

ORIGINAL ARTICLE

Zingiberene targets the miR-16/cyclin-B1 axis to regulate the growth, migration and invasion of human liver cancer cells

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Summary

Purpose: Liver cancer or hepatocellular carcinoma (HCC) is considered as one of the most frequent malignancies with significantly high morbidity and mortality across the globe. MicroRNAs (miRs) are regarded as important regulators of liver cancer formation and its development. However, the full biochemical mechanism of their role is still very less understood. The main objective of the current research work was to examine the role of miR-16/cyclin-B1 axis in liver cancer regulation and how this pathway along with liver cancer migration and invasion are targeted by zingiberene molecule.

Methods: Quantitative reverse transcriptase polymerase chain reaction was used to evaluate miR-16 expression in HCC cell lines. Western blotting was performed to evaluate the expression of the miR-16 target genes. Effects on cell migration and invasion were evaluated by in vitro wound healing assay and transwell Matrigel assay, respectively. Ef-

fects of zingiberene on HCC cell viability were evaluated by MTT assay.

Results: Zingiberene treatment led to downregulation of miR-16 in HepG2 human hepatocellular carcinoma cells, accompanied by induction of G0/G1 cell cycle arrest targeting cyclin B1 as direct target. These effects were also accompanied by inhibition of cell migration and invasion, indicating that miR-16 can have a significant role as liver cancer suppressor after zingiberene treatment. Luciferase reporter assay confirmed that miR-16, which was one of HCC down-regulated miRs, directly targeted Cyclin B1 in HCC cells

Conclusion: The current study indicates miR-16/cyclin B1 axis might have significant applications as a therapeutic target for patients with liver cancer.

Key words: zingiberene, miR-16, liver cancer, cell migration, cell invasion

Introduction

Liver cancer (LC) is a frequently occurring primary liver malignancy accompanied with high mortality globally [1]. LC ranks 5th among the frequently prevailing malignancies and 3rd in cancer-associated mortality worldwide [2]. LC incidence is more likely in males than in females with an

approximate ratio of 2.4:1. Several risk factors are responsible for tumorigenesis and development of LC including hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, fatty diet, high alcohol consumption and exposure to aflatoxin B1 [3-6]. Besides these major risk factors, molecular

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Received: 18/01/2020; Accepted: 09/02/2020

mechanisms of LC development remain weakly understood. So far, treatment options for LC are mostly unproductive and limited [7,8]. In early stage treatment of LC, the only available curative treatments are liver transplantation or surgical resection [9,10]. Presently there is only one therapeutic drug (Sorafenib) used for unresectable LC treatment with lower efficacy and poor survival rate [11]. miRs are endogenous noncoding and conserved small RNAs comprising of 22 nucleotides, and have been reported to be involved in controlling gene expressions through post-transcriptional silencing of genes in numerous biological systems. Accumulating evidence from various research data reveal *de novo* role of miRs in various types of LC including hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and hepatoblastoma [12-16]. MiRs have been reported to play both carcinogenic and anticarcinogenic role in LC due to their involvement in alterations of oncogene expressions [17]. Examples of LC suppressive miRs include miR-29, miR-101, miR-122, miR-145, miR-195, miR-214, miR-370 and miR-370 and those with oncogenic role are miR-21, miR-155, miR-221, miR-222 and miR-224 [18-20]. MiRs have been reported with cancer suppression through alterations in different cellular pathways which results in apoptotic, autophagic or necrotic cell death.

Natural products are a rich source of natural medicines and have been used since early times. Zingiberene, a naturally occurring monocyclic sesquiterpenoid, major constituent of ginger oil extracted from *Zingiber officinale* (predominant in South-East Asia), has been reported with several medicinal uses including carminative, anti-inflammatory, digestive and antiemetic [21-23]. Herein, the current study was framed to unveil the anti-cancer effects of naturally occurring Zingiberene molecule in HCC cells via targeting of miR-16/cyclin-B1 axis through regulation of cell growth, migration and invasion.

