

Oncomirs miRNA-221/222 and Tumor Suppressors miRNA-199a/195 Are Crucial miRNAs in Liver Cancer: A Systematic Analysis

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Abstract

Background The high mortality rate of hepatocellular carcinoma (HCC) is partly due to a lack of good diagnostic markers and treatment strategies. Recently, several microRNA (miRNA) profiling studies were conducted with HCC; however, their inconsistency means that their diagnostic or therapeutic value is debatable.

Aims This study aims to systematically evaluate the consistency of miRNAs from multiple independent studies.

Methods A systematic analysis of miRNAs from eligible publications was conducted, followed by real-time PCRs. The targets of highly consistent miRNAs were collected using online programs, followed by enrichment analyses for gene ontology terms and Kyoto encyclopedia of genes and genomes pathways.

Results In total, 241 differentially expressed miRNAs were reported in 13 HCC profiling studies, of which 137 were upregulated and 104 downregulated. Among consistently upregulated miRNAs (cutoff > fourfold), miRNA-222, miRNA-21, miRNA-221, miRNA-210, and miRNA-224 were found increased in 8, 6, 6, 5, and 5 different studies, respectively. Among 137 downregulated miRNAs, miRNA-195, miRNA-199a, miRNA-125b, and miRNA-99a were reported in 8, 8, 5, and 5 studies, respectively. These results were confirmed by real-time PCR. Enrichment analyses demonstrated that programmed cell death and proliferation play important roles during the interplay of miRNA with HCC.

Conclusions miRNAs most consistently related to HCC are oncomirs miRNA-221/222 and tumor suppressors miRNA-199a/195.

Yanhu Li and Chunhong Di have contributed equally to this work.

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Keywords Hepatocellular carcinoma · miRNA · Systematic study · Differential expression

Introduction

Hepatocellular carcinoma (HCC), cancer with an extremely poor prognosis, represents the fifth most commonly diagnosed cancer category and the third most common cause of cancer-related mortality worldwide. There are over 50 million cases of HCC every year in the world, and incidence of HCC in the USA has almost doubled or even tripled in recent decades [1–3]. Particularly because of the very poor prognosis and high fatality, the increasing incidence and high mortality rates of this debilitating cancer are almost equal worldwide. Indeed, HCC has become one of the major public health concerns in the world. Research on the etiology and pathogenesis of HCC has so far

revealed that HCC usually occurs in the setting of underlying liver diseases; for example, hepatitis B and C are the major causes of HCC [3]. Additionally, genetic disorders like hemochromatosis and $\alpha 1$ -anti-trypsin deficiency [4] and environmental factors such as alcohol abuse may increase the risk of HCC [5]. Despite some very promising advances in the understanding of HCC in recent years, the early diagnosis and treatment of HCC in the clinic is still quite challenging. The high mortality rate is largely due to its detection only at late stage with limited therapeutic options, which renders the disease a major challenge. MicroRNAs (miRNAs) regulate the expression of up to 30 % of protein-encoding genes by binding to specific mRNA targets and promoting their degradation and/or translational inhibition [6]. miRNAs play important roles in essential cellular processes including development, cell differentiation, inflammation, proliferation, and apoptosis [7]. miRNAs also play important roles in tumorigenesis [8], functioning as tumor suppressors or oncogenes (oncomirs), evidenced by the miRNA deregulation observed in various cancer types, including lung cancer [9]. Thus, miRNAs are potential biomarkers and intervention targets against various forms of cancer [10, 11]. Several studies have been conducted to search for biomarkers or therapeutic agents by identifying the differentially expressed miRNAs between hepatocellular carcinoma tissue samples and corresponding non-tumor liver tissue [12–24]. These studies generated hundreds of differentially expressed miRNAs; however, many of these are likely false positive. Additionally, only a small number of these may have clinical significance and act as diagnostic and prognostic biomarkers. Further, in miRNA-based therapeutics, miRNAs are tissue specific and their role depends on cellular context. For instance, overexpression of miRNA-143 in pancreatic xenografts can reduce tumor growth, but anti-miRNA-143 is required to suppress liver tumor formation [25]. It is therefore crucial to select both liver tissue-specific and consistently differentially expressed miRNAs to investigate their potential as biomarkers or therapeutic agents. Thus, it is very imperative to find the important and consistent miRNAs from a large number of candidate miRNAs in many individual and independent studies [26], and no HCC-related miRNAs have yet been identified as highly consistently and differentially expressed miRNAs in HCC from many independent miRNA profiling studies. Abnormal activation and inactivation of oncogenes and tumor suppressor genes are important factors leading to malignancy, including HCC. Clarifying the molecular mechanisms of HCC could provide a basis for HCC risk assessment, early diagnosis, effective treatment, and intervention.

In the present study, we conducted a systematic analysis to screen the most consistently and differentially expressed

miRNAs from a number of miRNA profiling studies in HCC patients. Further, we confirmed the expression of most up- or downregulated miRNAs by quantitative PCR in 6 pairs of HCC and non-tumor liver tissue samples.

Materials and Methods

Search Strategy

Candidate profiling studies of miRNAs published in English were collected from PubMed, EMBASE, Web of Science, using a publication date range from April 2006 to July 2014 (last accessed on January 15, 2015) and the following medical subject heading terms: “miRNA” or “microRNA” or “miR,” “hepatocellular carcinoma” or “liver cancer” or “HCC,” “profiling” or “microarray.”

Inclusion Criteria of the Literature

The inclusion criteria of the literature were based on previously published studies, mainly including miRNA profiling studies (miRNA microarray methods) in HCC patients’ tissues and their corresponding adjacent non-tumor tissues for comparison, and the validation method set. Therefore, miRNA profiling studies that used serum samples of HCC patients or liver-relevant cell lines, those that compared HCC biopsies from tumors with different stages of disease, or those using different miRNA techniques were excluded. Review articles were also excluded. The final flowchart was made based on the guidance of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [27].

Data Extraction

Two investigators (L.Y. and Y.C.) independently evaluated and extracted the data using standard protocols, and all discrepancies were resolved by the corresponding investigator (Y.Y.). From the full text and corresponding supplementary information, the following eligibility items were collected and recorded for each study: author, journal and year of publication, location of study, methods and characteristics of animal modeling, platform of miRNA expression profiling, and author defined cutoff criteria of statistically significant differentially expressed miRNAs.

Ranking

All the included microarray investigations [12–24] reported lists of differentially expressed miRNAs related to HCC (Table S1). For example, Murakami et al. [12] carried out comprehensive analysis of HCC-related miRNAs based

on a large number of miRNA profiling studies. The potential biomarkers for comparison groups were ranked by a method described previously [52], which has been used for profiling such as meta-analysis studies. Briefly, miRNAs were ranked to the criteria in the order of importance, such as the consistency, difference, and frequency of target miRNAs in included studies and their total sample size.

Validation of miRNAs Using Real-Time PCR

To confirm the profiling results, 31 fresh HCC tissue samples and their paired non-tumor liver samples were obtained from the Second Hospital of Hangzhou City, affiliated to Hangzhou Normal University School of Medicine. These patients developed HCC as a consequence of infection by hepatitis B virus (HBV) and were at early (A) or intermediate stages (B) as categorized by the Barcelona Clinic Liver Cancer (BCLC) staging system.

