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A Bayesian two-step integrative procedure incorporating prior knowledge for the identification of miRNA-mRNAs involved in hepatocellular carcinoma

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Abstract— **Recent studies have confirmed the role of miRNA regulation of gene expression in oncogenesis for various cancers. In parallel, prior knowledge about relationships between miRNA and mRNA have been accumulated from biological experiments or statistical analyses. Improved identification of disease-associated miRNA-mRNA pairs may be achieved by incorporating prior knowledge into integrative genomic analyses. In this study we focus on 39 patients with hepatocellular carcinoma (HCC) and 25 patients with liver cirrhosis and use a flexible Bayesian two-step integrative method. We found 66 significant miRNA-mRNA pairs, several of which contain molecules that have previously been identified as potential biomarkers. These results demonstrate the utility of the proposed approach in providing a better understanding of relationships between different biological levels, thereby giving insights into the biological mechanisms underlying the diseases, while providing a better selection of biomarkers that may serve as diagnostic, prognostic, or therapeutic biomarker candidates.**

Keywords: hepatocellular carcinoma, graphical models, integrative models, liver cirrhosis prior knowledge, Bayesian variable selection,

I. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and the third cause of cancer deaths worldwide [1]. In many cases, HCC occurs in people with liver cirrhosis (CIRR) which complicates the detection of symptoms during early stages. As a result, HCC is diagnosed at advanced stages qualifying it as an aggressive cancer.The known diagnostic markers have low sensitivity for early detection [2]. The identification of novel diagnostic biomarkers for early detection of HCC is therefore still an active research of area.

The role of microRNAs (miRNAs) in many biological processes such as differentiation, cell signaling, and pathways supporting cancer stemness is crucial. A better understanding of their implication in biological processes underlying diseases may be achieved through linking miRNAs to respective target genes. Many studies have established that miRNA-mRNA pairs play a critical role in the activation of oncogenic or carcinogenesis pathways as, for example, in prostate cancer, liver diseases or HCC. These results highlight the great utility of miRNAs as biomarkers of diagnosis/prognosis and disease progression. While several studies have reported miRNA-mRNA pairs with opposite expression patterns, experimentally validated results obtained in some cancers have also revealed dual-upregulation of miRNA-mRNA pairs. Although those results are promising, the characterization of relationships between miRNA and mRNA is still a challenge, notably because each miRNA has multiple mRNA targets and vice-versa. Innovative approaches are therefore required. In addition, it could be important to take into account the connections between mRNAs through miRNAs or other factors.

The advances of high-throughput technologies along with the development of relevant statistical and bioinformatics methods for analyzing omic data enhance the capacity to identify relevant molecular targets and may lighten the long process of identification. The integrative analysis of different sources of data has led to significant results by offering a better understanding of complex biological mechanisms through the discovery of new relationships between disease and biological features from different biological levels [3]. In addition, in various domains the integration of prior knowledge into statistical models has led to promising results.

Gaining insights into the mechanistic differences between HCC and CIRR contributes greatly to improving the detection of important biological features. In this paper, we introduce a Bayesian two-step integrative procedure extending the hierarchical integrative model (HIM) proposed by [4], [3], and adapted by [5], for analyzing miRNA-seq and mRNA-seq data from patients with HCC or CIRR. The goals are to improve the knowledge about the relationships between miRNAs and mRNAs, as well as among mRNAs after considering the effects of miRNAs, and to identify relevant disease-associated miRNA-mRNA pairs. In a first step, we combine a Bayesian variable selection approach integrating prior knowledge about the relationships between miRNA and mRNA with a Gaussian graphical model. Then, a second model integrating the information obtained in the first step is used to jointly analyze miRNAs and mRNAs and to discover miRNA-mRNA pairs discriminating HCC and CIRR.

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II. METHODS

A. Samples

Human liver tissues from 64 adult patients recruited at MedStar Georgetown University Hospital through a protocol approved by the Georgetown IRB were considered in this analysis. All subjects signed informed consent forms and HIPAA authorization forms. Table I provides the characteristics for the 39 HCC cases and 25 patients with CIRR whose samples have been analyzed by various platforms to acquire multi-omic data. 23% of HCC cases have histologically verified adjacent CIRR tissues. Diagnostic imaging criteria and/or histology that are well-established have been used to diagnose the HCC cases.

