Iranian Journal of Blood & Cancer

Iran J Blood Cancer, 2026, 18 (1), 64-84
https://ijbc.ir

Original Study

Integrative In Silico and Experimental Analyses Reveal the Therapeutic Potential of miR-217-5p in Hepatocellular Carcinoma Through KRAS Suppression and p53 Activation

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Citation Nisa U, Astuti I, Martien R, Rinaldi Maulana D, Ysrafil Y, Dwi Rizkita L. Integrative In Silico and Experimental Analysis of miR-217-5p Highlights KRAS Suppression and p53 Activation in HCC. Iran J Blood Cancer. 2026 March 31;18(1): 64-84.

Article info:
Received: 16 Dec 2025
Accepted: 15 Mar 2026
Published: 31 Mar 2026

Abstract

Background: Hepatocellular carcinoma (HCC) is one of the most lethal malignancies worldwide, and dysregulation of microRNAs has been increasingly recognized as a critical driver in its pathogenesis. Among them, miR-217-5p has been reported as a tumor-suppressive miRNA, yet its molecular role and therapeutic potential in HCC remain largely unclear.

Methods: In silico analyses were performed using a network pharmacology approach to construct a protein–protein interaction (PPI) network of hepatocellular carcinoma (HCC)-associated genes targeted by miR-217-5p, followed by hub gene identification using CytoHubba. Functional enrichment analyses, including Gene Ontology (GO) and KEGG pathways, were conducted using ShinyGO, while expression and survival analyses were validated using UALCAN, GEPIA2, and the Human Protein Atlas. In vitro validation was performed by delivering miR-217-5p mimics into HepG2 cells using chitosan nanoparticles (CS-NPs), followed by qRT-PCR analysis of endogenous miRNA expression, KRAS suppression, and p53 restoration.

Results: A total of 152 potential target genes of miR-217-5p in HCC were identified, which were predominantly enriched in key biological processes related to regulation of cell population proliferation, as well as cancer-associated pathways including PI3K–Akt signaling and hepatocellular carcinoma pathways. PPI analysis, following the removal of disconnected nodes, generated a highly interconnected network comprising 146 nodes and 1042 edges, indicating extensive molecular interactions among the identified targets. Furthermore, hub gene analysis of the PPI network identified PTEN, KRAS, ESR1, HIF1A, and CDH1 as central regulatory genes. Notably, in vitro validation demonstrated that miR-217-5p delivered via chitosan nanoparticles (CS-NPs) successfully restored endogenous miR-217-5p expression and significantly suppressed KRAS expression, exhibiting a strong inverse correlation ($r = -0.937$, $p < 0.0001$). In addition, p53 protein levels were markedly increased following treatment.

Conclusion: Our findings demonstrate that miR-217-5p exerts tumor-suppressive effects in HCC by directly targeting KRAS and restoring p53 expression. Delivery of miR-217-5p using chitosan nanoparticles represents a promising miRNA replacement therapy strategy and highlights its potential as both a therapeutic agent in HCC.

Keywords:
MiRNA-217
Chitosan Nanoparticle
HCC
HepG2
KRAS
P53

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1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and is one of the leading causes of cancer-related death worldwide (1). GLOBOCAN database statistics reflect that The disease ranks as the fourth leading cause of cancer-related death globally and is the fastest-growing cause of cancer mortality in the United States. Prognosis among cases of HCC is significantly based on the stage of the tumor; while over 70% of patients presenting in the early stage exhibit a five-year survival rate, this drops significantly to below 20% among advanced-stage cases (2, 3). In Indonesia, Survival rates are very poor among Indonesian cases, where merely 24% of patients survive a period of twelve months after diagnosis, substantially based on late-stage diagnosis as well as late onset of therapy (4). Preceding most cases of HCC is chronic liver disorder, most notably cirrhosis, making important improved methodologies in early diagnosis as well as therapeutic intervention very necessary (5, 6). Consequently, the determination of molecular mechanisms that govern hepatocarcinogenesis as well as tumor growth is vital to the generation of improved, effective therapies.

MicroRNAs (miRNAs) are significant post-transcriptional controllers of cancer biology. As short, endogenous non-coding RNA molecules, measuring about 17 to 25 nucleotides, the miRNAs control the expression of the target messenger RNAs (mRNAs) by binding to the 3′ untranslated region (3′UTR) thereof, leading to repression or degradation of the latter. In the scenario of HCC, the miRNAs control various biological processes, such as proliferation, angiogenesis, apoptosis, as well as regulation of the cell cycle (7-9). Malregulation of miRNA expression, most meaningfully repression of the tumor suppressor miRNAs (TS-miRNAs), leads to the pathological upregulation of the oncogenes, thus the onset as well as the expansion of tumors (10, 11).

One such TS-miRNA is miR-217-5p, which has been reported to be downregulated in HCC (12, 13). In silico and experimental analyses indicate that miR-217 targets the Kirsten rat sarcoma viral oncogene homolog (KRAS), a well-characterized oncogene involved in abnormal cell growth, differentiation, and malignant transformation. Elevated expression of KRAS has been correlated with increased tumor proliferation, progression, and poor patient survival in HCC (14-17). Notably, KRAS, the most frequently mutated isoform of the Ras family, can interact with the mechanistic target of rapamycin (mTOR) signaling pathway, thereby enhancing tumorigenicity and promoting metastasis (18). Furthermore, aberrant KRAS activity has been shown to suppress the tumor suppressor protein p53, which normally functions to induce cell cycle arrest, senescence, and apoptosis in response to oncogenic or genotoxic stress. Taken together, these findings suggest that dysregulation of the miR-217–KRAS–p53 axis constitutes pivotal molecular mechanism driving hepatocarcinogenesis. Targeting this axis may provide new therapeutic opportunities for restoring tumor suppressor activity and inhibiting oncogenic signaling in HCC (19).

Increasing evidence points to the involvement of miRNA dysregulation in the generation of cancer, indicating that miRNA-based replacement therapy is a promising anticancer strategy (20). However, rapid degradation and minimal cellular uptake restrict the systemic delivery of naked miRNAs. Although viruses and non-virus vectors are also under consideration, their applications are restricted due to toxicity, high expense, and non-specificity (21, 22). Nanotechnology-based carriers, such as chitosan nanoparticles (CS-NPs), hold great promise due to safety, biocompatibility, as well as the capability to cushion and deliver miRNAs into target cells through electrostatic interactions (22, 23). Former research has been successful in demonstrating the anticancer effect of CS-NPs as vehicles delivering tumor-suppressing miRNA, such as miR-122, into HCC models, ultimately yielding decreased proliferation, as well as increased apoptosis (24-29). Upon this body of evidence, the current research proposes to explore the involvement of miR-217-5p during the occurrence of hepatocellular carcinoma (HCC) as well as the therapeutic value of miRNA replacement therapy through the use of chitosan nanoparticles (CS-NPs) to deliver the miR-217-5p and upregulate the decreased expression, as well as dampen the cancerous signaling, within the cells responsible for HCC.

2. MATERIALS AND METHODS

2.1. Material

Human HCC cell line (HepG2) was provided by the Agency for the Assessment and Application of Technology Indonesia. Cells were cultivated under high glucose DMEM (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 1% streptomycin and 0.5% amphotericin B. The miR-217-5p was provided by Integrated DNA Technologies, while chitosan particles medium molecular weight.

2.2. Methods

2.2.1. In Silico Analysis

2.2.1.1 Identification gene involve in hepatocellular carcinoma

The analysis of genes associated with hepatocellular carcinoma (HCC) was carried out making full use of two open-access repositories, GeneCards (https://www.genecards.org/) (accessed August 2025) and OpenTargets (https://www.opentargets.org/) (accessed August 2025), by searching the keyword "hepatocellular carcinoma". Gene filtering obtained from GeneCards was carried out on the basis of the relevance score being >5, while those OpenTargets genes that had a global score >0.2 were included. Resulting genes were then merged, redundant entries being excluded to yield a non-redundant dataset that would be further analyzed (30).

2.2.1.2 Analysis target gene of miRNA-217-5p

The identification of miR-217-5p target genes was conducted to explore potential target gene regulate by this miRNA. Two independent tools were employed for this analysis. First, miRWalk 3.0 (http://mirwalk.umm.uni-heidelberg.de/), an accessible database providing machine learning–based predictions with high accuracy, simplicity, and updated content, was queried using the keyword "miR-217-5p" with a cut-off score of 0.95 (31). In addition, miRTarBase (https://mirtarbase.cuhk.edu.cn/), which contains experimentally validated miRNA–target interactions, was utilized to strengthen the prediction. The resulting gene lists from both databases were merged, and duplicate entries were removed (31, 32). Furthermore, all selected target of miRNA-217-5p were intersected with collected HCC related gene using Venny 2.1.0 to obtain common target gene of miRNA in HCC.

2.2.1.3 Enrichment Analysis : Gene Ontology and KEGG Pathway

To characterize the biological relevance as well as functional contribution among common target genes during cellular as well as molecular processes, Gene Ontology (GO) as well as KEGG pathway enrichment analysis were carried out. GO analysis was also performed to analyze enrichment on the basis of three dimensions, i.e., biological process (BP), molecular function (MF), as well as cellular component (CC). Concurrently, the Kyoto Encyclopedia of Genes and Genomes (KEGG) was also used to identify enriched pathways that corresponded to the confirmed common target genes among miR-217-5p during the whole process of HCC. Enrichment-based analysis was carried out under ShinyGO version 0.82 (https://bioinformatics.sdstate.edu/go/) based on a false discovery rate (FDR) threshold value of 0.05, after which the first 20 enriched categories were retrieved under each analysis (32).

