Egypt. J. Chem. **Vol. 68,** No. 7 pp. 483 - 493 (2025)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

High-throughput Sequencing for Differentially Expressed microRNAs in Hepatocellular Carcinoma

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Abstract

HCC's has low survival rate this is due to its late-stage diagnosis. Early diagnosis of cancer liver could be successfully managed with high cure rate exceeding 80%. We aimed to search for the top expressed miRNAs that contribute to the pathogenesis in HCC. The study included 3 male subjects. They were well-diagnosed HCC patients . We isolated total RNA from serum to be subjected to miRNAs profiling by High-throughput Sequencing. Furthermore, we performed Target gene prediction and functional annotation of the miRNAs to identify the functional role of the reported miRNAs. The top DEMs in HCC vs controls were hsa-let-7i-5p, miR-103-3p, miR-24-3p, miR-27a-3p, miR-423-5p and miR-27b-3p. The functional enrichment of those genes revealed the highly enriched KEGG pathways and GO terms. This study showed the top deregulated miRNAs in plasma of HCC patients, their target genes that might contribute in HCC pathway so they might be highly involved in the disease pathogenesis. Further functional studies are crucial to assess their functions and actual target genes.

Keywords: HCC-miRNAs- high-throughput- functional annotation

Introduction:

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer globally [1] and is especially common in Asia; HCC is most common in areas with a high prevelance of hepatitis B, which is a major risk factor for HCC. Monitoring high-risk groups like patients with chronic hepatitis and early identification of shifts from chronic hepatitis to HCC could enhance treatment success rates. Currently, the prognosis for hepatocellular carcinoma is poor as it is typically detected in advanced stages. Early diagnosis leads to successful treatment in over 80% of cases of liver cancer **[2].**

At present, α-fetoprotein (AFP) remains the sole biomarker utilized alongside ultrasound for monitoring HCC. AFP can enhance ultrasound sensitivity from 45% to 63%. Nevertheless, the effectiveness of these techniques is restricted due to the poor sensitivity of AFP (40–60%) and its inconsistent specificity [3]. Searching for genetic circulating biomarkers shows potential for success. Of significant interest are microRNAs (miRNAs) among biomarkers found in circulation.

MiRNAs are tiny noncoding RNAs that control gene expression after transcriptional level. Focus has been placed on possible biomarkers for cancer and other illnesses due to their existence and durability in body fluids like blood, serum, plasma, and urine [4].

Therefore, the exploration of diagnostic circulating miRNAs has been thoroughly studied in various tumor types, including HCC. Detecting and quantifying circulating miRNAs is difficult due to their lower concentrations compared to those found within cells and tissues. Current gold standard approaches, including next generation sequencing (NGS), allows for detection of low miRNA input levels, while other recently developed techniques mentioned above can identify attomolar ranges [5]. This attempts to avoid low concentrations, but because cancer is diverse and miRNA expression varies among populations, individuals, tissues, and cells, along with the dynamic regulation of miRNA, there is no consensus on reliable internal controls. Overcoming this challenge requires a combination of factors including consensus among scientific community on testing, validation studies, and accessible data-sharing platforms [6].

Recently, the microarray has been widely used as a high-throughput platform for identifying overall genetic changes during tumorigenesis due to its ability to analyze gene expression efficiently [7]. During research of differentially expressed genes (DEGs) and differentially expressed microRNAs (DEMs) of HCC have been carried out for years and have identified some of their roles in various pathways and biological processes, there are still uncertainties surrounding the interactions between DEGs and microRNAs due to difficulties in comparing results from separate studies [8]. Due to the utilization of bioinformatics techniques, it is now possible to analyze the data produced by microarray technology and identify the connections between DEGs and microRNAs. Specifically, we can study the pathways within the interaction network to better understand their potential roles in the pathogenesis of HCC [9].

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Receive Date: 07 October 2024, **Revise Date:** 27 October 2024, **Accept Date:** 13 November 2024
DOI: https://doi.org/10.21608/ejchem.2024.325959.10579

In the current study, we aimed to study the top deregulated miRNAs that might be the cause of pathogenesis in HCC. MiRNA high throughput sequencing was done. Moreover, we have performed Target gene prediction and functional annotation of the miRNAs to identify the functional role of the reported miRNAs.

Subjects and Methods:

The study included 3 male subjects. They were well-diagnosed HCC patients recruited from the outpatient clinic of Medical Services Unit at the National Research Centre (NRC), and from the hepatology clinics of the National Hepatology and Tropical Medicine Research Institute in the period from October 2021 to March 2022. After signing an informed consent by each participant, venous blood was collected.

The enrolled HCC patients were naïve; they did not receive any therapy for HCC. Patients with chronic debilitating diseases, metastasis or other malignant diseases were excluded from the study. The selection of HCC patients was based on the presence of hypoechoic hepatic focal lesion by abdominal ultrasonographic examination. Most of them have elevated serum alpha-fetoprotein levels. To confirm accurate diagnosis of HCC, triphasic spiral contrast CT scanning was done. All demographic, lab. and radiological data were recorded and presented in table1.

