detected as DEGs with mRNA microarray analysis. These DEGs were analyzed with Ingenuity Pathway Analysis (IPA) systems (available at: http://www.ingenuity.com/), an online functional analysis software that interprets the genes in the context of biological processes, pathways and molecular networks. The Entrez ID of each gene was mapped to its corresponding gene object in the IPA Knowledge Base. These genes were then used as the starting point for generating functions. The biological functions related to the input DEGs were explored. Fisher's exact test was used to evaluate the function changes. A function with a P value <0.05 was considered as significantly affected by the comfrey treatment.

RESULTS

Classification of the Samples According to Their miRNA Expression

To investigate the carcinogenic effect of comfrey exposure on miRNA expression in rat liver, we treated rats with a protocol similar to one that resulted in liver tumors [Hirono et al., 1978]. Six-week-old rats were fed a diet containing 8% comfrey for 12 weeks or the normal diet alone. Six animals from the comfrey treatment group and six animals from the normal diet feeding group were sacrificed and the livers were removed for microarray analysis [Mei et al., 2006]. The miRNA expression profiles for these samples were determined using LC Sciences microarrays and the raw data were deposited into Gene Expression

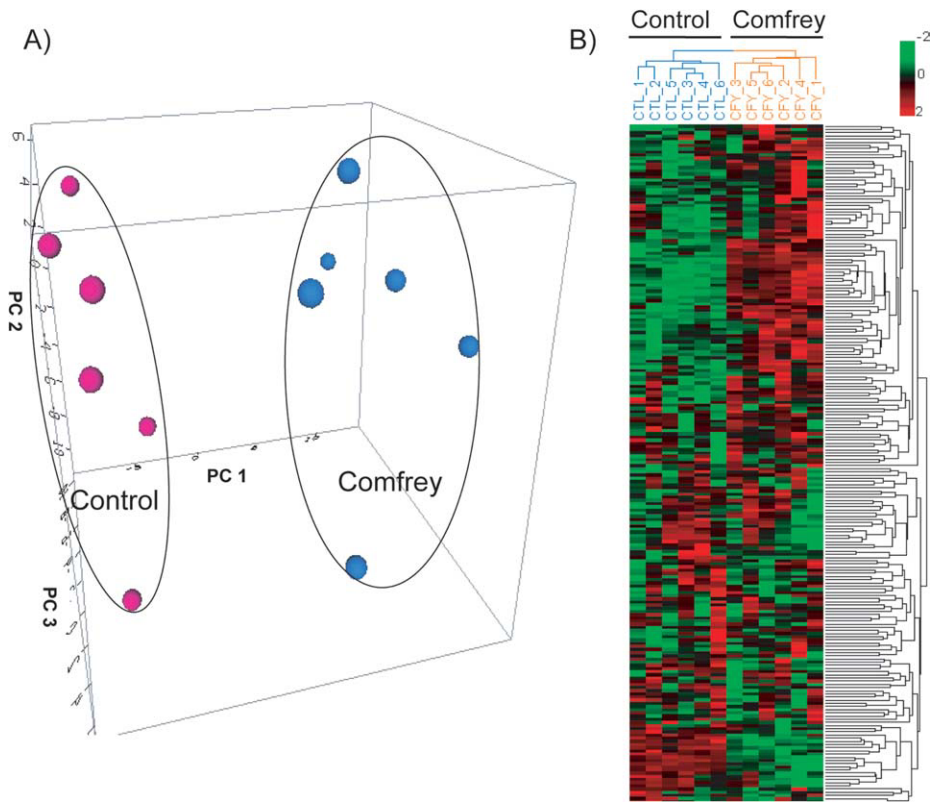

Fig. 1. Classification of samples according to expression intensity of the 215 detectable miRNAs. (**A**) Principal component analysis separates the 12 samples into two groups that are consistent with control and the treatment groups. (**B**) Hierarchical clustering analysis groups samples into comfrey-treated and control clusters.

