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Original Article

Investigating the effect of Curcumin on Long Noncoding RNAs NUTM2A-AS1 and HCG18 expression changes in Hepatocellular Carcinoma (HCC)

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Abstract

Hepatocellular carcinoma (HCC) is a common tumor in liver tissue. The lack of molecular markers for the diagnosis and evaluation of treatment methods is noticeable despite the progression of HCC diagnostic and treatment methods; therefore, the present study aimed to find molecular markers with key roles in the initiation and progression of HCC and investigate the impact of curcumin on their expression. To this end, bioinformatics studies were conducted to candidate two long noncoding RNAs (lncRNAs) related to HCC, which interacted with the highest number of microRNAs (NUTM2A-AS1 and HCG18), and five microRNAs (hsa-miR-15a-5p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-195-5p, and hsa-miR-424-5p), which interacted with most lncRNAs among candidate lncRNAs. Afterward, their expression was measured in hepG2 cancer cells and fibroblast cells treated with curcumin compared to the untreated samples. The expression of miRNAs and lncRNAs at half inhibition concentrations (IC50) of curcumin indicated that curcumin significantly increased the expression of miR-195, miR-15a/16, and miR-424 and reduced the expression of miR-15b-5P, NUTM2A-AS1 lncRNA, and HCG18 in comparison with a control group ($P \leq 0.05$). Obtained data showed that curcumin increases the expression of anti-cancer genes, miR-195, miR-15a/16, and miR-424, and decreases the activity of cancerous genes, miR-15b-5P, NUTM2A-AS1 lncRNA, and HCG18; therefore, it can be used as an anti-cancer agent in the treatment of HCC.

Keywords: Long noncoding RNAs (lncRNAs); Liver cancer; Curcumin.

1. Introduction

Hepatocellular carcinoma (HCC) is a common type of tumor in liver tissue and accounts for approximately 80% of hepatic malignancies in human societies [1]. In terms of prevalence, this malignancy is the seventh most common carcinoma among males and the ninth most common carcinoma among females. Differences among different populations, such as racial differences, ecological differences, lifestyles, and distribution of risk factors

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worldwide, have made differences in the prevalence of HCC in the world [1-4].

According to clinical experience, HCC is often resistant to chemotherapy and has a weak clinical response to routine chemotherapy methods [5]. This group of malignancies, particularly HCC, as the most common liver malignancy, is treated with a combination of medical treatments, liver transplantation, and surgical procedures [6]. Given different responses of these malignancies to existing treatments and the affectability of this treatment by the disease degree and severity, its course of recovery, and prognosis, experts have paid attention to the discovery and application of molecules related to this disease and the targeting the molecules by effective treatment methods to increase the patient's life expectancy. Furthermore, using cellular and molecular markers is clinically important to monitor patients' responses to treatment and estimate their disease prognosis [7, 8].

In recent years, researchers have considered identifying genes that are disease agents or related to cancers, owing to the importance of detecting cancer's cellular and molecular pathophysiology in their treatment and monitoring procedures [9]. Molecular markers are noticeable for the diagnosis and evaluation of the treatment method. Accordingly, it is crucial to develop strategies for early diagnosis, prognosis, prediction, and treatment goals of HCC patients [10].

MiRNAs and lncRNAs are molecular markers that have been recently considered in this field. MiRNAs are micro-noncoding RNAs that regulate biological processes such as cell cycle, proliferation, differentiation, apoptosis, stress tolerance, energy metabolism, and immune response. miRNAs regulate approximately 30% of human genes. Half of the genes are tumor-related. Dysregulation of miRNAs in tumor cells exhibits their modulatory effects on tumor growth. Some miRNAs may act as tumor genes, some as tumor suppressor genes, and others may play both roles [11]. Abnormal expression of miRNAs in cancers can be due to dysregulation of key transcription factors such as c-Myc and p53. Like protein-coding genes, miRNAs are believed to be susceptible to epigenetic modulation and can contribute to tumorigenesis [12]. lncRNAs are RNA transcripts not coded for proteins. They perform their specific functions based on their secondary structure and interact with other biological molecules such as RNA, DNA, protein, miRNAs, and other basic factors in normal cell biological activities. The molecules play a key role in biological processes by various mechanisms. Among the mechanisms are inhibiting miRNAs, contributing to chromatin rearrangement, and affecting protein stability. Inhibition of miRNA and many of these molecules are associated with human diseases such as gastric, breast, colon, and lung cancers [13].

