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Identification of novel microRNAs: Biomarkers for pathogenesis of hepatocellular carcinoma in mice model

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ABSTRACT

Hepatocellular carcinoma (HCC) is the most fatal cancer that has affected both male and female populations globally. With poor diagnosis and patient survival rates, it has become a global need for scientists to come to the aid. The main objective of the study was to profile the miRNAs in the serum of Control and DEN-treated mice at different time intervals (4 Weeks, 8 Weeks, 12 Weeks, and 16 Weeks) and identify HCC-associated miRNA as putative early biomarkers along with the miRNA regulated candidate gene which may be involved in HCC. Our study group involves 4,8,12, & 16 weeks 16-week-old treated male mice. Each group was sacrificed and analyzed for the stages of HCC. We employed *in silico* techniques for the small RNA-Seq and bioinformatics pipeline for further analysis. Our analysis revealed over 400 differentially expressed miRNAs in each treated sample and 10 novel miRNAs. The downstream analysis of these differentially expressed miRNAs, and their target genes opened an arena of different biological processes and pathways that these miRNAs affect during the development of HCC. The work has a promising role as the miRNAs predicted through this study can be used as biomarkers for early detection of HCC.

1. Introduction

Hepatocellular carcinoma (HCC), or liver cancer, is one of the most commonly occurring and frequently diagnosed cancers globally, wherein 70 %–80 % of the primary liver cancer cases are categorized under hepatocellular carcinoma. It ranks third among the cancers with a high mortality rate on the global scale [1,2].

The most common cause of HCC is cirrhosis, and the risk of HCC increases due to excessive alcohol intake, fatty liver disease, and the presence of certain Hepatitis viruses (strain B and C) [3–5]. The difficulties of diagnosing HCC in the early stages lead to a poor survival rate in patients. Given that the liver is an internal organ, diagnosis becomes onerous compared with other areas of the body, such as skin or mouth. Such limitations paved the way for discovering and exploiting biomarkers for preventive and diagnostic. Profuse multiplication of cancer cells, along with the byproducts therein, is a direct result of the upregulation of certain genes driving the proliferation. Deregulation of certain genes and other biological elements can also be observed along the process. Recent studies have inferred that detecting such entities in blood or cerebrospinal fluids helps identify the cancer as soon as possible, leading to the administration of the most effective diagnosis and prognosis. One such entity is microRNA [6].

MicroRNAs are small noncoding RNAs of length spanning 22–25 nucleotides [7]. They are responsible for the posttranscriptional degradation of mRNAs and are known as gene regulators [8]. Studies have shown deregulation of certain microRNAs in different diseases compared to the normal patient sample [9] and, therefore, have been used as a potent biomarker for specific conditions, such as epilepsy [10], Alzheimer's disease [11], other neurological disorders [12], cardiovascular diseases [13–15], and even cancer [16–18]. These circulating microRNAs can be classified as oncomiRs [19,20], and tumor suppressor miRNAs [21]. OncomiRs are those miRNAs that bind to the tumor suppressor genes and help in the progress of cancer, while tumor suppressor miRNAs are those miRNAs that bind to the oncogenes and help suppress cancer [22]. Certain miRNAs act as oncomiRs in certain cancers and as tumor suppressors in others. For example, miR-155 acts as oncomiR in pancreatic cancer and lymphomas [23,24]; in gastric cancer and melanomas, it acts as a tumor suppressor [25–27]. This study aims to identify differentially expressed microRNAs and their target protein in control and DEN-induced mice, demonstrating potential application as biomarkers for early detection, diagnosis, and prognosis of HCC.

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2. Methods

2.1. Experimental system

Swiss Albino (BALB/c) male mice of around 10–12 weeks old were procured from the Pasteur Institute, Shillong. Animals were bred at the animal house by random inbreeding, kept on basal diet ad libitum, and housed in plastic cages in a temperature-controlled animal room (21 ± 2 °C) with a 12 h light and dark cycle. The Institutional Ethics Committee approved the experimental protocols, and all guidelines were strictly followed as outlined in the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (Publication No. 86–23, revised 2011).

2.2. Carcinogen exposure protocol

Mice were exposed to a known hepatocarcinogen-diethyl nitrosamine (DEN) for a period of 4, 8, 12, & 16 weeks. DEN was prepared in Millipore water and administered intravenously at a dose of 20 mg/kg body weight at weekly intervals, starting with week 0 (the start of the experiment) and ending at week 16. Age and sex-matched untreated mice (n = 6) were used as controls. The DEN mice model was well established per previous publications and used in further study [28].

