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miR2Pathway: A novel analytical method to discover MicroRNA-mediated dysregulated pathways involved in hepatocellular carcinoma

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ABSTRACT

MicroRNAs (miRNAs) are small, non-coding RNAs involved in the regulation of gene expression at a post-transcriptional level. Recent studies have shown miRNAs as key regulators of a variety of biological processes, such as proliferation, differentiation, apoptosis, metabolism, etc. Aberrantly expressed miRNAs influence individual gene expression level, but rewired miRNA-mRNA connections can influence the activity of biological pathways. Here, we define rewired miRNA-mRNA connections as the differential (rewiring) effects on the activity of biological pathways between hepatocellular carcinoma (HCC) and normal phenotypes. Our work presented here uses a PageRank-based approach to measure the degree of miRNA-mediated dysregulation of biological pathways between HCC and normal samples based on rewired miRNA-mRNA connections. In our study, we regard the degree of miRNA-mediated dysregulation of biological pathways as disease risk of biological pathways. Therefore, we propose a new method, miR2Pathway, to measure and rank the degree of miRNA-mediated dysregulation of biological pathways by measuring the total differential influence of miRNAs on the activity of pathways between HCC and normal states. miR2Pathway proposed here systematically shows the first evidence for a mechanism of biological pathways being dysregulated by rewired miRNA-mRNA connections, and provides new insight into exploring mechanisms behind HCC. Thus, miR2Pathway is a novel method to identify and rank miRNA-dysregulated pathways in HCC.

1. Introduction

MicroRNAs (miRNAs) are short, non-coding RNAs about 22 nucleotides long, involved in the post-transcriptional regulation of gene expression. MiRNAs induce mRNA degradation or translational repression depending on the degree of homology to specific sequences, typically in the untranslated regions (UTRs) of their targets [1]. MiRNAs are able to impact the expression of one or more genes at a time. It is believed that more than 60% of human genes are regulated by miRNAs [2]. Hence, miRNAs can be important regulators of biological processes. For example, a single miRNA can control a complex biological pathway by simultaneously targeting multiple mRNAs within a pathway. The targeted mRNAs could be members of a cascade converging towards a functional endpoint in the same biological pathways or at the crosstalk between biological pathways [3].

Over the past years, research has demonstrated the role of miRNAs as key regulators that control a variety of fundamental biological processes involved in proliferation, apoptosis, cell growth, differentiation, invasiveness, motility, other oncogenic related processes, etc. [4–12]. For example, Lu and colleagues show that miRNA-21 can down-regulate the activity of the IL-12/IFN-γ pathway in lung cancer [7]. Other studies have found that miRNA-7 can simultaneously target multiple genes of the PI3-kinase/Akt pathway in hepatocellular carcinoma (HCC) and glioblastoma [8,9]. MiRNA-200 functions as a multifunctional tumor suppressor in meningiomas through multiple and simultaneous influences on the E-cadherin and Wnt/β-catenin signaling pathways [10]. MiRNA-106a has been shown to directly inhibit ULK1 mRNA expression levels in acute myeloid leukemia (AML) cells; MiRNA-106a can also target other members of the ULK1 complex, such as FIP200 and mAtg13 [6]. The C/EBP-α-PU.1 pathway is found to be regulated by miRNA-124 [12], and miRNA-1 has been suggested to inhibit the Pten/Akt pathway [11].

MiRNAs are often aberrantly expressed in tumor tissue even in early stages of a tumor and other conditions [13,14], which can make them valuable biomarker candidates, such as for Alzheimer's disease (AD) [15]. Furthermore, several studies have demonstrated a potential value of miRNA-based therapy in cancer [16–19]. A good example is the utility of anti-miRNA-21 in breast cancer, resulting in suppression of

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tumor growth in vivo and cell growth in vitro [20]. Therefore, miRNAs' potential as disease biomarkers and therapeutic agents places this group of small non-coding RNAs on the cutting-edge of biomedical research interest.

Based on these observations, an important question emerges: What degree of miRNA-mediated dysregulation of biological pathways is present in disease? Over the past decade, there has been a large volume of literature demonstrating that miRNAs dysregulate mRNA expression levels by their aberrant expression in disease [7–12,15–18,20,21]. Namely, the targeted mRNAs' expression might be aberrantly altered because they are incorrectly regulated by aberrantly expressed miRNAs. Based on this altered expression, several tools have been developed to detect miRNA-pathway associations [22–31]. These tools typically propose enrichment-based methods to study associations between miRNAs and pathways. These enrichment-based methods, however, have two common limitations. First, they study the association between miRNA and pathway based on enrichment analysis of targeted genes in a pathway. Hence, they ignore the topological importance of targeted genes within a pathway. For example, if one miRNA targets hub genes that have high topological importance, this miRNA might have higher association with the activity of this pathway than another miRNA that targets genes of low topological importance. Second, these methods do not aim to assess changes in pathways between disease and non-disease. Instead, they only focus on how miRNAs' aberrant expression affect the targeted genes in a pathway, and do not assess how rewired miRNA-mRNA connections influence a pathway.