The diagnosis of HCC was based on clinical and histological criteria. Clinical data for HCC patients were obtained by medical record review. Collection of samples from patients was approved by the Institutional Review Board of Hangzhou Normal University and performed after written informed consent was obtained. Total RNA was extracted from 6 pairs of matched human HCC specimens (including cancer and adjacent non-cancerous tissues) using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The differentially expressed amount of the miRNAs was validated in triplicate by real-time PCR. Briefly, reverse transcription from 3 µg RNA was carried out using SuperScript III First-Strand Synthesis System (Life Technologies) according to the manufacturer's protocol. Real-time PCR was performed using iQ SYBR Green SuperMix Kit (Bio-Rad, Hercules, CA, USA) with the iCycler sequence detection system (Bio-Rad) with specific primers (Table 1). Small nucleolar RNA U6 was used as an internal standard for normalization. The cycle threshold (C_T) value was calculated. The $2^{-\Delta C_T}$ ($\Delta C_T = C_{T_{miR}} - C_{T_{U6 RNA}}$) method was used to quantify the relative amount of miRNAs.

Identify the Experimentally Validated miRNA Target Genes

miRTarBase (mirtarbase.mbc.nctu.edu.tw), microRNA.org, and TargetScanHuman 6.2 were used to explore all the target genes of miRNAs and highlighted the genes that are experimentally validated by luciferase reporter assay, Western blot, or microarray experiments with overexpression or knockdown of miRNAs.

Enrichment Analysis

Enrichment analyses for gene ontology (GO) terms and KEGG pathways were carried out with Database for Annotation, Visualization, and Integrated Discovery (DAVID) [53]. For this, we listed all the target genes of all the differentially expressed miRNAs, including upregulated and downregulated miRNAs. The top 17 or 18 GO terms and KEGG pathways showing association with target genes were listed with GO terms, KEGG pathway, and number of genes in the GO term, number of genes in the KEGG pathways.

Statistical Analysis

Student's *t* test was used to compare values between two independent groups.

Results

Included Independent Studies

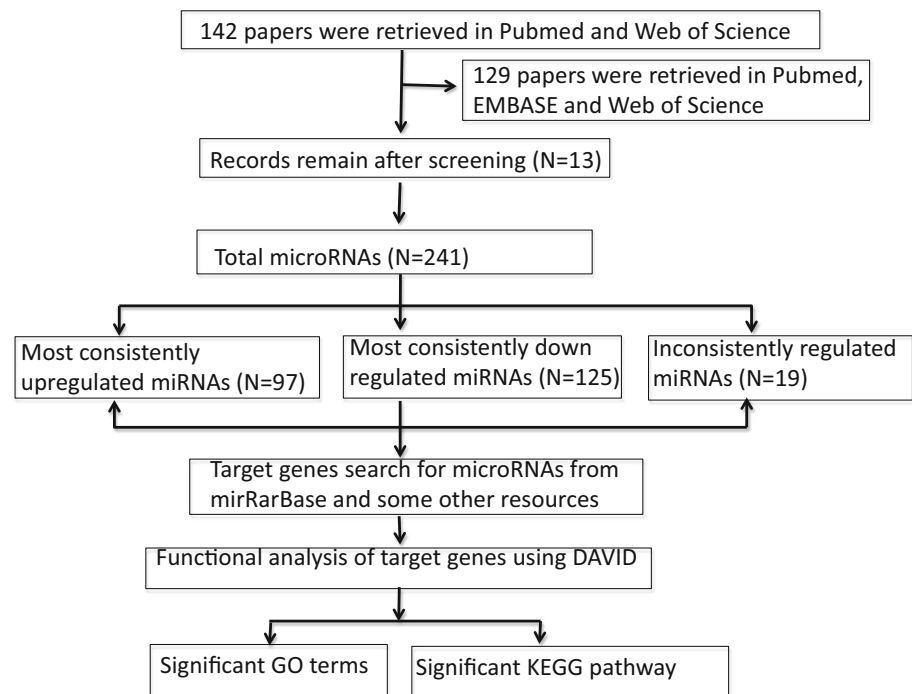
A total of 142 studies were recorded using PubMed, EMBASE, and Web of Science, 78 of which were excluded after screening the titles and abstracts and further 51 studies were excluded after reading the full text based on the inclusion and exclusion criteria. Only 13 independent studies (only the downregulated miRNAs were shown in study conducted by Peveling-Oberhag group [24]) were included in this systematic analysis. The detailed workflow used in our analysis is shown in Fig. 1, which is adapted and modified from PRISMA guidance [27]. The detailed characteristics of each study are given in Table 1 in descending order of year of publication.

Differentially Expressed miRNAs

These 13 studies from 13 different groups employed different platforms, different numbers of samples, and various statistical analyses for miRNA profiling analysis to compare HCC tissue with corresponding non-cancerous liver tissue. The number of differentially expressed miRNAs ranged from 6 to 38. A total of 239 differentially expressed miRNAs were reported in the 13 profiling studies (differentially expressed miRNAs in each study are detailed in supplemental data S1, S2, and S3). In total, 135 miRNAs are downregulated in HCC, and 104 miRNAs are upregulated. Among the 135 downregulated miRNAs, two were reported in 8 microarray studies (miRNA-195 and miRNA-199a), miRNA-125b and miRNA-99a were reported in five studies, and four miRNAs were reported in four studies (miRNA-126, miRNA-145, miRNA-378, and miRNA-

Table 1 Characteristics of miRNA datasets in hepatocellular carcinoma

Author	Year	Region	Platform	No. of tissues	Differently expressed miRNAs	Criteria	Upregulated miRNAs in HCC	Downregulated miRNAs in HCC
Han	2014	China	GPL10122 Platform	19 (9/10)	32	FDR < 0.05 Log FC > 1	14	18
Peveling-Oberhag	2014	Germany	Affymetrix GeneChip	12 (6/6)	8	$p < 0.05$		8
He	2014	China	Agilent-021	8 (6/2)	40	FDR < 0.05 Log FC > 1	4	36
Li	2014	China	Microcirculation pump	6 (3/3)	10	?	4	6
Karakatsanis	2013	Greece	Exiqon	158 (60/98)	9	$p < 0.05$	5	4
Alpini	2011	USA	SBI	38 (19/19)	6	$p < 0.05$	4	2
Yang	2010	China	miRCURYTM LNA Array	13 (8/5)	14	$p < 0.01$	6	8
Chung	2009	Korea	?	50 (25/25)	24	$p < 0.05$	17	7
Huang	2009	China	mirMASA technique	40 (20/20)	31	$p < 0.05$	12	19
Pineau	2009	USA	Exiqon	40 (20/20)	12	$p < 0.01$	11	1
Su	2009	China	Agilent	8 (5/3)	29	$p < 0.05$	14	15
Meng	2007	USA	GenePix 4000A array	6 (3/3)	15	$p < 0.05$	9	6
Murakami	2006	USA	Agilent	46 (24/22)	8	$p < 0.05$	3	5