Omnibus (Series accession numbers: GSE22655). Three hundred forty-nine miRNAs were evaluated and 215 of them were found to be expressed in at least one of the 12 samples (the intensity cutoff was 32). Only these expressed miRNAs were used for further analyses. The expression data for the detectable miRNAs were normalized with cyclic lowess and statistically analyzed with t-test (Supp. Info., Table I) to identify DEMs.

PCA was conducted to examine the relationship among the samples and to visualize clusters of the samples based on the variance-covariance structure of the miRNA expression in the 12 treated and control samples. A PCA three-dimensional view using the first three principal components for the miRNA expression profiles is displayed in Figure 1A. The captured variances reached 58%, indicating that these three components were able to represent most of the expression pattern of the individual samples. The PCA result clearly demonstrated that samples were grouped together according to comfrey treatment, with the six comfrey-treated samples well-separated from the six control samples (Fig. 1A). HCA also revealed distinct grouping of these 12 samples according to comfrey treatment (Fig. 1B), with the samples being separated into two main branches corresponding to the treatments.

MiRNAs Affected by Comfrey Treatment

Overall, microarray analysis identified a total of 45 miRNAs (Table I) whose expression was significantly changed by the comfrey treatment ($P < 0.01$). Twenty-nine of the 45 DEMs were up-regulated and 16 of them were down-regulated. The most up-regulated DEMs by the comfrey treatment were miR-34a, miR-200b, and miR-429 (23-, 11-, and 7-fold increase over the control, respectively) while the most down-regulated DEMs were miR-329 and miR-203 (22- and 7-fold decrease, respectively). To confirm our array expression results, seven of the DEMs (miR-34a, miR-200b, miR-214, miR-182, miR-92a, miR-125b-5p, and miR-203) were chosen for quantitative real-time PCR analysis. The trends and expression changes for either up- or down-regulation of miRNA expression determined by the real-time PCR measurement were consistent with the miRNA microarray finding (Fig. 2).

mRNAs Affected by Comfrey Treatment

Affymetrix mRNA expression analysis identified 2,898 mRNA probe sets ($q = 0$, false discovery rate of 0%) that were differentially expressed between the treatment