2.3. Serum collection

Blood was collected from mice of different experimental groups, age-matched control, and serum was separated from whole blood samples. Blood was collected by retro-orbital bleeding from the corner of the eyes using sterilized capillary tubes and kept for 1 h at 4 °C then, it was centrifuged at 2000×*g* for 30 min at 4 °C and serum was collected.

2.4. Isolation of RNA

RNA was isolated from the serum sample using a miRNeasy Serum/Plasma kit (Qigen, Hilden Germany) according to the manufacturer's instructions. Total RNA, including miRNA, was isolated and quantified using nanodrop (thermo scientific).

2.5. Small-RNA illumina library preparation

We used the NEBNext Small RNA Sample Preparation protocol to create a sequencing library. Most mature miRNAs have a 5′-phosphate and a 3′-hydroxyl group, allowing the Illumina adapters in this kit to attach directly to them. We followed the kit instructions for library preparation.

2.6. Bioinformatics analysis

2.6.1. Sample analysis

The RNA extracted from the control, represented as 'C' and DEN-exposed mice of 4, 8, 12, & 16 weeks, represented as '4W', '8W', '12W', and '16W', respectively, were sequenced using the Illumina HiSeq sequencing platform. The library type consisted of single-end reads of 50 base pairs. The sequencer produced sequence files for all 5 samples in FASTQ format, containing the reads and the quality scores.

2.6.2. Fastq quality checking

The read quality is an essential element to check before proceeding towards alignment. This step involves checking of quality parameters for the sequences obtained from the sequencer. The raw FASTQ files were checked for quality through FastQC tool 29, to determine the Base quality score distribution, the Average base content per read, the GC distribution in the reads, and the average read quality with Phred score for all the samples.

2.6.3. Adapter removal

Adapters are synthetically generated short oligonucleotide sequences (single or double-stranded) attached at the 5′ and 3′ of the ends of the sample to be sequenced (RNA) during the library preparation. These short sequences, when combined with primers, help with sequence amplification. It is necessary to remove these adapters before the alignment to avoid errors. In our study, the adapter sequences were removed from the raw reads using Cutadapt tool [29]. It is a useful computational tool that not only trims adapter sequences but also primers and other such sequences that naturally do not exist at the 3′ or 5′ end. The tool has an in-built program and a dataset of adapters generally used in the NGS sequencers, but it can also remove customized adapters. The software provides the user with commands that can be tweaked according to the need.

2.6.4. Alignment and differential expression analysis

The whole genome sequence (primary assembly: GRCm39) and the annotation file of a mouse were downloaded from Ensembl [30]. In the Subread package 32, the program Subread was used to create an index of the reference genome followed by the alignment, while the program featureCounts was used to generate read counts. Samtools [31]was used to sort and remove duplicates. The differential expression analysis of miRNAs was carried out by counting the aligned reads. This was done using the DESeq2 package [32] available in R. The package works based on binomial distribution and estimates the distribution variants between the sample reads. Before doing differential expression, variants/dispersion of the data were estimated. Differential expression was carried out by binomial Sample B and as a result, the mean read count of samples, fold change, and p-values were calculated. The p-value between samples shows the significance of differential expression.

2.6.5. miRNA identification

The differentially expressed miRNAs were annotated using miRBase [33] and miRDB [34]. These databases are used to functionally annotate the miRNAs as well as find the targets. The miRNAs that were searched for were usually length 17–24 bp. On the other hand, miRDeep2 pipeline [35], which is used for the prediction of novel miRNA, was used to find novel miRNAs. The unmapped reads file was aligned against the *Mus musculus* reference genome. The parameters used to filter the significant novel miRNAs were (a) miRDeep score > 4, (b) significant RNAfold: "yes", (c) no rfam alert, and (d) number of mature reads >10.

2.6.6. Target prediction

The miRNAs, after their annotation, were searched for their gene targets. miRTarBase [36], a database with a collection of 693,784 miRNA-gene interactions in mouse, was used for this purpose as it provides gene targets along with the experimental validations. The gene targets of miRNAs found through the low and high-throughput methods are reported well in this database. The miRNA-gene interaction network was created using the Cytoscape [37]. The downstream process of these genes interacting with miRNAs is important to decipher the functional role of the miRNAs found in this study. Gene Ontology and pathway analysis were done using the PantherDB [38].