Genes interact in complex networks that govern cellular processes. Researchers have discovered how rewired miRNA-mRNA connections influence biological processes in cancer. An important reason the rewired miRNA-mRNA connections influence biological processes in cancer is that miRNA-mRNA connections tend to be dynamic or condition-specific, or different between disease and non-disease. For example, Volinia and colleagues [32] have analyzed the genetic networks of miRNAs in cancer, and they suggested that in normal tissues, miRNAs are connected in networks and different cell types have different network connections. In cancer, they suggest that it is likely that normal network connections become disrupted or rewired, which might contribute to disease. In addition, Chen-Ching Lin, et al. [33] have identified a regulatory feedback loop between STAT1 and miRNA-155-5p that is consistently activated in cancer; they found that the rewired regulatory networks are highly associated with cancer. Sivan Elhanati, et al. [34] have found that miRNA-122 and SIRT6 negatively regulate each other's expression, and the connection between them is manifested in two physiologically relevant ways in the liver. First, they negatively regulate a similar set of metabolic genes and fatty acid β-oxidation. Second, the loss of a negative correlation between SIRT6 and miRNA-122 expression is significantly associated with better prognosis in HCC patients. In addition, there are also analytical approaches for exosomal miRNA expression analysis [35,36]. Thus, an increasing number of relevant studies suggest that rewired connections between miRNAs and genes are associated with disease. However, these studies mainly focus on rewired connections between miRNAs and genes, but do not analyze how much the rewired miRNA-mRNA connections are associated with dysregulation of biological pathways at the pathway-level.

A recent methodology developed by Kang et al. can analyze topological features of miRNA-target gene differential regulatory networks [37]. However, they use "degree" as the topological measurement in their study. "Degree" does not consider the topological weight of each gene in gene regulatory networks. Hence, we need to consider this in our study. Our analysis uses PageRank, an algorithm initially used by Google Search to rank websites in search engine results [38]. PageRank is a way of measuring the topological importance of nodes in a network. More generally, PageRank has been applied to other networks, e.g., social networks [39,40]. To date, several studies also use PageRank to analyze miRNA [41–43]. Xu et al. [41] focuses on miRNA-transcription factor (TF)-mRNA regulatory networks. They used their method to construct a miRNA-TF-mRNA regulatory network for clustering samples with different cancer subtypes, achieving the goal of cancer subtype classification. Noh et al. [42] focuses on identifying a set of miRNA-mRNA connections that are changed in Alzheimer's disease. Wang et al. [43] uses PageRank to rank miRNAs and mRNAs, separately, and to select the top ranked ones as biomarkers for ischemic stroke. Although these studies make use of PageRank, their focus is different from our approach.

Here, we propose a new PageRank-based method, miR2Pathway, to analyze and rank the disruption caused at the pathway network level by rewired miRNA-target gene connections, and then we apply it to study hepatocellular carcinoma (HCC). For example, in hypothetical case 1, a miRNA regulates several hub genes in a pathway in normal tissue, while it loses those regulatory connections in tumor tissue. In hypothetical case 2, this miRNA regulates the same number of non-hub genes in this pathway in normal tissue, while it loses those regulatory connections in tumor tissue. In this scenario, our hypothesis is that this miRNA has a larger influence on the activity of the pathway in case 1 than in case 2. This hypothesis is related to the basic idea of PageRank [38] that the topological importance of a node is high in a network if this node has connections to other nodes with high topological importance. As a PageRank-based approach, miR2Pathway focuses on quantifying the differential effects of miRNAs on the activity of biological pathways when miRNA-mRNA connections are altered from normal to HCC. miR2Pathway provides a new insight to explore mechanisms behind HCC. Thus, miR2Pathway is a novel method that can identify miRNA-dysregulated pathways in HCC. miR2Pathway has several characteristics that are different from previous methods [22–31]: (1) miR2Pathway can identify the relationship between a set of miRNAs and a set of pathways; (2) miR2Pathway focuses on identifying miRNA-mediated changes in pathways between control and case, while the other methods focus on finding pathways enriched in genes targeted by miRNAs; and (3) miR2Pathway focuses on identifying miRNA-mediated topological changes in pathways between control and case, while the other methods do not assess topological changes between control and case. These characteristics, particularly (2) and (3), make it difficult to directly compare miR2Pathway with other methods because miR2Pathway addresses a different question than the other methods.

2. Materials and methods

An overview of the miR2Pathway method is illustrated in Fig. 1. Briefly, gene and miRNA expression profiles are used to construct connections between miRNAs and genes for control and case, respectively. Subsequently, we compute the corresponding differential network between control and case for each miRNA-Pathway pair. For each miRNA-Pathway pair, we find the genes targeted by the miRNA in this differential network. Then, PageRank is applied to measure the topological influence (PageRank scores) of the targeted genes in this differential network, which quantifies the topological influence of the genes that are differentially targeted by the miRNA within this pathway. Next, we calculate the sum of PageRank scores of genes targeted by the miRNA in the differential network, which estimates the total differential influence of the miRNA on the activity of this pathway. Then, the same procedure is repeated for all miRNAs. We obtain a corresponding sum of PageRank scores for each miRNA. For a specific pathway, we then compute T, the total differential influence of all the miRNAs on this pathway, by adding all the sums corresponding to all the miRNAs. The T score reflects the degree of miRNA-mediated dysregulation of this pathway. We do this for all the pathways. Finally, we rank all the pathways by T score.