TABLE I. Differentially Expressed MicroRNAs Induced by Comfrey in Rat Livers

	Control		Comfrey				
MicroRNA	Mean	Standard deviation	Mean	Standard deviation	Fold-change	*P* value	Known targets
rno-miR-34a	49	25	1,128	423	22.8	0.00000	CCND1/Cyclin D1, CDK6, E2F3
rno-miR-200b	138	24	1,512	842	11	0.00022	ZEB1/TCF8, ZEB2/SIP1, ZEB1/TCF8
rno-miR-429	25	4	183	178	7.3	0.00194	ZEB1/TCF8, ZEB2/SIP1, ZEB1/TCF8
rno-miR-214	188	71	1,076	423	5.7	0.00001	PTEN
rno-miR-182	61	10	319	70	5.2	0.00000	
rno-miR-505	49	19	236	49	4.9	0.00053	
rno-miR-34c*	21	8	97	44	4.7	0.00015	
rno-miR-200a	25	8	114	75	4.6	0.00079	ZEB2/SIP1, ZEB1/TCF8, ZEB1/TCF8, ZEB1/TCF8, ZEB2/SIP1
rno-miR-210	35	9	107	64	3	0.00255	EFNA3
rno-miR-130a	54	18	160	70	3	0.00211	HOXA5, MEOX2/GAX, TAC1
rno-miR-183	56	13	161	49	2.9	0.00011	
rno-miR-199a-3p	428	142	1,198	299	2.8	0.00018	
rno-miR-146b	31	3	85	25	2.7	0.00098	
rno-miR-199a-5p	28	6	71	23	2.5	0.00059	
rno-miR-222	49	10	122	43	2.5	0.00061	KIT, CDKN1B/KIP1, p2, CDKN1C/p57, CDKN1B/KIP1, p2, CDKN1B/KIP1, p2, CDKN1B/KIP1, p2, KIT
rno-miR-181a	76	15	169	35	2.2	0.00004	BCL2, TCRalpha, CD69, HOXA11
rno-miR-99b	136	31	301	107	2.2	0.00153	
rno-miR-152	261	79	564	177	2.2	0.00207	
rno-miR-221	53	4	113	47	2.2	0.00959	KIT, CDKN1B/KIP1, p2, CDKN1C, CDKN1B/KIP1, p2, CDKN1B/KIP1, p2, CDKN1B/KIP1, p2, KIT
rno-miR-132	27	3	56	17	2.1	0.00146	RICS/p250GAP, PGC/RICS, 250GAP
rno-miR-148b-3p	28	5	56	17	2	0.00103	
rno-miR-93	83	25	164	51	2	0.00371	
rno-miR-23a	3,991	179	7,838	1,633	2	0.00056	FLJ13158, CXCL12
rno-miR-29a	4,577	850	8,846	1,955	1.9	0.00054	INSIG1, CAV2, BACE1, DNMT3A, DNMT3B
rno-miR-106b	103	19	189	57	1.8	0.00428	CDKN1A/p21
rno-miR-24	1,562	426	2,791	842	1.8	0.00876	KIAA0152, CDKN2A/INK4a p16, ALK4, DHFR, NOTCH1 MAPK14, KIAA0152
rno-miR-185	111	20	189	51	1.7	0.00525	
rno-miR-191	1,832	246	3,036	583	1.7	0.00039	
rno-miR-23b	5,724	453	8,950	1,364	1.6	0.00044	NOTCH1
rno-miR-130b	17	1	26	6	1.5	0.00469	
rno-miR-128	139	16	205	27	1.5	0.00037	SCP1
rno-miR-328	31	5	42	5	1.4	0.00712	
rno-miR-30c	4,091	518	5,253	433	1.3	0.00333	
rno-miR-323*	26	6	17	4	−1.6	0.00407	
rno-miR-125b-5p	5,175	577	3,154	512	−1.6	0.00030	
rno-miR-99a	580	67	345	123	−1.7	0.00874	
rno-miR-484	47	8	25	3	−1.8	0.00020	
rno-miR-92a	3,614	462	1,635	805	−2.2	0.00882	
rno-miR-296*	57	14	23	6	−2.5	0.00028	
rno-miR-150	1,114	230	407	173	−2.7	0.00095	MYB
rno-miR-92b	1,419	317	504	197	−2.8	0.00114	CrebA
rno-miR-10a-5p	725	118	228	114	−3.2	0.00404	
rno-miR-543	76	44	16	5	−4.7	0.00040	
rno-miR-203	352	134	49	25	−7.2	0.00011	
rno-miR-329	3,304	1,193	147	109	−22.4	0.00201	

The values shown here, including mean, standard deviation, fold change, and *P* values, were calculated based on the normalized intensities of the corresponding miRNA in each of the 12 samples. Known targets denote the genes which have been experimentally demonstrated to be targeted by the corresponding miRNA. Different target genes are separated by comma. The alternative names of one gene are separated by slash. The known targets information is derived from TarBase V5.0 (available at: http://diana.cslab.ece.ntua.gr/tarbase/) [Papadopoulos et al., 2009]. The miRNAs without known targets have no entries in the known targets column.