Methods

MiR analysis through real-time quantitative PCR

Using TRIzol reagent (Invitrogen, Carlsbad, USA) complete RNA was obtained from HCC HepG2 cells. Assisted with AMV reverse transcriptase (GIBCO, Galthersburg, MD, USA) complementary DNA was created. The following primers were utilized to perform PCR reactions: 5'-GGTATTTTGGTCTGACTGCTTGC-3' (reverse) and 5'-GACCTGTGTCAAGCTTTCTCTG-3' (forward) for CCNB1; 5'-GAAGGCTGGGGCTCTCATTT-3' (reverse) and 5'-CAGGAGGCATTGCTGATGAT-3' (forward) for GAPDH. Using $2^{-\Delta\Delta CT}$ method the relative expression of CCNB1 was normalized to endogenous GAPDH control. Ex-

traction of miR was performed using miR isolation kit (miRNeasy KIT, Qiagen, Germany) sticking tightly to the manufacturer's instructions.

Cell cytotoxicity and colony formation assay

MTT cell cytotoxicity assay was performed to detect the viability of HCC cells after Zingiberene treatment. Briefly, with an initial concentration of 1×10^5 cells per each well, cells were seeded into 96-well plates and incubated at 37°C in a 5%CO₂ incubator. Afterwards, cells were treated with changing Zingiberene concentrations (0, 10, 25, 40 and 100 µM) for 24 h and 48 h respectively and incubated under the same environment. Next, 5mg/ml of MTT standard solution was put into each well replacing cultural media and each well was incubated for a further 4 h. To each well 150 µL of DMSO was added and subjected to oscillations for about 10 min. Finally, using ELISA reader, absorbance was measured at 620 nm with a reference wavelength set at 490nm. Clonogenic assay was performed to check the effect of Zingiberene on colony formation potential of HCC cells. Six-well plates were used for seeding Zingiberene-treated cells with 600 cells per each well and for two weeks. After completion of culturing cell, colonies were fixed in cold methanol followed by crystal violet staining (0.1%). Colonies with over 50 cells were considered for counting.

Study of cell structural changes

Cell structural changes in HCC cells after Zingiberene treatment were studied through phase contrast inverted microscopy. In brief, human HCC cells were incubated after seeding in 12-well plates with an initial concentration of 4×10^5 per well. Cells were exposed Zingiberene in variant concentrations (0, 10, 25 and 100 µM). Treatment was followed by removal of RPMI-1640 and cells were washed using phosphate buffered saline (PBS). Structural changes were recorded through phase contrast inverted microscope (Leica DMI 3000B, Germany) at a magnification of 200x.

Acridine orange (AO)/ethidium bromide (EB) staining and annexin V-FITC/PI dual staining for apoptosis analysis

Apoptotic analysis of HCC cells was performed through fluorescence microscopy via performing AO/EB and annexin V-FITC/PI staining assays. For AO/EB staining, cells were processed with 0.25% trypsin at the logarithmic phase of growth and with 10% fetal calf serum (FCS) as culture media and then cells were put onto the 96-well plates with a density of 2×10^5 cells/well and incubated. HCC cells were exposed to variant doses of Zingiberene molecule (0, 10, 25 and 100 µM). Afterwards, cells were exposed to 20 µl trypsin followed by transference to glass slides quickly after cells sloughed off. Each suspension on glass slides were supplied with 100 µg/ml AO and 100 µg/ml EB and covered with a coverslip. Finally, apoptotic cell morphology was recorded with fluorescent microscope (Olympus, Japan). For annexin V-FITC/PI assay, Annexin V Apoptosis Detection Kit (BD, Biosciences, San Jose, USA) was used for about 15 min, strictly following the manufacturer's instructions at room temperature, followed by

flow cytometric analysis for apoptotic cell percentage determination.

Distribution of cell cycle phases

80% confluence after Zingiberene treatment at variant doses (0, 10, 25 and 100 μM) the HCC cells were subjected to PBS washing twice. Cells fixed with cold ethanol overnight at 4°C followed by filtration using cell strainer (0.05 mm). Afterwards, cells were incubated with PBS comprising 0.2% (v/v) Triton X-100, 100 $\mu\text{g/mL}$ RNase A and 50 $\mu\text{g/mL}$ PI and finally examined through flow cytometry (C6, BD, NJ, USA).

Wound healing migration assay

HCC cells were plated onto 6-well plates at an initial density of 1×10^5 cells/well reaching early confluence. Incubation lasted overnight. A pipette tip was used to scratch a wound in HCC cell unilayer, followed by mild PBS washing and incubation with serum-free RPMI-1640 medium. The healing ability of HCC cells was recorded by capturing individual sections after 0 and 72 h of Zingiberene treatment (0, 10, 25 and 100 μM).