2.1. Data

For illustration, we use miRNA and mRNA expression data (RNA-seq) from The Cancer Genome Atlas (TCGA) hepatocellular carcinoma

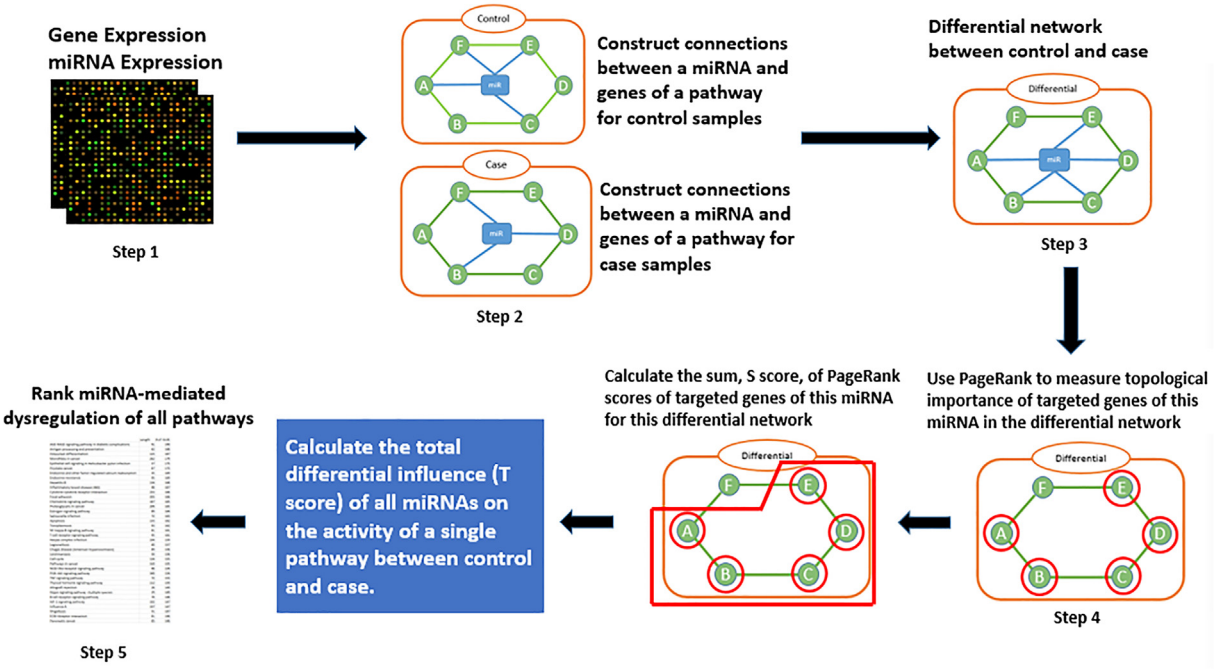

Fig. 1. An overview of miR2Pathway.

(HCC) study (https://cancergenome.nih.gov/). The dataset contains expression levels for 1046 miRNAs and 20,531 mRNAs. We apply miR2Pathway to analyze four datasets: 50 HCC samples and 50 tumor-adjacent normal samples, 34 hepatitis C-induced HCC samples and 34 tumor-adjacent normal samples, 22 hepatitis B-induced HCC samples and 22 tumor-adjacent normal samples, and 50 alcohol-induced HCC samples and 50 tumor-adjacent normal samples. Tumor-adjacent normal samples in the following sections will be referred to as normal samples. Pathway information from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [44] is used.

2.2. Construct connections between each miRNA and genes of each pathway

2.2.1. Predicted and validated miRNA targets

Five miRNA target site prediction programs (DIANA [28], Targetscan [2], PicTar [45], Miranda [46] and miRDB [47]) are employed to obtain putative miRNA target genes for all 1046 miRNAs. These five programs are included in the "miRNAtap" R package. Therefore, we implement the miRNA target site prediction by using miRNAtap. Genes are potential targets when they are identified by at least two of five programs. Three validated miRNA target site databases (miRecords [48], miRTarBase [49] and TarBase [50]) are used to obtain validated miRNA target genes for all 1046 miRNAs. These three databases are included in the "multiMiR" R package and database. We select validated target genes when they are present in at least one of these three databases.

2.2.2. Statistical analysis of miRNAs and target genes

miRNA expression is negatively correlated with mRNA expression. To statistically identify miRNA-mRNA connections in a regulatory network, we define a statistical connection between a miRNA and its target genes if the Pearson's correlation between their expression levels is less than a series of cutoffs (-0.4, -0.3, -0.2, -0.1) and the corresponding p-value of the Pearson's correlation is < 0.05. We implement it through a built-in function called "cor.test()" in the statistical software package R (https://www.r-project.org/). Case and control samples are each assigned a statistical connection.

2.2.3. Identification of connections between miRNAs and mRNAs

We use the intersection of the sets from **Step 2.1** and **Step 2.2** as the identified miRNA-mRNA connections, which are used for the downstream analysis.

Note, for Steps 2.1–2.3, we only determine the connections between miRNAs and target genes. For the construction of pathways, we directly obtain gene-gene connections within pathways from KEGG [44]. We pre-define the topology of pathways from KEGG and observe alterations of miRNA-mRNA connections in our study. Our analysis quantifies the degree by which miRNAs differentially influence the activity of each pathway between control and case.

2.2.4. Construct miRNA-Pathway regulatory networks

Next, we construct the miRNA-Pathway networks, i.e., construct each network consisting of a single miRNA and a single pathway. First, we obtain the gene list of a specified pathway from KEGG. Second, for each miRNA, we select the identified miRNA-mRNA connections specific for this pathway. Finally, we merge these identified miRNA-mRNA connections into the topology of this pathway derived from KEGG. The result is a miRNA-Pathway network. We perform this process separately for case and control samples.

2.3. Differential networks for miRNA-Pathway pairs

Based on the miRNA-Pathway networks constructed separately for control and case samples, we can easily find the corresponding differential networks between control and case samples (see Fig. 2).