Fig. 1 Flowchart for this systematic analysis

497). Among the 104 upregulated miRNAs, we identified 7 (36.5 %) that are reported to be affected in at least four times (miRNA-21, miRNA-25, miRNA-93, miRNA-210, miRNA-221, miRNA-222, miRNA-224), miRNA-222 was found increased in 8 different studies, two miRNAs were reported in 6 studies (miRNA-21 and miRNA-221), and miRNA-210 and miRNA-224 were reported to be

upregulated in 5 studies. Interestingly, miRNA-222 and its paralog miRNA-221 were represented 14 times in our selected studies. Consistently expressed miRNAs, the corresponding microarray study, and the total number of tissue samples are shown in Table 2. We also noticed that the differential expression of these selected miRNAs was very inconsistent in our selected profiling studies. For

Table 2 Consistently reported up-/downregulated miRNAs in profiling studies (HCC tissue vs. normal)

Upregulated miRNA	Frequency [references]	No of tissue samples (HCC patients/healthy)	Downregulated miRNA	Frequency [references]	No of tissue samples (HCC patients/healthy)
miRNA-222	8 [26–28, 32–37]	407 (228/179)	miRNA-199a	8 [25–31, 33, 38, 40]	183 (97/86)
miRNA-221	6 [26, 28, 32, 34–36, 39]	373 (209/164)	miRNA-195	8 [25, 27–31, 33, 37, 38, 40]	184 (97/87)
miRNA-21	6 [26, 32, 34–37, 39]	352 (198/154)	miRNA-125b	5 [26–28, 34, 40]	104 (53/51)
miRNA-210	5 [26, 27, 29–33]	202 (145/57)	miRNA-99a	5 [27, 33, 37, 39, 40]	58 (31/27)
miRNA-224	5 [25, 27, 28, 32, 37]	225 (121/104)			

example, in the groups of inconsistently reported miRNAs, miRNA-122 was reported downregulated in two studies but upregulated in one study; miRNA-130a was increased in one study but downregulated in 3 studies (Table 3), respectively.

Validation of the Selected miRNAs in HCC Patients

To validate the expression of the nine most consistently reported miRNAs (miRNA-199a, miRNA-195, miRNA-125b, miRNA-99a, miRNA-222, miRNA-221, miRNA-21, miRNA-210, and miRNA-224), the expression of these miRNAs in HCC biopsies and adjacent non-cancerous tissues was compared in 31 HCC patients using real-time

PCR with specific primers (Table 4). The results showed that the expression of miRNA-199a, miRNA-195, miRNA-125b, and miRNA-99a was downregulated in HCC tissues, whereas the other five miRNAs (miRNA-222, miRNA-221, miRNA-21, miRNA-210, and miRNA-224) were upregulated significantly in HCC patients (Fig. 2), which were consistent with those of the corresponding original profiling studies.

Primary miRNA Analysis

The identified and selected miRNAs all play specific roles in hepatocellular homeostasis, liver development, or pathogenesis of HCC. Particularly, all these miRNAs are either oncogenic miRNAs or tumor suppressors. Specifically, miRNA-222, miRNA-221, miRNA-21, miRNA-210, and miRNA-224 are oncomirs, and miRNA-199a, miRNA-195, miRNA-125b, miRNA-99a are tumor suppressors. These selected oncomirs are overexpressed consistently in the selected profiling studies; however, all the tumor suppressors are consistently inhibited in all the corresponding studies.

Target Genes of Differentially Expressed miRNAs

After we identified the most consistently downregulated or upregulated miRNAs in HCC, we screened their potential target genes with programs such as miRTarBase

Table 3 Inconsistency of differentially expressed miRNAs

miRNA	No. of in upregulated miRNA	No. of in downregulated miRNA
miRNA-122	1	2
miRNA-130a	1	3
miRNA-146	1	2
miRNA-27a	1	2
miRNA-376	1	2
miRNA-520c	1	1
Let-7a	1	1

Table 4 Primers used for real-time PCR

Primers	Sequence
miRNA-222	For 5'-CGCAGCTACATCTGGCTACTG-3' Rev 5'-GTGCAGGGTCCGAGGT-3'
miRNA-224	For 5'-CACTAGTGGTTCCGTTTAGTAG-3' Rev 5'-TTGTAGTCACTAGGGCACC-3'
miRNA-21	For 5'-GGGCTTTCAAGTCACTAGTG-3' Rev 5'-GGGCTTTGTAGTCACTAGGGC-3'
miRNA-125b	For 5'-TGCCTCTCTCAGTCCCTGAG-3' Rev 5'-AGCAGACTCGCAGCTCCCA3'
miRNA-22	For 5'-TGAACATCCAGGTCTGGGGC-3' Rev 5'-GAGAACATGTTCCAGGTAGCC-3'
miRNA-99a	For 5'-CCCATTGGCATAAACCCG-3' Rev 5'-CGGGTTTATGCCAATGGG-3'
miRNA-199a	For 5'-AGGAAGCTTCTGGAGATCCTGC-3' Rev 5'-TGCTCTCCCTTGCCAGTCTAAC-3'
miRNA-195	For 5'-AGCTTCCCTGGCTCTAGCAG-3' Rev 5'-CACCACCCTGCCTGGAGCAG-3'
miRNA-210	For 5'-ACCCGGCAGTGCCTCCAGGCG-3' Rev 5'-GGGTGCGCTGCCAGGCAC-3'
U6	For 5'-GCGCGTCGTGAAGCGTTC-3' Rev 5'-GTGCAGGGTCCGAGGT-3'