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the National Research Centre with registration number: 20018 and all patients signed a written informed consent.

- **MiRNA extraction**

We isolated total RNA from serum to be subjected to miRNAs profiling by NGS. To harvest cell-free plasma, 2 ml of blood samples were centrifuged at 4500 ppm for 10 minutes, and the supernatant plasma was quickly removed and stored immediately at -80˚C until analysis. Total short RNA extraction was carried out using miRNeasy kit (Qiagen) according to the manufacturer's protocol designed for serum/plasma samples. Total RNA quantity was evaluated using a Fluorimeter Denovix for quantification before Next Generation Sequencing (NGS). Purified RNA was stored at -80˚C until processed.

- **MiRNA high throughput sequencing**
  - **Library preparation**

RNA samples (2.5 ng) were used as input material to prepare libraries for RNA sequencing. Sequencing libraries were constructed and generated using the NEBNextR UltraTM small RNA Sample Prep Kit for Illumina R (NEB, USA), according to the manufacturer's instructions. Index codes were added to attribute sequences to each individual sample. Finally, polymerase chain reaction (PCR) products were purified using the AMPure XP system (AMPure XP system); library quality was evaluated by checking the size distribution of the final library using the Bioanalyzer DNA assay. The concentration was assessed using the Qubit fluorometric assay for DNA. After this final step, the library was sequenced using MiSeq flow cell.

  - **NGS**

Quality of samples were checked using fastqc and multiQC. Samples adaptors were trimmed using cutadapt then aligned using bowtie to mirbase database. All counts files were then merged together using a python script. Output counts data were then analyzed using R. The R script performed the following; 1. Quantile normalization. 2.Calculating log2 fold change and difference between normalized counts. 3.Plotting top 20 miRNAs according to sorted absolute difference of counts and lollipop plot of top 10 miRNAs according to log2 fold change. The DEMs were functionally annotated and a heatmap of the enriched pathways of them was drawn.

- **Target gene prediction and functional annotation of the miRNAs**

The Mienturnet online tool was used to extract the target genes of the deregulated miRNAs from miRTarBase database to help understand the pathophysiology of both diseases.

Results:

i. MiRNA high-throughput sequencing

Total of three HCC patients compared to two controls were included in miRNAs sequencing. The results of the studied cases are shown in the following figures and tables, where the quality of the sequencing in the 3 patients is summarized in table 1 and figure 1.

ii. MiRNA high-throughput sequencing

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Table 1: summary of sequencing quality in 3 HCC patients

Samples	% Dups	% GC	Number of reads
Sample 1	64.7%	51%	1,200,000
Sample 2	60.0%	54%	800,000
Sample 3	58.1%	55%	700,000

Figure 1: FastQC mean quality scores

The mapping step began with the Alignment. The cleansed reads provided by the previous step were mapped to the reference genome of homo sapiens. The mapping quality of the 3 samples are shawn in figure 2. For the Quantification step, the results of the Alignment step were mapped to known mature and precursor miRNAs, acquired from the miRBase database, and were transformed into raw and normalized Reads Per Million (RPM), counts per known miRNA as well as log2 of RPM.