B. miRNA-seq and mRNA-seq data

RNA samples extracted from the 64 liver tissues were analyzed by Illumina Hiseq 4000 using 150 bp paired-end (PE150) for RNA-seq expression profiling and by Illumina NextSeq 550 platform using 2x150 bp paired-end (PE150) for miRNA-seq expression profiling. More details are available in [6]. RNA-seq and mi-RNA-seq data were Gaussianized before applying the subsequent analyses. We used the function `huge.npn` from the R package `huge` [7] which consists of applying a nonparanormal transformation that estimates the Gaussian copula by marginally transforming the variables using smooth functions.

In this paper we focused on a subset of 106 mRNAs from the mRNA-seq data that are selected in previous comparisons using the same dataset and are known to have some association with liver disease [8], [5], [6], [9]. Student t-tests with multiple testing adjustment were used to identify miRNAs with significant changes in their levels between HCC and CIRR. Using a p-value cut-off of 0.05 after false discovery rate correction, a total of 261 miRNAs out of the 2195 miRNAs from the miRNA-seq data were selected.

In order to integrate prior knowledge into statistical models, scores measuring the belief in the association between mRNAs and miRNAs were computed with Ingenuity Pathway Analysis (IPA) Target filter analysis tool [10], which extracts experimentally verified and predicted associations between mRNA-miRNA pairs from multiple sources such as TargetScan Human or TarBase [11], [12]. Four values corresponding to different levels of confidence were considered based on IPA calls: 1 for experimentally observed associations, 0.75 for high predicted links, 0.5 for moderate predicted links, and 0 for associations that have not been experimentally observed or predicted.

C. Bayesian two-step integrative procedure

The proposed integrative model consists of two submodels (Fig. 1): a mechanistic submodel that relates miRNA and mRNA and a clinical submodel that relates the phenotypic outcome to mRNA and miRNA expression levels.

TABLE I
CHARACTERISTICS OF PATIENT-DERIVED SAMPLES

		HCC (N=39)	CIRR (N=25)	p-value
Age	Mean(SD)	62.02 (11.46)	50.05 (12.1)	0.0013
Gender	Male	77%	72%	0.7683
Race	EA	41%	64%	
	AA	33%	32%	
	Asian	26%	0%	
	other	0%	4%	

a) Mechanistic submodel: Bayesian variable selection using spike-and-slab prior [13], which places a discrete mixture distribution on the regression coefficients, is used to identify miRNAs associated to each mRNA. Similarly to [14], prior knowledge was integrated into the model by including scores in the variable prior inclusion probabilities. miRNAs with posterior inclusion probability greater than 0.2 were selected. The expression level of an mRNA can thus be decomposed into two parts: the fitted values correspond to the part of the mRNA accounted for by miRNAs and the residuals corresponding to the remaining part explained by other unmeasured factors.

In order to study the relationships between mRNAs after adjusting for miRNAs, an undirected graph based on residuals is estimated by using a Gaussian graphical model (GGM) [15]. GGM has been widely used to estimate partial correlations, which correspond to correlations between variables corrected for all other variables under investigation. Thus, contrary to Pearson correlations which translate marginal relationships between variables, partial correlations help distinguish direct from indirect relationships between variables. An attractive aspect of partial correlations is their visualization via an undirected graph, where nodes are the variables and edges the dependencies between them. The absence of edges correspond to a conditional independence of two variables given the remaining variables. A lasso graphical algorithm [16] was used to estimate sparse undirected graph via the R package `huge`. The optimal regularization parameter was selected by using the stability approach to regularization selection (stars). A graph structure for gene expressions adjusted for miRNAs effects was therefore estimated, the corresponding graph is a covariate-adjusted Gaussian graph or conditional Gaussian graph [17]. We will refer to it as the adjusted graph. Note that the estimated partial correlation between two genes are now corrected for all other genes being analyzed and all miRNAs.