2.2.1.4 Analysis of Protein – Protein Interaction (PPI) Network

Protein–protein interaction (PPI) network analysis was carried out to depict the interactions and relationships between the common target genes of miR-217-5p in HCC. The PPI network was created based on the STRING database (https://string-db.org/), which combines protein interaction data both from predictive models and experimental verifications. Analysis was performed based on a medium-confidence score value of 0.400, as well as an FDR threshold value of 5%. The output PPI network was then exported as a file in the TSV (Tab Separated Values) format, followed by visualization, again based on the Cytoscape software, to enable additional network analysis and interpretation (31)

2.2.1.5 Identification hub gene and their interaction with miR-217-5p

Determination of hub genes was conducted to identify the core targets of miR-217-5p in HCC. Hub gene identification was performed using the CytoHubba plugin (http://apps.cytoscape.org/apps/cytohubba), a widely used open-source platform for subnetwork regulation analysis. In this study, hub genes were ranked based on multiple centrality measures, including Degree (DC), Betweenness (BC), and Closeness (CC), which are considered powerful indicators for prioritizing essential proteins as suggested in previous studies (33-35). The top five genes identified from each centrality parameter were then intersected using Venny 2.1.0 to obtain the core hub proteins. Furthermore, the hub genes were visualized alongside their interactions with target genes, including binding scores derived from MiRWalk and miRTarBase predictions, to strengthen the validation of their regulatory relevance in HCC.

2.2.1.6 Analysis Expression of selected hub gene

Analysis Expression of selected hub gene was carried out by examining their expression levels in liver hepatocellular carcinoma (LIHC) through the UALCAN platform (http://ualcan.path.uab.edu), an online interactive website offering comprehensive analysis of gene expression data in TCGA. Each hub gene was interrogated separately within

the dataset of LIHC to determine differential expression between the tumor as compared to the normal tissues. To verify the results further, gene expression patterns were also investigated through the use of GEPIA2 (http://gepia2.cancer-pku.cn), a powerful web-based tool that combines the TCGA and the GTEx dataset. Analysis comprised differential expression profiling based on a log2FC threshold value of 1 as well as jitter size of 0.4, together with overall survival (OS) analysis based on the median group threshold. Expression levels were depicted as boxplots, while the outcomes related to survival were analyzed based on the use of the Kaplan–Meier survival curve. A threshold of significance of $p \leq 0.05$ was used in both the expression as well as the survival analysis (32, 33).

2.2.1.7 In silico immunohistochemical analysis of selected hub gene

Verification of protein expression was also performed to determine the expression patterns of the core proteins that were uncovered in cancerous and normal liver tissues. Immunohistochemical (IHC) data provided in the Human Protein Atlas (HPA) database (https://www.proteinatlas.org/) were used to do so, as they depict high-resolution protein expression signatures both within normal tissues, as well as within tumor tissues. It was also contrasted between normal tissues and hepatocellular carcinoma (HCC) tissues, to identify differential expression on the protein level, after complementing the findings of the transcriptomics, thereby validating the biological significance of the hub genes (32).

2.2.2. In Vitro Analysis

2.2.2.1 Nanoencapsulation and evaluation using electrophoresis

Preparation of chitosan–miRNA nanoparticles was achieved through the method of ionic gelation. In short, 28 µL of chitosan solution (0.2%) was combined with 22 µL of sodium tripolyphosphate (Na-TPP) (0.05%) at the ratio of 5:1, which was optimized before as the most effective ratio (9, 23). Incubation was performed during 5 minutes, followed by adding to 3 µM miRNA solution, incubating again during 20 minutes. Then, 18 µL chitosan–miRNA nanoparticle product was combined together with 3 µL loading dye (6×). Electrophoretic analysis was then conducted on the gel containing 2% agarose during 50 V, 400 mA during 20 minutes to analyze nanoparticle generation as well as stability (9, 36).

2.2.2.2 Characterizations CS-NPs-miR-217-5p

Morphological as well as physicochemical characterizations of the nanoparticles were performed using Scanning Electron Microscopy (SEM, JSM-6510 LA, JEOL Ltd., Akishima, Tokyo, Japan) operating at 15 kV, aided by 5000× as well as 30,000× magnification. Elemental composition as well as chemical structure of the nanopowder were determined using energy-dispersive X-ray spectroscopy (EDS). Concurrently, the particle distribution based on size was determined using dynamic light scattering (DLS) operating on a Zetasizer Nano ZSP (Malvern Panalytical, UK). Nanoparticles were suspended in dialyzed water, where the default values corresponding to the dielectric constant, the refractive index, as well as the viscosity of the water, used to estimate the zeta potential.

2.2.2.3 Cell cultures

2.2.2.3.1 HepG2 cell culture and seeding for treatment

Second generation cells were gathered and plated into 96-well plates at the rate of 6×10^3 cells/well. Subsequently, the cells were cultivated for 24 h under the routine culture condition (37 °C, 5% CO_2) containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with full growth solution.

2.2.2.3.2 cell treatment

Treatment of cells was conducted when the cell reached 70-80% confluence in each T-flask. HCC cells were planted with 5 × 105 cells per well in 6-well plates and subsequently incubated at 37°C for 24 hours in an incubator at 370C with CO2 flow of 5% before treatment. Furthermore, cell washes with PBS 1X were followed by the treatment of CS-NPs-miRNA-217 that were prepared in concentrations of miRNA 30 nM, 60 nM and 120nM for the treatment groups and naked miRNA-217 for the control group. The experiments were performed in triplicate. Cells that were treated with the test solution were incubated for 24 hours in a CO2 incubator. After the treatmen for 24 h, cells were collected for further tests.

2.2.2.4 Quantification of miRNA and mRNA expression by qPCR

Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), and 10 ng RNA was reverse transcribed into cDNA using the miRCURY LNA™ RT Kit (Qiagen) following the manufacturer's protocol. Primers for KRAS, β-actin (internal control), miR-16-5p (reference miRNA), and miR-217 were obtained from GeneCraft (Jakarta) and Genetika Sciences (Jakarta). Primer sequences

were as follows: KRAS (forward: 5'-GACTGTGTTTCTCCCTTCT-3'; reverse: 5'-TGGCAAATACACAAAGAAAG-3'), β-actin (forward: 5'-GGGAATTCAAAACTGGAACGGTGAAGG-3'; reverse: 5'-GGAAGCTTATCAAAGTCCTCGGCCACA-3'), miR-16-5p (5'-UAGCAGCACGUAAAUAUUGGCG-3'), and miR-217 (5'-UACUGCAUCAGGAACUGAUUGGA-3'). Quantitative PCR was performed using the SensiFAST™ SYBR® Green Kit on a Bio-Rad CFX96 C.1000 system. Cycling conditions were 95 °C for 2 min, followed by 39 cycles of 95 °C for 5 s and 60 °C for 5 s (ramp rate 1.6 °C/s). Relative expression levels were calculated using the 2^−ΔCt method (37)

2.2.2.5 In Vitro Immunocytochemistry Analysis of p53 Expression

The expression of the p53 protein was analyzed by immunocytochemistry as per the procedure described by De Falco et al. (38). in 2004, with some modifications. Briefly, HepG2 cells (2.5×10^5) were plated on coverslips laid on the bottom of the 6-deep well plates, followed by culture at 37 °C under 5% CO_2 atmosphere. Cells were then treated with chitosan-entrapped miR-217 (60 and 120 nM) for 24 h. After that, cells were fixed with cold methanol (400 µL) for 15 min, followed by incubation in 500 µL of H_2O_2 solution (1:9 in H_2O) for 5–10 min to inhibit the endogenous peroxidase activity. Primary anti-human p53 antibody (1:100) was introduced (100 µL/well) and kept at 4 °C for 96 h. Cells were washed using PBS, followed by incubation with the biotin-labelled secondary antibody for 10 min, kept at room temperature, then incubated using streptavidin. Visualization was carried out using 100 µl DAB chromogen, followed by counterstaining the nuclei with Mayer's hematoxylin for 5 min. Stained slides were observed under the optical microscope at a magnification of 400×. Quantitative analysis on the expression of p53 was carried out from five randomly picked areas/treatment group (39, 40).

2.2.3. Data Analysis

Each experiment was carried out in triplicate, and the data are presented as mean ± standard deviation (SD). Normality of the data was determined using Shapiro–Wilk tests. Comparison between groups was carried out using analysis of variance (one-way ANOVA) following pairwise post hoc tests (Bonferroni). Moreover, Pearson or Spearman correlation tests were used to identify the correlation between the expression levels of miR-217 and KRAS mRNA expression. A p-value ≤ 0.05 was significant.

3. RESULTS

3.1. In Silico Analysis

3.1.1 Identification gene involve in hepatocellular carcinoma and targeted by miR-217-5p

Gene mining of hepatocellular carcinoma (HCC) was carried out based on two open-access high-quality databases, GeneCard (cut-off score > 5) and OpenTarget (global score > 0.2). We obtained 797 genes retrieved from GeneCard and 695 genes retrieved from OpenTarget based on the inclusion criteria. Following the removal of duplicates, a total number of 1,289 non-redundant genes were short-listed as being related to HCC (Supplementary File 1). Correspondingly, the potential target mRNAs of miR-217-5p were predicated based on miRWalk and miRTarBase, leading to the identification of 1,475 original target mRNAs after the removal of duplicates (Supplementary File 2). Intersecting the HCC-related genes and the predicated miR-217-5p targets, we obtained 152 common genes, reflecting the likely regulatory functions of miR-217-5p on HCC (**Figure 1A**).

3.1.2 Protein – Protein Interaction (PPI) network

Protein protein interaction analysis indicated the construction of highly complex network consisting of hundreds of nodes with strong connectivity (**Figure 1B**). Cytoscape was then used to further visualize the network, and proteins outside the main cluster were excluded to gain more representative interaction network. As a result, the PPI network comprised 146 protein nodes with a total of 1,042 edges (**Figure 1C**). The high connectivity suggests that the intersected proteins do not work independently rather work as part of an interconnected biological system. Large number of edges also confirms the hypothesis that these proteins play important roles during the pathogenesis of HCC and are potential targets modulated by miR-217-5p.