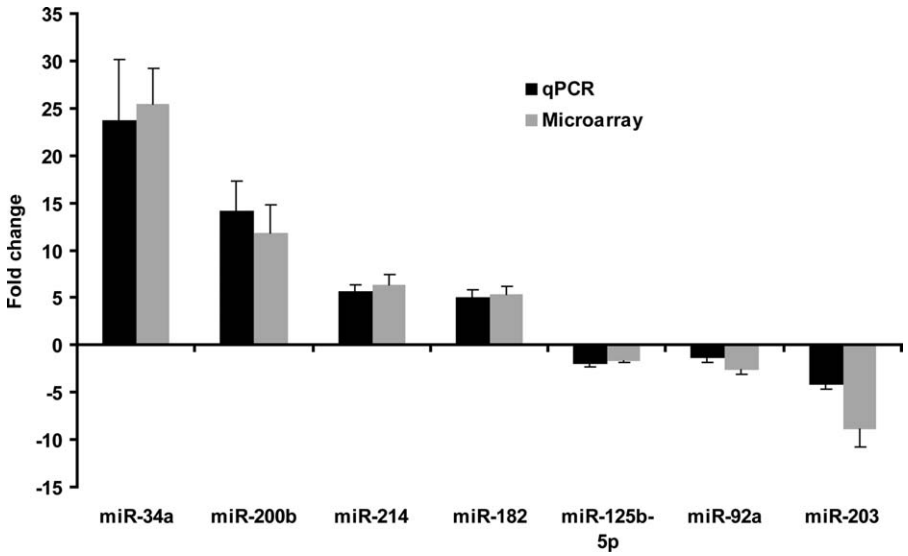

Fig. 2. Comparison of the expression of seven miRNAs measured by quantitative real-time PCR (qPCR) and microarray ($n = 6$).

and control groups (Supp. Info., Table II). These probes were mapped to 1,921 unique genes with valid Entrez gene IDs. To examine the functions of these DEGs, they were uploaded into the IPA software. There were 1,641 genes that qualified for IPA Network analysis and 1,521 genes that qualified for IPA Function/Pathway analysis. The analysis results indicated that the altered functions and pathways were related to toxicity and carcinogenicity of comfrey in rat liver, including liver cancer development (Supp. Info., Table III), which is consistent with our previous finding using a different microarray platform [Mei et al., 2006].

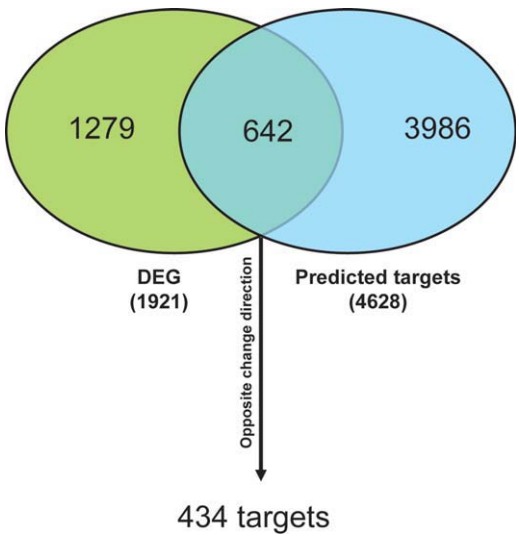

Fig. 3. MiRNA target gene selection. After comfrey treatment, 1,921 differentially expressed genes (DEGs) were identified by microarray analysis. Using TargetScan software, the 45 differentially expressed miRNAs (DEMs) were predicted to interact with 4,628 target mRNAs. There were 642 genes in common between these two gene sets. Four hundred thirty-four of these DEGs had expression changes in the opposite direction compared with the targeting DEMs. These genes were considered as the DEM-targeted genes.