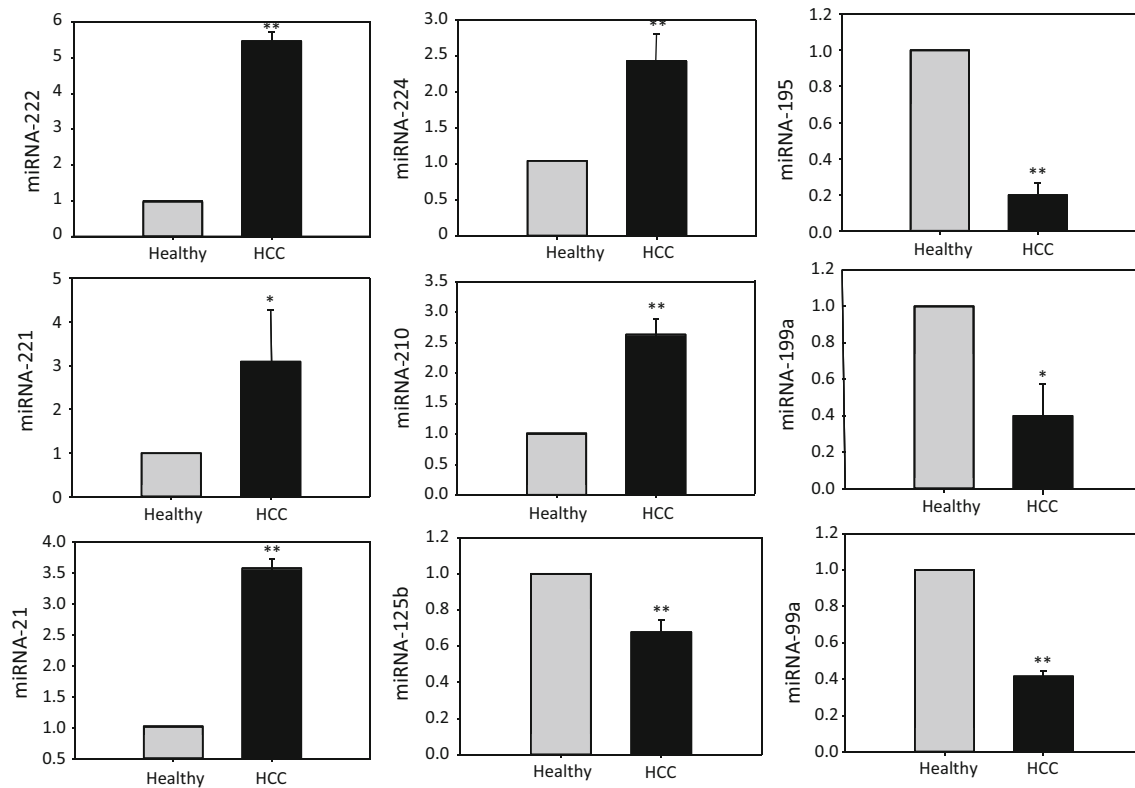


Fig. 2 Real-time PCR analysis of the most consistent differentially expressed miRNAs. Expression levels of miR-222, miR-221, miR-21, miR-224, miR-210, miR-199a, miR-99a miRNA-125b, and miR-195 in HCC and adjacent non-cancerous tissue samples were analyzed by real-time PCR, and U6 here functions as an internal control. The

expression of miR-222, miR-221, miR-21, miR-224, and miR-210 was significantly higher in HCC tissues, while the expression of miR-199a, miR-195, miR-99a, and miR-125b was significantly reduced. Here, values represent mean \pm SE of triplicate of experiments. * $p < 0.05$; ** $p < 0.01$

(mirtarbase.mbc.nctu.edu.tw), microRNA.org, and TargetScanHuman 6.2, focusing on the genes which were confirmed by either real-time PCR, Western blot, microarray, or luciferase assay. As a result, we identified 142 and 267 target genes (409 in total) corresponding to those downregulated and upregulated miRNAs in HCC (Table 5, supplemental data S1, S2, and S3), respectively.

Enrichment Analysis Result

We used the DAVID program to build up the molecular networks corresponding to the target genes of downregulated and upregulated miRNAs. The top 10 of 428 GO terms for upregulated miRNAs are shown in Table 6 ($p < 10E-9$, Benjamini $< 1.0E-6$ false discovery rate (FDR) $< 1.0E-5$). Predicted target genes are mainly involved in cancer-related cellular processes including programmed cell death and proliferation probably through regulating cell cycle and cell differentiation. The top 10 of 670 GO terms for downregulated miRNAs are listed in Table 7 ($p < 8E-15$, Benjamini $< 1.0E-11$, FDR $< 1.0E-10$); predicted target genes are also involved in cancer-related cellular process including apoptosis, proliferation, cellular biosynthetic

process, and metabolic process. Table 8 ($p < 1.0E-16$, Benjamini $< 1.0E-13$, FDR $< 1.0E-13$) shows the top 10 of 818 GO terms for combined target genes of all upregulated or downregulated differentially expressed miRNAs; similarly, predicted target genes are mainly involved in cancer-related cellular processes including apoptosis, proliferation, cellular biosynthesis, and metabolism. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways combined with different functional and signaling transduction pathways with number of target genes were analyzed, as shown in Table 9 (25 KEGG pathways are related to target genes of upregulated miRNAs), Table 10 (44 KEGG pathways for downregulated miRNAs), and Table 11 (46 KEGG pathways for all selected miRNAs). These KEGG pathways are very similar to, and largely overlap with, relevant signaling pathways and cellular functions, even though the target genes themselves may function quite differently. Among these, the cell cycle pathways seem unique and important for both cell proliferation and apoptosis as listed as example in Figs. 3, 4, and 5. Importantly, the KEGG pathways in cancer from total target genes, similar to the ones from upregulated or downregulated genes, were combined with various types of pathways related to tumorigenesis of cancers, like cell

Table 5 Selected miRNAs and their predicated target genes

MicroRNAs	Predicted targets
Upregulated miRNAs	
miRNA-222	TRPS1, CERS2, RECK, DICER1, ETS1, DIRAS3, TCEAL1, CORO1A, TP53, SELE, PTEN, BBC3, ESR1, Kit, ICAM1, FOS Bcl2l11, Cdkn1b, TNFSF10, TIMP3, PPP2R2A, TMED7, Cdkn1c, FOXO3, MMP1, SOD2, STAT5A
miRNA-221	CDKN1B, BCL2L11, BMF, FOXO3, Cdkn1c, kit, Psmc9, HOXB5, TMED7, DDIT4, BNIP3L, TBK1, MYBL1, DKK2, CREBZF, BRAP, USP18, ARIH2, BBC3, HMGXB4, TIMP3, TNFSF10, ICAM1, FOS, DDIT4, BNIP3, NAIP, ESR1, TICAM1, MDM2, PTEN, SELE, TP53, CORO1A, TCEAL1, DIRAS3, ETS1, DICER1, TRPS1, CERS2, SOX9, FN1
miRNA-21	BTG2, CDKAP1, PDCD4, LRRFIP1, CDC25A, PELI1, PDCD4, TP63, PTEN, HNRPK, TGFBR2, TGFBR2, SMARCA4, SPRY1/2, PRARA, TIMP3, RHOB, MSH2/6, ANP32A, BCL2, ANP32A, BMPR2, MARCK5, ANKRD46, RECK
miRNA-210	TCF7L2, HOXA9, TP53I11, PIM1, HOXA1, FGFRL1, RAD52, NPTX1, EFNA3, BDNF, PTPN1, P4HB, UBQLN1, SERTAD2, SEH1L, NCAM1, MID1IP1, MDGA1, KIAA1161, ISCU, HOXA3, GPD1L, DENND6A, CPEB2, CDK10, ABCB9, CBX1, XIST, TNPO1, SMCHD1, PTAR1, NIPBL, MIB1, HECTD1, ELK3, DDAH1, CLASP2, CHD9, ATP11C, APC, E2F3, ACVR1B, MRE11A, XPA, UCP2, MNT, AIFM3, CASP8AP2, VMP1, TFRC, NFKB1, BCL2
miRNA-224	KLK10, CXCR4, CDC4, AP2M1, NIT1, FOSB, NCOA6, API5, EYA4, EDNRA, DIO1, SMAD4, PEBP1
Downregulated miRNAs	
miRNA-99a	RAVER2, FGFR3, SERPINE1, IGF1R, mTOR, SNF2H
miRNA-199a	SIRT1, HIF1A, MET, KRT7, CD44, EZH2, IKBKB, CCNL1, MAPK9, MAPK1, AKT1, MAPK8, MAPK14, LIF, DDR1, EDN1, MAP3K11, HIF1A, SOX9, FUT4, UBE2G1, UBE2I, SMARCA2, SMAD1, RUNX1, COX2, CAV2, TMEM54, SMAD4, SULT1E1, GPR78, ERBB2, UNG, CAV1, ET1
miRNA-195	WEE1, E2F3, CDK6, CCND1, ARL2, CCND1, VEGFA, CCL4, KRT7, BCL2, RAF1, RUNX2, SLC2A3, TBCCD1, CCND3, CDK4, SIRT1, BACE1, CDC42, CAB39, CHUK, TAB 3, MBD1, CCNE1, BCL2L2, ARL2
miRNA-125b	BMPR1B, EIF4EBP1, HMGA2, HMGA1, GLI1, NKIRAS2, TP53, SMO, VDR, SGPL1, BAK1, ERBB3, ERBB2, BMF, KLF13, NTRK3, LIN28A, CBF, AKT1, CYP24A1, RAF1, SMO, PRDM1, IRF4, GRIN2A, CDKN2A, LIN28A, MAP2K7, JUB, KRT7, TNF, TP53INP1, E2F3, TRIM71, IGF2, LIN28B, BAK1, BBC3, BMF, KLF13, TEF, STAT3, BAK1, JUN, JUND, PPP1CA, PPKRA, PRKRA, PPP1CAB, SRF, NKX2-5, PRPF8, BCL2, ETS1, RPS6KA1, TNFAIP3, PIGF, BCL3, TBC1D1, DGAT1, FGFR2, SUV39H1, ARID3B, SMAD4, MCL1, IL6R, STARD13, ABTB1, CBF, HK2, MMP13, SNAI1, MAPK14, MUC1, NES