3. Results

Illumina platform. High throughput sequencing technology creates hundreds of millions of sequences in a single run, which must be subjected to quality control checks to guarantee that the raw data is accurate and free of errors or biases before biological inferences can be drawn. FastQC was utilized to check the quality of raw data, which provides a modular collection of studies that rapidly assess the data's quality and flag issue areas. Using the FastQC program, the samples' sequenced reads (50bp) were initially evaluated for adapter dimers, duplication, repeats, base biases, and GC content. Reads with a lower Phred score Q30 were omitted from the sample. Both the 5′ and 3′

sequence adapters were trimmed using Cutadapter as the default option. Adapter-trimmed reads with≥17bp were used to filter t-RNA, rRNA, siRNA, snRNA, snoRNA, and piRNA; the reads were then aligned with the mouse genome's whole sequence. The most recent version of the mouse genome's primary assembly was aligned with the five samples, which were further processed to eliminate duplicates and sorted. The reads were counted using the featureCounts software. The unaligned or unmapped reads were also processed through the miRDeep2 pipeline for the novel miRNAs. Using the DESeq2 package in R, differential expression analysis was performed with a significance of log2FoldChange greater than or equal to 1 and a p-value less than or equal to 0.05. MirTarBase was utilized to determine the interaction between miRNAs and mRNAs.

3.1. Sample analysis

The 5 samples 'C', '4W', '8W', '12W', and '16W were checked for their quality. The fastq files include the sequence with phred quality score. The raw reads summary provided with sequence length, size, GC content, phred quality score, etc.

3.2. Fastq quality checking

This step involves checking the quality parameters for the sequences obtained from a sequencer. The FastQC tool returns a summary of the read quality that includes many parameters, such as the Base quality score distributions, the Average base content per read, the GC distribution in the reads, etc., most of which for all the 5 samples were of good quality and within the range. There were duplicates present in all the samples, which might arise during sequencing for various reasons. Other parameters were within the acceptable range.

3.3. Adapter removal

Adapter sequences were removed from the raw reads using the cutadapt tool. As a result, it generates different lengths of sequence reads. The in-built list of adapters provided with this stand-alone tool was used. The adapters in the sample matched with the list and were removed, followed by a second check for the quality of the reads. The reads before and after adapter removal showed a significant change in the overall quality.

3.4. Differential expression studies

The differential expression analysis of miRNAs shows more than 300 significantly expressed miRNAs for different time points. The difference between the control sample and DEN-treated mice for different time points shows a clear difference in the number of miRNAs expressed. A heat map for all the four-week samples for the common and highly significant miRNAs is plotted using the ggplot2 in R (Fig. 1). The Differential expression analysis results are provided as supplementary files.

3.4.1. Target prediction for known miRNAs

The miRNAs, after their annotation, were searched for its gene targets miRTarBase (http://mirtarbase.cuhk.edu.cn/) a database that has a collection of 693,784 miRNA-gene interactions in mouse, was used to for this purpose as it provides gene targets along with the experimental validations [36]. The miRNA-gene interaction network was created using the Cytoscape [37]. The downstream process of these genes interacting with miRNAs is essential to decipher the functional role of the miRNAs found in this study. Gene Ontology and pathway analysis were done using the PantherDB [39].

Targets were predicted for the known miRNAs through the most significant targets that were validated through four or more experimental techniques were taken into consideration. The network created through Cytoscape software version 3.7.0 showed the RNA-mRNA interaction (Fig. 2). Some of the miRNAs that were interacting with the driver genes, like ACVR1B and IRS2, were magnified, and their interaction was shown (Fig. 2). The network analyser analyzed the network, which summarised this undirected network, showing the number of nodes and edges along with other parameters. There were numerous gene targets for the respective miRNAs, which were further studied for their functional role.

3.4.2. Gene ontology and pathway analysis

The gene Ontologies like the Biological processes: 'Positive Regulation Of Metabolic Process' (GO:0009893), 'Regulation Of Developmental Process' (GO:0050793); Molecular Function: 'Protein Binding' (GO:0005515), 'Enzyme Binding' (GO:0019899); Cellular Component: 'SMAD Protein Complex' (GO:0071141), 'Bcl-2 Family Protein Complex' (GO:0097136), 'Wnt Signalosome' (GO:1990909), and Pathways like 'Gonadotropin-releasing hormone receptor pathway' (P06664), 'CCKR signalling map' (P06959), 'Apoptosis signalling pathway' (P00006), 'Inflammation mediated by chemokine and cytokine signalling pathway' (P00031), 'Wnt signalling pathway' (P00057), shows indirectly the functional role of these miRNAs targeting these particular genes. The functional annotation (biological process, cellular component, and molecular functions) of the known miRNA, which was common in all the treated groups, is shown in Fig. 3.