Target Analysis of Differentially Expressed miRNAs

The functions of most miRNAs have not been well defined. One current approach for miRNA functional analysis is in silico prediction of miRNAs target genes. Because miRNAs exert their gene regulatory activity primarily by base-pairing to the 3′ untranslated region (UTR) of their target mRNA [Lewis et al., 2003; Krek et al., 2005; Lewis et al., 2005; Ragan et al., 2009], computation methods are used to find potential strong interactions. Although there are two major mechanisms by which miRNAs regulate gene expression, degrading target mRNA or suppressing target mRNA translation, recent studies have shown that the former is the primary mechanism, accounting for most (>84%) of the decreased protein production [Lim et al., 2005; Li et al., 2009a; Sun et al., 2009]. Thus, investigating mRNA level alterations of miRNA target genes may reveal the functions of the miRNAs. To identify the target genes of the DEMs, we performed a search using TargetScan and found 4,628 mRNA targets that have unique valid Entrez gene IDs (Supp. Info., Table IV). The computation-based target gene prediction methods still have some drawbacks at present, and cannot provide full confidence that a gene with the binding site of a miRNA at its 3′ UTR will ultimately be targeted by the miRNA [Thomas et al., 2010]. The TargetScan method was chosen here because its prediction results were demonstrated to be more consistent than other methods with experimental results [Baek et al., 2008]. Thus, the software provides candidate mRNA targets for follow-up analysis. Considering that some of

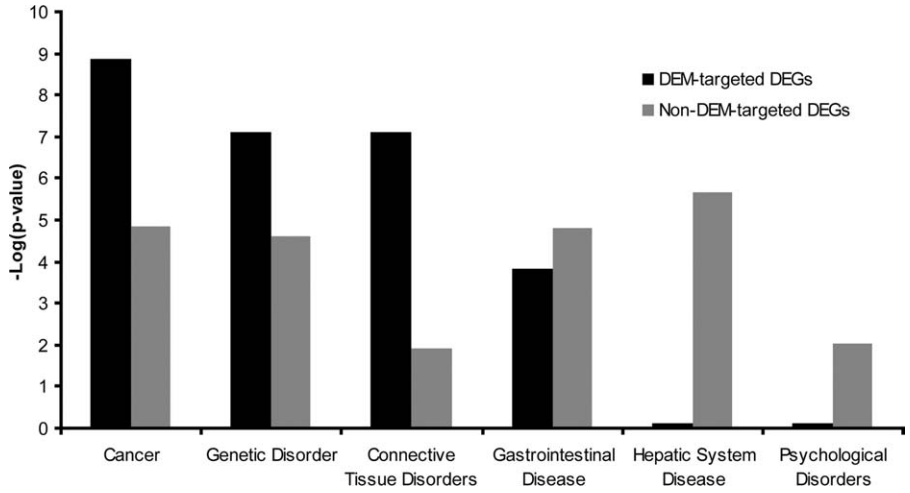

Fig. 4. Functions of differentially expressed genes (DEGs) that are targeted by differentially expressed miRNAs (DEMs) and comparison with DEGs that are not targeted by DEMs. The 434 DEGs targeted by the DEMs and the 1,487 DEGs not targeted by the DEMs were analyzed by Ingenuity Pathway Analysis software to reveal the biological functions. The top most significant functions are shown. The *y*-axis is the negative log 10 transformed Fisher's exact test *P* value.

these predicted genes may not be expressed in liver or in this life stage (e.g., many genes are expressed only during fetal development), we filtered the genes using the following criteria: (1) predicted by the TargetScan software to be targeted by DEMs, (2) differentially expressed by comfrey treatment, and (3) exhibiting expression changes in the opposite direction from the miRNA. Using these criteria, 434 target genes were identified for further functional analysis (Fig. 3 and Supp. Info., Table V).