Notably, all of the networks in our study are based on correlation; thus, all of them are undirected graphs.

2.4. Measure the differential influence of miRNAs on the activity of pathways

2.4.1. Measure the differential influence of a single miRNA on a single pathway

To summarize, up to this point the algorithm finds the differential miRNA-Pathway network, which provides information about the differential influence of a miRNA on the activity of a pathway between control and case. The null hypothesis is that there is no difference in the

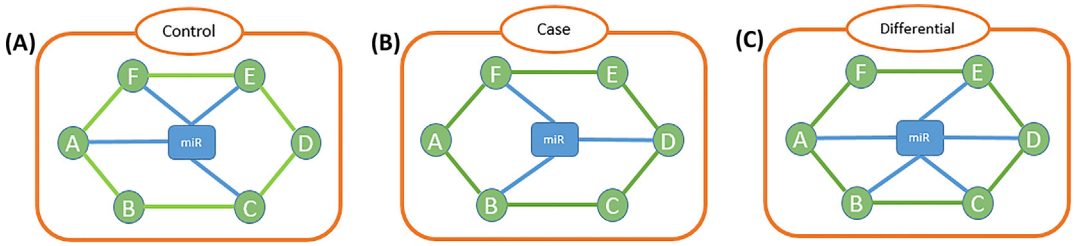

Fig. 2. Construction of differential networks. (a) is a miRNA-Pathway network for control. (b) is the corresponding miRNA-Pathway network for case. (c) is the differential miRNA-Pathway network between control and case. A differential connection is constructed if it appears in either (a) or (b) but not in the other. The green round nodes represent genes and the blue rectangular node represents a miRNA.

miRNA-Pathway network between control and case, indicating that the differential miRNA-Pathway network has an isolated miRNA. In other words, the single miRNA has no differential influence on the activity of this pathway. Conversely, if this miRNA has many connections in the differential miRNA-Pathway network between control and case, and, even more importantly, has differential connections with hub genes in this pathway, it suggests that this miRNA has a large differential influence on the activity of this pathway between control and case samples.

In this step, we measure the differential influence of a miRNA on the activity of this pathway by using PageRank [38] (see Eq. (1)).

$$S_{i,j} = PR(TG)_{i,j,1} + PR(TG)_{i,j,2} + ...+PR(TG)_{i,j,k} \quad (1)$$

In Eq. (1), $S_{i,j}$ is the sum of PageRank (PR) scores of targeted genes (TG) for miRNA i and pathway j in the differential miRNA-Pathway network. The letter k denotes the number of genes targeted by miRNA i in the corresponding differential miRNA-Pathway network. $S_{i,j}$ quantifies the differential influence of miRNA i on the activity of pathway j between control and case samples. Since PageRank considers the sum of PageRank scores of all the nodes in a network equal to 1, $S_{i,j}$ ranges from 0 to 1. When $S_{i,j} = 0$, it indicates that miRNA i does not differentially regulate genes of the pathway j between control and case. When $S_{i,j} = 1$, it indicates that miRNA i differentially regulates all genes of the pathway j between control and case.

2.4.1.1. The PageRank algorithm. The PageRank algorithm is used by the Google search engine to rank the importance of web pages. It is based on the assumption that the importance of a web page is high in a network if this web page has connections to other nodes of high importance. This idea is naturally applied to analyzing biological networks, where the importance of a gene is high if this gene is connected to other genes of high importance. In our study, the gene-gene network is an undirected graph where a node represents a gene and the edges can be defined by prior knowledge (e.g., KEGG database).

The output from the PageRank algorithm is a probability distribution representing the likelihood that a person randomly clicking on links will arrive at any particular web page. A probability is a numeric value between 0 and 1. The sum of probabilities for all web pages is equal to 1. The probability of a web page is proportional with the time spent at the web page when a person surfs the web. This idea can also be intuitively extended to ranking genes in gene networks where the probability of a gene is proportional to the time a research scientist spends looking at and returning to the same gene when analyzing research results. For additional details of PageRank, please refer to [38].

2.4.2. Measure the total differential influence of a set of miRNAs on a single pathway

For the same pathway, we repeat Step 4.1 for all miRNAs of interest. We obtain different $S_{i,j}$ (i = 1,2,…,M, where M is the number of miRNAs and j is the index of the pathway) for different miRNAs. Then, we assess the total differential influence of all the miRNAs on this pathway by adding up all the $S_{i,j}$ scores (see Eq. (2)).

$$T_j = S_{1,j} + S_{2,j} + ...+S_{M,j} \quad (2)$$

In Eq. (2), T_j is the sum of all the S scores corresponding to all M miRNAs for pathway j. The T score quantifies the total differential influence of all the miRNAs on the activity of a single pathway between control and case. If T_j is larger, it suggests that miRNAs differentially regulate a larger number of genes and/or differentially regulate hub genes in pathway j between control and case. Hence, the T score can reflect how much miRNAs dysregulate a single pathway.

2.5. Rank pathways based on T score

We repeat Step 4 to obtain a corresponding T score for each pathway. Finally, we rank all pathways by their T scores, which are measures of the degree of miRNA-mediated dysregulation.

2.6. Software tools

All the analyses are conducted using the R programming language. We use the following R Bioconductor packages: parallel for parallel computing, graphite for pathway databases, igraph for PageRank function, graph for visualization, and miRNAtap and multiMiR for predicted and validated miRNA targets.