3.5. Novel miRNA identification

The differentially expressed miRNAs were annotated using miRbase and miRDB (see Fig. 4). These databases are used to functionally annotate the miRNAs and find the targets. The unaligned reads of precursor miRBase-21 were taken for novel miRNA prediction using the

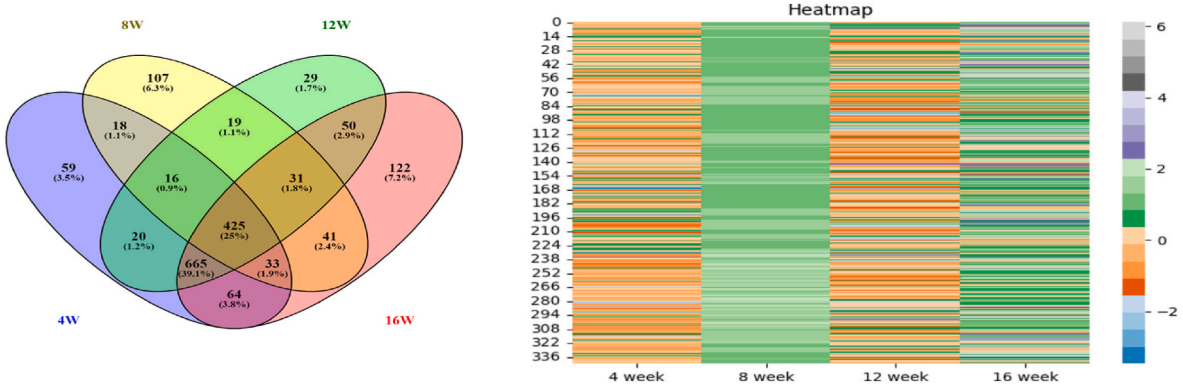

Fig. 1. Venn diagram of common miRNAs between all the groups and Heat map showing the differentially expressed **common miRNAs** in all the four groups of 4W, 8W, 12W, 16W. The color scales showed the range of expression values (Positive values: Upregulated miRNAs; Negative values: Downregulated miRNAs).