Cell invasion assay

Cell invasion potential of HCC cells after Zingiberene treatment of variant concentrations (0, 10, 25 and 100 μM), was estimated via transwell assay with Matrigel. The upper chamber was filled only with RPMI-1640 medium and nearly 200 μL of cell culture. The lower chamber was placed with only 600 μL of the same medium containing 10% FBS, followed by incubation for 24 h at 37°C. Additionally, the cells were fixed in methanol and stained with crystal violet staining dye. An inverted microscope was utilized to analyze and count the invaded cells at a magnification of 200x.

Western blotting analysis

Cultured HCC cells were treated with variant doses of Zingiberene (0, 10, 25 and 100 μM), followed by lysing with RIPA lysis buffer. Ten μg of total protein were subjected to 8% SDS-PAGE gel. Thereafter, proteins were subjected to electrophoresis followed by primary and secondary antibody treatment, respectively. Proteins were finally transferred to PVDF membranes and protein bands were visualized under enhanced chemiluminescence.

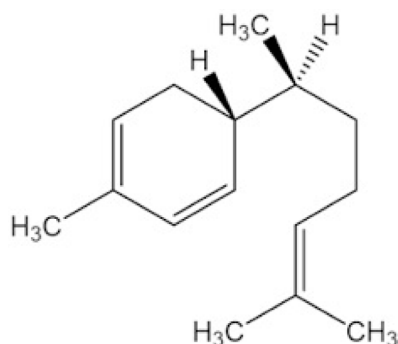


Figure 1. Chemical structure of Zingiberene molecule.

Statistics

All the experimental data are presented as mean $\pm$ SD. One-way analysis of variance (ANOVA) for analysing data and Students-Newman-Keuls test for multiple comparisons was carried out. Statistically significant difference was set at $p < 0.05$.

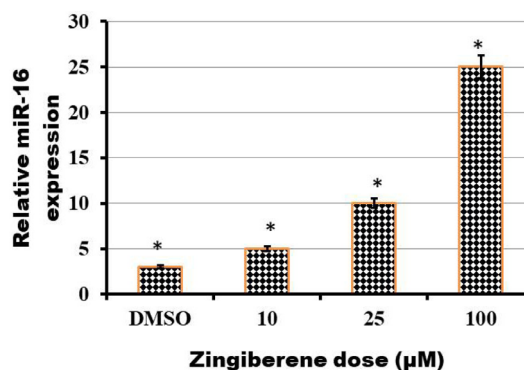


Figure 2. Real-time quantitative PCR analysis of HCC cells treated with Zingiberene molecule at indicated doses. Data are shown as mean $\pm$ standard deviation ($n=4$). The experiments were performed in triplicate (* $p < 0.05$).

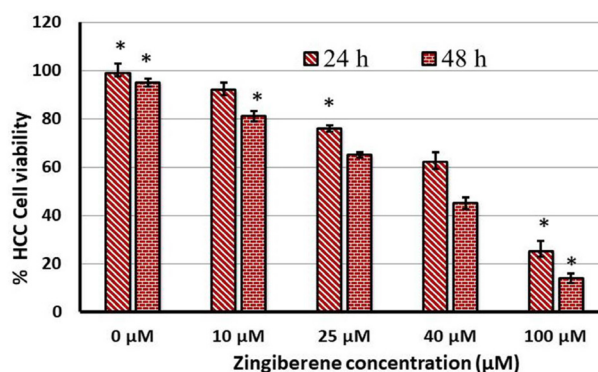


Figure 3. Impact of increased miR-16 expression (increased Zingiberene concentration) on cell viability of HCC cells at indicated doses with exposure time intervals of 24 h and 48 h. Data are shown as mean $\pm$ standard deviation (with repetitions $n=4$). The experiments were performed in triplicate (* $p < 0.05$).

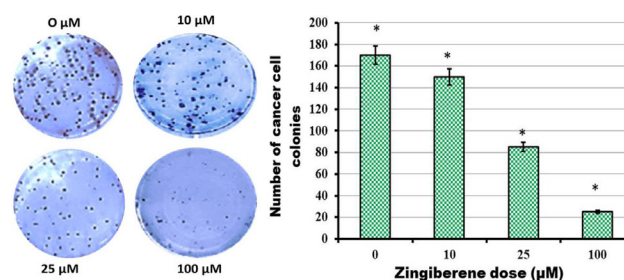


Figure 4. Clonogenic assay for determination of colony inhibition effects of Zingiberene in HCC cells. Data are shown as mean $\pm$ standard deviation (with repetitions $n=4$). The experiments were performed in triplicate (* $p < 0.05$).