Table 6 Top 10 of 428 GO terms of target genes related to upregulated miRNAs

Term	Gene count	<i>p</i> value	Benjamini	FDR
Regulation of programmed cell death	33	6.1E−14	1.0E−10	1.0E−10
Regulation of cell death	33	6.8E−14	5.8E−11	1.1E−10
Regulation of apoptosis	32	3.0E−13	1.7E−10	5.1E−10
Regulation of cell proliferation	28	2.0E−10	8.6E−8	3.4E−7
Programmed cell death	24	9.7E−10	3.3E−7	1.2E−5
Regulation of cell cycle	18	2.0E−9	5.7E−7	1.2E−5
Negative regulation of programmed cell death	18	6.9E−9	1.7E−6	1.6E−5
Negative regulation of cell death	18	7.1E−9	1.5E−6	1.6E−5
Induction of apoptosis	17	9.2E−9	1.7E−6	2.7E−5
Induction of programmed cell death	17	9.6E−9	1.6E−6	3.0E−5

cycle (such as ARF, SMAD4, p53; Fig. 5), adherens junction (such as SMAD4, CDC42, and PTPN1; Supplemental data S4), cytokine–cytokine receptor interaction (such as ERBB2, IGFR, and Met; Supplemental data S5), and some signaling pathways like mTOR (such as mTOR, IKK, and Bcl-2; Supplemental data S6), Toll-like receptor (AP-1, Erk, NF-κB; Supplemental data S7), MAPK (such as Raf, Erk, and c-Fos; Supplemental data S8), p53 (such as p53, p14, and MDM2; Supplemental data S9), apoptosis (Trail, TNF, BCL-2; Supplemental data S10), VEGF signaling pathways (such as VEGF, TGF, and HIF; Supplemental data S11),

TGF-β (such as TGF-βR1, TGF-βR11, and SMAD4; Supplemental data S12), and WNT (APC, JNK, p53; Supplemental data S13). All these pathways work synergistically to evade apoptosis and increase proliferation.

Discussion

In the current study, we conducted a systematic analysis to establish the concept that only a few consistently differentially expressed miRNAs may be of significance for

Table 7 Top 10 of 670 GO terms of target genes related to downregulated miRNAs

Term	Gene count	<i>p</i> value	Benjamini	FDR
Regulation of cell proliferation	38	4.9E−19	1.0E−15	8.4E−16
Positive regulation of macromolecule metabolic process	38	8.1E−18	8.4E−15	1.4E−14
Positive regulation of cell differentiation	21	2.2E−15	1.5E−12	3.8E−12
Positive regulation of cellular biosynthetic process	32	2.3E−15	1.2E−12	4.0E−12
Positive regulation of biosynthetic process	32	3.4E−15	1.4E−12	5.9E−12
Regulation of apoptosis	34	3.9E−15	1.3E−12	6.7E−12
Positive regulation of macromolecule biosynthetic process	31	5.0E−15	1.6E−12	8.6E−12
Regulation of programmed cell death	34	5.2E−15	4.8E−12	9.0E−12
Regulation of cell death	34	5.7E−15	1.9E−11	1.0E−11
Positive regulation of developmental process	22	7.8E−15	3.6E−11	1.3E−11

Table 8 Top 10 of 818 GO terms of target genes related to all selected differentially expressed miRNAs

Term	Gene count	<i>p</i> value	Benjamini	FDR
Regulation of programmed cell death	61	1.2E−24	3.1E−21	2.1E−21
Regulation of cell death	61	1.4E−24	1.9E−21	2.6E−21
Regulation of apoptosis	60	4.6E−24	3.9E−21	8.1E−21
Regulation of cell proliferation	59	9.9E−24	6.4E−21	1.7E−20
Positive regulation of macromolecule metabolic process	57	2.2E−20	1.1E−17	3.9E−17
Positive regulation of developmental process	33	9.4E−19	4.0E−16	1.7E−15
Positive regulation of cell differentiation	30	3.3E−18	1.2E−15	5.8E−15
Positive regulation of cellular biosynthetic process	46	1.9E−17	6.1E−15	3.3E−14
Regulation of biosynthetic process	47	6.3E−17	1.0E−14	6.4E−14
Positive regulation of cell cycle	33	1.6E−16	2.9E−14	2.0E−13

Table 9 The 25 KEGG pathways of target genes related to upregulated miRNAs

Term	Gene count	<i>p</i> value	Benjamini	FDR
Pathways in cancer	19	2.9E−10	2.0E−8	3.0E−7
Chronic myeloid leukemia	9	3.6E−7	1.2E−5	3.7E−4
Colorectal cancer	8	1.1E−5	2.6E−4	1.1E−2
Prostate cancer	8	1.6E−5	2.8E−4	1.1E−2

Table 10 The 44 KEGG pathways of target genes related to downregulated miRNAs

Term	Gene count	<i>p</i> value	Benjamini	FDR
Pathways in cancer	31	1.1E−17	1.1E−15	1.2E−14
Focal adhesion	17	1.4E−8	2.0E−7	1.6E−5
Neurotrophin signaling pathway	14	1.4E−8	1.8E−7	1.6E−5
MAPK signaling pathway	19	2.1E−8	2.1E−7	2.3E−5
T cell receptor signaling pathway	12	2.8E−7	2.0E−6	3.1E−4
erbB signaling pathway	11	3.3E−7	2.2E−6	3.6E−4
mTOR signaling pathway	9	5.5E−7	3.5E−6	6.2E−4
Toll-like receptor signaling pathway	11	1.3E−6	7.9E−6	1.5E−3
NOD-like receptor signaling pathway	9	2.2E−6	1.2E−5	2.5E−3
p53	9	4.5E−6	2.2E−5	5.0E−3

tumorigenesis and development of HCC. The potential targets of these selected miRNAs conduct the signaling pathways critical for programmed cell death and

proliferation. Together, this research sheds some light on the potential of certain miRNAs as biomarkers or therapeutic target for HCC.