3.1.3 Functional Enrichment Analysis : Gene Ontology and KEGG Pathway

To determine the potential biological functions of miR-217-5p, functional enrichment analysis was carried out, involving biological processes (BP), molecular functions (MF), cellular components (CC), as well as signal pathways. We determined that the 152 target mRNAs of miR-217-5p expressed in hepatocellular carcinoma (HCC) were involved

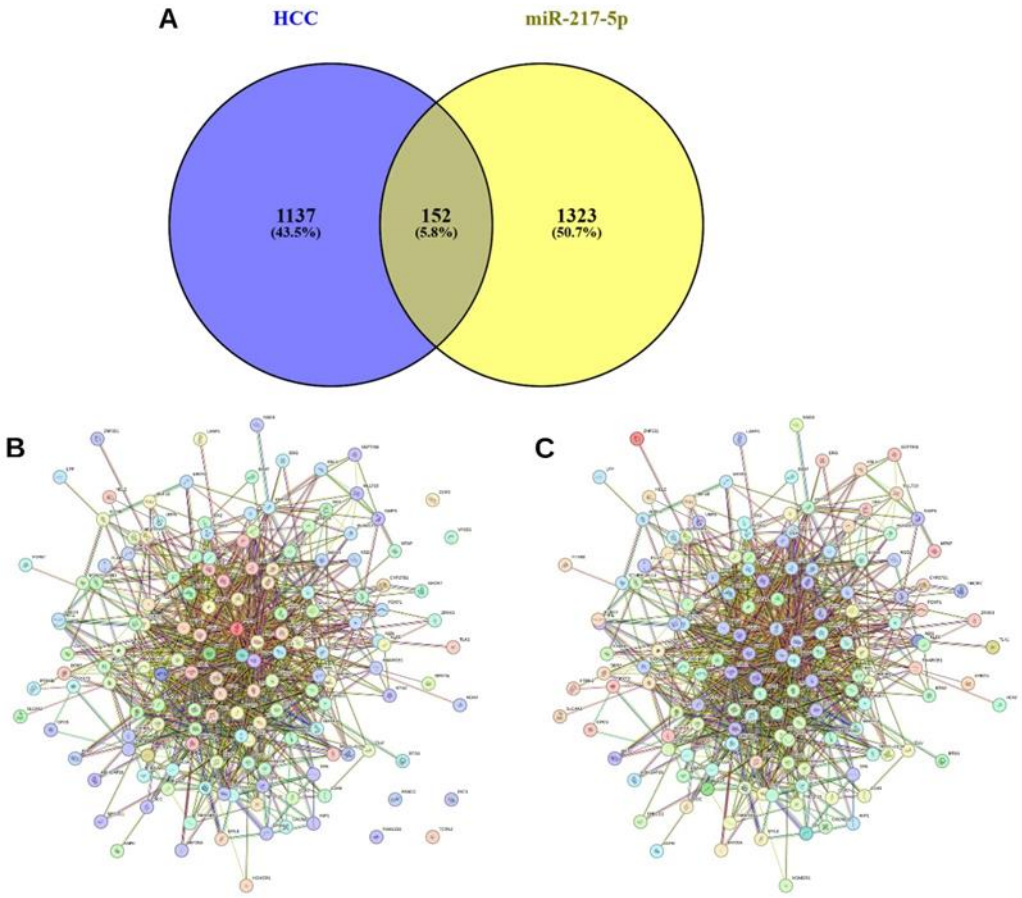


Figure 1. **(A)** Intersection of target gene of miR-217-5p with protein involved in HCC results 152 gene nodes gene. **(B)** Protein Protein Interaction (PPI) Network of the 152 gene nodes gene **(C)** Protein Protein Interaction (PPI) Network of the 146 nodes gene after removing.

in more than 1,000 biological processes. Top three significantly enriched BP are regulation of cell population proliferation (GO:0042127; fold enrichment = 5.6), cell population proliferation (GO:0008283; fold enrichment = 5.0), as well as regulation of programmed cell death (GO:0043067; fold enrichment = 4.9). These processes are significant contributors to the establishment of HCC, suggesting the potential oncogenic regulation function of miR-217-5p. Additional enriched biological processes are provided in **Figure 2A** as well as listed in **Table 1**.

In addition to biological processes, enrichment analysis also indicated that there are significant associations between miR-217-5p target genes and 146 molecular functions (MF) in human beings, most of which are hypothetically related to the pathogenesis of HCC. Top three most enriched MFs were β-catenin binding (GO:0008013; fold enrichment = 15.2), RNA polymerase II-specific DNA-binding transcription factor binding (GO:0061629; fold enrichment = 6.5), and DNA-binding transcription factor binding (GO:0140297; fold enrichment = 6.3). Interestingly, β-catenin, as a classical oncogene, also serves as a transcription factor, as well as a cell adhesion molecule, highlighting the latter's pivotal role during the development of hepatocellular carcinoma. These findings indicate the potential regulating role of miR-217-5p on key molecular mechanisms during tumorigenesis. Details of enriched MFs are shown in **Figure 2B**, while shortlists are included in **Table 2**.

Enrichment analysis of the cellular component (CC) category indicated that there was strong enrichment of miR-217-5p target mRNAs in 95 CC terms. Among these, the most significant enrichment was caveola (GO:0005901)

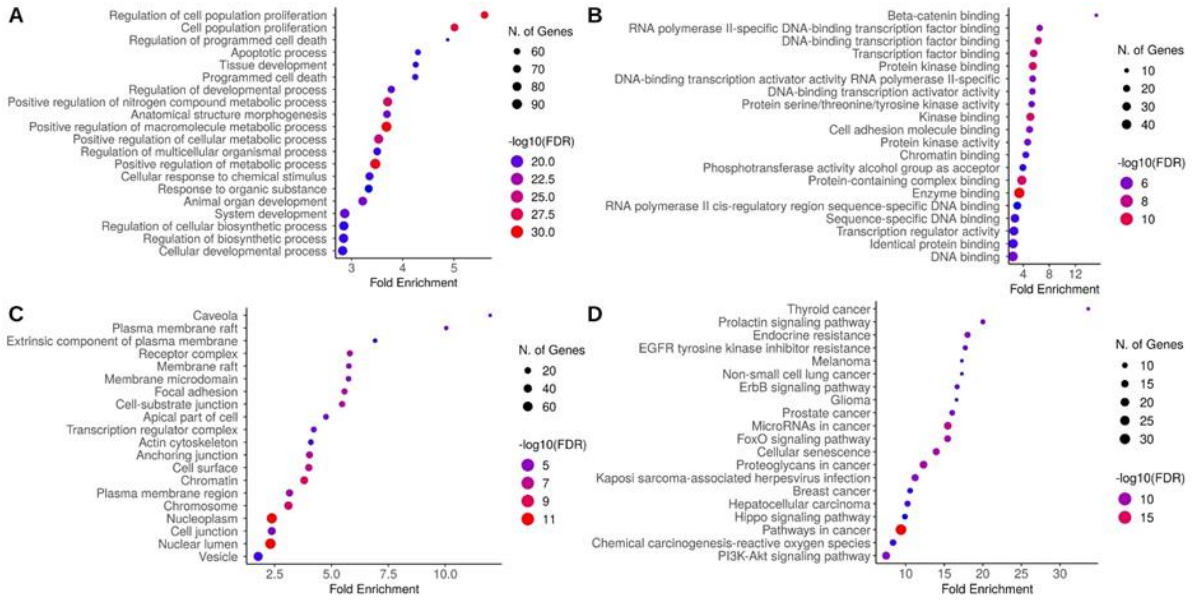


Figure 2. Functional enrichment analysis results of 152 target gene miR-217-5p in HCC.
(A) Top 20 of enriched biological process **(B)** Top 20 of enriched molecular function **(C)** Top 20 of enriched cellular component **(D)** Top 20 of enriched KEGG Pathway**.**

Table 1. Top 20 of Enriched Biological Process associated with 152 target of miR-217-5p in HCC

Pathways	Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment
Regulation of cell population proliferation	2.70E-30	66	1835	5.6
Cell population proliferation	2.90E-29	69	2143	5
Regulation of programmed cell death	5.20E-20	51	1627	4.9
Apoptotic process	4.20E-20	57	2065	4.3
Tissue development	5.30E-20	57	2086	4.3
Programmed cell death	3.50E-20	58	2127	4.2
Regulation of developmental process	1.70E-20	65	2680	3.8
Positive regulation of nitrogen compound metabolic process	7.70E-27	81	3403	3.7
Anatomical structure morphogenesis	4.10E-21	68	2867	3.7
Positive regulation of macromolecule metabolic process	5.80E-31	91	3847	3.7
Positive regulation of cellular metabolic process	6.80E-26	82	3617	3.5
Regulation of multicellular organismal process	4.70E-20	68	3024	3.5
Positive regulation of metabolic process	2.70E-30	93	4181	3.5
Cellular response to chemical stimulus	4.20E-20	71	3300	3.3
Response to organic substance	9.60E-20	70	3269	3.3
Animal organ development	1.70E-21	78	3774	3.2
System development	6.10E-21	85	4617	2.9
Regulation of cellular biosynthetic process	5.90E-20	82	4482	2.8
Regulation of biosynthetic process	4.20E-20	83	4545	2.8
Cellular developmental process	5.20E-20	83	4576	2.8

Table 2. Top 20 of Enriched molecular function associated with 152 target of miR-217-5p in HCC.