Results

Effect of Zingiberene molecule on expression of miR-16

Real-time quantitative PCR analysis was performed to check the effect of Zingiberene (Figure 1) on miR-16 expression. There was a substantial increase in the miR-16 on treatment expression. The miR-16 expression at control (only DMSO) was found to be very low, nearly 2.5, but on increasing the Zingiberene dose to 10 μ M its expression began

to rise from 2.5 to 5. At a concentration of 100 μ M the expression reached 25. Thus it is evident that on increasing Zingiberene concentration there is a swift increase in the expression of miR-16 (Figure 2).

Effects on cell growth and colony formation of HCC cells by increased miR-16 expressions mediated by Zingiberene

The effect on cell growth of HCC cells by Zingiberene was assessed through MTT cell growth assay. On treatment with variant doses there was a remarkable reduction observed in cell viability of target cells. Cell viability was observed for both concentration and time dependence and the results revealed that on increasing the concentration from control to 100 μ M, cell viability reduced from 100% to nearly 25% on 24-h exposure and on 48-h exposure the viability dropped upto 15%. Hence it is clear that Zingiberene molecule led to both dose as well as time-dependent inhibition of cell viability (Figure 3). Furthermore, the impact of Zingiberene on colony formation potential of HCC cells was evaluated through clonogenic assay and the results revealed that after two weeks of drug treatment there was a significant suppression on colony formation with increasing molecule doses. The number of cell colonies reduced from 170 to 25 (Figure 4). Hence, from MTT and clonogenic assay data it can be concluded that on increasing Zingiberene concentration miR-16 expression was associated with suppression of cell viability and colony formation.

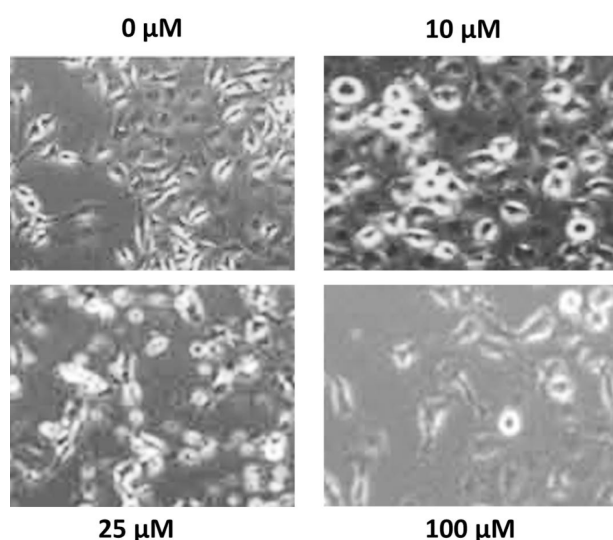


Figure 5. Cellular structure change determination via phase-contrast microscopy in Zingiberene-treated HCC cells. The experiments were performed in triplicate ($p < 0.05$).

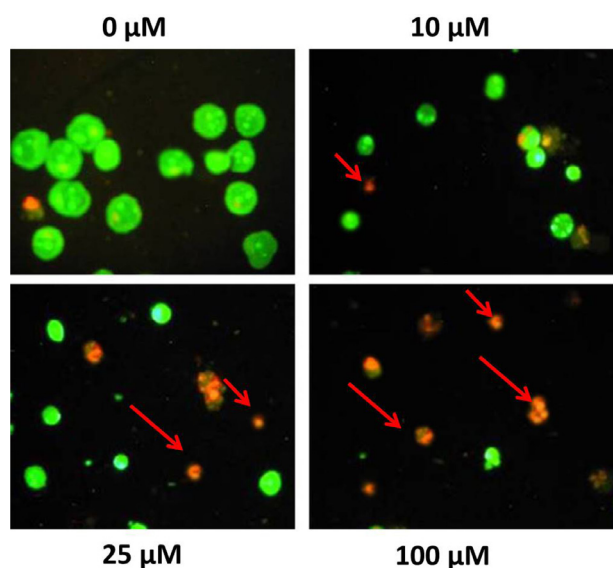


Figure 6. Apoptotic cell morphology via AO/EB staining at indicated doses of Zingiberene molecule. The arrows show early apoptotic, late apoptotic and necrotic cells in the Zingiberene-treated group. The experiments were performed in triplicate ($p < 0.05$).