b) Clinical submodel: The linear predictor of the probit model is modeled in terms of the mRNA expression profiles and the miRNA effects on disease status. The former may be decomposed into two parts corresponding to modulation via miRNAs (G_{miRNA}) and via other factors than miRNAs ($G_{\overline{miRNA}}$). The associated model is given by (1):

$$probit P(Y=1) = G_{miRNA} + G_{\overline{miRNA}} + \overline{\text{mRNA}} + \overline{\text{miRNA}} \quad (1)$$

where $\overline{\text{mRNA}}$ corresponds to the set of mRNAs that had

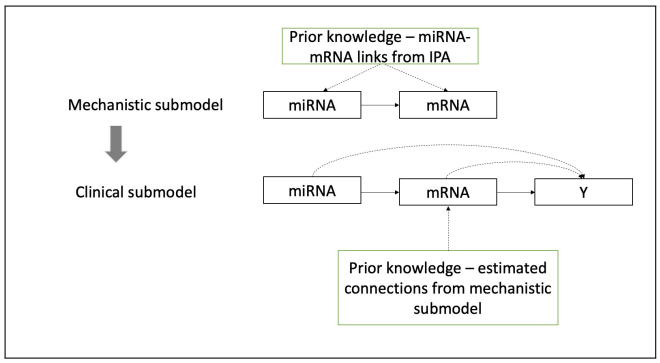

Fig. 1. Bayesian two-step integrative procedure

no related miRNA in the mechanistic submodel and $\overline{\text{miRNA}}$ corresponds to the set of miRNAs not found to be associated with any of the mRNAs in the mechanistic submodel. To simultaneously select relevant variables and account for the adjusted graph, a spike-and-slab approach integrating the dependence structure between mRNAs estimated in the mechanistic submodel is applied [18]. In addition to selecting variables associated to the outcome this approach encourages choosing variables that have dependence structure. Variables with posterior inclusion probability greater than 0.1 were selected. As a result mRNAs, miRNAs, and miRNA-mRNA pairs associated with HCC status were identified.

III. Results

A. Mechanistic submodel

The mechanistic submodel relating each of the 106 mRNAs to the 261 miRNAs using a spike-and-slab variable selection method integrating prior knowledge about their relationships identified 371 miRNA-mRNA pairs. A subset of 166 miRNAs were related to at least one mRNA. Of the selected pairs, there were 22 experimentally verified pairs. For example, *CAT* was found to be associated with 9 miRNAs, 4 of which (hsa-miR-101-5p, hsa-miR-421, hsa-miR-4327, hsa-miR-4686) are known to target that mRNA.

Relationships between genes before adjusting for miRNAs were also investigated by estimating an undirected graph with a lasso graphical model. We refer to this as the unadjusted graph. The resulting graph contained 497 edges. *ADAMTS13*, *ECM1*, and *PTH1R* were identified as the most connected genes with 29 related edges. The adjusted graph estimated by considering mRNA expressions adjusted for miRNA effects contained 101 edges, 86 of which are in common with the unadjusted graph (see Table II). Thus, the majority of gene-gene interactions were not maintained after accounting for the miRNA regulation of these genes. The results are in line with those observed in previous studies: the edge number is reduced when accounting for potential confounder covariates. As an example, we focus on 5 genes that are connected in the unadjusted graph (left panel of Fig. 2) and conditionality independent in the adjusted graph (middle panel of Fig. 2). We also display the adjusted graph with the associated miRNAs (right panel of Fig. 2) for a better understanding. The direct relationship observed between *SMPD3* and *WDR66* in the unadjusted graph is mainly due to the regulation by a common miRNA (hsa-miR-200a-5p) modulating the expressions of both genes. Similarly, the edge that was present between *LILRB5* and *PIGU* in the unadjusted graph disappears in the adjusted graph. Accounting for miRNA effects helps to clarify genes that are co-regulated by miRNA versus genes that are interacting through other mechanisms. Unchanged connections across the unadjusted and adjusted graphs evidence dependence due to biological factors other than miRNAs. For example, *TERT* and *STAB2* remain connected in both graphs (left panel of Fig. 3). The adjusted graph with the associated miRNAs is shown in the right panel of Fig. 3.

The same approaches (spike-and slab prior integrating prior knowledge and estimation of unadjusted and adjusted graphs) were applied separately on HCC cases and patients with cirrhosis. The results are reported in Table II. We observe that the number of connections in the unadjusted graphs, when considering the two groups independently, is reduced in particular for CIRR group. A denser graph in HCC cases may indicate that the considered subset of genes is mostly connected in HCC.