Higher knowledge about the etiology of HCC at genetic, epigenetic, and molecular levels, which can be utilized to find new diagnostic-treatment methods for personalized medicine, is a way that has developed the finding of new treatments and increased the effect of adjuvant therapies. Given the significance of molecular and cellular biomarkers, the present study sought to

investigate the roles of two lncRNAs, AS1-NUTM2A and HCG18, in hepatocellular carcinoma by emphasizing the importance of lncRNAs in HCC. According to the possible roles of existing markers in HCC, the present study investigated the impact of a chemical with anti-cancer properties (curcumin) on the Hep-G2 cell line as an appropriate representative for HCC. It also examined the companionship and co-expression of the selected network and the mechanisms of action of these molecular markers on the diagnosis and treatment of this cancer.

2. Materials and Methods

2.1. Bioinformatics analysis

A list of lncRNAs involved in liver cancer with the most miRNAs-binding sites was prepared in this study using the bioinformatics technique. Accordingly, a dataset containing the complete list of lncRNA-miRNA interaction was first prepared from the web server StarBase v2.0 and then lncRNAs with differential expression in HCC. The Cancer RNA-Seq Nexus web server and a complete list of miRNAs with changed expression in liver cancer obtained from the dbDEMC 2.0 web server were employed to prepare this dataset. After that, a list of lncRNAs involved in liver cancer with the furthest miRNA-binding sites was obtained by combining the three mentioned datasets to create a new dataset. The lncRNAs were then ranked from the highest to the lowest based on the number of targeted miRNAs to select the appropriate lncRNA. LncRNA filtering in terms of expression was the final step. In other words, including a lncRNA in the list was not the reason for their selection for laboratory study; hence, miRNAs, which interacted with lncRNAs, were detected, and their expression correlation with desired lncRNAs in the cell line was investigated. Therefore, a dataset containing the complete list of mRNA-miRNA interaction was prepared from the dataset of the StarBase v2.0 web server, and then the dataset of mRNAs with differential expression in HCC was prepared (the whole data belonged to TCGA) to find some proteins that were probably in the regulatory pathway of the selected lncRNAs. Afterward, as mentioned earlier, the three datasets were mixed to create a new dataset, and the mRNA with the highest binding site with miRNA was selected as a candidate gene.

2.2. Cell lines and cell culture

The hep-G2 cell line, as a representative of HCC, and the fibroblasts (primary cells), as a normal cell line, were purchased from the Iranian Biological Resource Center. This is an immortal cell line that was derived in 1975 from the liver tissue of a 15-year-old boy with well-differentiated hepatocellular carcinoma. These cells are epithelial in terms of morphology and secrete different types of main plasma proteins, for example, albumin and acute phase proteins of fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferrin, and plasminogen. HepG2 cells are a suitable model for the intracellular study of human liver cells in laboratory conditions due to their high degree of morphological and functional differentiation in vivo. These cells can be used to study human liver diseases and as a model system for studying liver metabolism and toxicity of Xenobiotics, understanding hepatocarcinogenesis, and drug target studies.