Pathways	Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment
Beta-catenin binding	6.10E-07	9	92	15.2
RNA polymerase II-specific DNA-binding transcription factor binding	3.00E-07	16	381	6.5
DNA-binding transcription factor binding	2.10E-09	21	518	6.3
Transcription factor binding	2.10E-09	23	639	5.6
Protein kinase binding	4.10E-10	26	739	5.5
DNA-binding transcription activator activity RNA polymerase II-specific	9.60E-07	17	485	5.5
DNA-binding transcription activator activity	9.90E-07	17	489	5.4
Protein serine/threonine/tyrosine kinase activity	2.80E-06	16	470	5.3
Kinase binding	4.10E-10	27	824	5.1
Cell adhesion molecule binding	6.30E-07	19	594	5
Protein kinase activity	1.30E-06	19	632	4.7
Chromatin binding	6.00E-06	18	636	4.4
Phosphotransferase activity alcohol group as acceptor	1.10E-05	19	748	4
Protein-containing complex binding	4.10E-10	36	1477	3.8
Enzyme binding	2.40E-12	49	2237	3.4
RNA polymerase II cis-regulatory region sequence-specific DNA binding	1.30E-05	25	1258	3.1
Sequence-specific DNA binding	7.60E-06	31	1765	2.7
Transcription regulator activity	7.60E-06	34	2056	2.6
Identical protein binding	6.50E-06	37	2342	2.5
DNA binding	1.50E-06	42	2707	2.4

(false discovery rate [FDR] = 6.00×10^{-5}; fold enrichment = 12). It is recognized that caveolae, flask-shaped evaginations of the plasma membrane, play multiple roles during hepatocellular carcinoma (HCC), such as modulating signal transduction, facilitating endocytosis, and promoting tumor growth. Other significantly enriched CC terms comprised plasma membrane raft (GO:0044853) and extrinsic component of plasma membrane (GO:0019897), indicating that the regulation impacts caused by miR-217-5p are likely related to cellular architectures of the membrane. Enriched CCs are shown in detail **(Figure 2C)** and summarized **(Table 3)**.

Enrichment analysis of the pathways also revealed that the 152 miR-217-5p target genes significantly participated in 152 human signaling pathways. Various important pathways were significantly enriched, comprising the prolactin signaling pathway, the resistance to endocrine, EGFR tyrosine kinase inhibitor resistance, the ErbB signaling pathway, the PI3K-Akt signaling pathway, and notably, the hepatocellular carcinoma (HCC) pathway **(Figure 2D and Table 4)**. In the HCC pathway, various genes were found to regulate downstream processes vital to cancer development, including increased proliferation, changed differentiation, immortalization of cells, as well as continuous cell cycle expansion **(Figure 3)**. These results strongly indicate that miR-217-5p plays an oncogenic role through the regulation of various oncogenic signal transductions, ultimately enforcing the initiation and development of HCC.

Table 3. Top 20 of Enriched cellular component associated with 152 target of miR-217-5p in HCC.

Pathways	Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment
Caveola	6.00E-05	7	91	12
Plasma membrane raft	4.70E-05	8	124	10
Extrinsic component of plasma membrane	5.20E-04	8	180	6.9
Receptor complex	9.60E-07	16	429	5.8
Membrane raft	1.90E-05	13	351	5.8
Membrane microdomain	1.90E-05	13	352	5.7
Focal adhesion	7.90E-07	17	475	5.6
Cell-substrate junction	9.10E-07	17	484	5.5
Apical part of cell	5.10E-05	14	458	4.8
Transcription regulator complex	8.10E-05	15	553	4.2
Actin cytoskeleton	2.40E-04	14	532	4.1
Anchoring junction	4.40E-07	24	926	4
Cell surface	6.00E-08	27	1050	4
Chromatin	1.60E-09	34	1392	3.8
Plasma membrane region	4.50E-06	27	1332	3.2
Chromosome	6.00E-09	40	2003	3.1
Nucleoplasm	1.10E-11	70	4581	2.4
Cell junction	3.30E-05	35	2293	2.4
Nuclear lumen	9.10E-12	74	4973	2.3
Vesicle	2.70E-04	51	4466	1.8

Table 4. Top 20 of Enriched KECGG Pathway associated with 152 target of miR-217-5p in HCC

Pathways	Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment
Thyroid cancer	2.40E-09	8	37	33.7
Prolactin signaling pathway	1.50E-08	9	70	20
Endocrine resistance	1.00E-09	11	95	18
EGFR tyrosine kinase inhibitor resistance	4.10E-08	9	79	17.7
Melanoma	3.00E-07	8	72	17.3
Non-small cell lung cancer	3.00E-07	8	72	17.3
ErbB signaling pathway	6.50E-08	9	84	16.7
Glioma	3.70E-07	8	75	16.6
Prostate cancer	1.50E-08	10	97	16
MicroRNAs in cancer	8.90E-13	16	161	15.5
FoxO signaling pathway	1.30E-10	13	131	15.4
Cellular senescence	9.40E-11	14	156	14
Proteoglycans in cancer	2.10E-11	16	202	12.3
Kaposi sarcoma-associated herpesvirus infection	1.00E-09	14	194	11.2
Breast cancer	4.90E-07	10	147	10.6
Hepatocellular carcinoma	1.90E-07	11	167	10.3

Hippo signaling pathway	8.80E-07	10	157	9.9
Pathways in cancer	8.50E-20	32	530	9.4
Chemical carcinogenesis-reactive oxygen species	3.20E-07	12	223	8.4
PI3K-Akt signaling pathway	3.60E-09	17	354	7.5

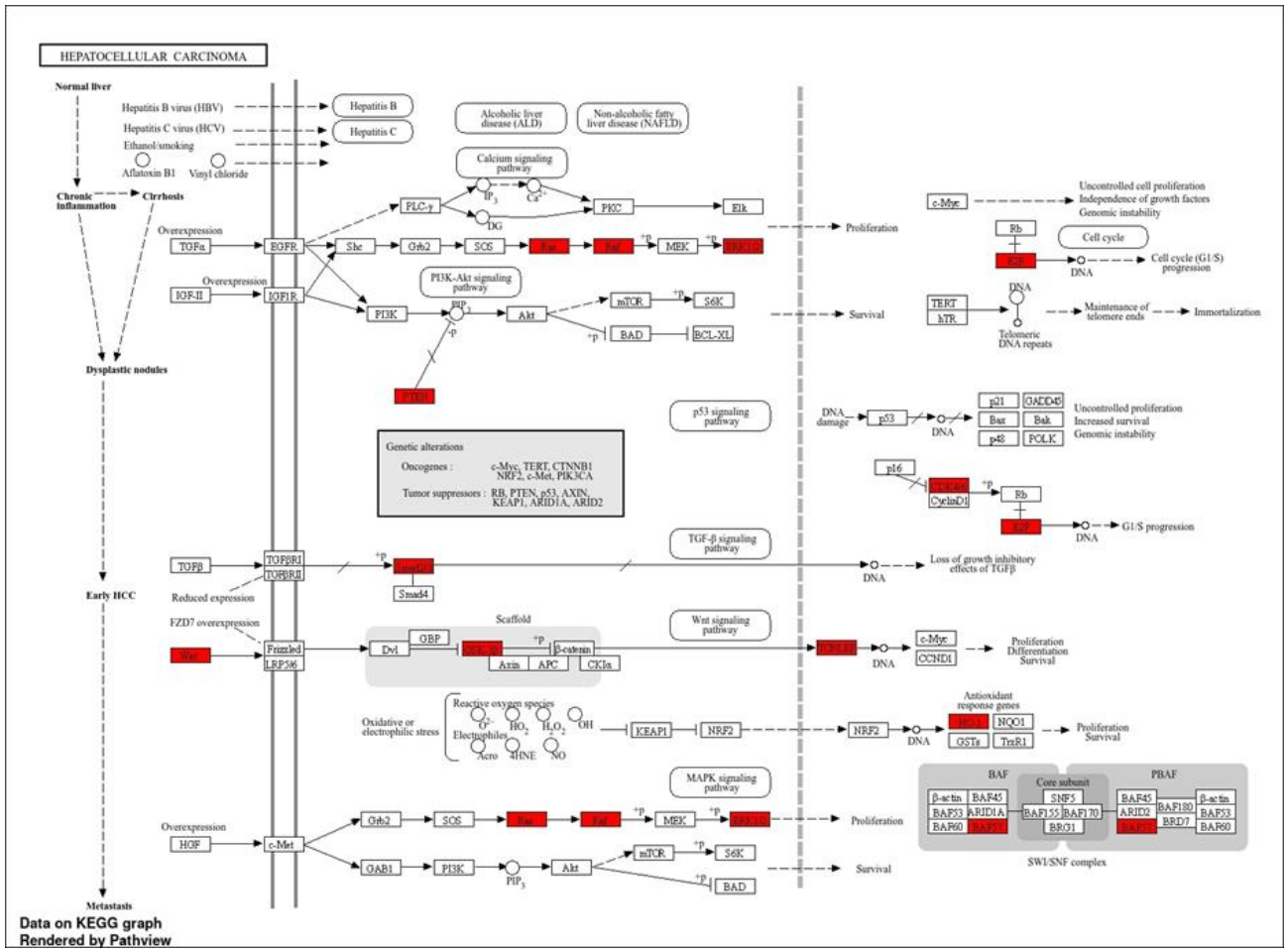


Figure 3. Hepatocellular cancer pathway as one of top 20 enriched pathway obtained KEGG. The red color were target gene of miR-217-5p in HCC.