3. Results

First, we applied miR2Pathway to analyze HCC and normal samples. We use, as an example, the interaction between miRNA-122 and the "MicroRNAs in cancer" pathway. MiRNA-122 is reported to be specific to liver cancer in several studies [51–54], and "MicroRNAs in cancer" is a miRNA-related oncogenic pathway. We are interested in seeing how miRNA-122 differentially influences the activity of this pathway between normal and HCC states. We directly obtained the topological structure of this pathway from the KEGG database. Namely, there are 262 genes and 518 connections between genes in this pathway. For gene names, see Supplementary Table S1.

3.1. Construct connections between miRNA-122 and genes in the "MicroRNAs in cancer" pathway for normal and HCC

Based on the topological structure of this pathway, we need to know which gene(s) is/are targeted by miRNA-122 in this pathway for normal and cancer samples, separately. After we complete **Step 2.3**, we identify connections between miRNA-122 and genes in the "MicroRNAs in cancer" pathway. In Supplementary Fig. S1, as an example, we only show the identified connections between miRNA-122 and genes based on predicted targets using a correlation cutoff of -0.4.

Supplementary Fig. S1 shows that miRNA-122 targets one gene (geneID is 6541 and gene symbol is CAT-1 or SLC7A1) in normal samples, and it does not target this gene in HCC samples. Very interestingly, several studies show that CAT-1/SLC7A1 is a well-known target gene of miRNA-122 [54–58]. CAT-1/SLC7A1 is an important protein for liver tissue. It is a carrier protein required in the

regenerating liver for the transport of cationic amino acids and polyamines in the late G1 phase – a process that is essential for liver cells to enter mitosis. Also, CAT-1/SLC7A1 is involved in amino acid metabolism [55]. Several studies show that miRNA-122's loss of function has been observed in liver cancer [51,53,59,60]. Thus, this result suggests that miRNA-122's loss of function probably leads to loss of a connection between miRNA-122 and CAT-1/SLC7A1 in the HCC samples. Therefore, this result is consistent with the evidence from prior literature.

3.2. A differential network between normal and cancer samples

Based on the results illustrated in Supplementary Fig. S1, we can easily obtain the differential network between normal and HCC samples, whose topological structure is the same as Supplementary Fig. S1(a).

3.3. Measure the differential influence of miRNAs on the specified pathway

Based on the differential network above, we assess the topological influence of genes dysregulated by miRNA-122 in this pathway by calculating PageRank scores of targeted genes in the differential network. Namely, the PageRank score of the CAT-1/SLC7A1 gene is 0.00264, which is also shown in Supplementary Table S1. In Supplementary Table S1, we also compute and include eigenvector centrality, which is another topological measure of the "MicroRNAs in cancer" pathway, which was previously discussed in the literature [61]. However, we only discuss and use PageRank scores for genes in each pathway in our study.

We repeat this process to measure the differential influence of all other miRNAs on the activity of the "MicroRNAs in cancer" pathway, then we sum up the differential influence of all the miRNAs as a total T score. This total score, T = 0.139, is the degree of miRNA-mediated dysregulation of the "MicroRNAs in cancer" pathway.

3.4. Rank miRNA-mediated dysregulation of all pathways

Similarly, we obtain a corresponding T score for each pathway. Then, we rank pathways using the T scores and show the top 50 pathways in Table 1, using a Pearson correlation cutoff value of −0.4. As mentioned in Materials and Methods, we also tested several other correlation cutoffs: −0.3, −0.2, −0.1. These other results are very similar to the results from Table 1 and are included in Supplementary Tables S4, S5 and S6, respectively.

Interestingly, we found that a large number of pathways are associated with cancer in general, as shown in Table 1. Some of these pathways are specific to liver cancer. First, the "MicroRNAs in cancer" pathway is listed in the top 50 pathways, which suggests that miRNA-mediated dysregulation is able to contribute to cancer [62–64]. The FoxO signaling pathway is top-ranked and known to be involved in the regulation of the cell cycle, apoptosis, and metabolism. The second ranked pathway, circadian rhythm, is well known to be implicated in cancer [65,66]. The Hedgehog signaling pathway is a major regulator of many fundamental processes in vertebrate embryonic development, including stem cell maintenance, cell differentiation, tissue polarity and cell proliferation [67–70]. The Notch signaling pathway is one of the most commonly activated signaling pathways in cancer, and it plays a key role in cell proliferation, differentiation and survival [71–73]. The Hippo signaling pathway is reported to be able to control organ size through regulating cell proliferation and apoptosis [74,75]. The Wnt signaling pathway is a well-known pathway specific to liver cancer. The deregulation of the Wnt signaling pathway is an early event in hepatocarcinogenesis. This pathway plays a critical role in developing and regenerating the liver and promoting tumor formation in this organ [76–78]. The MAPK signaling pathway is involved in the regulation of survival, cellular growth, and gene expression [79]. Deregulation of the MAPK signaling pathway can lead to uncontrolled or increased cell proliferation and resistance to apoptosis [80,81]. The mTOR signaling pathway is a well-known cancer-associated pathway. Alterations of the mTOR signaling pathway have significant effects on cancer progression. The major components of the mTOR signaling pathway are critical effectors in cell signaling pathways commonly deregulated in cancers [82–84]. The Rap1 signaling pathway is very important in basic cellular functions (e.g., formation, junctions and control of cell adhesion), cellular migration, and polarization [85]. Rap1 plays key roles during cell invasion and metastasis in various cancers [85,86]. The p53 signaling pathway is a very important oncogenic pathway, and it can regulate apoptosis and the cell cycle and help prevent cancer. The P53 protein, a major component of the p53 signaling pathway, is the most frequently altered gene in cancer [87].