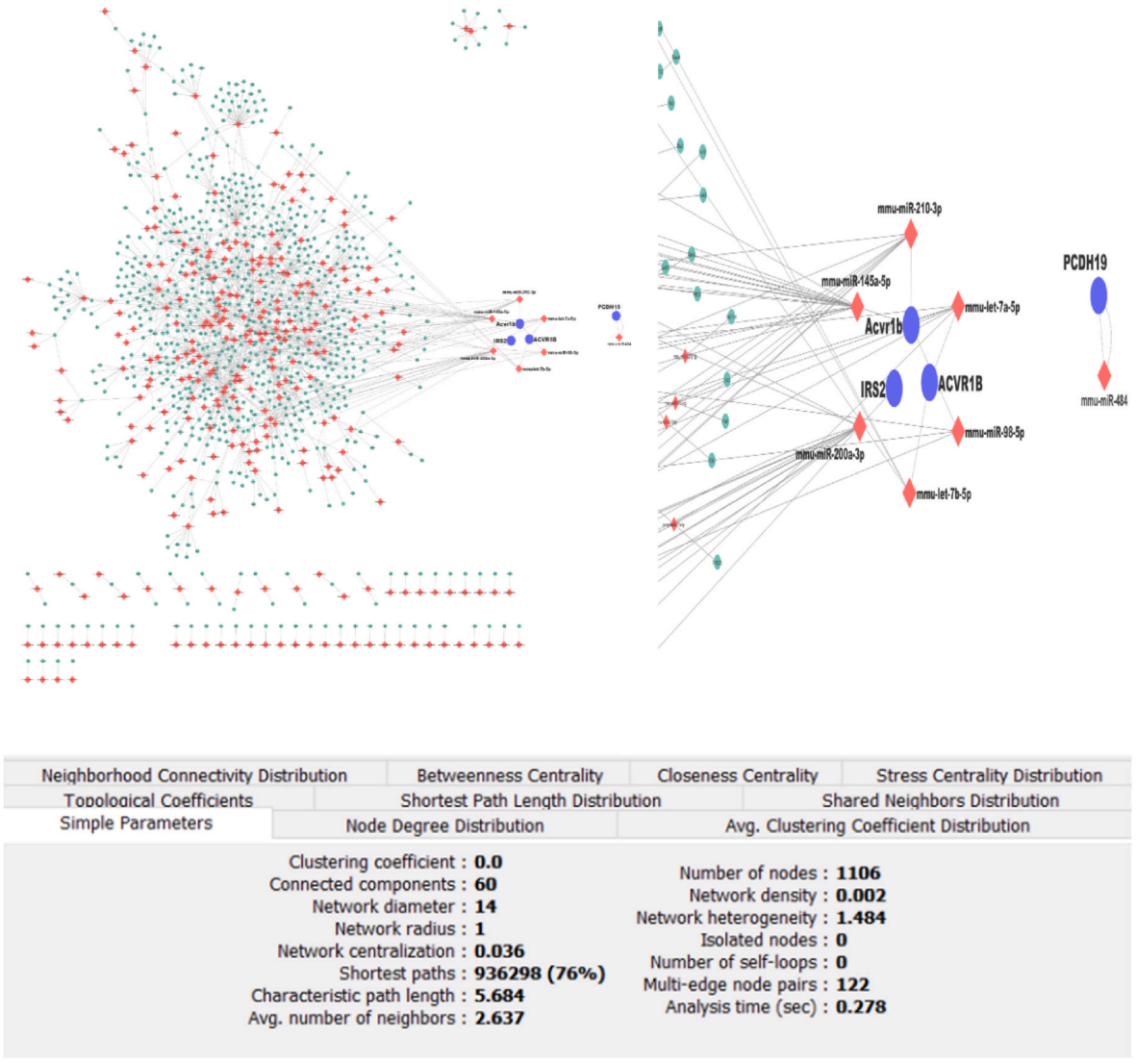

Fig. 2. miRNA-gene interaction network. Nodes are represented as miRNAs and genes, while the edges represent their interaction. The diamonds represent miRNA and the spheres represent the genes.

Musmusculus reference genome. The parameters used to filter the significant novel miRNAs were (a) miRDeep score > 4, (b) significant RNAfold: "yes", (c) no rfam alert, and (d) number of mature reads >10. Their secondary structure prediction was done using an RNA fold program from the Vienna package with default parameter. Then, we found that 13 miRNAs were common among all the treated groups.

3.5.1. Target prediction for novel miRNAs

The novel miRNA targets were predicted using miRDB (www.mirdb.org) tool [40]. The gene targets were filtered using a parameter of Target Score > 80, which were the most significant targets. Few miRNAs were seen to interact with the HCC-specific driver genes like NC_000068.8_12839 interacted with ELF3 and NAT2; NC_000072.7_19170 with GOLPH3 and CHD3; NC_000067.7_11671 with ITFG1, WWP1, and FOXP1. The list of top 10 the novel miRNAs and their targets are provided as supplementary files (Supplementary Table 1).

Similarly, a network of the top 5 novel miRNAs interacting with their gene targets was created. Few miRNAs in the network were seen to interact with the HCC-specific driver genes, like NC_000068.8_12839 interacted with ELF3 and NAT2; NC_000072.7_19170 with GOLPH3 and CHD3; NC_000067.7_11671 with ITFG1, WWP1, and FOXP1 (Fig. 5) (see Fig. 6).

3.5.2. Gene ontology and pathway analysis

The functional annotation of the novel miRNA targets revealed numerous processes. A gene ontology enrichment analysis was done against the mouse genome using Fisher's Exact as the test type and FDR and Bonferroni correction. The top 10 Gene Ontologies showed highly significant Biological processes, like 'Nervous System Development' (GO:0007399), 'Neurogenesis' (GO:0022008); Molecular Function: 'Protein Binding' (GO:0005515), 'Olfactory Receptor Activity' (GO:0004984); Cellular Component: 'Intracellular Anatomical Structure' (GO:0005622), 'Neuron Projection' (GO:0043005), and Pathways like 'Cadherin Signaling Pathway' (P00012), 'CCKR signalling map' (P06959), 'Wnt Signaling Pathway' (P00057), 'Angiogenesis' (P00005).

4. Discussion

The functional significance of the gene targets was examined. Gene ontology and pathway analyses aid in comprehending the significance of these miRNAs. The 4W showed the greatest number of significant interactions, while 8W, 12W and 16W showed miR-484 interacting with PCDH19. miR-484 has been studied before for being a biomarker in