We also observed than the sizes of the adjusted graphs are smaller than that of the unadjusted graphs in each disease group. To compare the graphs between the two groups we performed a differential network analysis via the R package `iDINGO` [19]. Partial correlations were computed by using a Gaussian graphical model based on the raw gene expressions and the gene expressions adjusted for miRNAs effects. Fig. 4 presents the partial correlations in HCC versus CIRR. Pairs with a p-value less than 0.05 and with absolute values of the differential scores and partial correlations greater than 3 and 0.1, respectively, are labeled on the plots. While most pairs of genes have similar partial correlations for the unadjusted graphs, partial correlations for HCC and CIRR are more contrasted for the adjusted graphs. Among the selected mRNA-miRNA pairs in both groups, 29 are in common. Of those, 8 are also identified when analyzing the two groups. For example the pair *BCL9* - hsa-miR-378d is identified in all three analyses. We emphasize that the results obtained for each group analyzed separately need to be considered with caution since the small sample size may lead to a lack of statistical power and to computational instability.

TABLE II
Number of edges in the estimated unadjusted and adjusted graphs.

	Undajusted graph	Adjusted graph	Common edges
HCC +CIRR	497	101	86
HCC	247	171	74
CIRR	92	59	4

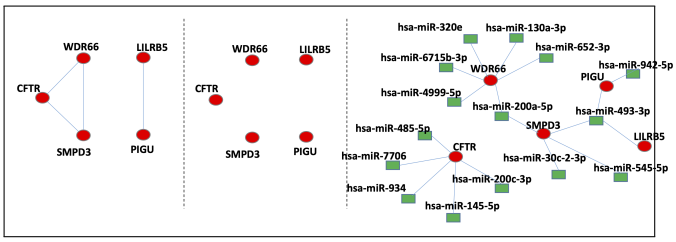

Fig. 2. Unadjusted graph (left), adjusted graph (middle), and adjusted graph with associated miRNAs (right)

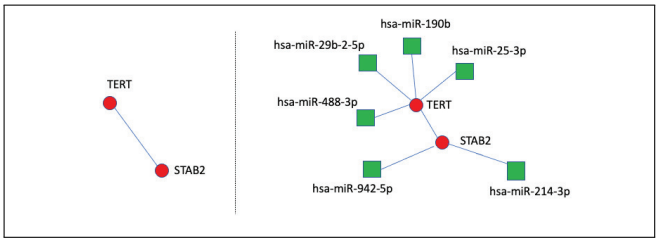

Fig. 3. Adjusted graph (left) and adjusted graph with associated miRNAs (right)

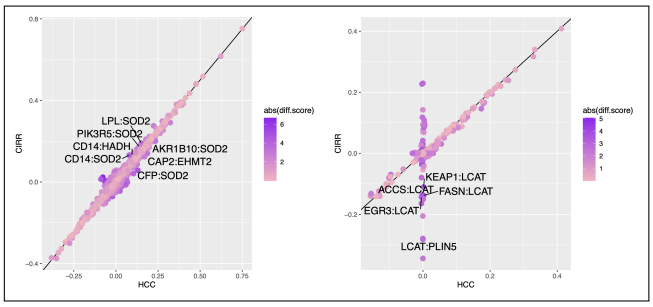

Fig. 4. Partial correlations for HCC versus CIRR based on unadjusted graphs (on left) and adjusted graphs (on right)

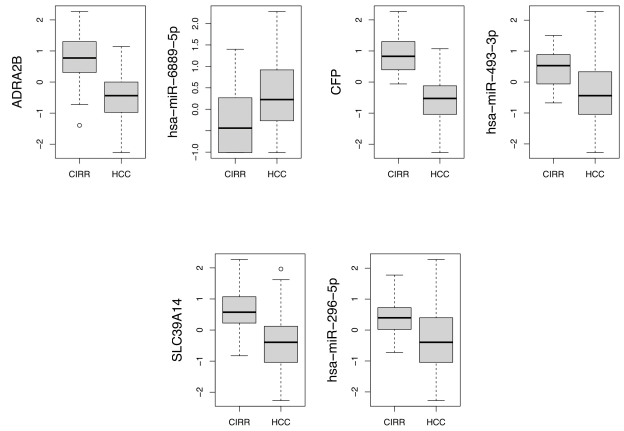

Fig. 5. Boxplots of miRNA and mRNA expressions across disease status for three experimentally verified pairs.