Interestingly, Hepatitis C and Hepatitis B pathways appear within the top 50 pathways in Table 1. It is well known that Hepatitis C and

Table 1
Top 50 pathways ranked by T score comparing normal with HCC samples, using a Pearson's correlation cutoff of −0.4.

		Gene count (L)	T score
1	FoxO signaling pathway	126	0.652
2	Circadian rhythm	31	0.418
3	Hedgehog signaling pathway	47	0.375
4	Notch signaling pathway	48	0.354
5	Hippo signaling pathway -multiple species	29	0.333
6	Dorso-ventral axis formation	13	0.315
7	Cytosolic DNA-sensing pathway	21	0.265
8	Thyroid cancer	28	0.257
9	Shigellosis	51	0.253
10	Inflammatory bowel disease (IBD)	48	0.242
11	RNA degradation	18	0.217
12	Toll-like receptor signaling pathway	104	0.207
13	AGE-RAGE signaling pathway in diabetic complications	91	0.203
14	Wnt signaling pathway	137	0.177
15	MAPK signaling pathway	252	0.175
16	Cocaine addiction	42	0.168
17	mTOR signaling pathway	144	0.159
18	Oocyte meiosis	120	0.155
19	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	10	0.154
20	Epithelial cell signaling in Helicobacter pylori infection	37	0.153
21	Insulin resistance	94	0.151
22	Pancreatic cancer	65	0.142
23	Steroid biosynthesis	20	0.142
24	MicroRNAs in cancer	262	0.139
25	HTLV-I infection	194	0.137
26	Progesterone-mediated oocyte maturation	89	0.129
27	Adipocytokine signaling pathway	63	0.128
28	Rap1 signaling pathway	208	0.127
29	Acute myeloid leukemia	57	0.115
30	Hepatitis C	97	0.114
31	Ether lipid metabolism	44	0.110
32	Hepatitis B	134	0.107
33	Leishmaniasis	50	0.101
34	Glyoxylate and dicarboxylate metabolism	26	0.097
35	Herpes simplex infection	104	0.095
36	Pantothenate and CoA biosynthesis	16	0.094
37	Huntington's disease	27	0.092
38	Vascular smooth muscle contraction	114	0.091
39	Antigen processing and presentation	62	0.091
40	Breast cancer	143	0.088
41	Calcium signaling pathway	179	0.085
42	Long-term potentiation	67	0.085
43	Chagas disease (American trypanosomiasis)	89	0.084
44	Estrogen signaling pathway	89	0.082
45	RIG-I-like receptor signaling pathway	48	0.082
46	p53 signaling pathway	68	0.080
47	Osteoclast differentiation	123	0.079
48	Toxoplasmosis	93	0.077
49	Gap junction	88	0.076
50	Vasopressin-regulated water reabsorption	22	0.075

Hepatitis B are major risk factors for liver cancer [88–91].

Table 1 also includes several pathways that are immune- and inflammatory- related, such as the Toll-like receptor signaling pathway, HTLV-I infection, Antigen processing and presentation, and the RIG-I-like receptor signaling pathway. It is well documented [92–95] that the immune system plays a key role in the development and progression of cancer, and inflammatory responses play critical roles at different stages of cancer development, including initiation, promotion, malignant conversion, invasion, and metastasis. In addition, inflammation affects immune surveillance and response to therapy.

Table 1 also includes several other tumor-associated pathways, such as thyroid cancer, pancreatic cancer, acute myeloid leukemia and breast cancer.

3.5. miR2Pathway for cancer subtype analysis

We then apply miR2Pathway to all the pathways from KEGG comparing normal and hepatitis B-induced HCC samples. Table 2 lists the top 50 pathways of miRNA-mediated dysregulation for this analysis. As before, here we only show and analyze the results for hepatitis B-induced HCC using a Pearson's correlation cutoff of −0.4. The other results, based on different Pearson's correlation cutoffs (−0.3, −0.2, −0.1), are shown in Supplementary Tables S7, S8 and S9, respectively.

We find that most of the top 50 pathways from Table 2 overlap with the results from Table 1. The non-common pathways are marked in bold font. Some of these non-common pathways are theoretically specific to hepatitis B-induced HCC. As we know, hepatitis B-induced HCC is mainly caused by the hepatitis B virus, hence, the pathways unique to Table 2 might be involved in inflammatory response and be immune-system related. Interestingly, we find many of the non-common pathways are, indeed, inflammation- and immune-system related, such as the TNF signaling pathway, the Fc epsilon RI signaling pathway, Salmonella infection, Viral carcinogenesis, Bacterial invasion of epithelial cells, and the Inflammatory mediator regulation of TRP channels. The selective presence of inflammation-related pathways in Table 2 suggests that miR2Pathway can identify pathways specific to subtypes of HCC.

We also identify in Table 2 several other pathways that are involved in metabolism, such as D-Glutamine and D-Glutamate metabolism, Sulfur metabolism, the Insulin signaling pathway and Type II diabetes mellitus. Three of these four pathways are related to hepatitis B virus infection. Several studies show that glutamine synthesis and metabolism are potential markers of HCC patients infected by hepatitis B [96,97]. Very interestingly, some studies show that a hepatitis B virus infection can contribute to the impairment of insulin signaling [98,99]. It is reported that a hepatitis B virus infection rate is higher in type II diabetes mellitus patients compared with healthy controls, suggesting that the hepatitis B virus infection is associated with type II diabetes [100].

