MicroRNAome of Splenic Macrophages in Hypersplenism due to Portal Hypertension in Hepatitis B Virus-Related Cirrhosis

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MicroRNAs (miRNAs) are a recently discovered class of post-transcriptional regulators of gene expression with critical functions in health and disease. Their role in the pathogenesis of hypersplenism, however, is completely unknown. To determine whether miRNA expression is altered in splenic macrophages associated with hypersplenism due to portal hypertension in hepatitis-B-virus (HBV)-related cirrhosis, we analyzed the entire miRNAome in macrophages from normal and portal hypertensive spleen samples by microarray and Real-Time PCR. In this study, we identified 99 miRNA differences in expression in splenic macrophages associated with hypersplenism due to portal hypertension in HBV-related cirrhosis. Among the miRNAs identified in this study, hsa-miR-615–3p was significantly up-regulated in hypersplenism. Dynamic changes in miRNA expression occurred during the pathogenesis of portal hypertension-induced hypersplenism in HBV-related cirrhosis. The miRNAs then are novel regulatory RNAs in hypersplenism in patients with HBV-related cirrhosis. Exp Biol Med 233:1454–1461, 2008

Key words: hypertension; portal; hypersplenism; microRNAs; microarray analysis

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Introduction

MicroRNAs (miRNAs) are a class of noncoding RNAs that are ~22 nucleotides in length (1). They specifically regulate the expression of many protein-coding genes by targeting their messenger RNAs through pairing interactions in a combinatorial fashion (2–4). Since the discovery of the first miRNA, *lin-4* (5), considerable progress has been made in the elucidation of their precise functions. miRNAs are involved in various important biological processes, such as developmental timing and patterning, apoptosis, hematopoietic differentiation, cell proliferation, organ development, as well as tumorigenesis and other diseases (6–8). Moreover, it is estimated that 10–30% of all protein-coding genes are regulated by miRNAs in the human genome (9). The progressive discovery of new miRNA functions implies their association with the regulation of almost every aspect of cell physiology and pathology.

Currently, much is yet to be learned concerning the pathogenesis of hypersplenism due to portal hypertension (HS-PHT) in patients with hepatitis-B-virus (HBV)-related cirrhosis. It is generally accepted that cytopenia results predominantly from the increased phagocytosis and destruction of hemocytes in splenic macrophages (Mϕ) (10). However, no research has been undertaken with regard to the role of miRNAs in HS-PHT. Consequently, the present study investigated the miRNAome of splenic Mϕ in HS-PHT using miRNA microarray techniques.

Materials and Methods

Patients. Two groups of patients were studied (Table 1). Twenty patients (median age 44.6 years, range 27–69 years; twelve males and eight females), with HS-PHT in our hospital during September 2006 to March 2007, were included as the HS-PHT group. As splenectomy is commonly performed in patients with portal hypertension

Table 1. Description of the Patients

Patient number	Platelet count ($\times 10^9$/L)	Erythrocyte count ($\times 10^{12}$/L)	Leukocyte count ($\times 10^9$/L)	Analysis	
				Microarray	PCR
HS-PHT					
1	26	2.98	2.10	√	√
2	53	2.94	2.32	√	√
3	27	3.11	2.86	√	√
4	22	3.16	1.26	√	√
5	39	2.22	1.66		√
6	52	3.51	2.99		√
7	40	2.94	1.86		√
8	34	5.10	1.09		√
9	22	3.7	1.77		√
10	39	2.83	1.65		√
11	32	3.45	1.38		√
12	69	2.92	1.73		√
13	33	2.68	1.42		√
14	29	2.86	2.45		√
15	38	3.41	3.21		√
16	44	3.23	2.80		√
17	18	2.53	1.92		√
18	42	3.21	3.14		√
19	35	2.42	2.50		√
20	32	2.30	2.62		√
Control					
1	178	4.26	9.52	√	√
2	216	4.46	10.14	√	√
3	158	4.74	10.42	√	√
4	182	3.98	8.52	√	√
5	162	4.50	8.69		√
6	193	3.86	9.31		√
7	172	4.83	8.37		√
8	169	4.78	8.94		√