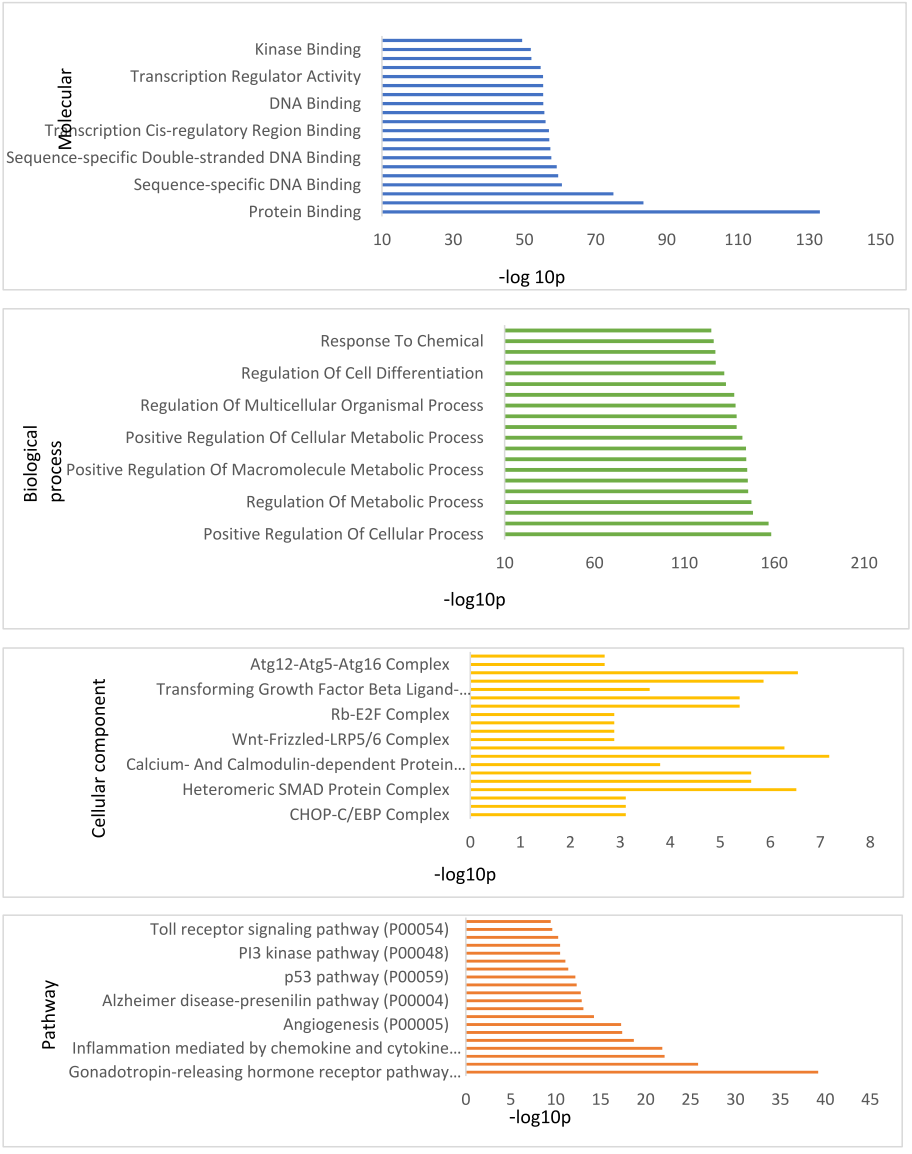

Fig. 3. Gene ontology and pathway analysis of known miRNAs predicted target gene. Above figures show top 20 Biological, Molecular function, Cellular component, and Pathways of target gene.

Samples	Number of Novel miRNAs
4 Week	43
8 Week	49
12 Week	51
16 Week	105
Total	**248**

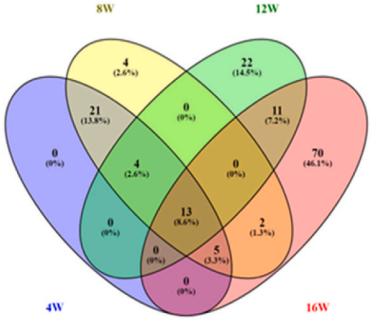

Fig. 4. Supplementary Table 1 showing the number of putative novel miRNAs in all four treated groups and Venn diagram showing common novel miRNAs in all treated groups.