Both cell lines were cultured according to the protocol presented by ATCC (HB-8065, HYR0103) [14]. Accordingly, both lines were cultivated at 37ºC, 95% humidity, and 5% CO_2 pressure. Cell passage was performed when their density reached 90% using 0.05% Trypsin-EDTA (Pan Biotech, Germany). It is worth noting that DMEM (Dulbecco's Modified Eagle Medium; Pan Biotech, Germany) [15, 16] was applied for both cell lines according to the ATCC protocol, and 10% FBS and 1% Pen-strep was added to the culture medium.

2.3. Cell viability determination by cell counting

A total of 100 µl uniform cell suspension was poured into a test tube under sterile conditions, and then Trypan blue dye was added equal to its volume, and a drop of the resulting mixture was taken after a few minutes and pipetting to measure the cell viability percentage. Then, cell counting was performed from the Neubauer lam and lamellar in the boxes related to white blood cell count. Finally, the cell viability percentage was obtained by counting the cells that turned blue using the following equation. Viability (%) = live cells/live cells and dead cells × 100

2.4. Cytotoxicity assay by MTT

The MTT-colorimetric method was used to investigate the cytotoxic effects of pharmaceutical compounds on the growth and proliferation of cancer cells and determine the IC50 of the compounds [17]. Curcumin was purchased from Sigma-Aldrich Corporation, and both normal and cancer cells were treated with different concentrations based on previous articles (10, 20, 30, 40, 50, and 60 µM) for 24, 48, and 72 hours [18]. The culture medium was drained, and 100 µl of the prepared concentrations of curcumin was added instead. The following equation calculated the viability percentage of treated cells:

% Cell viability= (mean absorbance of treated cells/mean absorbance of control cells) × 100

The data (toxicity percentage) of the samples and controls (untreated cells) were examined using the Pharm-PCS statistical package [19], and the exact level of corresponding IC50 was obtained to determine the 50% lethality dose of the drugs on cancer cell lines.

2.5. RNA extraction and cDNA synthesis

Genomic RNA was extracted and purified from the cell line according to the instructions of the RNA extraction kit, called QIAamp® miRNeasy Mini Kit, with the catalog number 217004 made by Qiagen Company, using the column-based method [20]. The samples were kept at -70ºC until conducting the tests after extraction. Furthermore, the RevertAID TM Firs Standard cDNA synthesis kit (Fermentase) was utilized for cDNA synthesis based on the manufacturer's instructions [21].

2.6. Gene expression assessment using the Real-Time PCR method

The Rotor gene device by Qiagen Company was used to assess gene expression. The primers of each gene were also designed using the Primer 3 software and synthesized and used by Bioneer Company (Germany) (**Table 1**). The reactions were based on the use of cyber probes. Real-time PCR data was analyzed based on the threshold cycle comparison.

Table 1. List of primers used in the study.

Primer	Forward	Reverse
miR-15a-5p	GCAGGGTCCGAGGTA	CGTGGCCGTTAAACTTCTGC
miR-15b	ACGGTTAGCAGCACAT	GCGCCCAATACGACCAAATC
mir-16-5p	GGCATAGCAGCACGTA	AATGGGCAGCCGTTAGGAAA
miR-424-5p	GGAACAGCAGCAATTC	ATCACTGTAAAACCGTT
miR-195-5p	GCCAATATTTCTGTGCTGCTA	GACGCACCGCAGTGTTC
NUTM2A-AS1	CATTATCTCACTGCCTCCGGT	GTGGAGGCTCCCGAGAGAAG
HCG18	AGGCTGTCTAGTCCAGGCTC	GAAGTGACGTGCCAGCTTAG

The present study measured the difference of the threshold cycles obtained from the test samples (cells treated with the drug) and the control samples (cells not treated with the drug), and then the ratio of target to reference gene (GAPDH) was measured using the ΔΔCt equation as follows:

ΔCt=Ct target-Ct reference, ΔΔCt=ΔCt test sample- ΔCt Control sample, Relative expression: $2^{-\Delta\Delta Ct}$

After the reaction, Ct was measured using the Rest 2009 software, ΔΔCt was calculated for each sample, and then the change in the expression of each sample was estimated using the GraphPad Prism 6 software.