and hypersplenism in China, all patients in the study underwent splenectomy and pericardial devascularization. Free portal vein pressure was measured by placing a catheter connected to a water-based pressure manometer in the main omental vein during the procedure. Based on this method, 13 to 24 cm of H_2O is considered as normal, whereas >30 cm of H_2O indicates portal hypertension. In addition, the diagnosis of cirrhosis and portal hypertension was made based on supporting evidence including clinical features, abnormal laboratory tests, and ultrasonography. In this regard, hypersplenism was defined by anemia, leukopenia, or thrombocytopenia with splenomegaly; a normal or hypercellular bone marrow; and normalization of the peripheral blood cell counts after splenectomy. Conversely, eight patients (median age 35.8 years, range 18–43 years; six males and two female) with traumatic rupture of the spleen were enrolled in the control group. Hepatitis, cirrhosis, history of hypersplenism, and abnormalities in postoperative laboratory findings and pathological examinations were absent in the participants. All patients provided written informed consent, while the hospital ethics committee approved the protocol.

Sample Preparation and RNA Extraction. Splenic Mϕ were isolated and purified by anchored cultivation from all the patients, and total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) as previously described (11, 12).

LNA-Based miRNA Microarray. The profiling of miRNA in RNAs from both splenic Mϕ in HS-PHT ($n=4$) and the normal group ($n=4$, mixed into a sample pool) was performed using the miRCURY™ LNA microRNA Array (Version 8.1 Exiqon, Vedbaek, Denmark). The total RNA from the HS-PHT and control groups were labeled with Hy5™ and Hy3™ fluorescent dye, respectively, using the miRCURY™ LNA Array labeling kit (Exiqon, Denmark). The Hy3™-labeled samples and the Hy5™-labeled reference pool RNA samples were mixed pair-wise and were hybridized to the miRCURY™ LNA array, which contains capture probes targeting all human miRNA listed in miRBASE Version 8.1 (13, 14). The hybridization was performed according to the miRCURY™ LNA array manual using a Tecan HS4800 hybridization station (Tecan, Austria), the slides scanned by Axon 4000B (Axon, Union City, CA), and the resulting images analyzed with species-specific GenePix® Array Lists files by GenePix 4.0 (Axon Instruments, Union City, CA). The signal from each spot was analyzed with Spotfire 8.0 (TIBCO, Palo