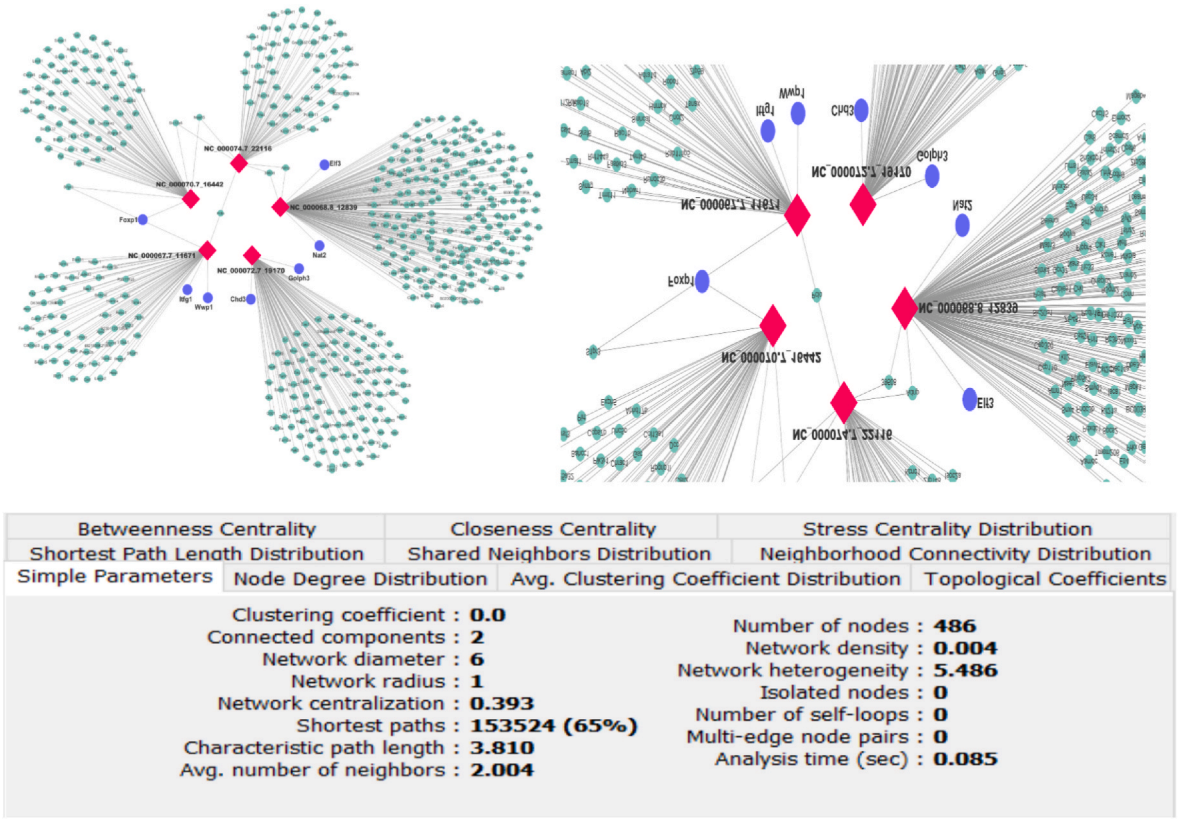

Fig. 5. Novel miRNA-gene interaction network of specific novel miRNAs and genes playing a role in the development of HCC. The diamond nodes represent miRNAs that interact with the spheres representing the driver genes. The network parameters, for the miRNA-gene interaction network.

different health diseases and HCC [41,42]. PCDH19 inhibits the epithelial-mesenchymal transition and cell migration in HCC [43]. The overexpression of miR-484 by the 16th Week of the DEN treatment might be responsible for degrading the PCDH19 post-transcriptionally, which in turn might increase the possibility of cell migration and EMT in HCC as previously reported by Xiaobin Yao and group [44].

Certain genes called driver genes are responsible for driving the process of cancer. HCC-specific driver genes were compared with the known and novel miRNA targets. There were two driver genes, ACVR1B and IRS2, that were found to be interacting with miR-210-3p, miR-145a-5p, let-7a-5p, let-7b-5p, and miR-98-5p, in the 4W. Activin receptor type-1B precursor (ACVR1B) also increases tumorigenesis [45], while IRS2 is also reported in the progression of hepatocellular carcinoma [46]. miR-98-5p is downregulated in the 4th week, which might increase the driver gene ACVR1B or IRS2 level leading to HCC. A study proves that the downregulation of miR-98-5p is seen to increase metastasis [47]. In our study, miR-98-5p proves to be a tumor suppressor miRNA whose downregulation contributes to the carcinogenesis of HCC [48]. Another miRNA, let-7a-5p, which acts as tumor suppressor miRNA by inhibiting invasion and migration in liver cells [49] is downregulated in the 4th week.

However, the target driver genes were different in the case of novel miRNAs. The miRNAs in the network were seen to interact with the HCC-specific driver genes like NAT2NC_000068.8_12839 interacted withELF3 and NAT2; NC_000072.7_19170 with GOLPH3 and CHD3; NC_000067.7_11671 with ITFG1, WWP1, and FOXP1; NC_000070.7_16442 with FOXP1, etc. These driver genes had an active role to play in driving the process of HCC. ELF3 promotes Epithelial-Mesenchymal Transition in HCC [50], whereas NAT2 is also seen in HCC patients having a history of smoking. NAT2 polymorphism increases the risk of HCC [51]. Golgi phosphoprotein 3 (GOLPH3) is seen to promote HCC aggressiveness by activating the NF kappa β pathway [52], while WWP1 induces cell proliferation [53]. FOXP1, which is seen to be interacting with two novel miRNAs NC_000067.7_11671 and NC_000070.7_16442 induces cell growth and proliferation [56], which indicates the roles of these two novel miRNAs.