2.7. Statistical analysis

Each test was repeated three times to avoid user error. Data analysis was conducted using the chi-square test, Fisher's exact test, independent t-test, and one-way analysis of variance (ANOVA) by the SPSS 22 software.

3. Results and Discussion

3.1. Cell viability analysis

The effects of curcumin toxicity on cell lines were first investigated. To this end, the cytotoxic effect of curcumin was evaluated at concentrations of 10, 20, 30, 40, 50, and 60 μM against the hepG2 cancer cell line of HCC during 24, 48, and 72 hours, and the viability of cancer cells treated with 10 and 20 μM did not indicate any significant difference at 24, 48, and 72 hours. Moreover, the viability rate of the treated cancer cells decreased drastically at concentrations of 30, 40, 50, and 60 μM over time so that the lowest viability rate occurred 72 hours after the treatment; however, the difference was statistically significant (P<0.001) (**Figure 1 A**). The evaluation of the cytotoxicity effect of curcumin at the concentrations mentioned above and time intervals against the liver fibroblast cell line indicated that the treatment of cancer cells with curcumin at different concentrations during the first 24 hours had no significant effect on reducing the viability of fibroblast cells, and only 60 μM curcumin could significantly reduce the viability of fibroblast cells at 24 hours. Furthermore, the treatment of cancer cells at different concentrations of curcumin for 48 and 72 hours significantly decreased the viability of fibroblast cells; thus, increasing the concentration decreased the viability rate (P<0.001) (**Figure 1 B**). Accordingly, 50 μM curcumin at a 24-hour time interval was its optimal concentration to reduce the cancer cell viability, and it had no significant effect on fibroblast cells.

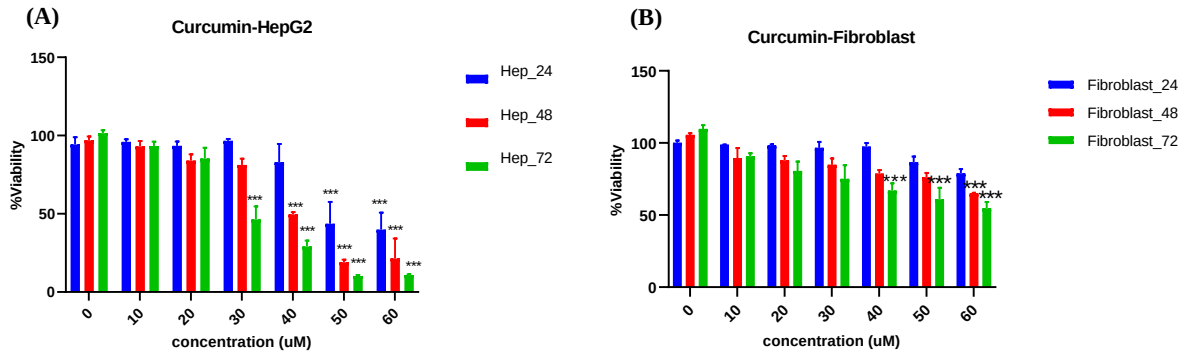

Figure 1. (A) The cytotoxic effect of 10, 20, 30, 40, 50 and 60 µM curcumin concentrations on HepG2. (B) Fibroblast during 24, 48, and 72 hours. A significance level of P<0.05 was considered with three repetitions. *** indicates P<0.05.