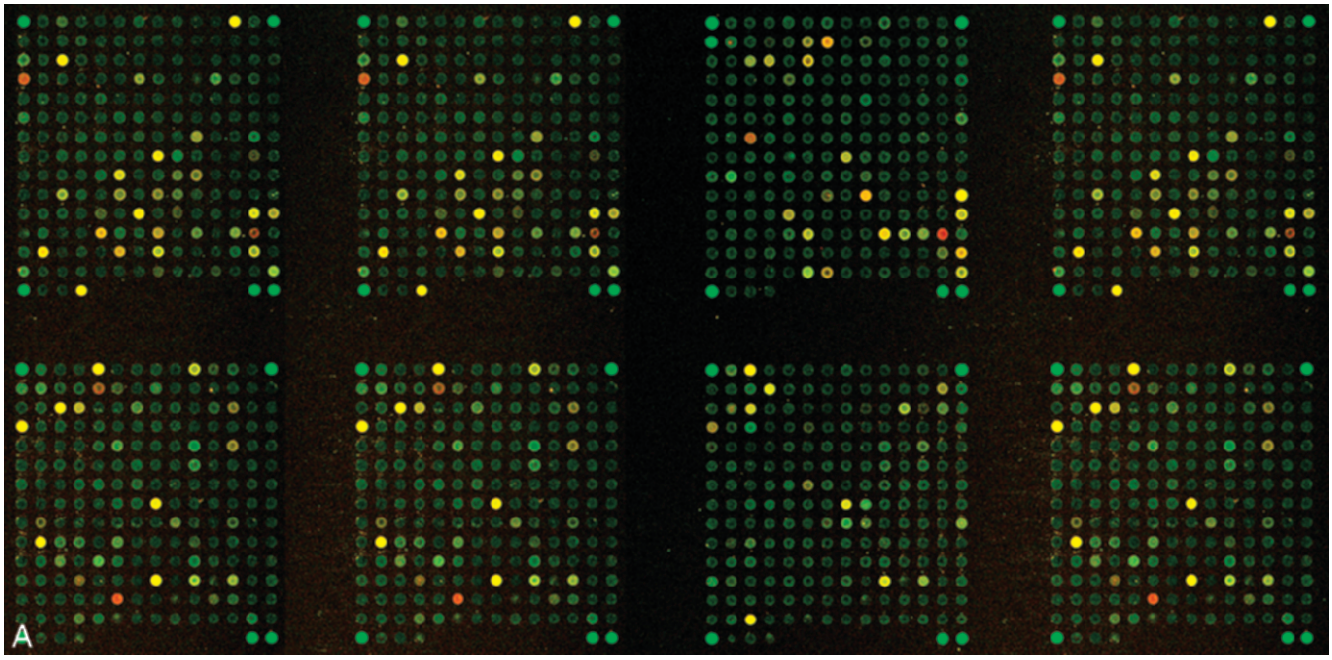


Figure 1. Comparison of expression profiles of miRNAs between HS-PHT and normal splenic Mϕ. (A) MicroRNA (miRNA) array comparison of splenic Mϕ from normal spleens ($n=4$) and spleens with HS-PHT ($n=4$). Total RNA from splenic Mϕ was labeled and hybridized to microarrays containing probes corresponding to known miRNA sequences. (B) The heat map summarizes the biological replicates for the splenic Mϕ specimens, the four experiments are grouped as experiments 1, 2, 3, and 4 and then subheadings of four replicates per experiment represent the technical replicates of the same sample. Color intensity is scaled within each row so that the highest expression value corresponds to bright red and the lowest to bright green. Gene names are listed to the right. The hsa-miR-615, in the heat map, has been renamed as hsa-miR-615–3p in miRBase (release 10.0: August 2007). (C) Venn diagram for the miRNAs expression. 292 miRNAs were expressed in splenic Mϕ, and 99 miRNAs were differentially expressed between HS-PHT and normal group.

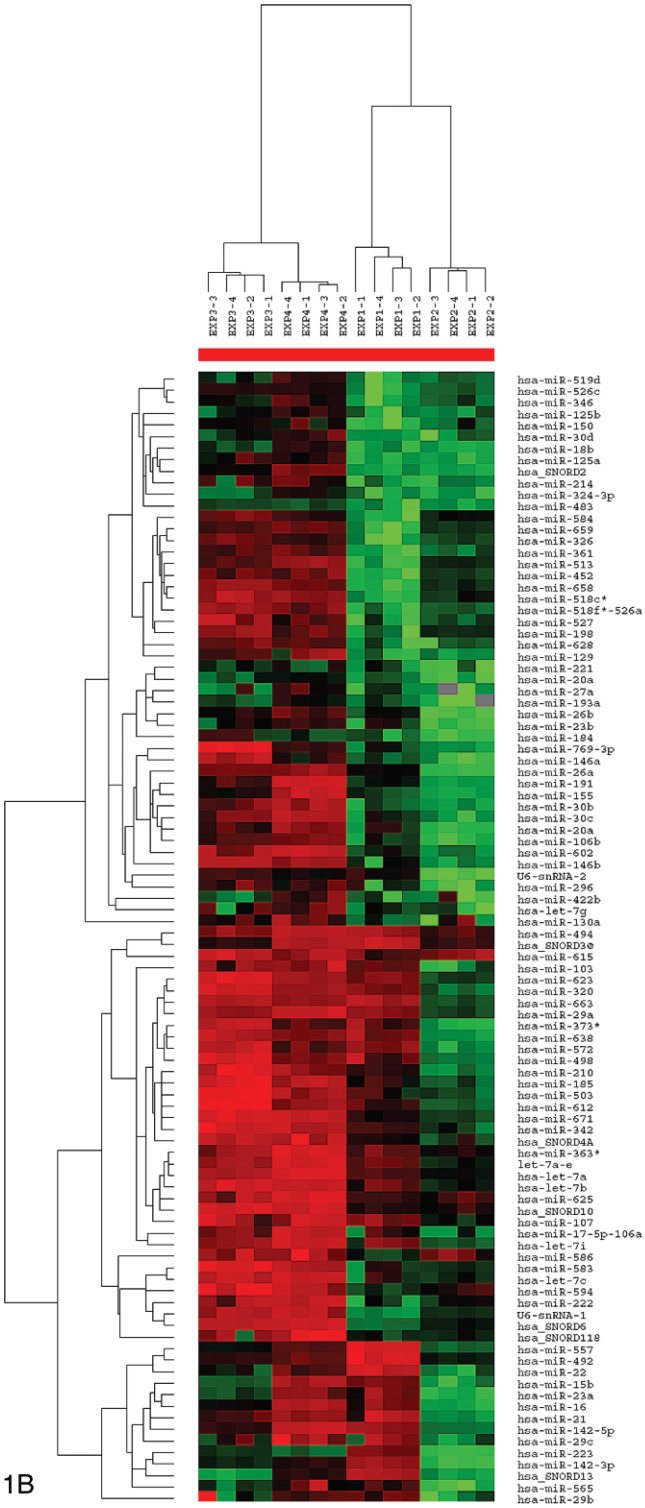
Alto, CA). Afterwards, the resulting signal intensity values were normalized to per-chip median values and then were used to obtain the geometric means and standard deviations for each miRNA. Statistical comparisons were performed next by using the Analysis of Variance (ANOVA) statistic. For visualization of differentially expressed miRNAs, a heat map was generated using TreeView (http://jtreeview.sourceforge.net).