Functional Analysis of the Differentially Expressed miRNAs

The 434 mRNA target genes were inputted into Ingenuity database for functional analysis. Assuming that these genes are the target genes of the DEMs, the functions of these genes should reflect those of the DEMs. The Ingenuity database had information on 391 of these genes. The functional analysis showed that cancer was the top functional category significantly related to these genes (Fig. 4). Many well-studied tumorigenesis-related genes (e.g., *Notch1* and *Mafb*), cell growth-related genes (e.g., *Ctsb* and *Fgfr1*), transformation-related genes (e.g., *Ccnd1* and *Rhob*), and metastasis-related genes (e.g., *Casp3* and *Cd44*) were found in this gene list (Supp. Info., Table VI). In total, 164 of the 391 miRNA target genes were cancer-related, with the *P* value of the Fisher's exact test reaching 1.42E-9 (Fig. 4). Besides cancer, miRNA target genes, such as *Rgs5*, *Basp1*, *Egr1*, *Itga7*, and *Oxct1*, also showed close association with genetic disorder (Fig. 4), which is associated with comfrey-induced carcinogenesis [Mei et al., 2005]. To examine whether these DEM-targeted DEGs had functional specificities, the non-DEM-targeted DEGs were also functionally analyzed. The top function for these genes was hepatic system disease that reflected comfrey's hepatic toxicities (Fig. 4). Overall, this analysis indicated that among genes whose expression was significantly altered by comfrey, the genes involved in cancer formation and growth were more likely to be targeted by the miRNAs.

DISCUSSION

Our results demonstrated that comfrey treatment significantly changed miRNA expression levels in rat liver. In both the PCA and HCA analysis with the 215 detectable miRNAs, the six comfrey-treated samples clustered together and were well-separated from the six control samples that also clustered together (Fig. 1). Forty-five miRNAs, accounting for 21% of the detectable miRNAs, were deregulated at the significance level of $P < 0.01$ (Table I). TaqMan real-time PCR confirmed the comfrey-induced expression changes identified by the microarray analysis, with the change in direction and fold-change being similar between the measurements of these two technologies (Fig. 2).

Changes in expression of protein-encoding mRNAs were also found after exposure to comfrey (Supp. Info., Table II). These changes were consistently detected by four microarray platforms including Applied Biosystems, Affymetrix, Agilent, and GE Healthcare as presented in our previous papers [Guo et al., 2006; Li et al., 2009b]. The Affymetrix data were used here for in-depth analysis in combination with miRNA expression data to explore the functions of the DEMs.

In this study, the relationship between expression levels of mRNA and miRNA at a genomic scale was analyzed. The recent finding that mRNA degradation, not protein synthesis inhibition, is the predominant mode of action of miRNAs provides grounds for this kind of interrogation [Lim et al., 2005; Li et al., 2009a; Sun et al., 2009]. Among the 1,921 DEGs that were induced by comfrey

treatment in rat liver, 434 of them have predicted binding sites of the DEMs, indicating the possibility that these genes might be deregulated via DEM actions. Furthermore, we found that cancer-related genes dominated the types of genes predicted to be regulated by miRNAs. According to the analysis using the Ingenuity Pathway database, the top function associated with the DEM-targeted DEGs was cancer, while the top function associated with non-DEM-targeted DEGs was hepatic system disease (Fig. 4). These data suggest that the DEMs are mainly involved in regulation of the expression of the protein-coding genes associated with carcinogenesis. The relationship between miRNA and their targeted mRNA is very complicated, however, with multiple miRNAs often targeting the same mRNA and a particular miRNA targeting many mRNAs [Lim et al., 2005]. Thus, the computational approach we used here may not capture all the genes that are targeted by DEMs and confirmation of the functional role of the miRNAs will require further mechanistic experiments.

The carcinogenicity of comfrey in rat liver was demonstrated as early as 1978 by Hirono et al. [1978]. ACI rats were treated with different doses of comfrey leaves and root in the diet for up to 600 days. The first hepatocellular carcinoma appeared 7 months after treatment in the group fed 8% comfrey roots, and ultimately 79% of rats in this group developed liver tumors [Hirono et al., 1978]. The carcinogenic effects of comfrey are likely due to PAs, a class of chemicals with potent tumor-inducing activities as demonstrated in ACI rats and F344 rats by several groups [Chen et al., 2010]. Comfrey contains several types of PAs, such as intermedine, lycopsamine, and symphytine. All of these PAs possess the structural features that have been demonstrated to be closely associated with PA-induced hepatotoxicity in rats and mice [Frei et al., 1992]. These structural features include (1) a double bond in the 3-pyrroline ring, (2) one or two hydroxyl groups attached to the pyrroline ring, (3) one or two ester linkages between the base and necic acid, and (4) the presence of a branched chain on the acid moiety [Frei et al., 1992; Prakash et al., 1999]. Previous studies showed that the average levels of PA-derived DNA adducts after exposure to the comfrey root extract and comfrey compound oil were 22 adducts/10^8 nucleotides and 32/adducts/10^8 nucleotides, respectively [Chou and Fu, 2006]. The DNA adducts can further result in mutations and tumors [Mei et al., 2005]. The comfrey-induced DNA adducts, gene mutation and tumors may be associated with the mRNA/miRNA expression observed in this study, and the deregulated mRNA/miRNA in turn can contribute to the process of tumor initiation, promotion and progression.