We then apply miR2Pathway to analyze other subtypes of HCC, such as hepatitis C-induced and alcohol-induced HCC. The top 50 pathways are listed in Supplementary Table S2 and S3, respectively.

In Supplementary Table S2, we include the top 50 pathways from the miR2Pathway analysis of hepatitis C-induced HCC using a Pearson's correlation cutoff of -0.4. Again, we find that most of the pathways overlap with those from Table 1. The pathways unique to Supplementary Table S2 are, theoretically, specifically associated with hepatitis C virus (HCV) infection. Interestingly, we discover that several non-common pathways from this table are also listed among the non-common pathways from Table 2 (hepatitis B-induced HCC), such as D-Glutamine and D-Glutamate metabolism, bacterial invasion of epithelial cells and the insulin signaling pathway. HCV infection might increase glutamine use and dependence, and inhibiting glutamine metabolism attenuates HCV infection and the oxidative stress associated with HCV infection [101]. Some studies show that HCV can induce insulin resistance (IR), thereby contributing to steatosis, progression of fibrosis and HCC [102,103]. Additionally, Bacterial invasion of epithelial cells is an inflammation-related pathway, which is specifically associated with this subtype of liver cancer. The results for hepatitis C-induced HCC based on different Pearson's correlation cutoffs (−0.3, −0.2, −0.1) can be found in Supplementary Tables S10, S11 and S12, respectively.

In Supplementary Table S3, we include the top 50 pathways from the miR2Pathway analysis of alcohol-induced HCC using a Pearson's correlation cutoff of -0.4. Again, we find that most of the pathways overlap with Table 1. Among the pathways unique to Supplementary Table S3 and not found in Table 1, we find many pathways associated with inflammation and the immune system, such as Pathogenic Escherichia coli infection, the TNF signaling pathway, the B cell receptor signaling pathway and the GnRH signaling pathway. Some studies have shown that the major mechanisms of alcohol-induced HCC include

Table 2
Top 50 pathways ranked by T score comparing normal with hepatitis B-induced HCC samples using a Pearson's correlation cutoff of −0.4. The bold-font pathways are not overlapped with the top 50 pathways in Table 1.

		Gene count (L)	T score
1	Circadian rhythm	31	4.603
2	FoxO signaling pathway	126	4.547
3	Hedgehog signaling pathway	47	4.281
4	Dorso-ventral axis formation	13	3.619
5	GnRH signaling pathway	85	3.516
6	Toll-like receptor signaling pathway	104	2.513
7	Wnt signaling pathway	137	2.398
8	MAPK signaling pathway	252	2.388
9	Rap1 signaling pathway	208	2.379
10	Epithelial cell signaling in Helicobacter pylori infection	37	2.083
11	Shigellosis	51	2.058
12	Renal cell carcinoma	57	2.043
13	Notch signaling pathway	48	1.948
14	Estrogen signaling pathway	89	1.896
15	Calcium signaling pathway	179	1.796
16	Hippo signaling pathway -multiple species	29	1.733
17	Gap junction	88	1.694
18	Thyroid cancer	28	1.671
19	AGE-RAGE signaling pathway in diabetic complications	91	1.629
20	HTLV-I infection	194	1.612
21	**Bladder cancer**	29	1.394
22	Vascular smooth muscle contraction	114	1.343
23	**TNF signaling pathway**	72	1.282
24	**Amyotrophic lateral sclerosis (ALS)**	36	1.230
25	**D-Glutamine and D-glutamate metabolism**	4	1.181
26	Cocaine addiction	42	1.179
27	**Sulfur metabolism**	9	1.171
28	MicroRNAs in cancer	262	1.157
29	**Fc epsilon RI signaling pathway**	61	1.143
30	**Long-term depression**	59	1.125
31	Chagas disease (American trypanosomiasis)	89	1.108
32	**Salmonella infection**	72	1.098
33	RNA degradation	18	1.083
34	**Tight junction**	125	1.077
35	**Insulin signaling pathway**	139	1.010
36	Leishmaniasis	50	0.986
37	**Viral carcinogenesis**	6	0.959
38	mTOR signaling pathway	144	0.944
39	**Adherens junction**	71	0.924
40	Oocyte meiosis	120	0.916
41	**Bacterial invasion of epithelial cells**	57	0.913
42	**Tuberculosis**	173	0.912
43	Vasopressin-regulated water reabsorption	22	0.897
44	RIG-I-like receptor signaling pathway	48	0.893
45	**Inflammatory mediator regulation of TRP channels**	91	0.879
46	**Retrograde endocannabinoid signaling**	59	0.853
47	**Type II diabetes mellitus**	47	0.843
48	Progesterone-mediated oocyte maturation	89	0.826
49	**Thyroid hormone synthesis**	46	0.823
50	**Glutamatergic synapse**	89	0.815

pathways of the immune system and inflammation, reviewed in [104]. Additionally, very interestingly, Glutamatergic synapse and GABAergic synapse are two of the non-common pathways unique to this subtype of HCC. Several studies show that alcohol induces many neuroadaptative changes in the CNS involving both glutamatergic and GABAergic synaptic transmission [105,106]. The results for alcohol-induced HCC based on different Pearson's correlation cutoffs (-0.3, -0.2, -0.1) are shown in Supplementary Tables S13, S14 and S15, respectively.