3.2. Gene expression changes

According to the results of the bioinformatics method, five miRNAs, namely hsa-miR-15a-5p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-195-5p, and hsa-miR-424 5p, and two lncRNAs, namely NUTM2A-AS1, and HCG18, were selected as candidates. Accordingly, changes in the expression of miRNAs hsa-miR-15a-5p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-195-5p, and hsa-miR-424-5p, and lncRNAs NUTM2A-AS1 and HCG18 were studied hepG2 cancer cells and normal fibroblast cells treated with IC50 of curcumin. Based on the findings, the expression of miRNAs 15 a-5p, 16, 195, and 424 significantly increased in both cancer and normal cell lines compared to the control group (untreated cells) (P<0.001). On the contrary, the expression of miR-15b-5p and lncRNAs NUTM2A-AS1 and HCG18 significantly decreased in both cell lines treated with curcumin compared to the control group (P<0.001) (**Figure 2**).

The results of the present study indicated that curcumin at high concentrations significantly decreased the viability of cancer cells. It should be noted that obtained data showed that an IC50 of curcumin could increase the expression of miR-15a/16, miR-195, and miR-424 and decrease the expression of miR-15b-5P and lncRNAs NUTM2A-AS1 and HCG18.

Curcumin is a polyphenol extracted from the rhizome and root of turmeric. It has potent anti-inflammatory, antioxidant, and anti-tumor properties and low cytotoxicity for normal cells. Given the high prevalence and lethality of HCC, the development of safe and effective therapies to improve the effectiveness of the HCC treatment has always been essential.

It has been reported that curcumin can prevent the proliferation of various cancers, including HCC [22]. You et al. also reported that it suppressed the HCC growth by reduction of SREBF1. Curcumin can also decrease the proliferation of HCC SMMC-7721 cells by regulating the AMPK signaling pathway. Another study reported that curcumin significantly inhibited HCC growth and induced HCC cell apoptosis in vivo and in vitro, indicating that the induced apoptosis may be a primary mechanism for curcumin-mediated growth inhibition [23-26]. The present study indicated that curcumin could have a growth-inhibitory effect on cancer cells at high concentrations, and the effect was dose-dependent and time-dependent and had no cytotoxic effect on HepG2 cancer cells at low concentrations.

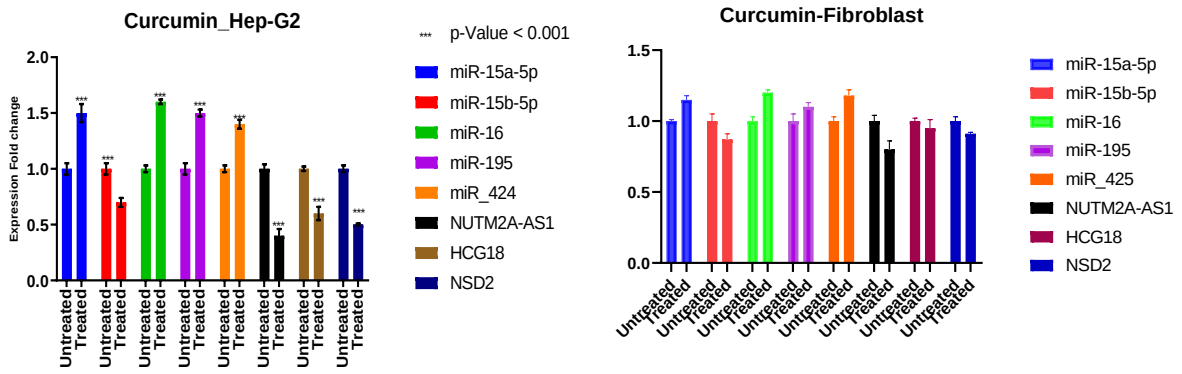

Figure 2. Expression changes of hsa-miR-15a-5p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-195-5p and hsa-miR-424-5p and NUTM2A lncRNAs -AS1 and HCG18 in HepG2 cancer cells (A) and fibroblasts (B) treated with IC50 concentration of curcumin with three repetitions. Untreated: Control cells (Cells not treated with curcumin), Treated: Cells treated with curcumin.

However, it significantly decreased the viability of normal cells at all its concentrations at time intervals of 48 and 72 hours and had a more toxic effect against normal cells at higher concentrations.