B. Clinical submodel

The clinical submodel selected 21 mRNAs, 5 miRNAs and 66 miRNA-mRNA pairs. Among them, 17 genes have expression levels that are modulated by miRNAs leading to 66 miRNA-mRNA pairs, and 4 have expression levels modulated by biological features other than their associated miRNAs. 5 miRNAs (hsa-miR-150-3p, hsa-miR-193-3p, hsa-miR-3192-3p, hsa-miR-365a-3p, hsa-miR-548ao-3p) are found to be directly associated to HCC (see Table A1 in the Appendix). Three of the 66 disease associated miRNA-mRNA pairs that are experimentally verified are shown in Table A1 in bold face (*ADRA2B* - hsa-miR-6889-5p, *CFP* - hsa-493-3p, *SLC39A14* - hsa-296-5p). There are also a few molecules identified in the selected pairs that are involved in experimentally verified pairs. For example miRNA hsa-miR-7-5p, which is found to be related to ABCG5 in our model, is also targeting TLR4. These two genes belong to the LXR/RXR pathway. Fig. 5 presents boxplots of expression levels across disease status associated to the three experimentally verified pairs. While *ADRA2B* and hsa-miR-6889-5p have opposite expression patterns, dual-downregulation is observed for *CFP* and hsa-493-3p, and *SLC39A14* and hsa-296-5p.

A pathway analysis of the miRNAs and mRNAs selected from the clinical submodel was performed using the IPA tool. Fig. 6 represents the top 10 pathways using the molecules selected in the clinical submodel. The overlapping canonical pathways are shown as a network where each pathway is a single node colored proportionally to the p-value, where brighter red represents a more significantly enriched pathway. As shown in Fig. 6, LXR/RXR activation pathway, Hepatic Fibrosis/Hepatic Stellate Cell Activation, Role of MAPK Signaling, and INOS Signaling pathways are found to be significantly enriched in our analysis. Accumulating evidence demonstrated that LXR is a potential prognostic marker and exerted significant anti-tumor effect in HCC. Our previous studies have also reported these pathways to be significantly enriched in HCC [9], [20].

Fig. 7 shows the molecule interaction network generated using the miRNAs and mRNAs from the clinical model. The molecules in this network are mainly involved in cancer, organismal injury and abnormalities, reproductive system disease, and gastro intestinal diseases. We also note a few overlaps with the miRNA-mRNA relationships identified with our analysis. For example, miR-146a-5p and LBP pair overlaps in our model and the network generated by IPA. Down regulation of miR-146a-5p and its targets in HCC have been reported to play a tumor-suppressive role [21].

IV. Discussion

With regards to the mechanistic model, the integration of prior knowledge identified 22 experimentally verified miRNA-mRNA pairs. The proposed approach was also able to identify 349 novel miRNA-mRNA pairs, some of which were revealed to be associated with the disease status in the clinical submodel. The integration of prior knowledge into the analysis helps complement the information in the data and can reinforce the existing evidence as well as lead to new discoveries.

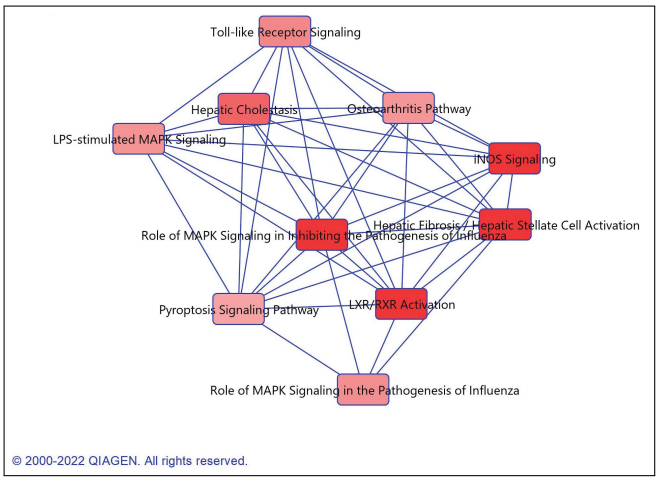

Fig. 6. Top 10 pathways represented by the molecules selected from the clinical model. The connections show that one or more molecule is common across multiple pathways. The darker red color shows that the pathways has higher significance