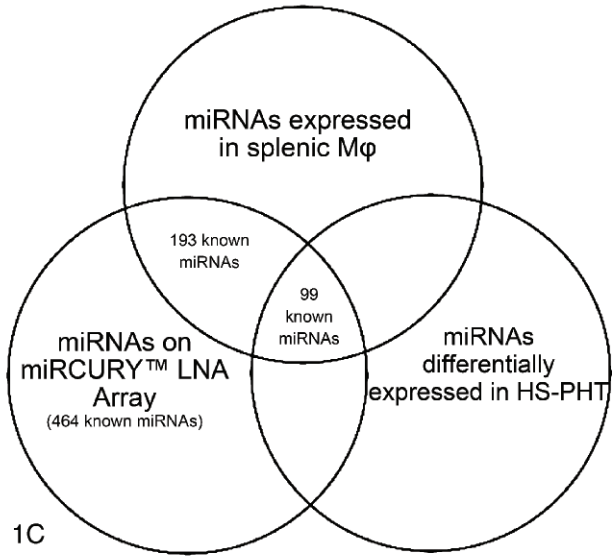
Quantitative Real-Time PCR. Quantification of miRNAs by TaqMan® Real-Time PCR was carried out as described by the manufacturer (Applied Biosystems, Foster City, CA). In brief, 10 ng of template RNA was reverse transcribed employing the TaqMan® MicroRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems). A 1.5 ml RT product was introduced into the 20 ml PCR reactions which were incubated in 96-well optical plates on the Applied Biosystems 7300 Sequence Detection system at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Target miRNAs expression was normalized between different samples based on the values of U48 RNA expression. Student's *t* test was then used to determine the statistical significance of data.

Prediction of miRNA Target (9, 15–17). The miRNA predicted targets were analyzed by way of four programs: TargetScan (http://www.targetscan.org), PicTar (http://pictar.bio.nyu.edu/), miRanda (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl), and miRBase targets database (http://microrna.sanger.ac.uk). The predicted target genes were then distributed into classes by the molecular function of gene ontology (GO) on Ensembl GOView (Ensembl release 45, June 2007, http://www.ensembl.org/Homo_sapiens/goview?acc=GO). The experimentally supported miRNA targets were later analyzed using Tarbase (http://www.diana.pcbi.upenn.edu/ tarbase.html).

Results

Marked Difference in the miRNA Expression of Splenic Mϕ. Analysis of the microarray data showed that the miRNAs are expressed in a non-random manner between splenic Mϕ from the HS-PHT and normal group (Fig. 1A). We identified that 292 miRNAs were expressed in splenic Mϕ, and 99 miRNAs were differentially expressed between HS-PHT and normal group (Fig. 1B, C; Suppl Table S1, which is available in the online version of the journal). Among the miRNAs identified, two findings were noted in comparison with the normal spleen: (I) consistent up-regulation of miRNAs in the HS-PHT group (e.g. miR-494, miR-615–3p, miR-363, miR625), and (II) consistent miRNAs down-regulation in HS-PHT (e.g. miR-324–3p, miR-483, miR-20a), as compared to normal spleen. However, only hsa-miR-615–3p (MIMAT0003283, miRBase release 10.0: August 2007) was significantly up-regulated in the HS-PHT group.