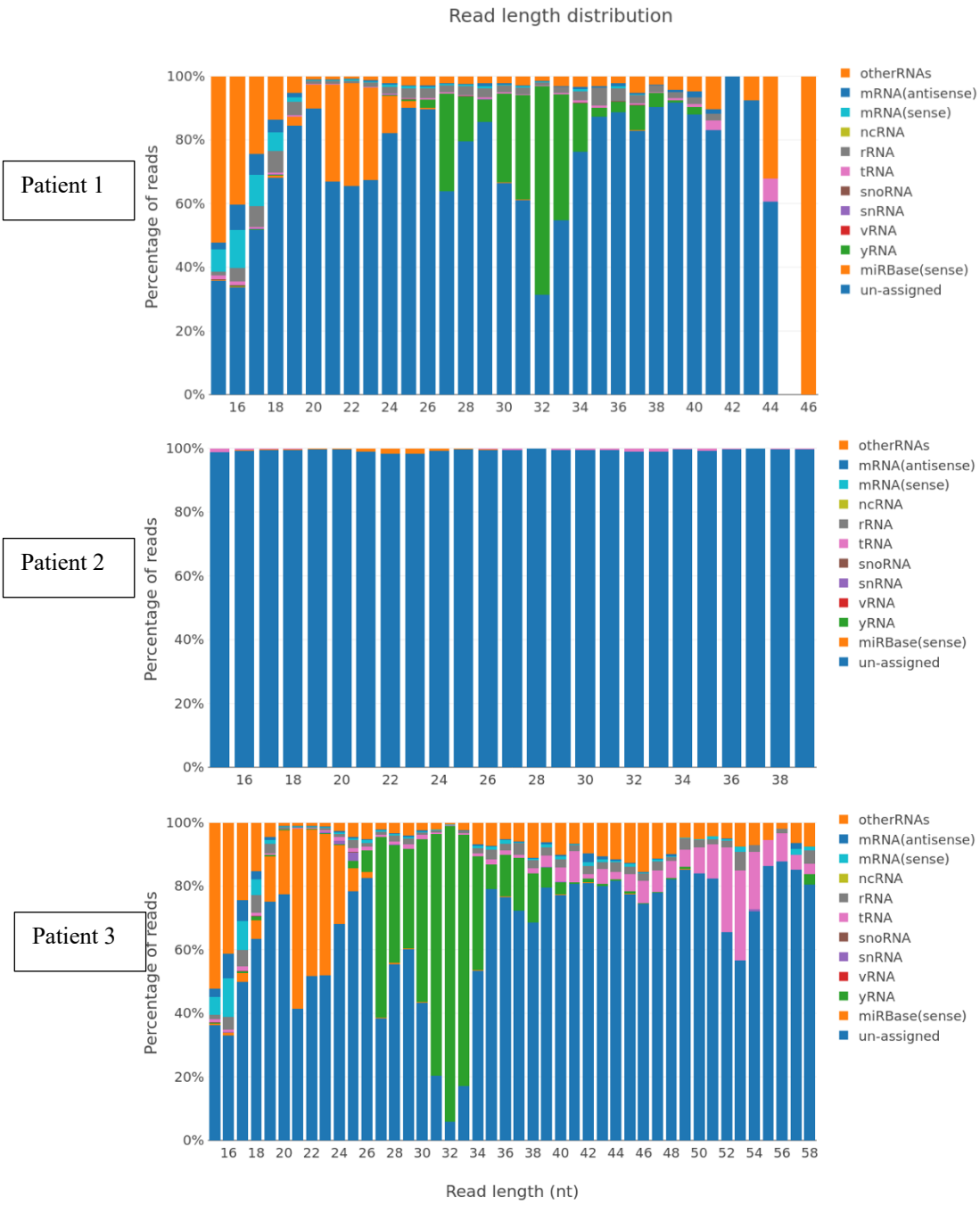

Figure 2: Mapping quality for all HCC patients

3- MiRNA Counting per sample

The top deregulated miRNAs for each patient and the differences in count of each miRNA between the patient and controls are listed in table 2. As regard to the results of all the recruited cases in comparison to the controls, the top deregulated miRNAs that were found in all the cases are: let-7b-5p, let-7g-5p, miR-423-5p, miR-451a, miR-486-3p miR-486-5p, (downregulated), let-7a-5p, let-7i-5p, miR-21-5p, miR-122-5p and miR-320a-3p (upregulated).