3.1.4 Hub Gene Identification

To determine the relative significance of nodes in the PPI network and determine the most promising core genes, hub analysis, as provided by the plugin package CytoHubba, was used to analyze the PPI network in Cytoscape. Three parameters related to centrality—degree, betweenness, and closeness—were used to rank each gene based on the highest topology significance. These parameters automatically ranked the nodes based on their connectivity as well as their capability to act as central regulators within the network (34). It was presumed that the disruption of those genes that interacted extensively would cause extensive disruption across the network. The first five genes ranked by each parameter of centrality indicated significant overlap. Intersections among the results obtained using all three parameters regularly indicated five hub genes: PTEN, KRAS, ESR1, HIF1A, and CDH1 (**Table 5, Figure 4**). These proteins are postulated to be key regulators of hepatocellular carcinoma (HCC) and are potential targets that could be therapeutically moderated by miR-217-5p. It is

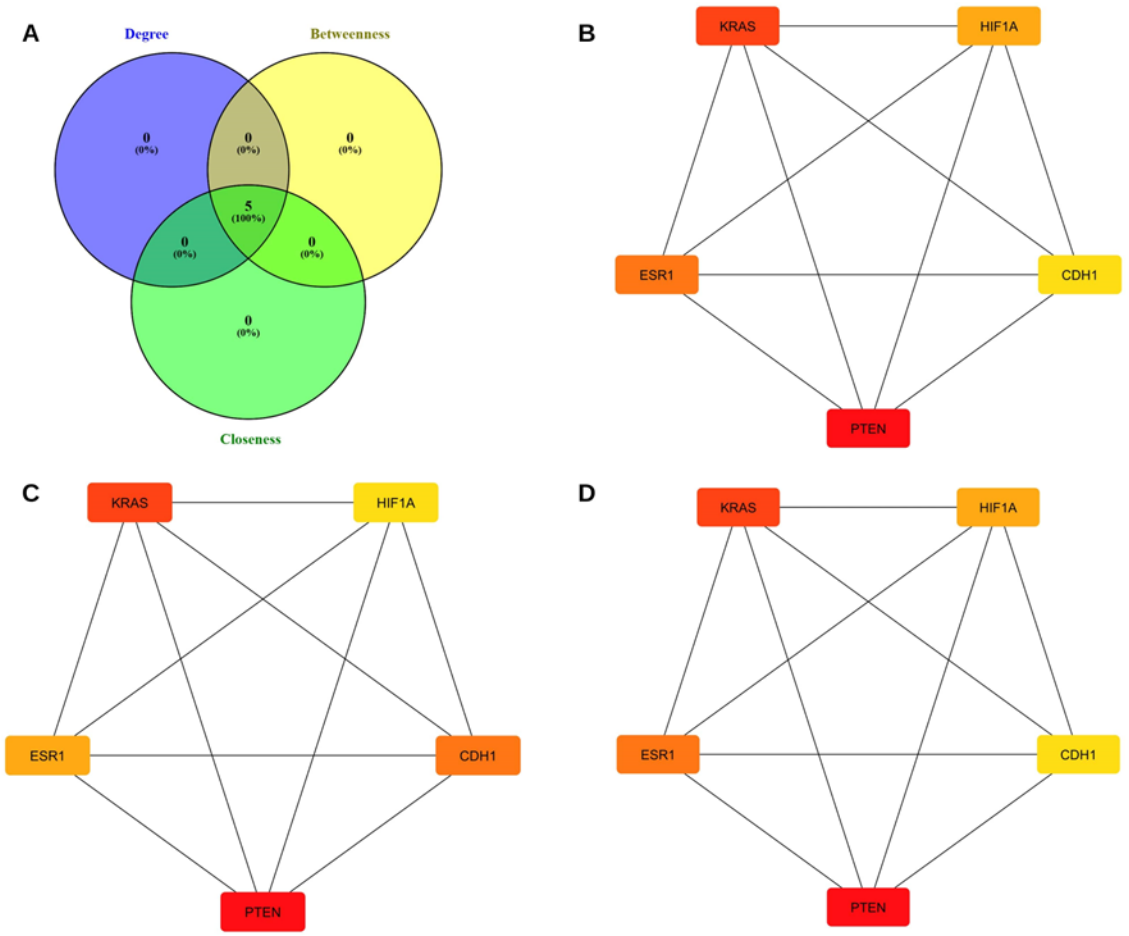
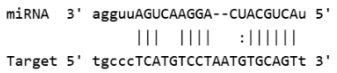
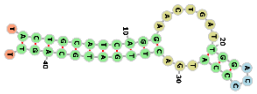
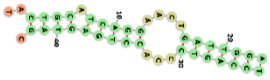
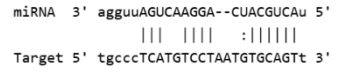
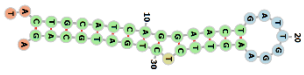


Figure 4. Hub gene results.
(A) Intersection of top 5 hub gene in each centrality parameter obtain five core gene targeted by-miR-217-5p against HCC.
(B) top 5 hub gene according degree centrality
(C) top 5 hub gene according betweenness centrality
(D) top 5 hub gene according closeness centrality.

Table 5. Summary score of gene in each centrality parameters obtained in identification hub

Gene	Degree Centrality	Betweenness Centrality	Closeness Centrality
PTEN	65	2018.614	103.8333
KRAS	62	1625.834	102.5
ESR1	59	1366.522	100.5
HIF1A	57	1085.952	99.5
CDH1	52	1419.71	96.66667

Table 6. **miR-217-5p interaction with core target gene dan binding energy mereka masing masing**

miR	Protein	Binding Energy (kcal/mol)	Visalisasi prediksi interaksi
miR-217-5p	PTEN	-14.8	
miR-217-5p	KRAS	-18.0	
miR-217-5p	ESR1	-19.8	
miR-217-5p	HIF1A	-14	
miR-217-5p	CDH1	-21.7	

notable that the appearance of the hub proteins PTEN and KRAS aligns with their involvement, as depicted in the schematic diagram of the HCC pathway **(Figure 3)**, where both proteins play key roles driving the growth and survival of cancer cells.

3.1.5 Binding analyses of miR-217-5p with Hub Genes

To authenticate the regulatory effect of miR-217-5p on the hub genes that were indentified, binding assays were conducted through miRWalk and miRTarBase. The findings verified that all the hub genes (PTEN, KRAS, ESR1, HIF1A, and CDH1) had potential binding with miR-217-5p **(Table 6)**. Interestingly, the binding interaction between miR-217-5p and CDH1 had the highest binding affinity, where the binding energy was remarkably low (−21.7 kcal/mol), proving the interaction to be highly stable. These observations also authenticate the postulation that miR-217-5p possesses the capability to modulate the progression of HCC through the direct regulation of the core oncogenic as well as tumor-suppressor genes.

3.1.6 Validation of hub gene related hepatocellular carcinoma targeted by miR-217-5p

3.1.6.1 prediction of expression level of hub gene

To more fully characterize the expression level of the screened hub genes during hepatocellular carcinoma (HCC), expression analysis was also carried out on UALCAN and GEPIA2 web servers. Gene expression during HCC tissues (LIHC group) was compared to that during normal liver tissues. both of tools gave the same gave the same output, showing that PTEN, KRAS, HIF1A, and CDH1 were upregulated during HCC, while ESR1 was decreased **(Figures 5–6)**.

In the GEPIA2 analysis, the upregulation of PTEN, KRAS, HIF1A, and CDH1 was insignificant. Conversely, ESR1 exhibited statistically significant downregulation during HCC tissues as opposed to the normal controls **(Figure 6)**. These results indicate a differential regulation of the miR-217-5p target hub genes, wherein ESR1 downregulation may contribute significantly to the progressiveness of HCC.

3.1.6.2 In Silico Immunohistochemical expression of Hub Gene Expression

Immunohistochemical (IHC) Data from the Human Protein Atlas was analyzed to confirm the expression patterns of the five hub genes (PTEN, KRAS, ESR1, HIF1A, and CDH1) among normal liver tissues vs. hepatocellular carcinoma (HCC) tissues **(Figure 7a)**. There were noticeable expression disparities across the protein panel. PTEN had negative to very feeble staining within the HCC tissues, compared to moderate staining within normal hepatocytes, corroborating its presumed loss of tumor suppressive capability within liver cancer. Conversely, KRAS had significantly upregulated expression, showing strong diffuse cytoplasmic staining within carcinoma cells, yet expression