To confirm the results obtained by microarray profiling, we performed quantitative real-time PCR analysis of hsa-miR-615–3p and hsa-miR-24 expression on the RNA samples obtained from HS-PHT and normal group (Fig. 2). In accordance with the microarray data, quantitative real-time PCR results showed significantly ($P < 0.01$) increased hsa-miR-615–3p levels in splenic Mϕ from the HS-PHT group when compared with normal spleen. The expression of hsa-miR-24 was with no difference between two groups. Taken together, HS-PHT is characterized by a distinct miRNA expression profile in comparison with normal spleen.

Details of hsa-miR-615–3p. hsa-miR-615–3p belongs to the miR615 family, which has a seed region of

CCGAGCC. The sequence of hsa-miR-615 is located in chromosome 12:52,713,537–52,714,560 and has overlapping transcripts with intron1 of Homobox protein C5 (HOXC5). It used to be named hsa-miR-615, but because another mature miRNA sequence (hsa-miR-615–5p) has been found in the *Homo sapiens* miR-615 stem-loop in miRBase (release 10.0: August 2007), it has been renamed as hsa-miR-615–3p (13, 14, 18, 19).

Target Prediction of hsa-miR-615–3p. Ten conserved target genes were found by TargetScan (release 4.0), with a total of 10 conserved sites and 1 poorly conserved site (Table 2) (15). There were also 1041 *Homo sapiens* transcript identifiers found in the miRBase targets database (Table 3). Conversely, there was no information about target prediction on PicTar or miRanda, nor any experimentally supported miRNA targets on Tarbase.

Discussion

Hypersplenism is a clinical syndrome common in PH due to hepatic cirrhosis. It is characterized by splenomegaly as well as the associated destruction of one or more cell lines in the peripheral blood (20). Nonetheless, there are little data present about the pathogenesis of hypersplenism in PH. It is generally accepted that cytopenias result predominantly from the increased phagocytosis and destruction of hemocytes in splenic Mϕ. Our prior studies have indicated that phagocytosis of Mϕ was augmented; the rate of phagocytosis and the index of phagocytosis of splenic Mϕ were notably in negative correlation with the count of leukocyte and platelet in peripheral blood. Likewise, we have also identified 121 differentially expressed genes by cDNA microarray in the splenic Mϕ of hypersplenism due to PH, including 21 known up-regulated and 73 known down-regulated known genes. These differently expressed genes were related with ionic channel and transport proteins, cyclins, cytoskeletons, cell receptors, cell signal conduct, metabolism, immunity, and so on (21–23). However, the mechanism for increased phagocytosis in hypersplenism and the molecular regulation in splenic Mϕ are yet unknown.

The spatial and temporal expression patterns of miRNAs can provide clues for their possible functions. Profile studies have already shown that many miRNAs are specific for organs, cell types and developmental stages. In fact, a number of different methods, including Northern blotting, oligonucleotide microarrays, and quantitative PCR have been developed for miRNA profiling study. Microarray-based miRNA profiling assays, in particular, is an efficient approach in screening, in a parallel fashion, the expression of a large number of miRNAs through extensive sample (24–27).

Recently, a new microarray platform (miChip) using LNA-modified capture probes showed increased sensitivity and specificity (26, 28, 29). Therefore, the miRNA expression analysis was carried out using the miRCURY™

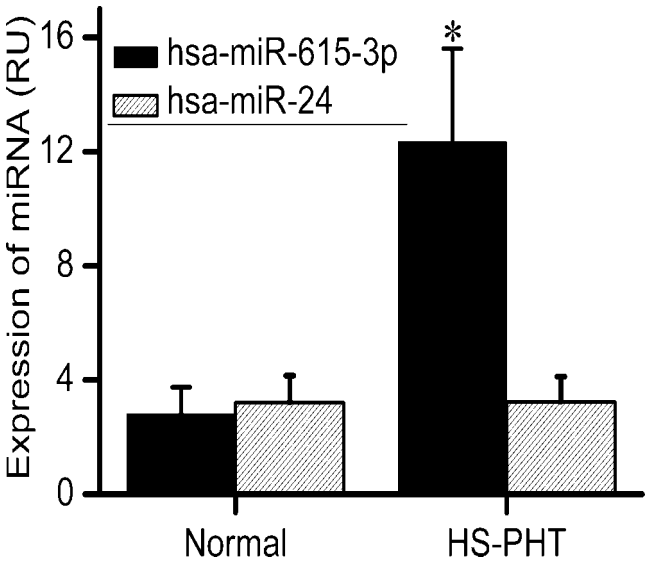


Figure 2. The expressions of the functionally active, mature forms of hsa-miR-615–3p and hsa-miR-24 analyzed using quantitative real-time PCR in the splenic Mϕ from normal spleens ($n = 8$) and spleens with HS-PHT ($n = 20$). Data are expressed in relative units compared to U48 RNA. * $P < 0.01$.