Table 2: top 25 miRNA count in each patient

Patient 1		Patient 2		Patient 3		Controls		
miRNA name	Count	miRNA name	Count	miRNA name	Count	miRNA name	Count	Pt vs control
hsa-miR-122-5p	697	hsa-miR-122-5p	65384	hsa-miR-486-5p	42098	hsa-miR-423-5p	2906.8	-559.03
hsa-miR-483-5p	601	hsa-miR-486-5p	4863	hsa-miR-92a-3p	29283	hsa-miR-103a-3p	798.7	-424.43
hsa-miR-122b-3p	260	hsa-miR-122b-3p	4854	hsa-miR-486-3p	9969	hsa-miR-486-3p	2263.7	-1410
hsa-miR-486-5p	202	hsa-let-7b-5p	3996	hsa-let-7b-5p	6333	hsa-miR-23a-3p	495.8	184.2
hsa-miR-451a	178	hsa-miR-423-5p	3890	hsa-miR-122-5p	4774	hsa-let-7b-5p	4704	-683.73
hsa-let-7a-5p	150	hsa-miR-320a-3p	3720	hsa-miR-451a	3428	hsa-let-7i-5p	252.8	290.333
hsa-miR-92a-3p	108	hsa-miR-92a-3p	3708	hsa-miR-423-5p	2841	hsa-let-7a-5p	1666.1	416.033
hsa-miR-423-5p	93	hsa-miR-451a	3545	hsa-miR-320a-3p	1418	hsa-let-7f-5p	1593.8	-878.13
hsa-let-7b-5p	77	hsa-let-7a-5p	2608	hsa-let-7a-5p	1253	hsa-let-7g-5p	1265.3	-263.43
hsa-miR-23b-3p	60	hsa-miR-21-5p	1290	hsa-miR-484	1212	hsa-let-7d-5p	648.9	-253.73
hsa-miR-26a-5p	59	hsa-miR-486-3p	1276	hsa-let-7d-3p	656	hsa-miR-122-5p	1957.5	20843.8
hsa-let-7f-5p	57	hsa-let-7g-5p	1186	hsa-let-7g-5p	616	hsa-miR-21-5p	174.4	135.433
hsa-miR-486-3p	52	hsa-miR-320b	1057	hsa-miR-122b-3p	601	hsa-miR-92a-3p	26356.8	-15999
hsa-miR-21-5p	37	hsa-miR-23b-3p	1039	hsa-miR-320b	565	hsa-miR-25-3p	1840.4	-1534.7
hsa-miR-27b-3p	33	hsa-let-7f-5p	956	hsa-miR-191-5p	462	hsa-miR-486-5p	37343	-22457
hsa-let-7g-5p	23	hsa-miR-26a-5p	926	hsa-miR-150-5p	450	hsa-miR-1260a	126.65	937.45
hsa-miR-3184-3p	23	hsa-miR-191-5p	903	hsa-miR-3184-3p	416	hsa-miR-483-5p	16.2	12678.3
hsa-miR-320a-3p	22	hsa-let-7i-5p	795	hsa-miR-885-5p	364	hsa-miR-103b	798.7	-424.43
hsa-let-7d-5p	19	hsa-miR-423-3p	794	hsa-miR-423-3p	362	hsa-miR-451a	9130.6	-6210.8
hsa-let-7i-5p	19	hsa-miR-23a-3p	752	hsa-miR-16-5p	354	hsa-miR-122b-3p	593.95	12199.8
hsa-let-7e-5p	18	hsa-miR-24-3p	722	hsa-miR-25-3p	354	hsa-miR-3960	79.3	1573.37
hsa-miR-93-5p	18	hsa-miR-103a-3p	653	hsa-miR-197-3p	340	hsa-miR-4502	42.7	423.867
hsa-miR-22-3p	17	hsa-let-7d-5p	618	hsa-miR-125b-5p	340	hsa-miR-1-3p	458.9	-417.37
hsa-let-7c-5p	14	hsa-miR-223-3p	613	hsa-miR-21-5p	339	hsa-miR-320a-3p	2144.8	1786.43
hsa-miR-103a-3p	14	hsa-miR-3184-3p	582	hsa-let-7i-5p	327	hsa-miR-3184-3p	2906.8	-559.03

4- Downstream analysis

In this part, the top deregulated miRNAs that were found in all the cases were picked up for further analysis. Target enrichment of deregulated miRNA in all patients was done by mienturnet as shown in figure 3. The Hub genes were MDM4, MYC, SOCS1 and NAA30. Then a network of the top deregulated miRNAs and their highly enriched target genes (genes targeted by 3 or more of the DEMs) was drawn as a network to emphasis each miRNA and its targets as shown in figure 4 and table 3.