Table 11 The 46 KEGG pathways of target genes related to all selected differentially expressed miRNAs

Term	Gene count	<i>p</i> value	Benjamini	FDR
Pathways in cancer	44	5.1E–23	5.7E–21	5.7E–20
Colorectal cancer	17	2.9E–11	5.4E–10	3.3E–8
Toll-like receptor signaling pathway	16	4.8E–9	6.8E–8	5.5E–6
MAPK signaling pathway	24	1.7E–8	1.7E–7	1.9E–5
Focal adhesion	19	4.2E–7	3.6E–6	4.7E–4
erbB signaling pathway	13	4.3E–7	3.0E–6	4.9E–4
T cell receptor signaling pathway	14	6.9E–7	4.6E–6	7.9E–4
NOD-like receptor signaling pathway	11	9.5E–7	5.9E–6	1.1E–3
mTOR signaling pathway	10	1.8E–6	1.1E–5	2.1E–3
Cell cycle	14	3.7E–6	2.0E–5	4.2E–3

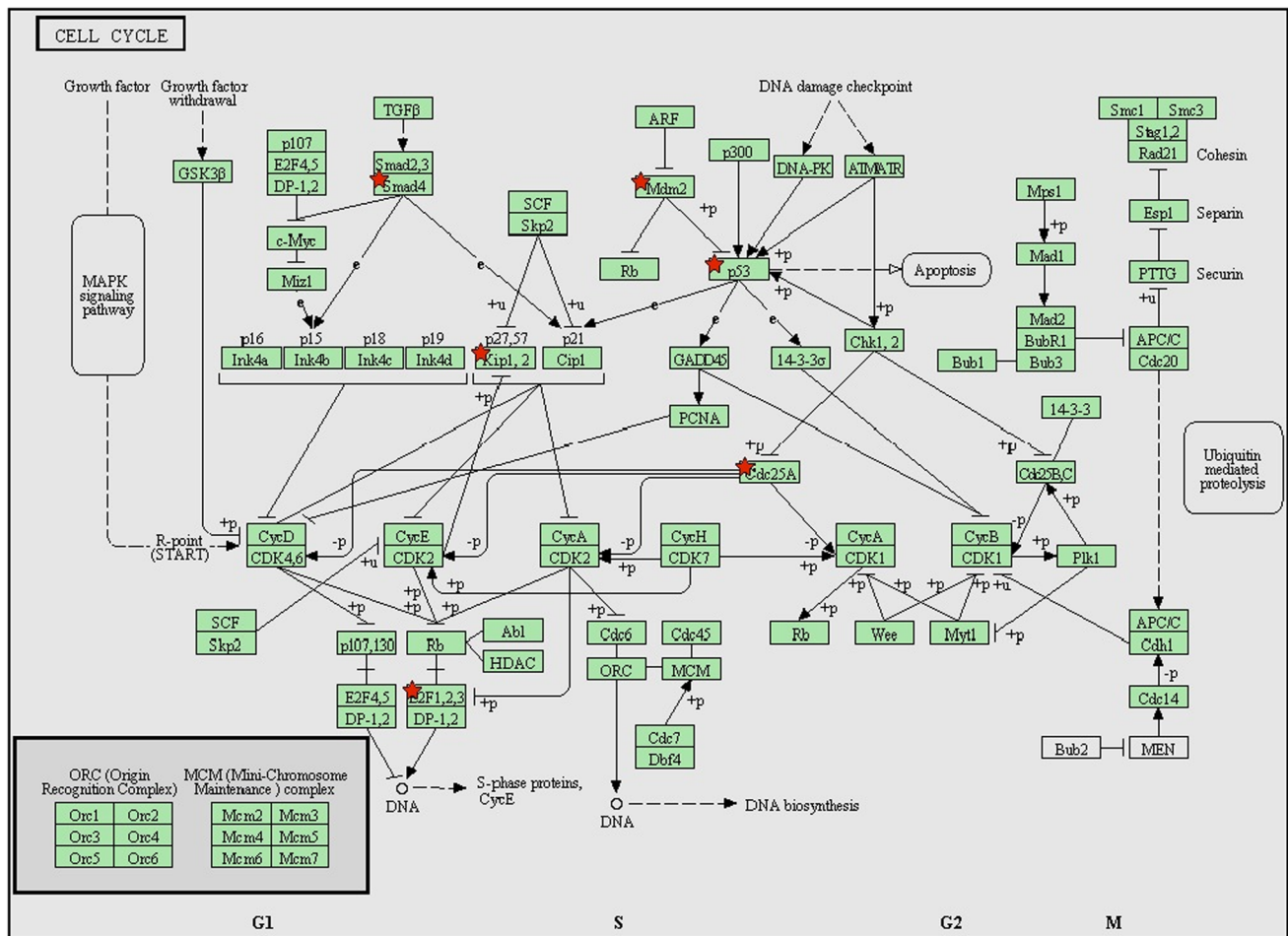


Fig. 3 KEGG pathway (pathways in cancer) analysis with predicated target genes from upregulated miRNAs. These pathways in cancer are a combination of many different but relevant pathways, such as

MAPK signaling pathway, mTOR signaling pathway, and apoptosis signaling pathway. *Green boxes with red star* represent the potential genes targeted by selected miRNAs

The usage of many conventional tumor biomarkers, such as ovarian cancer biomarker CA 125 and HCC biomarker alpha-fetoprotein (AFP), is limited due to their poor sensitivity and specificity. For example, only 50 % of patients with early-stage ovarian cancer have elevated levels of

CA125, AFP with variety of sensitivity (20–90 %) for HCC [28]. Thus, there is a critical need for reliable biomarkers that allow high-sensitivity diagnosis and precise monitoring of the HCC pathogenic process and novel therapeutic alternatives to treat HCC. Many miRNAs are