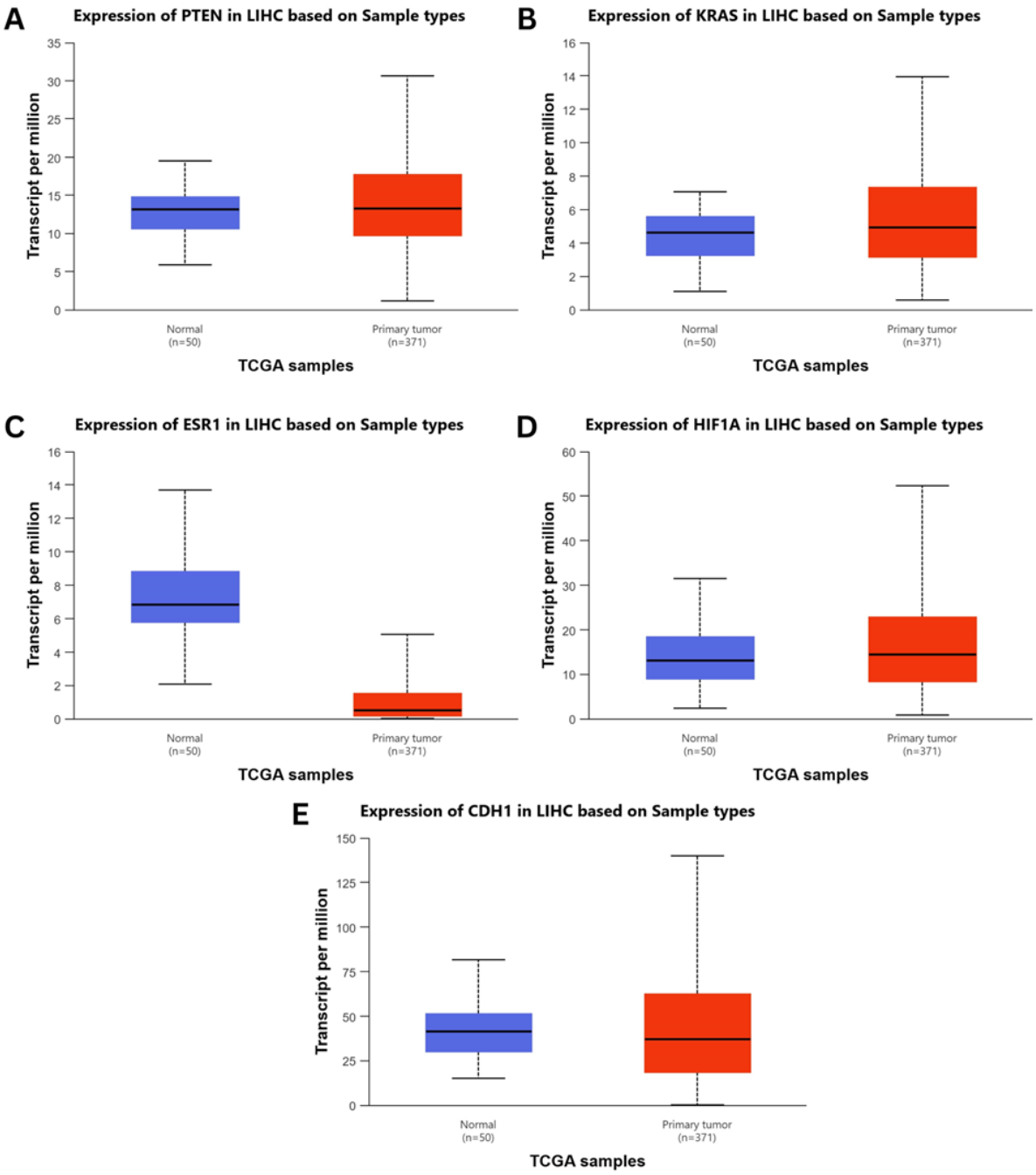


Figure 5. **Expression pattern of core/hub gene in LIHC/HCC vs normal sample resulted by UALCAN.**
(A) Expression PTEN **(B)** Expression KRAS **(C)** Expression ESR1 **(D)** Expression HIF1A **(E)** Expression CDH1.

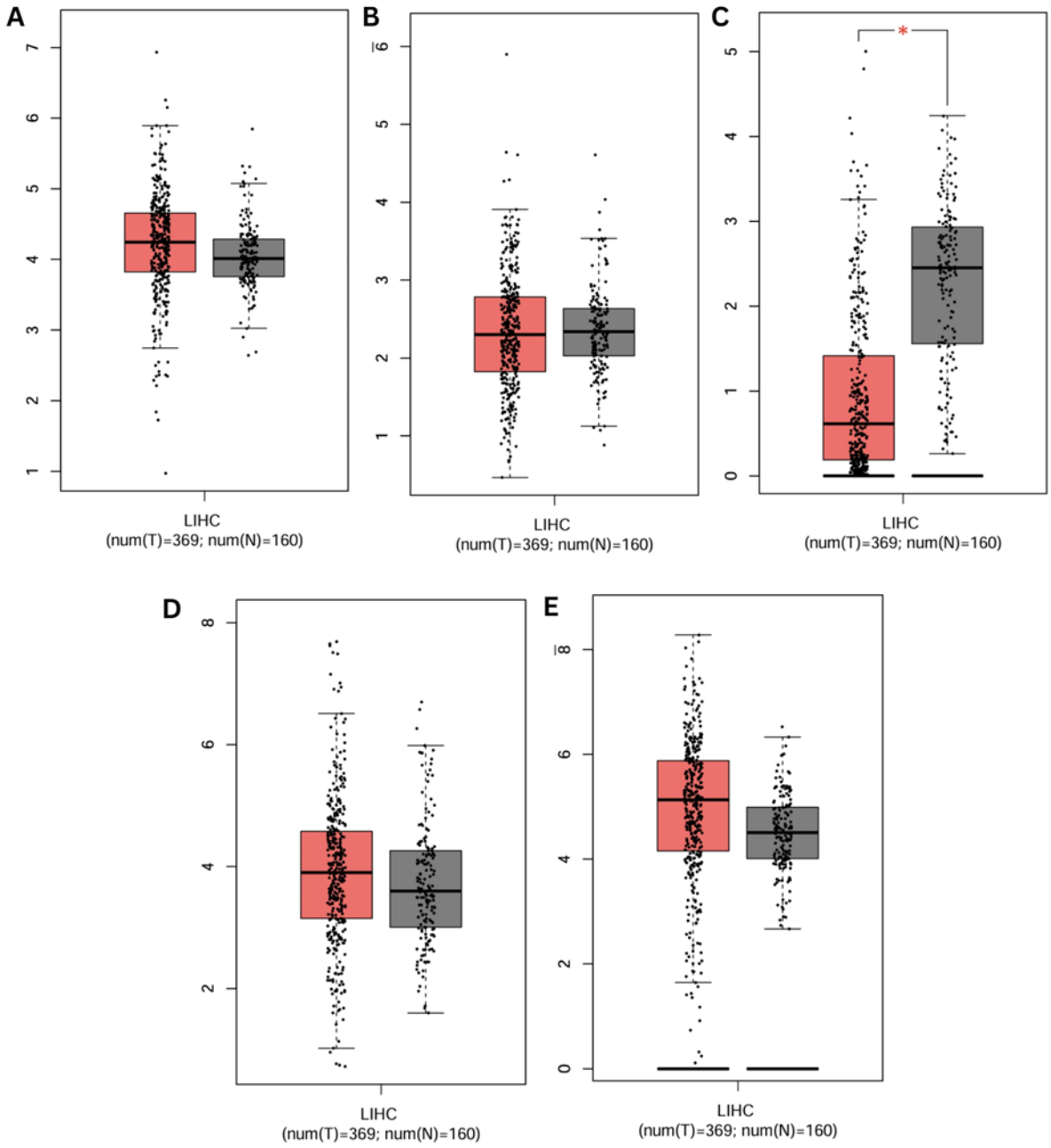


Figure 6. **Expression level (Transcript per million (TPM) of core/hub gene in LIHC/HCC vs normal sample resulted by GEPIA2.** **(A)** Expression PTEN **(B)** Expression KRAS **(C)** Expression ESR1 **(D)** Expression HIF1A **(E)** Expression CDH1

within natural liver was feeble. ESR1 presented an impressive differential expression, wherein weak staining was observed within natural tissue, yet upregulated to moderate or strong cytoplasmic staining was evident within the HCC specimens. HIF1A expression was typically low to undetectable within both natural as well as tumor tissues, indicating that rather than the expression levels, the activity of this protein is likely controlled through the influence of the microenvironment, such as hypoxia. CDH1, which was notably undetectable within natural hepatocytes, presented upregulated membranous staining within the HCC tissues, validating the disruption of cellular adhesion regulation during the process of hepatocarcinogenesis. Overall, these observations point towards the critical disparities observed in the expression patterns of hub gene proteins between natural as well as tumor tissues, ascribing additional insights into the molecular processes that govern the onset as well as advancement of the HCC.

3.1.6.3 analysis survival

Kaplan–Meier survival analysis was used to determine the prognostic value of the five candidate hub genes among the patients in the Liver Hepatocellular Carcinoma (LIHC) cohort. The findings indicated that the expression levels of PTEN and CDH1 were not significantly related to overall survival ($p = 0.69$ and $p = 0.36$, respectively) and, therefore, they do not act as prognostic markers among this cohort. Conversely, KRAS and HIF1A expressed a tendency to be risk factors as their hazard ratios (HR) were more than 1 (HR = 1.4). Higher expression levels of the both genes indicated lower survival, albeit the outcomes did not gain statistical significance ($p = 0.05$ and $p = 0.089$, respectively).
Unexpectedly, the expression of ESR1 had a statistically significant relationship with the patients' prognosis. ESR1 high expression corresponded to a favorable overall survival ($p = 0.00069$; HR = 0.55), suggesting the potential protective role of ESR1 on LIHC. These findings indicate ESR1 as a likely biomarker that implies the prognosis of HCC, while the role of KRAS and HIF1A must be confirmed before they are determined to be of prognostic value **(Figure 7b)**.

3.2. In Vitro Validation Analysis

3.2.1 Preparation of chitosan nanoparticles (CS-NPs) of miR-217-5p

In order to facilitate the release of miR-217-5p into the cells, the miRNA was entrapped into chitosan nanoparticles (CS-NPs). Formulation of the CS-NPs-miR-217-5p was carried out through the method of ionic gelation, a rapid and convenient procedure to package nanoparticles. Under this method, sodium tripolyphosphate (TPP) acted as a cross-linking molecule under a chitosan-to-TPP ratio of 5:1. Nanoparticles were formed due to the complexation between the protonated amine groups (NH_3^+) based on the polyanionic groups offered by TPP, where chitosan was dissolved under acidic (1% acetic acid) conditions. We decided upon this ratio based on previous optimizing research, which suggested that the composition was able to effectively entrap, as well as deliver, miRNAs to their site-specific targets within the cells, including cancer cells (9, 23). Encapsulation efficiency of miR-217-5p in chitosan nanoparticles was first assayed by gel electrophoresis (**Figure 8**). Naked miRNAs, during this assay, did not show significant migration away from the loading wells, most likely because they were being degraded without the protection offered by the nanoparticle. Conversely, the miRNAs that were embedded inside the CS-NPs were distinctly observed around 29 bp, validating successful encapsulation. Entrapment efficiency was then measured following the procedure outlined under our previous publications. Moreover, endogenous expression of miR-217, as outlined subsequently under section 2.7, was also determined to confirm the success of transfection (41).
Morphological description of chitosan nanoparticles (CS-NPs) was performed through scanning electron microscopy (SEM), which gives the structure based on the interactions between the electron beam and the surface of the sample (42). Analysis through SEM indicated that the CS-NPs had mostly spherical morphologies. Corresponding spherical shapes were also evidenced in the case of nanoparticles containing mimic miR-217-5p, as presented in **Figure 9**. Distribution sizes of particles were also determined using dynamic light scattering (DLS), which indicated an average particle size of 413.3 ± 5.77 nm, showing no particles larger than 500 nm. These indicated results agreed with observations obtained through SEM, validating that the particles retained a homogenous distribution size smaller than 500 nm.