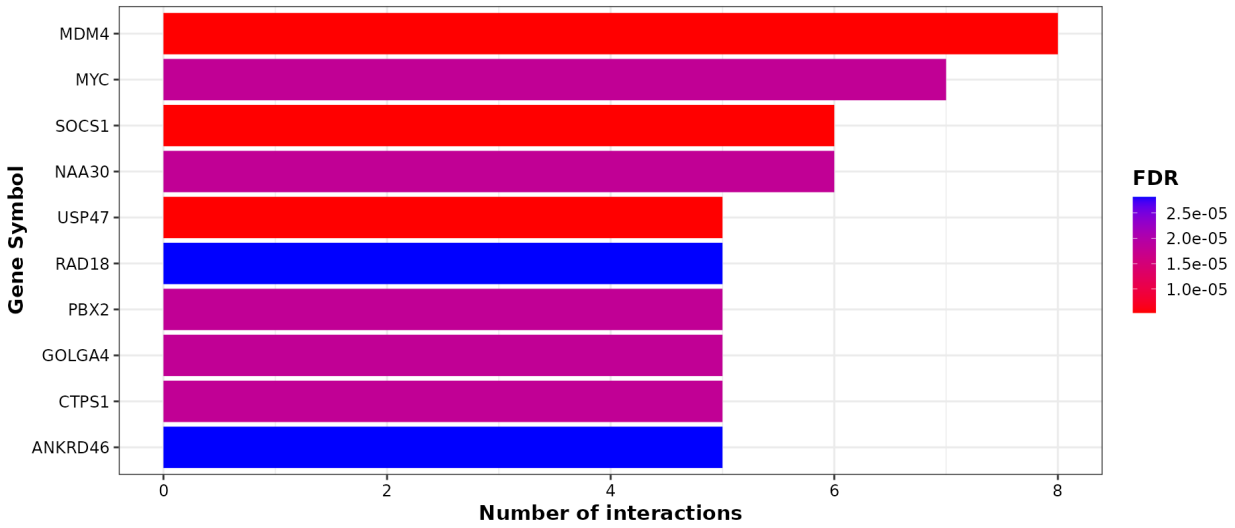

Figure 3: miRNA-Target enrichment in miRTarBase database

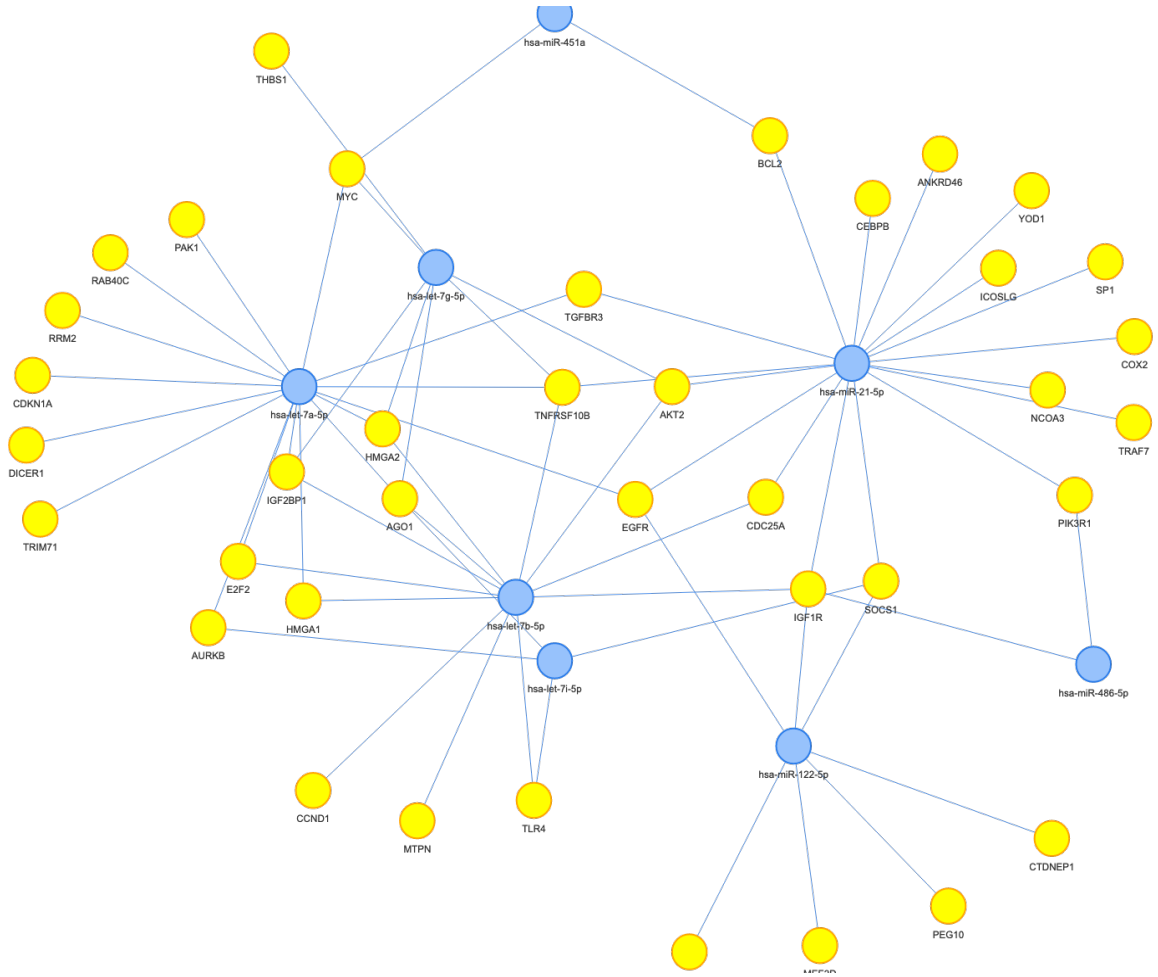

Figure 4: Network of the deregulated miRNAs and their highly enriched target genes

Table 3: the deregulated miRNAs and their highly enriched target genes

hsa-miR-21-5p	hsa-let-7a-5p	hsa-let-7b-5p	hsa-miR-122-5p	hsa-let-7g-5p	hsa-miR-451a	hsa-let-7i-5p	hsa-miR-486-5p
CDC25A	MYC	CDC34	IGF1R	MYC	ABCB1	TLR4	IGF1R
BCL2	ITGB3	IGF2BP1	CCNG1	HMGA2	MMP2	SOCS1	PIK3R1
JAG1	PRDM1	HMGA1	GYS1	IGF2BP1	MMP9	IL13	
SPRY2	TRIM71	MTPN	UBAP2	CDKN2A	BCL2	AGO1	
TIMP3	HMGA2	HMGA2	MAPK11	IL13	MYC	AURKB	
MTAP	HMGA1	CDC25A	TPD52L2	AGO1	IL6R		
MARCKS	DICER1	CCND1	EGLN3	CASP3	ADAM10		
NCOA3	HRAS	LIN28A	ADAM10	THBS1	OXTR		
PCBP1	LIN28A	PRDM1	WNT1	AKT2	MAP3K1		
TGFBR3	CASP3	HRAS	SOCS1	TNFRSF10B	IL6		
TIAM1	CASP8	AGO1	MEF2D				
MEF2C	IL6	IGF1R	CTDNEP1				
EIF4A2	E2F2	AKT2	SPRY2				
ANKRD46	IGF2BP1	TLR4	PEG10				
EGFR	CDC34	TNFRSF10B	EGFR				
SP1	CCR7	EZH2					
DOCK5	CDKN1A	E2F2					
SMARCA4	EGFR						
SOX2	RRM2						
PIK3R1	AGO1						
AKT2	EZH2						
YOD1	UHRF1						
HPGD	PAK1						
TNFRSF10B	AURKB						
IGF1R	RAB40C						
ICOSLG	TNFRSF10B						
CEBPB	TGFBR3						
CADM1	WNT1						
PSMD9							
COX2							
ABCB1							
OXTR							
SOCS1							
CASP8							
TRAF7							