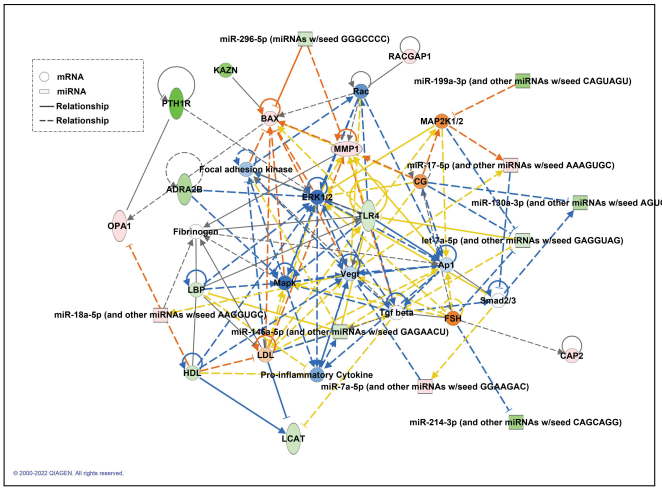

Fig. 7. Network generated using the miRNAs and mRNAs selected from the clinical model using IPA. Green molecules are downregulated and red molecules are upregulated.

Through the estimation of adjusted and unadjusted graphs we pointed out that, although a direct application of GGM on gene expressions data provided some insights into gene regulation at the expression level, adjusting for the effects of miRNAs on mRNA expressions improved the understanding of relationships between genes. The results revealed some genes with unchanged connections in the adjusted and unadjusted graphs indicating genes that are dependent conditionally to other genes and all miRNAs. On the other hand, some connections disappeared once the effects of miRNAs are accounted for, underlying a dependence most likely due to miRNAs. As a result a finer understanding of dependence sources between genes was achieved.

The clinical submodel selected 21 mRNAs, 5 miRNAs, and 66 miRNA-mRNA pairs associated to disease status. As shown in the network and pathway analyses, the approach helped us to narrow down to the most important mRNAs and miRNAs as well as miRNA-mRNA pairs that are more relevant to study HCC. The identified molecules are involved in pathways known to play an important role in the pathogenesis of HCC, including the LXR/FXR Activation, iNOS Signaling, and Hepatic Fibrosis / Hepatic Stellate Cell Activation Pathways. These results underlined the potential of integrating the proposed approach in selecting markers associated with HCC in a larger study containing more samples and molecules.

V. CONCLUSIONS

The identification of relevant biomarkers of HCC is essential to obtain a better diagnosis in early stages and improve understanding of complex biological mechanisms underlying HCC. In this paper, we proposed a Bayesian two-step integrative procedure extending the initial approach developed by [4], [3]. The extension lies in the integration of knowledge from various sources at the different stages of the modeling. Here, the mechanistic submodel used prior knowledge from an external database, through IPA target filter analysis in this case. The clinical submodel integrated as prior knowledge the connections estimated by the graphical lasso in the mechanistic submodel. In addition to helping statistical models lessen the challenge of ill-posed problems, these types of prior knowledge integration into the analysis provide a better understanding of relationships between biological features (between miRNAs and mRNAs, as well as between mRNAs in our application). They also help identify biologically relevant biomarkers for the phenotype under investigation. The findings, of course, need to be experimentally validated to confirm their potentials as diagnostic or prognostic biomarkers.

The methods implemented in this study led to the identification of key miRNA-mRNA pairs and pathways that are potentially associated with HCC. The results highlight the biological relevance of studying molecular interactions and the need for integrating prior knowledge when analyzing data from mRNA-seq and miRNA-seq. Additional studies focused on comprehensive and integrative analysis of larger datasets are necessary to explore the interactions.