3.6. Analysis of the miR2Pathway using validated target genes

All of the above results are based on predicted target genes. To demonstrate the robustness of the miR2Pathway, we also employ validated miRNA target site databases. The results for HCC, hepatitis B-induced HCC, hepatitis C-induced HCC and alcohol-induced HCC based on Pearson's correlation cutoff of -0.4 are shown in Supplementary Tables S16, S17, S18 and S19, respectively. One can observe that the pathway ranks are very similar to the corresponding results for the miR2Pathway analysis using predicted target genes as listed in Tables 1 and 2, Supplementary Tables S2 and S3, demonstrating that miR2Pathway is also robust to including validated target genes in the analysis.

4. Discussion

We propose a PageRank-based method, called miR2Pathway, to measure and rank the degree of miRNA-mediated dysregulation of biological pathways in HCC. miR2Pathway can help explore how much miRNAs differentially influence the activity of biological pathways between two classes of phenotypes. The basic idea of PageRank is that the topological importance of a node is high in a network if this node has connections with other nodes with high topological importance [38]. This idea can be applied to miRNA-mediated dysregulation of biological pathways. For example, a miRNA has a larger differential influence on a pathway if it regulates more genes, particularly hub genes, in the differential network between cases and control. Based on this observation, we assess the differential influence of a miRNA on the activity of a pathway between normal and HCC by adding up the PageRank scores of genes targeted by this miRNA in the corresponding differential network, which we call S score. Then, we assess the differential influence of all other miRNAs on the activity of the same pathway. We sum up the S scores for all miRNAs to obtain the T score, which measures the total differential influence of all the miRNAs on the activity of this pathway. Similarly, we calculate corresponding T scores, which are measures of the degree of miRNA-mediated dysregulation, for each pathway. Finally, we rank all the pathways by their T scores. The miR2Pathway method focuses on quantifying the differential effects of a set of miRNAs on the activity of biological pathways when miRNA-mRNA connections are altered from normal to HCC.

Our use of PageRank to study the effect of miRNA-mRNA connections at the pathway level is novel. Previous uses of PageRank to study miRNA have focused on clustering analysis for disease subtype classification in cancer [41], and identification of hub genes in Alzheimer's Disease [42] and ischemic stroke [43].

In our application of miR2Pathway to study HCC and its subtypes, we find that many highly ranked pathways are tumor-associated, such as the FoxO signaling pathway, circadian rhythm, the Wnt signaling pathway, the MAPK signaling pathway, the mTOR signaling pathway, the p53 signaling pathway, etc. We also find that many highly ranked pathways are HCC-specific, such as Hepatitis B, Hepatitis C, etc. These results suggest that these important pathways are dysregulated by rewired miRNA-mRNA connections in cancer. Additionally, many other pathways identified by miR2Pathway are associated with inflammation, the immune system, metabolism, etc., which directly link to the occurrence and progression of HCC. We also find that the "MicroRNAs in cancer" pathway is listed in the top 50 pathways, which is consistent with the fact that miRNAs are involved in cancer. Therefore, miR2Pathway can quantify and rank the dysregulation of these biological pathways.

Further, we apply miR2Pathway to analyze three subtypes of HCC: hepatitis B-induced HCC, hepatitis C-induced HCC, and alcohol-induced HCC. By comparing each subtype of HCC with HCC, we check whether the non-common pathways from each subtype of HCC are indeed related to each specific subtype of HCC. For hepatitis B-induced HCC and hepatitis C-induced HCC, both hepatitis B and hepatitis C viruses are strongly linked to inflammation response and the immune system. Similarly, among the non-common pathways for hepatitis B-induced HCC and hepatitis C-induced HCC, we find several inflammation- and immune-related pathways, such as the TNF signaling pathway, the Fc epsilon RI signaling pathway, salmonella infection, viral carcinogenesis, bacterial invasion of epithelial cells, and inflammatory mediator regulation of TRP channels. Notably, viral carcinogenesis is strongly associated with virus-induced HCC. Similarly, for alcohol-induced HCC, we find several pathways related to the immune system and inflammation, such as Pathogenic Escherichia coli infection, the TNF signaling pathway, the B cell receptor signaling pathway and the GnRH signaling pathway. Previous studies have shown that these immune system and inflammation pathways are related to the major mechanisms of alcohol-induced HCC [104].

5. Future directions

In this study, we apply miR2pathway to HCC as a proof of concept. We intend to study other cancers and diseases to assess the generalizability of our method. We apply miR2Pathway to pre-defined biological pathways from the well-curated KEGG pathway database. In addition to this application, miR2Pathway can also be applied to any set of genes of interest, such as functional gene networks. Moreover, miR2Pathway could also be used to assess differential influence of other regulatory factors, e.g., transcriptional factors (TFs) or circular RNAs, on biological pathways. These areas could be interesting to further explore.

6. Conclusion

In summary, miR2Pathway is a novel method that can be used to assess the total differential influence of miRNAs on the activity of biological pathways between control and case. The total differential influence can reflect the degree of miRNA-mediated dysregulation of biological pathways. In turn, one can explore disease-associated biological pathways. We apply this method to study HCC and its subtypes, and find that a number of highly ranked biological pathways are involved in cancer generally, while some pathways are specific to HCC. Also, we find that many highly ranked pathways are related to inflammation, the immune system and metabolism, which are directly associated with the pathogenesis of cancer. miR2Pathway is also able to identify pathways specific to HCC subtypes. miR2Pathway is a new method to explore dysregulated pathways by analyzing rewired miRNA-mRNA connections.

7. Availability

R software to carry out the miR2Pathway computations is available via http://www.dinulab.org/tools.

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Competing interests

The authors have declared no competing interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbi.2018.03.013.

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