The significant highly enriched KEGG pathways that are related to the highly deregulated miRNAs in our HCC cases are listed in figure 5. It emphasizes that most of the DEMs are related to ErbB and JAK-STAT signaling pathways and different types of cancers especially colorectal cancer and melanoma. In the same context, the Diseases ontology database show that target genes are related to different cancers figure 6

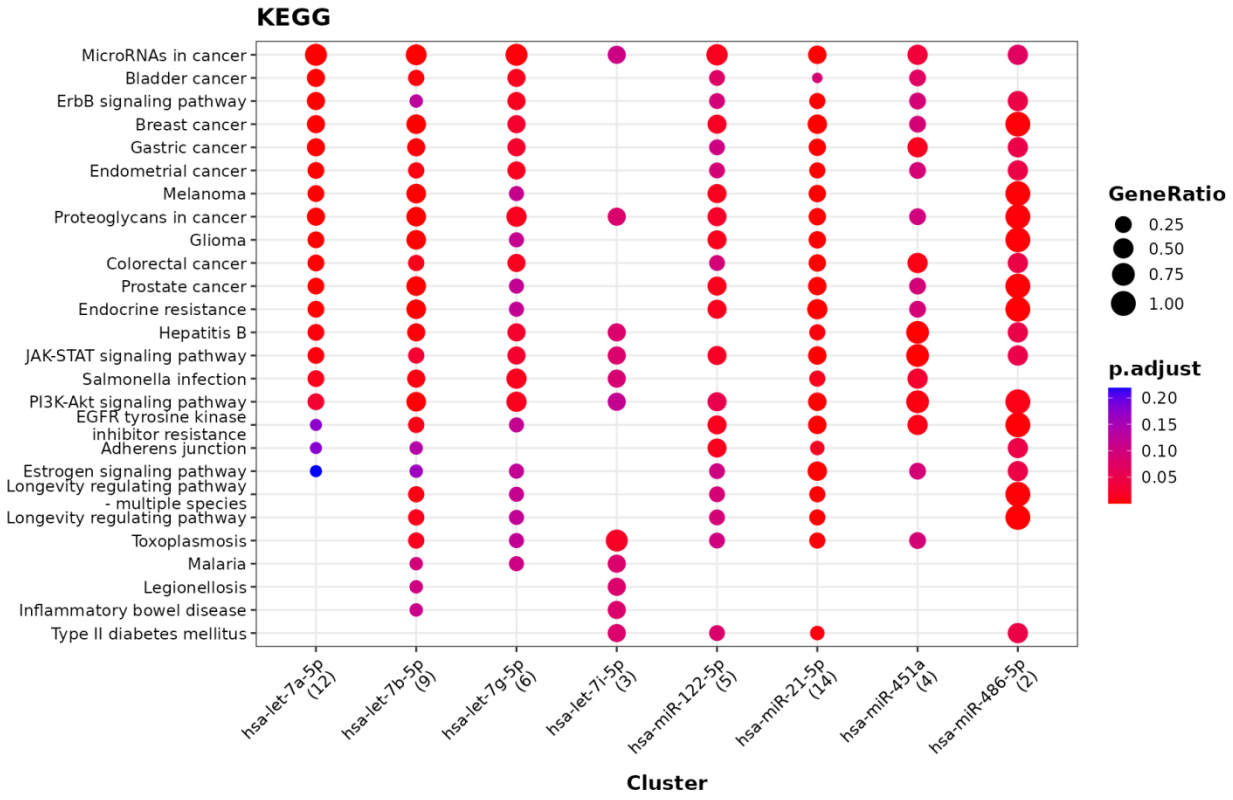

Figure 5: enriched KEGG pathways

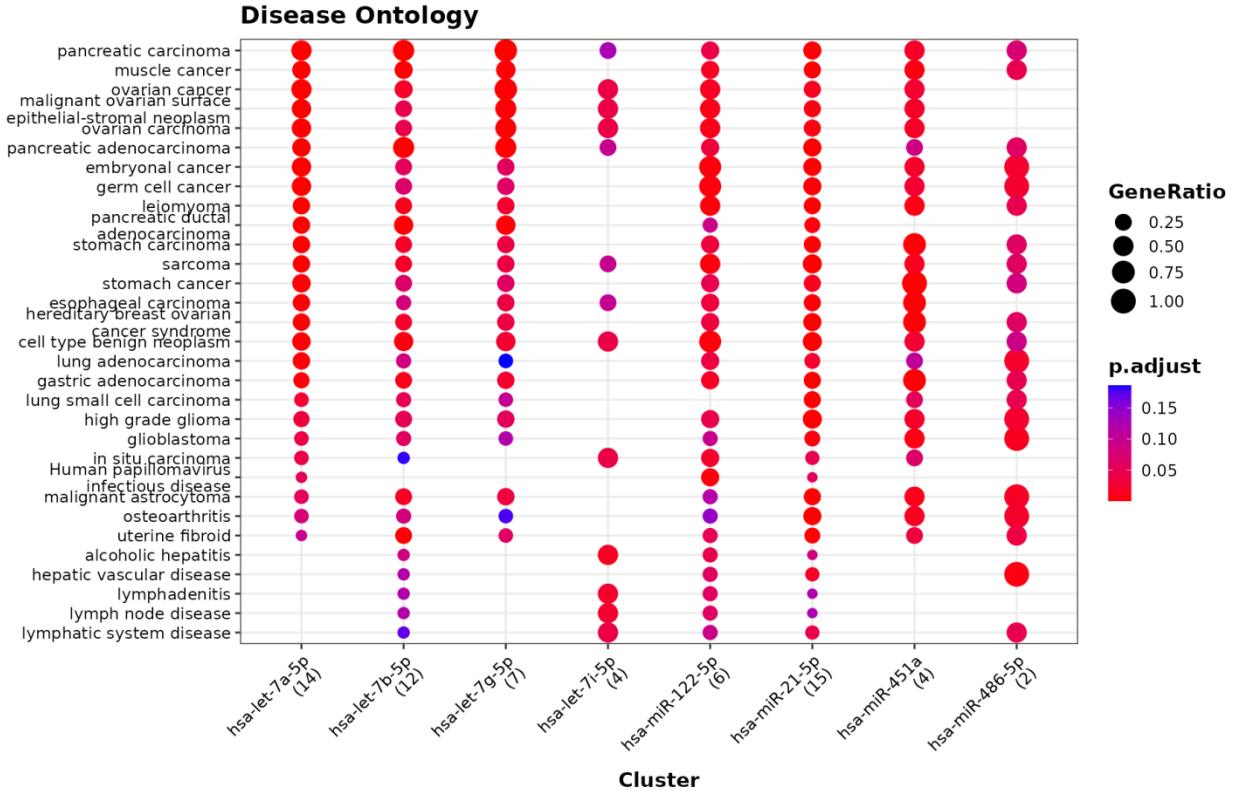

Figure 6: enriched Diseases pathways

Discussion

The identification of miRNA paved the path for comprehending the molecular processes of gene regulation and novel ideas in HCC pathogenesis. Thus, miRNAs could potentially serve as new novel biomarkers and targets for therapeutics [10].

As regard to the results of all the recruited cases in comparison to the controls, the top deregulated miRNAs that were found in all the cases are: let-7b-5p, let-7g-5p, miR-423-5p, miR-451a, miR-486-3p miR-486-5p, (downregulated), let-7a-5p, let-7i-5p, miR-21-5p, miR-122-5p and miR-320a-3p (upregulated).

Through literature review, we could divide those miRNAs into 2 groups; miRNAs that act as tumor suppressors (miR-122-5p, miR-486-5p, miR-451a, miR-320a-3p) and miRNAs that increase HCC progression (let-7i-5p, miR-486-3p, miR-21-5p, miR-423-5p).

All tumor suppressors miRNAs are found down regulated in HCC in comparison to adjacent tissue. MiR-122-5p is thought to function as a tumor suppressor by modulating genome replication [11]. MiR-486-5p inhibits HCC progression by targeting PIK3R1 and phosphatidylinositol 3-kinase-AKT activation [12]. Exosomal miR-451a targets LPIN1 to inhibit hepatocellular tumorigenesis by regulating tumor cell apoptosis and angiogenesis [13].

Research has indicated that miR-320a acts as a tumor inhibitor in (HCC). miR-320a inhibits the growth of HCC cells by directly targeting c-Myc. Research indicates that miR-320a could be a promising focus for treating HCC [14]. In our results, miR-486-5p and miR-451a are downregulated in HCC, which goes with being tumor suppressors.