Furthermore, the slope of cytotoxicity induction was lower in normal cells by curcumin, consequently

The viability of normal cells was almost six times higher at a concentration of 60 µM after 72 hours than that of cancer cells, indicating that this substance was much more toxic against cancer cells than normal cells at high concentrations. The present study also indicated that high concentrations of this compound could induce apoptosis in cancer cells. The results are also evident according to a comparison of IC50s of curcumin. Xu et al. (2013) reported that curcumin inhibited proliferation and induced apoptosis of HCC in a concentration-dependent manner [27]. HSU et al. (2015) investigated the impact of curcumin, radiation, and a mixture of both on cell viability, NF-kB activation, apoptosis, and the expression of NF-kB downstream effector proteins using the MTT assay. Their results indicated that curcumin significantly increased radiation-induced cytotoxicity and MMP reduction and inhibited the radiation-induced NF-κB activity and the expression of NF-κB downstream effector proteins in HCC cells. Hence, they suggested that curcumin could increase the anti-cancer effects of radiation by suppressing the activation of NF-κB [28]. Sanaei et al. (2018) reported that 1, 5, 10, 25, and 50 µM curcumin had anti-cancer effects against HCC [29]. Kavousi et al. (2018) also considered curcumin a potent agent capable of inhibiting cell growth and inducing apoptosis in HCC cells [29].

Given that numerous miRNAs, lncRNAs, and proteins contribute to tumor development and cause many malignancies. Since liver cancer is common worldwide [12, 13], the present study investigated the interactions of miRNA-lncRNA and miRNA-miRNAs with differential expression in HCC using a bioinformatics study. According to the findings, biomarkers, including five miRNAs, namely hsa-miR-15a-5p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-195-5p, and hsa-miR-424-5p, and two lncRNAs, namely NUTM2A-AS1 and HCG18, played significant roles in HCC.

According to the results of studies, the expression of hsa-miR-15a-5p significantly decreased in tumor tissues and colon cancer cells [30]. Dong et al. (2019) reported that, unlike miR-15a-5p, miR-15b-5p was often upregulated in liver cancer cells and tissues, and a high expression level of miR-15b-5p was associated with the tumor-node-metastasis stage, invasion, and low prognosis. Furthermore, the overexpression of miR-15b-5p increased the HCC cell proliferation and invasion in vitro. The results also indicated that miR-221-3p and miR-15b-5p might play crucial roles in the occurrence of liver cancer and could accelerate its progress [31]. MiR-195 is another agent in malignancies such as gastric, breast, colon, and cholangiocarcinoma. Chen et al. (2019) considered miR-195 a critical regulator in HCC-lung metastasis using the BEL-7402 lung metastasis-HCC cell line. Furthermore, the expression of miR-195 decreased in HCC tissues and cell lines; thus, miR-195 inhibited cell proliferation by targeting AEG-1 in HCC. In this study, the lower miR-195 expression was associated with HCC progression [32]. MiR-16 often plays a protective role in many types of cancer, such as chronic lymphocytic leukemia, prostate cancer, hepatocellular carcinoma (HCC), breast cancer, ovarian cancer, non-small cell lung cancer (NSCLC), gastric cancer, pituitary adenoma, and multiple myeloma. MiR-16 is generally considered a key tumor suppressor miRNA. It can modulate the cell cycle, inhibit the cell proliferation, reduce cell invasion, promotes cell apoptosis, and suppress tumorigenesis in various cancers by targeting Bcl-2, CCND1 (cyclin D1), CCND3 (cyclin D3), CCNE1 (cyclin E1), CDK6 (cyclin-dependent kinase 6), and WNT3A (Wingless Type MMTV Integration Site Family, Member 3A) [33]. MiR-424-5p is introduced as an inhibitory factor of HCC in regulating various physiological activities. Piao et al. reported that the miR-424-5p expression decreased in HCC patients, and it caused the apoptosis of HCC cells by targeting and inhibiting the expression of YAP1 [34]. Li et al. (2019) reported that WEE1 was a target of miR-424-5p, and its overexpression caused the proliferation, migration, and invasion of HCC cells [35]. Another study acknowledged that miR-424-5p was a tumor suppressor gene, could inhibit the invasive ability of HCC cells by direct regulation of TRIM29, and could reveal its potential as a new prognostic indicator [36]. In the present study, curcumin could increase the expression of miR-15a/16, miR-195, and miR-424 and decrease the expression of miR-15b-5P. According to the results of studies, the higher expression of miR-15a/16, miR-195, and miR-424 played a protective role in the incidence and treatment of HCC. Moreover, a higher expression of miR-15b-5P increased the incidence of HCC.