LNA microRNA Array, which has a normalized 72°C melting temperature (24). Among 292 miRNAs expressed in splenic Mϕ, 99 miRNAs were identified as differentially expressed between HS-PHT and normal spleen, suggesting that miRNAs may be involved in the pathogenesis of HS-PHT.

Dysregulation of miRNA levels would be anticipated to affect the translation of multiple protein coding genes. With this in mind, even though minimal information about miRNA target genes hampers a full understanding of the biological functions of miRNAs, the prediction of miRNA targets can provide an alternative approach. Because of the general existence of endotoxemia and hormone metabolic abnormalities in patients with HS-PHT, we hypothesized that TRIM8 and LCoR were the most likely targets of hsa-miR-615–3p. They might regulate the activation of splenic Mϕ in pathological "homeostasis" and might as well be involved in the pathogenesis of HS-PHT (30–34).

In addition, we also have compared the miRNA expression to the previous expression array data. We tested whether the differentially expressed genes in cDNA microarray could be the targets of miRNAs by analysis in TargetScan program. The results showed that there was some relationship between the differentially expressed genes and miRNAs (Suppl Table S2, which is available in the online version of the journal). It is interesting that the up-regulation of hsa-mir-625 was in coincidence with the down-regulation of ARAF1 (NM_001654, Homo sapiens v-raf murine sarcoma 3611 viral oncogene homolog 1) and MPL (NM_005373, Homo sapiens myeloproliferative leukemia virus oncogene), and the down-regulation of

Table 2. Targets of hsa-mir-615 Predicted by TargetScan[a]

Target gene	Ensembl ID	Molecular function	Conserved sites		Poorly conserved sites		Total context score
Ligand dependent nuclear receptor corepressor (LCOR)	196233	GO:0003677 DNA binding	8mer	1			−0.48
Microtubule-associated protein tau (MAPT)	186868	GO:0017124 SH3 domain binding GO:0019899 enzyme binding GO:0008034 lipoprotein binding GO:0008017 microtubule binding	7mer-m8	1			−0.19
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 11 (PSMD11)	108671	GO:0004672 protein binding	7mer-1A	1			−0.18
Kruppel-like factor 13 (KLF13)	169926	GO:0003677 DNA binding GO:0003702 RNA polymerase II transcription factor activity GO:0046872 metal ion binding	7mer-1A	1			−0.11
Tripartite motif-containing 8 (TRIM8)	171206	GO:0046872 metal ion binding GO:0004672 protein binding GO:0008270 zinc ion binding	7mer-m8	1			−0.08
Cdc42 guanine nucleotide exchange factor (GEF) 9 (ARHGEF9)	131089	GO:0005089 Rho guanyl-nucleotide exchange factor activity	7mer-m8	1			−0.04
Protein kinase C and casein kinase substrate in neurons 1 (PACSIN1)	124507	GO:0016301 kinase activity GO:0005515 protein binding GO:0004672 protein kinase activity	7mer-m8	1			−0.01
SH3 and multiple ankyrin repeat domains 3 (SHANK3)	99882	GO:0004601 peroxidase activity GO:0004672 protein binding	7mer-m8	1	7mer-1A	1	0.01
Insulin-like growth factor 2 (somatomedin A) (IGF2)	167244	GO:0008083 growth factor activity GO:0005179 hormone activity GO:0005159 insulin-like growth factor receptor binding GO:0018445 prothoracicotrophic hormone activity	7mer-1A	1			0.10

[a] The 10 conserved target genes found by TargetScan were ranked on the total context score. The last six digits of the Ensembl ID are shown (ENSG00000#). 8mer: An exact match to positions 2–8 of the mature miRNA (the seed + position 8) with a downstream 'A' across from position 1 of the miRNA. 7mer-m8: An exact match to positions 2–8 of the mature miRNA (the seed + position 8). 7mer-1A: An exact match to positions 2–7 of the mature miRNA (the seed) with a downstream 'A' across from position 1 of the miRNA (32).