On the other hand, miR-486-3p, miR-21-5p, miR-423-5p are miRNAs that increase HCC progression and mostly upregulated in HCC tissue. Moreover, miR-21-5p exhibited increased levels in multiple cancer types and facilitated the development of tumors. High miR-21-5p expression was observed in both HCC tissues and cell lines. Additionally, miR-21-5p directly affected FAS ligand (FASLG) and reduced the responsiveness of HCC cells to cisplatin (DDP) therapy [15]. MiR-423-5p is an oncogenic factor and commonly increased in gastric cancer. Its levels were notably higher in HCC versus noncancerous tissues, and this increase was linked to a lower chance of recurrence-free survival. Functional analysis indicated that miR423-5p increased the proliferation, invasion, and migration capacity of HCC cells [16]. Additionally, miR-486-3p played a significant role in controlling resistance to sorafenib in HCC by directly targeting FGFR4 and EGFR, presenting a promising HCC therapeutic target [17]. In our results, let-7i-5p and miR-21-5p are upregulated in HCC, which goes with previous studies.

Significantly, let-7 family expression was decreased in HCV-related HCC. The levels of let-7 family expression were found to be inversely related to the severity of liver fibrosis caused by HCV infection, and reduced let-7 family expression influenced liver fibrosis by activating the TGF-β pathway in HSCs. [18] However, Liu et al. provided evidence that let-7i-5p functions as a tumor promoter in renal carcinoma which goes with our results. [19]

In order to have a deep comprehension of the proteins recognized and measured in the data, we conducted in depth annotations on their functions and features through GO and KEGG pathway analysis on the circRNAs parent genes and miRNAs target genes.

Numerous bioinformatics algorithms have been developed to handle miRNA research data. Many of them are using various functions to identify or validate individual miRNA, predict target sequences, identify miRNA expression, determine signaling pathways, metabolic pathways, analyze regulatory networks, identify miRNA-transcription factor interactions, and investigate links to human disorders [20, 21].

Out of total 236 KEGG pathways, 234 (99.15%) of them have miRNA sources and targets in humans; highlighting the significant influence of miRNA-mediated regulation in biological pathways. There are 65,473 miRNA gene interactions in the 234 KEGG pathways controlled by miRNAs [22].

Analysis of KEGG pathways revealed enrichment of target genes of miRNAs in metabolic pathways and various signaling pathways as shown in figure 3. In the same context, previous studies reported that the Hub genes were MDM4, MYC, SOCS1 and NAA30. MDM4 expression was thought to be a potential mechanism for p53 dysregulation in fibrolamellar HCC [23]. C-Myc is HCC pathogenesis, and it is upregulated in about 30% of human HCC tissue samples [24]. SOCS1 downexpression increase susceptibility to HCC through increasing CDKN1A levels [25]. N-Terminal Acetyltransferases are involved in different cancers. NAA30 with glioblastoma and NAA20 in HCC [26].

The functional enrichment of the hub DEMs showed that the enriched pathways/diseases are ErbB and JAK-STAT signaling pathways and different types of cancers especially colorectal cancer and melanoma as shown in figures 7 and 8.

ErbB family proteins are significantly involved in the development, advancement, and metastatic spread of HCC, and are linked to a poor negative prognosis [27]. JAK-STAT signaling pathway has been found crucial in liver regeneration and gluconeogenesis. It is also activated in HCC leading to dysregulation [28, 29].

To best of our knowledge, we did not find Egyptian researches about high Throughput Sequencing among HCC Egyptian patients. This novel technique paves the way for applying personalized medicine for diagnosis prognosis and treatment of HCC. As established, single microRNA can target several genes. High Throughput Sequencing can help us to detect hundreds of microRNAs in a single run. Through application of bioinformatics, using identified microRNAs to determine their target genes and explore the role of contributing genes to solve the mystery of HCC pathogenesis. Furthermore, this evidence-based research will lead to development of genetic signature for liver carcinogenesis and pave the way for development of tailored diagnostic and therapeutic interventions.

Conclusion

High Throughput Sequencing is a novel technology that enables the sequencing of multiple samples in a single run, which raises the sequencing speed and reliability compared to conventional sequencing techniques. The High Throughput Sequencing provides the ability to obtain large amounts of genetic information quickly and to understand the definite roles of genes in development of HCC. In this study, a number of DEMs in HCC were unraveled, together with their target genes. Further study will be done to search for the reported microRNAs among large sample of HCC patients.

Authors' contributions

W E and K A designed the study and wrote the proposal**.** W E was the principal investigator while K A was the co- principal investigator**.** Both supervised all activities done in the current study. A H , A A , M T, Y H. selected the enrolled patients, collect blood samples and recorded all demographic, clinical and investigations data of selected patients. N E helped in molecular investigations, bioinformatics techniques and biostatistics . All authors contribute in writing the manuscript.

Funding:
National Research Center and Science And Technology Development(STDF), ID:22917.

Institutional review board statement: This study was reviewed and approved by the Institutional Ethical Committee of National Research Center, ID:20018.

Informed consent statement: All study participants, provided informed written consent prior to study enrollment.

Declaration of competing interest: The authors have no conflicts of interest to declare.

Data availability:

No new data were generated or analyzed in the current manuscript.

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