NUTM2A-AS1 is a long noncoding RNA (lncRNA) with higher expression in liver and lung cancers. Additionally, the oncogenic role of HCG18 as a lncRNA in gastric cancer can be observed through the P13k/Akt pathway and the miR214-3p/CENPM axis in HCC [37, 38]. Studies indicate that NUTM2A-AS1 is upregulated, and its promoter region is hypomethylated in lung adenocarcinoma. NUTM2A-AS1 hypomethylation and its upregulation may facilitate its role as an oncogene. However, there is no report of the

underlying molecular mechanism for the effects of NUTM2A-AS1 on liver cancer. Studies show that the programmed death ligand one partially decreases the tumorigenesis of gastric cancer cells and the drug resistance of NUTM2A-AS1 and miR-376a. Recently, it has been reported that miR-376a is associated with NUTM2A-AS1 and is critical for NUTM2A-AS1-induced tumorigenesis [39]. Other studies have also reported that lncRNA molecules are abnormally expressed in tumors where they can act as oncogenes or tumor suppressors depending on the type of cancer. A study on the number of aberrantly-expressed lncRNA molecules demonstrated that NUTM2A-AS1 was upregulated in some cancers and might act as an oncogene [39]. LncRNA HLA complex group 18 (HCG18) acts as an oncogene in colorectal and nasopharyngeal carcinoma. HCG18 contributes to the growth and metastasis of colorectal cancer cells through the regulation of miR-1271 and MTDH/Wnt/β-catenin pathways. According to a study, HCG18 is significantly upregulated in HCC cells and tissues, and its expression negatively correlates with the prognosis of HCC patients. Accordingly, HCG18 can act as an oncogene in HCC. The results also indicate that HCG18 is strongly expressed in HCC tissues, and the silencing of HCG18 prevents proliferation and migration while inducing the apoptosis of HCC cells [38]. HCG18 promotes gastric cancer proliferation by absorbing miR-141-3p and miR-197-3p. It also contributes to lung adenocarcinoma progression through the miR-34a-5p/HMMR axis and can accelerate the colorectal cancer invasion by a miR-1271/MTDH/Wnt/β -catenin dependent method. Another group of studies detected HCG18 as one of the 30 upregulated lncRNAs in breast cancer by analyzing the data of two groups in the Cancer Genome Atlas (TCGA) [40]. According to the studies mentioned above, the expression of these lncRNAs increases in cancer cells, and they act as oncogenes in cancerous cells. Their expression is expected to decrease after treatment with anti-cancer agents, which is consistent with our results.

4. Conclusion

Based on the results, curcumin was a key anti-cancer agent for treating liver cancer. The results were also analyzed by examining the expression of some lncRNAs and miRNAs, and it was found that the IC50 of curcumin could increase the expression of miR-15a/16, miR-195, and miR-424 and decrease the expression of miR-15b-5P, lncRNAs NUTM2A-AS1, and HCG18. Thus, the present study introduced curcumin as a potential anti-cancer agent against the HCC cell line by affecting changes in the expression of miRNAs and lncRNAs.

Conflict of interest

The authors declare to have no conflict of interest.

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