Metabolic activation of PAs by cytochrome P450s [Fu et al., 2004; Guo et al., 2007; Mei et al., 2007; Chen et al., 2010] to form pyrrolic esters is considered to be the primary pathway for the genotoxicity and carcinogenicity of PAs [Chen et al., 2010]. Both our previous study [Guo et al., 2007] and this study (Supp. Info., Table II) showed that a large number of P450 genes were dysregulated by the comfrey treatment by enhancing their expression level. There were 22 P450 genes identified as DEGs (Supp. Info., Table II), although only one of them, the Cyp26b1 gene, was predicted to be targeted by several miRNAs (Supp. Info., Table V).

One example of miRNA involvement in the carcinogenesis of comfrey is the deregulation of miR-34a. MiR-34a expression was dramatically increased by comfrey treatment with a fold-change reaching 22.8, the largest change among the 45 DEMs. We also observed that miR-34a was highly induced in mouse liver after exposure to *N*-ethyl-*N*-nitrosourea (ENU), and rat livers after exposure to riddelliine or aristolochic acid (manuscripts in preparation). ENU, riddelliine, and aristolochic acid are all genotoxic carcinogens. On the other hand, induction of miR-34a was not observed in mouse liver after treatment with the nongenotoxic carcinogens propiconazole and triadimefon [Ross et al., 2010], indicating miR-34a might be a good responder to genotoxic carcinogen exposure.

The miR34 family genes are the direct transcription targets of the tumor suppressor gene *p*53 [Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Welch et al., 2007]. Its promoter region contains a palindromic sequence that matches the canonical *p*53 binding site and can be bound by *p*53 as shown by chromatin immunoprecipitation [He et al., 2007; Raver-Shapira et al., 2007]. Up-regulation of miR-34a can cause cell-cycle arrest in the G1 phase [He et al., 2007; Raver-Shapira et al., 2007]. In addition, introduction of miR-34a and miR-34b/c into primary human diploid fibroblasts induced cellular senescence [He et al., 2007] and re-expression of miR-34a in tumor cells induced apoptosis [Chang et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007]. In this study, we found that many miR-34a-targeted DEGs are also related to the functions of the *p*53 gene (Supp. Info., Table V), such as aldolase A (*Aldoa*) [Borlon et al., 2008], serpin peptidase inhibitor (*Serpine1*) [Qi et al., 2008], Notch homolog 1 (*Notch1*) [Lefort et al., 2007], and SRY-Box 4 (*Sox4*) [Pan et al., 2009]. Thus, induction of miR-34a appears to be directly involved in regulation of DNA repair, cell proliferation, and other carcinogenic processes caused by the comfrey treatment. However, among the three known targets of miR-34a (Table I), only one gene, cyclin D1 (Ccnd1), showed significant expression change and was down-regulated by more than fourfold. This is consistent with the fact that miR-34a was up-regulated by more than 22-fold.

In conclusion, the data showed that comfrey treatment altered the expression level of a large number of miRNAs and their predicted target mRNAs. In silico functional analysis of the DEM-targeted DEGs suggests that miRNAs are involved in the carcinogenic response to the comfrey treatment.

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