3.2.2 CS-NPs-miR-217-5p Restores Endogenous miR-217-5p and Suppresses KRAS

As indicated in **Figure 9**, the treatment with CS-NPs-miR-217-5p significantly enhanced the expression of the endogenous miR-217-5p along with decreased KRAS mRNA expression among the treatment groups as compared to the control. KRAS mRNA was notably

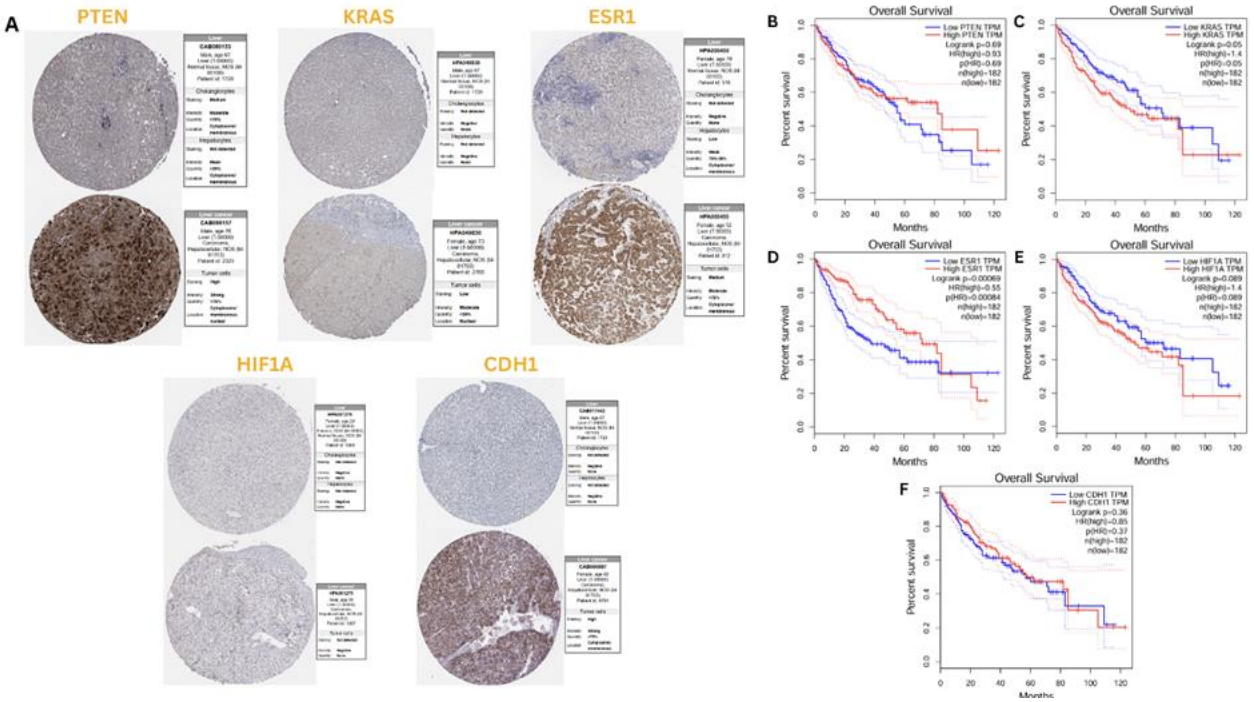


Figure 7. (A) Differential expression profiles of hub genes in normal liver and liver hepatocellular carcinoma (LIHC) tissues analysed by HPA. Kaplan–Meier overall survival analyses based on the expression levels of each hub gene in LIHC, including (B) PTEN, (C) KRAS, (D) ESR1, (E) HIF1A, and (F) CDH1, generated using GEPIA2.

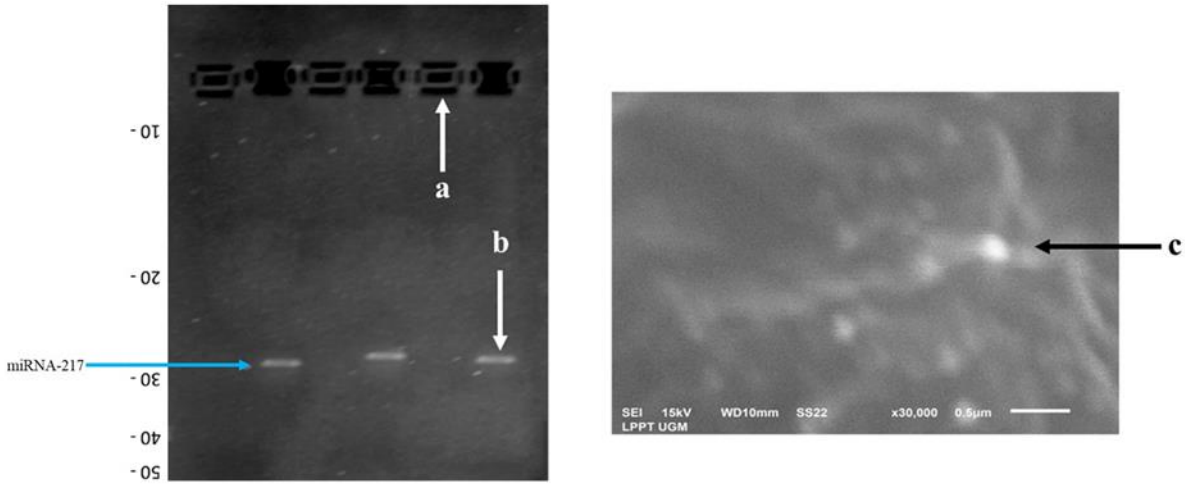


Figure 8. Electrophoresis of chitosan-miR-217 nanoparticles on 2% agarose gel showing **(a)** chitosan nanoparticles loaded with miR-217 and **(b)** naked miR-217. Nanoparticle morphology was characterized by SEM **(c).**

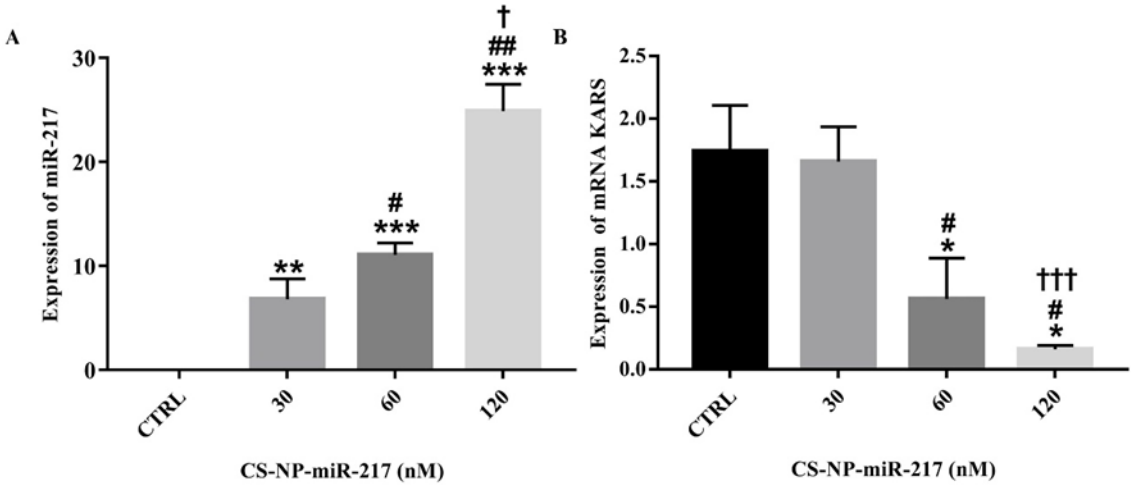


Figure 9. Relative expression levels of (A) miR-217-5p and (B) KRAS mRNA in HepG2 cells following treatment with CS-NP-miR-217 at concentrations of 30, 60, and 120 nM compared with control cells (treated with naked mimic miR-217). Data were analyzed using one-way ANOVA followed by Bonferroni post hoc tests ($p < 0.05$, 95% confidence interval). Statistical significance: *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ vs. control; #$p < 0.05$, ##$p < 0.005$ (30 nM vs. 60 and 120 nM); +$p < 0.05$, +++$p < 0.001$ (60 nM vs. 120 nM).

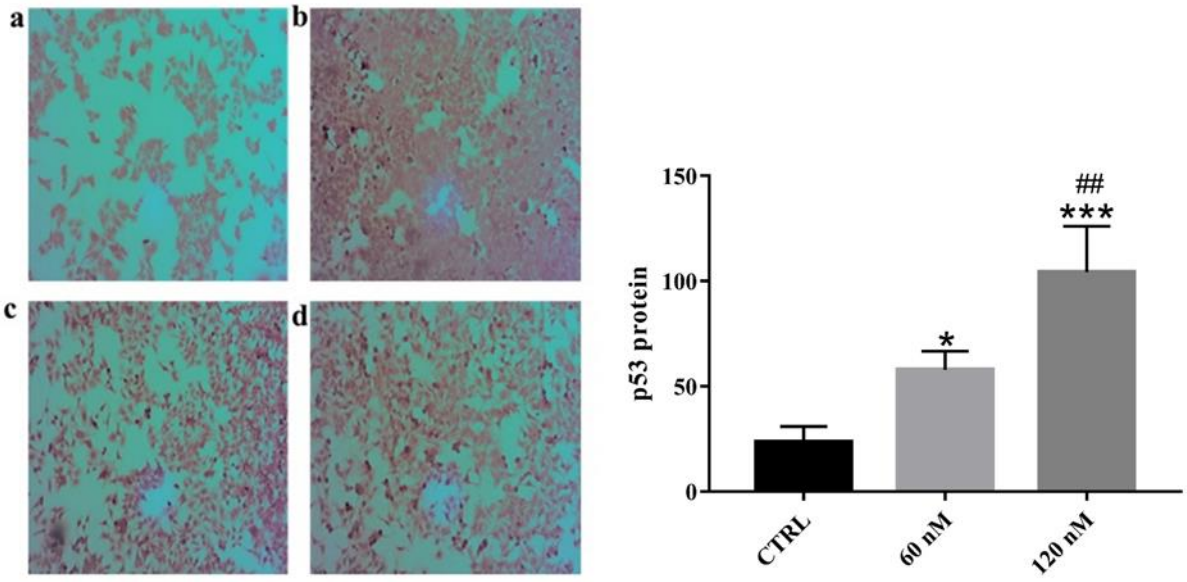


Figure 10. Immunocytochemical analysis of p53 expression in HepG2 cells: (a) control without miR-217 and without anti-p53 antibody, (b) control without miR-217 but with anti-p53 antibody, (c) CS-NP-miR-217 at 60 nM , and (d) CS-NP-miR-217 at 120 nM. (e) Quantification of p53 expression levels. Data represent mean ± SD of three independent experiments. Statistical significance was determined by one-way ANOVA with $p < 0.05$ (*$p < 0.05$, ***$p < 0.001$ vs. control; ##$p < 0.005$ between 60 nM and 120 nM groups).

suppressed, especially in the case of the HepG2 cells. Analysis based on the post hoc tests by Bonferroni indicated the significant differences among the 60 nM as well as the 120 nM group as compared to the control ($p < 0.05$), while the group that was treated with a concentration of 30 nM did not indicate any statistically significant decrease.