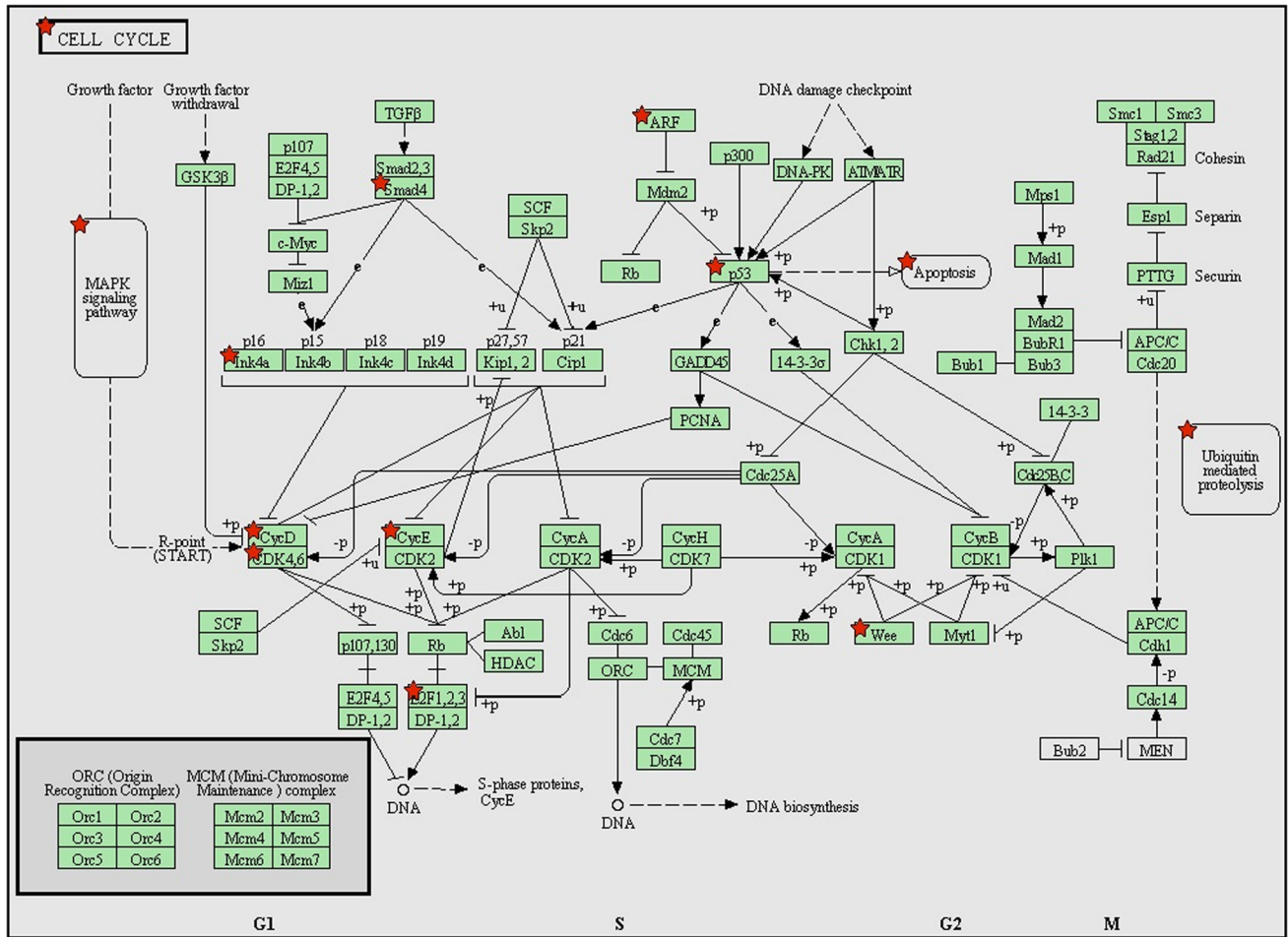


Fig. 4 KEGG pathway (pathways in cancer) analysis with predicated target genes from downregulated miRNAs. These pathways in cancer are a combination of many different but relevant pathways, such as

MAPK signaling pathway, mTOR signaling pathway, and apoptosis signaling pathway. Green boxes with red star represent the potential genes targeted by selected miRNAs

deregulated significantly even in early stages of tumorigenesis and metastasis of many cancer categories, exhibiting their diagnostic and therapeutic potential. However, as miRNA profiling studies have been performed on various platforms using diverse processing methods by investigators, consistency is lacking between these studies. This may result in inconsistent miRNA expression profiles [12–24].

miRNAs can act as both tumor suppressors and oncogenes and are important in the tumorigenesis and development of HCC. By systematic analysis, we found that five of the screened miRNAs (miRNA-221/222, miRNA-21, miRNA-224, miRNA-210) exhibit enhanced expression and act as oncomirs, and four of the screened miRNAs in 13 investigations (miRNA-199a, miRNA-99a, miRNA-195, miRNA-125b) decreased markedly, functioning as tumor suppressors. Oncomirs miRNA-221 and its paralog miRNA-222 are the most consistently expressed miRNAs in 13 profiling studies (14 times) and can aggravate the

tumorigenesis of HCC by regulating cell growth, cell cycle, or apoptosis by directly targeting p27, p57, PUMA, Ki67, NF-κB, STAT3, CDKN1B, and CDKN1C [31]. Oncogenic miRNA-21 has been reported in a wide range of tumor types with mechanisms underlying the inhibition of apoptosis and the promotion of cell proliferation by targeting at PTEN, PDCD4, and TIMP3 [13, 32]. Particularly, miRNA-21 is upregulated during HCC migration and invasion [33], implying that it may be a novel marker for HCC progression and treatment. Besides being upregulated in HCC, hypoxia-inducible miRNA-210 can increase the metastatic potential of HCC cells by promoting entry into the cell cycle and inhibiting apoptosis [34] by acting on the cell cycle regulator E2F3, homeobox proteins (HOXA1, HOXA9), and the myc antagonist MNT [35, 36]. miRNA-224, another important oncomir, is upregulated and persists throughout HCC development, acting to inhibit apoptosis [37]. In hepatitis B virus (HBV)-related HCC, accumulation of miRNA-224 has a significant role in promoting cell