Table 3. Classification of 1041 Predicted hsa-mir-615 Targets in miRBase Target Database[a]

GO ID	Molecular function	Genes	
	None/unknown	384	36.90%
	Known function	657	63.10%
GO:0016209	Antioxidant activity		2 (0.2%)
GO:0004601	Peroxidase activity	2	
GO:0015457	Auxiliary transport protein activity	1	1 (0.1%)
GO:0005488	Binding	12	327 (31.4%)
GO:0030246	Carbohydrate binding	2	
GO:0003682	Chromatin binding	3	
GO:0043167	Ion binding	94	
GO:0008289	Lipid binding	10	
GO:0003676	Nucleic acid binding	56	
GO:0000166	Nucleotide binding	51	
GO:0001871	Pattern binding	3	
GO:0005515	Protein binding	69	
GO:0005102	Receptor binding	25	
GO:0043021	Ribonucleoprotein binding	1	
GO:0008430	Selenium binding	1	
GO:0003824	Catalytic activity	7	170 (16.3%)
GO:0016491	Oxidoreductase activity	12	
GO:0009055	Electron carrier activity	3	
GO:0004386	Helicase activity	2	
GO:0016787	Hydrolase activity	67	
GO:0016853	Isomerase activity	5	
GO:0016874	Ligase activity	7	
GO:0008639	Small protein conjugating enzyme activity	3	
GO:0016740	Transferase activity	63	
GO:0004803	Transposase activity	1	
GO:0030234	Enzyme regulator activity		36 (3.5%)
GO:0008047	Enzyme activator activity	5	
GO:0004857	Enzyme inhibitor activity	14	
GO:0030695	GTPase regulator activity	16	
GO:0019208	Phosphatase regulator activity	1	
GO:0060089	Molecular transducer activity		53 (5.1%)
GO:0004871	Signal transducer activity	4	
GO:0004872	Receptor activity	48	
GO:0005057	Receptor signaling protein activity	1	
GO:0003774	Motor activity		1 (0.1%)
GO:0003777	Microtubule motor activity	1	
GO:0005198	Structural molecule activity	8	16 (1.5%)
GO:0005201	Extracellular matrix structural constituent	3	
GO:0005200	Structural constituent of cytoskeleton	1	
GO:0005212	Structural constituent of eye lens	2	
GO:0003735	Structural constituent of ribosome	2	
GO:0030528	Transcription regulator activity	1	12 (1.2%)
GO:0003702	RNA polymerase II transcription factor activity	5	
GO:0003709	RNA polymerase III transcription factor activity	2	
GO:0016563	Transcriptional activator activity	1	
GO:0003711	Transcriptional elongation regulator activity	2	
GO:0016564	Transcriptional repressor activity	1	
GO:0045182	Translation regulator activity	1	1 (0.1%)
GO:0005215	Transporter activity	15	38 (3.7%)
GO:0005275	Amine transporter activity	3	
GO:0005386	Carrier activity	1	
GO:0015267	Channel or pore class transporter activity	2	
GO:0015075	Ion transporter activity	12	
GO:0005326	Neurotransmitter transporter activity	1	
GO:0015932	Nucleobase, nucleoside, nucleotide and nucleic acid transporter activity	3	

[a] The number and percentage of transcripts annotated with various Gene Ontology molecular function categories are shown for targets of hsa-mir-615. One Ensembl gene identifier can map to several Refseq identifiers or Ensembl transcript identifiers corresponding to different splice forms of a gene. The amounts of genes refer to the transcript identifiers.

hsa-mir324-3p, 20a were also in coincidence with MED23 (NM_004830, Homo sapiens mediator complex subunit 23).

Taken together, miRNA expression patterns distinguish HS-PHT from normal spleen. The results reported here reveal a new layer of regulatory mechanisms in the pathogenesis of HS-PHT in HBV related cirrhosis. Because miRNAs are master switches that ultimately affect complex cellular processes and functions through the regulation of several proteins, miRNA-based therapies may be more effective than drugs targeting single proteins.

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