5. Conclusion

The concept of identifying HCC biomarkers is not new, and numerous advances have been witnessed in recent years in this arena. With this in mind, we devised this work to profile and identify miRNAs as biomarkers in mice treated with DEN at various time points. This work identified the top 10 new miRNAs based on their interactions with genes and pathways, demonstrating their relevance in HCC. However, additional investigation and validation are required for these miRNAs' diagnostic or prognostic relevance.

Author contributions

Conceptualization Lakhon Kma & Shivani Priya methodology, formal analysis, investigation, data curation, writing original draft by Shivani Priya re. Both authors have read and agreed to publish a version of the manuscript.

Institutional review board statement

The animal study protocol was approved by the Institutional Ethical Committee of North Eastern Hill University Shillong.

Disclosure statement

No potential conflict of interest is reported by the authors.

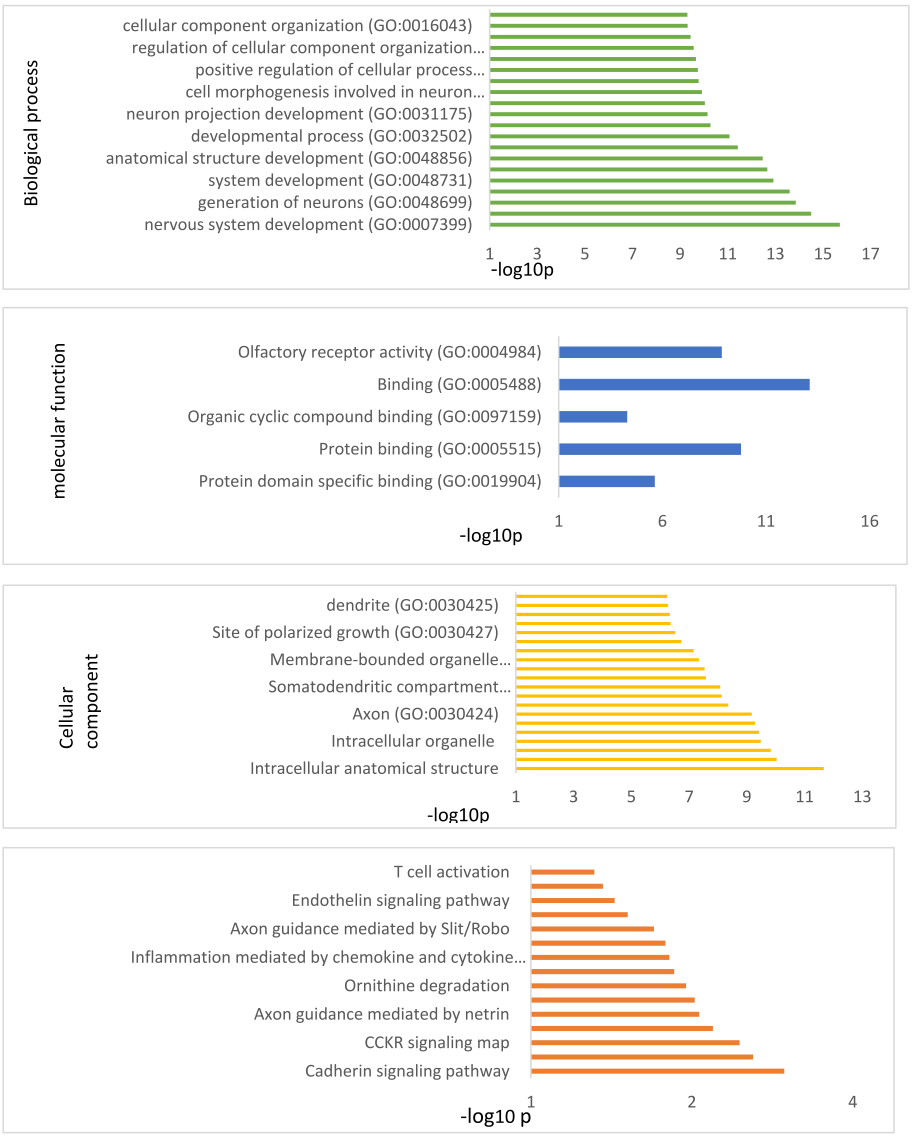

Fig. 6. Gene ontology and pathway analysis of novel miRNAs predicted target gene. Above figure shows top 10 biological process, molecular function, and cellular component of target gene.

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Declaration of competing interest

Please consider the following information regarding potential conflicts of interest and financial contributions to this work. Alternatively, we can confirm that there are no conflicts of interest associated with this publication and no significant financial support that could have influenced its outcome. We also confirm that all named authors have read and approved the manuscript and that there are no other qualifying authors who have not been listed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101896.

Data availability

Data will be made available on request.

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