APPENDIX

ACKNOWLEDGMENT

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TABLE A1

MRNAS, MIRNAS, AND MIRNA-MRNA PAIRS IDENTIFIED BY THE CLINICAL SUBMODEL. AN UPWARD ARROW DENOTES A POSITIVE FOLD CHANGE AND A DOWNWARD ARROW DENOTES A NEGATIVE FOLD CHANGE WHEN COMPARING HCC VS CIRR.

miRNA - mRNA pair			miRNA - mRNA pair			miRNA - mRNA pair		
ABCG5	↓	hsa.miR.16.5p ↑	RACGAP1	↑	hsa.miR.10a.5p ↓	SLC39A14	↓	hsa.miR.4484 ↓
ABCG5	↓	hsa.miR.7.5p ↑	ABCG5	↓	hsa.miR.378d ↓	TLR4	↓	hsa.miR.10a.3p ↓
ADRA2B	↓	hsa.miR.15b.3p ↑	ADRA2B	↓	hsa.miR.143.5p ↓	TLR4	↓	hsa.miR.10a.5p ↓
ADRA2B	↓	**hsa.miR.6889.5p** ↑	ADRA2B	↓	hsa.miR.675.3p ↓	TLR4	↓	hsa.miR.214.3p ↓
IFITM10	↓	hsa.miR.556.3p ↑	ADRA2B	↓	hsa.miR.6827.3p ↓	TLR4	↓	hsa.miR.376a.5p ↓
KAZN	↓	hsa.miR.942.5p ↑	CFP	↓	hsa.let.7e.5p ↓	TLR4	↓	hsa.miR.376c.5p ↓
PSD3	↓	hsa.miR.425.5p ↑	CFP	↓	hsa.miR.199a.3p ↓	TLR4	↓	hsa.miR.377.3p ↓
PTH1R	↓	hsa.miR.1283 ↑	CFP	↓	hsa.miR.214.3p ↓	TLR4	↓	hsa.miR.450b.5p ↓
PTH1R	↓	hsa.miR.15b.5p ↑	**CFP**	↓	**hsa.miR.493.3p** ↓	TLR4	↓	hsa.miR.5690 ↓
PTH1R	↓	hsa.miR.425.5p ↑	CFP	↓	hsa.miR.497.3p ↓	CCNA2	↑	hsa.miR.15b.3p ↑
PTH1R	↓	hsa.miR.6715b.3p ↑	IFITM10	↓	hsa.miR.424.5p ↓	CCNA2	↑	hsa.miR.18a.5p ↑
SLC39A14	↓	hsa.miR.6747.5p ↑	IFITM10	↓	hsa.miR.675.3p ↓	CCNA2	↑	hsa.miR.20a.5p ↑
SLC39A14	↓	hsa.miR.93.3p ↑	KAZN	↓	hsa.miR.214.3p ↓	RACGAP1	↑	hsa.miR.454.5p ↑
TLR4	↓	hsa.miR.365b.3p ↑	KAZN	↓	hsa.miR.490.3p ↓	RACGAP1	↑	hsa.miR.4677.5p ↑
TLR4	↓	hsa.miR.4327 ↑	KAZN	↓	hsa.miR.6827.3p ↓	mRNA		miRNA
TLR4	↓	hsa.miR.520d.3p ↓	LBP	↓	hsa.miR.146b.5p ↓	BAX	↑	hsa.miR.193b.3p ↑
CCNA2	↑	hsa.miR.139.3p ↓	LCAT	↓	hsa.miR.1275 ↓	CAP2	↑	hsa.miR.3192.3p ↑
EBF2	↑	hsa.miR.130a.3p ↓	LCAT	↓	hsa.miR.130a.3p ↓	NDRG3	↑	hsa.miR.365a.3p ↑
H2AFX	↑	hsa.miR.101.5p ↓	LCAT	↓	hsa.miR.4686 ↓	MT1L	↓	hsa.miR.548ao.3p ↓
H2AFX	↑	hsa.miR.139.5p ↓	PSD3	↓	hsa.miR.378d ↓			hsa.miR.150.3p ↓
MMP1	↑	hsa.miR.10a.5p ↓	PTH1R	↓	hsa.miR.130a.3p ↓			
MMP1	↑	hsa.miR.200c.3p ↓	PTH1R	↓	hsa.miR.214.5p ↓			↑ Up-regulatioon
MMP1	↑	hsa.miR.3614.5p ↓	PTH1R	↓	hsa.miR.3117.3p ↓			↓ Down regulation
MMP1	↑	hsa.miR.381.3p ↓	PTH1R	↓	hsa.miR.490.3p ↓			mRNAs directly associated to Y
OPA1	↑	hsa.miR.378d ↓	**SLC39A14**	↓	**hsa.miR.296.5p** ↓			miRNAs directly associated to Y
RACGAP1	↑	hsa.miR.101.5p ↓	SLC39A14	↓	hsa.miR.378d ↓			

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