In addition, correlation analysis indicated significant inverse correlation between the expression of miR-217-5p and the levels of KRAS mRNA among the treatment groups. Pearson's correlation test gave an r value of –0.937, also showing a p value of 0.0001, demonstrating as very strong the negative correlation between the relative expression levels of miR-217-5p and KRAS ($p < 0.05$).

3.2.3 CS-NPs-miR-217-5p Enhances p53 Expression

Several studies have reported that the expression of KRAS, PTEN, HIF1A, CDH1, and ESR1 in HCC or LIHC is closely associated with alterations in p53 expression as well as disease progression. p53 functions as a tumor suppressor that regulates the expression of multiple oncogenes, and its downregulation or mutation often leads to oncogene expansion. In this study, we investigated the potential role of miR-217 in enhancing p53 expression using immunocytochemistry. As shown in **Figure 10**, p53 protein expression in HepG2 cells was evaluated following treatment with 60 nM and 120 nM mimic miR-217. Positive p53 staining was visualized as brown coloration in cells treated with CS-NPs-miR-217 (**Figure 10c–d**), indicating an upregulation of p53 expression compared with the controls (**Figure 10a-b**). Quantitative analysis was performed by assessing five microscopic fields per slide for each group. Statistical analysis using one-way ANOVA revealed significant differences in p53 expression among the groups ($p < 0.05$).

4. DISCUSSION

Recent advances in transcriptomics have provided novel insights into the pathomechanisms of a number of diseases, including cancer. Among them, the role of microRNAs (miRNAs) has been increasingly recognized in cancer initiation, development, and severity. Similarly, in hepatocellular carcinoma (HCC), these small non-coding RNAs play roles in diverse oncogenic pathways, such as cell cycle control, proliferation, apoptosis, necroptosis, autophagy, metastasis, tumor angiogenesis, and drug resistance, in addition to shaping the tumor microenvironment (TME) to support cancer progression (43). This regulatory role is via the ability of miRNAs to bind the mRNA of their target gene, leading to translational inhibition or degradation of mRNA (44). Because of their significant roles in cancer processes, numerous researchers worldwide have directed efforts toward the development of therapeutic strategies against these molecules (43). In the present study, we evaluated the role of miR-217-5p in HCC molecular mechanisms and ascertained if it may act as a candidate for miRNA replacement therapy. We were prompted by evidence showing that miR-217-5p, a tumor-suppressor microRNA in HCC, is highly downregulated in this cancer.

The in silico analysis performed in the present work revealed that 152 HCC-involved genes might be targeted by miR-217-5p, indicating the important regulatory role of this miRNA through the translational repression of a wide array of genes implicated in various biological mechanisms. The target genes participate in various biological processes and signalling pathways, including those implicated in cancer development. As shown by **Figure 2A**, these genes are involved in different biological processes, among which the regulation of cell population proliferation was identified as the most impacted process, encompassing 68 out of the 152 analyzed genes. The process is especially critical in HCC, where it is accountable for the proliferation of cancer cells through the synergistic action of a number of genes. In the present study, genes such as WNT1 and HNF4α were identified as the key regulators in this pathway**.** These findings are consistent with previous observations that have indicated that both WNT1 and HNF4α play central roles in HCC cell proliferation, and their suppression by miRNAs can effectively suppress cancer cell growth (45, 46). In addition to these two genes, several other genes are also implicated in this process. The fact that our findings are consistent with existing literature is further confirmation that miR-217-5p is able to target genes participating in these pathways, thereby having a part in suppressing cellular proliferation.

These findings are consistent with the KEGG pathway analysis, which identified that several of the miR-217-5p target genes are involved in significant signaling pathways, including the PI3K-Akt signaling pathway and the hepatocellular carcinoma pathway. Tian et al. demonstrated that dysregulation of the PI3K-Akt signaling pathway in HCC plays a critical role in promoting tumor growth and survival by modulating basic cellular processes such as proliferation, metabolism, apoptosis, and motility (47). Moreover, the hepatocellular carcinoma (HCC) pathway was among the most enriched signaling pathways, containing 11 genes, such as KRAS (**Figure 3 and Supplementary Figure 6**). As indicated, these genes are

involved in the proliferation of cancer cells. This finding is consistent with previous reports that the expression of KRAS and its mutant alleles can enhance the proliferation of hepatocytes and hepatocellular carcinoma cells (48). Interestingly, in the current study, hub gene analysis identified KRAS as one of the five central targets of miR-217-5p in HCC, which further supports the fact that its miRNA regulation may be a promising novel therapeutic strategy. Furthermore, expression profiling and survival analysis indicated that KRAS is overexpressed in hepatocellular carcinoma tissues, where it is linked to poor prognosis and reduced overall survival. The observation agrees with the gene's role in promoting proliferation and other processes in cancer cell formation. In line with these, Human Protein Atlas data also showed that KRAS is intensely and diffusely cytoplasmic in tumor cells, but its expression in normal liver tissue is low (**Figure 7A**). To validate these findings, HCC cells were transfected with mimic miR-217-5p in order to ascertain its potential for miRNA replacement therapy and to explore its effect on KRAS, which was identified as an important target gene.

For the intracellular delivery of the mimic miRNA, chitosan nanoparticles (CS-NPs) were utilized as a carrier system. CS-NPs have been shown to efficiently shield miRNAs against degradation by nucleases, facilitating efficient delivery to target cells (49). During cellular internalization, the chitosan nanoparticles (CS-NPs) are exposed to lysozyme, an intracellular enzyme with the ability to degrade chitosan. Lysozyme breaks the glycosidic bonds of the chitosan polymer so that miR-217-5p encapsulated in it is released into the cytosol, where it is free to perform its regulatory role on target gene expression (50). As illustrated in **Figure 9**, CS-NPs-miR-217-5p treatment of HepG2 cells resulted in an increase in intracellular miR-217 levels with a concomitant decrease in KRAS expression. This downregulation is hypothesized to be caused by direct targeting of KRAS by miR-217-5p, leading to its degradation and suggesting a putative inhibition of cancer cell growth. These findings are also supported by viability assays reported in our previous study, where a reduction in HepG2 cell viability was noted with treatment (41).

Our findings are also consistent with previous reports demonstrating that miR-30a also suppresses KRAS expression in HCC cell lines. Kun Zhou et al. (51) in 2017 depicted that miR-30a suppresses KRAS protein expression through inhibiting its translation. KRAS, a RAS family proto-oncogene protein, is a central regulator of several cellular functions. KRAS dysregulation can disrupt intracellular signaling pathways like RAF/MEK/ERK and PI3K/Akt/mTOR, which in turn will lead to aberrant regulation of cell proliferation, differentiation, and survival (52, 53). A number of reports have indicated that KRAS expression in HCC or LIHC is tightly linked to changes in p53 expression and disease progression. p53, a classical tumor suppressor, controls the expression of various oncogenes, and its downregulation or mutation tends to result in oncogene expansion. In this study, we found that miR-217-5p treatment upregulated p53 expression in HepG2 cells (**Figure 10**). This upregulation is most likely mediated by the downregulation of KRAS, as KRAS was determined to negatively regulate p53. Restoring p53 function therefore has anti-proliferative effects, as evidenced by the decreased viability of HCC cells observed in our previous experiments (19, 41, 54).

In Summary, our study highlights the significant role of miR-217-5p as a tumor suppressor in HCC based on its ability to target oncogenic genes, mainly KRAS, which is a core gene discovered in our in silico and hub analysis. Functional enrichment revealed that miR-217-5p regulates key biological processes, particularly cell population proliferation, with high involvement in the PI3K-Akt and HCC-specific pathways. Experimental verification using CS-NPs as a delivery system demonstrated that miR-217-5p mimics greatly increased intracellular concentrations of miRNA, suppressed KRAS expression, and subsequently reduced HepG2 cell viability. Furthermore, miR-217-5p-mediated inhibition of KRAS was accompanied by a corresponding increase in p53 expression, which suggests tumor suppressor pathway restoration and cancer cell proliferation inhibition. These findings not only provide solid evidence that miR-217-5p is an essential regulator in HCC development at the molecular level but also present miR-217-5p replacement therapy as a new potential therapeutic strategy and prognostic biomarker for HCC treatment.

Acknowledgment

Thanks to the LRT, LPPT, and Pharmacology staff of technicians of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Conflict of interest

None declare.

Funding

None declare.

Ethical statement

The approval for conducting this research was granted by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia with reference number: KE/FK/0918/EC.

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