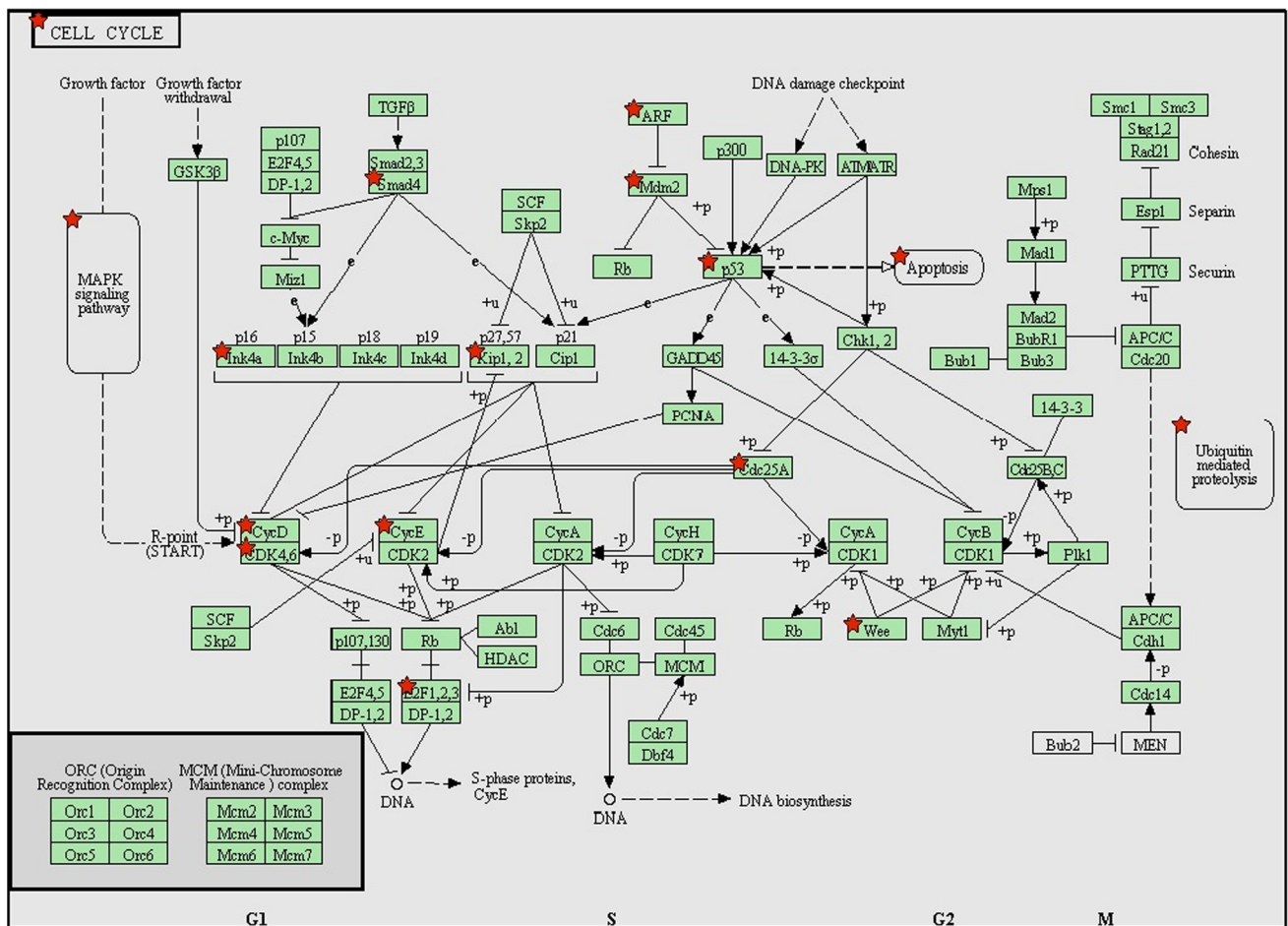


Fig. 5 KEGG pathway (pathways in cancer) analysis with predicated target genes from all selected differentially expressed miRNAs. These pathways in cancer are a combination of many different but relevant

pathways, such as MAPK signaling pathway, mTOR signaling pathway, and apoptosis signaling pathway. *Green boxes with red star* represent the potential genes targeted by selected miRNAs

proliferation, migration, and invasion by targeting SMAD4 [38].

miRNAs can also function as tumor suppressors in different cancer types by inhibiting the oncogenic process and preventing tumor development. In our systematic study, both miRNA-199a and 99a consistently decreased in 8 out of 13 studies. As a tumor suppressor, miRNA-199a negatively regulates oncogene c-Met, an important oncogene involved in HCC invasion and metastasis [39]. Overexpression of miRNA-199a decreases invasion and proliferation of HCC cell lines [40]. Additionally, miRNA-199a regulates the tumor suppressor mitogen-activated protein kinase kinase kinase 11 in gastric cancer [41]. miRNA-99a functions as another tumor suppressor, inhibits HCC growth, and correlates with prognosis of HCC patients [42]. Furthermore, miRNA-99a can inhibit proliferation of c-Src-transformed cells and prostate cancer cells by targeting mTOR [43]. miRNA-195 is downregulated frequently in multiple cancer types, including HCC, indicating its growth-suppressive function [44]. miRNA-

195 has been shown to block the G₁/S transition of the cell cycle by targeting CCND1/3, CDK4/6, and E2F3 [44, 45] and to promote apoptosis by suppressing the expression of BCL2 and BCL-w [46, 47]. miRNA-125b performs tumor suppressor functions in several cancers including hepatocellular carcinoma, by suppressing HCC cell proliferation and metastasis [48]. In our analysis, there are several other miRNAs that may contribute to a lesser extent to HCC development and progression, such as upregulated miRNA-93 and miRNA-95 or downregulated miRNA-145 and 497. Taking miRNA-145 as an example, the tumor suppressor miRNA-145, normally transcribed together with miRNA-143, regulates renewal and differentiation of cancer-related stem cells (CSCs) by negatively regulating the expression of certain key genes in stem cells such as CD44, Oct4, Sox2, and Klf4 [49].

miRNAs function as oncogenes or tumor suppressors by involving multiple molecular mechanisms. By enrichment analysis, we found these oncomirs and tumor suppressors can target various substrate genes. It is of great significance

that these target genes cluster with cancer-related cellular process, including apoptosis and proliferation. This is in agreement with the fact that apoptosis and proliferation play checkpoint roles in tumorigenesis of most, if not all, cancers [50, 51], which further highlight the importance of these miRNAs as potential biomarkers or therapeutic alternatives. KEGG pathway analysis using DAVID demonstrated that the target genes of these miRNAs build up very meaningful networks, combined with different pathways and molecule–molecule interactions. For example, the cell cycle and p53 signaling pathways contribute to apoptosis inhibition and stimulate proliferation; the Wnt signaling pathway is vital for the integrity of endothelial cells and for HCC invasion and metastasis; the inhibition of mTOR signaling pathway can reduce hepatocyte apoptosis; and the MAPK signaling pathway is crucial for hepatocytes proliferation. All these pathways work together to evade apoptosis and increase proliferation. Several other pathways that related to the block of differentiation, resistance to chemotherapy, failed repair of genes, insensitivity to anti-growth signals are also implicated. The pathways related to these miRNAs and target genes exactly mimic the pathways involved in HCC tumorigenesis. This makes these miRNAs even more important as potential biomarkers and therapeutic candidates for clinical application. Extensive studies should focus these miRNAs to establish them as biomarkers for cancer detection and progression.

Limitations

Several limitations of this research should be considered when interpreting the results due to some unsolvable reasons. Firstly, our literature searching was based on English databases only, and as a result, language bias may present. Secondly, our study only included Chinese, Korean, American, German, Greece, and Japanese populations, so the result may not be applicable to other populations such as Latin American and African.

Conclusions

We performed a comprehensive literature search in multiple databases by limiting publication language, date, and key words. By systematic analysis, we identified the most consistently expressed upregulated (5) and downregulated (4) miRNAs, and further confirmed by real-time PCR, specially, highlight will be given to miRNA-221/222, miRNA-195, and miRNA-199a since they consistently exhibited differential expression (14 and 8 studies, respectively). By DAVID analysis, we showed that the target genes are mainly involved in the regulation of

apoptosis and proliferation mediated by the combined action of several signaling pathways.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest (financial or otherwise).

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