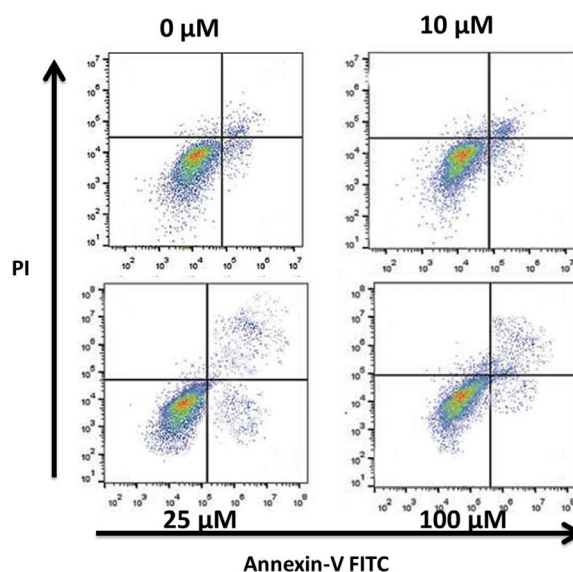


Figure 7. Counting the number of apoptotic cells via annexin V-FITC/PI dual staining assay after Zingiberene exposure at variant doses. Data are shown as mean $\pm$ standard deviation (with repetitions $n = 4$). The experiments were performed in triplicate ($p < 0.05$).

Determination cellular morphology after Zingiberene treatment of HCC cells

Cellular morphology of HCC cells was examined through phase-contrast microscopy. Microscopic study revealed that HCC cells treated with variant doses of Zingiberene underwent substantial morphological changes (Figure 5). On Zingiberene exposure at a dose of 10 μ M HCC cells condensed and retract from other surrounding cells. RPMI-1640 culture medium could be seen with floating cells at a concentration of 25 μ M and after incubation with 100 μ M of Zingiberene cells lost their normal morphology and a sum of more cells appeared floating over the culture RPMI-1640.

Zingiberene treatment of HCC cells resulted in apoptotic cell death mediated via targeting of miR-16/cyclin-B1 axis

Apoptosis analysis was performed by AO/EB and annexin V-FITC/PI dual staining. The results AO/EB staining revealed substantial morphological changes and identification of early apoptotic cells (yellow-green fluorescent nuclear location at one side of cells) at a drug concentration of 10 μ M, late apoptotic cells (nucleus located in bias and orange fluorescence) at a drug concentration of 25 μ M and necrotic cells (revealing confusing outline, increased volume and orange-red fluorescence) at a drug concentration of 100 μ M (Figure 6). An-

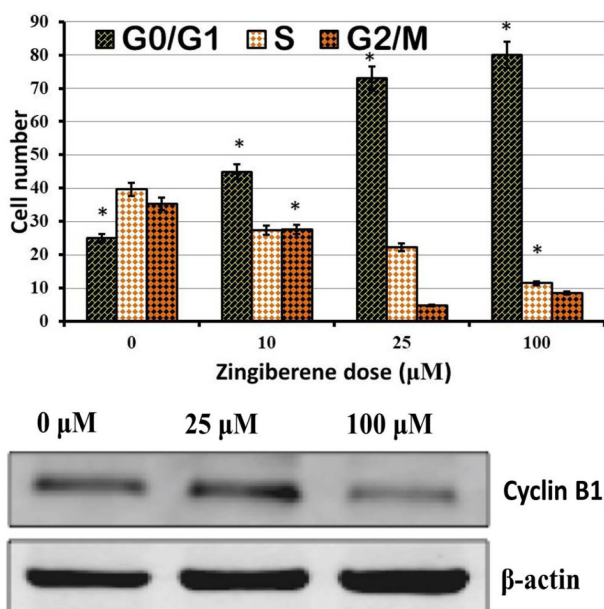


Figure 8. A: Flow cytometric analysis of Zingiberene treated HCC cells for cell cycle phase distribution. **B:** bold Western blotting was used to monitor the expression of Cyclin B1 and the results show its downregulation on Zingiberene exposure. B-actin acted as normalization control. Data are shown as mean $\pm$ standard deviation (with repetitions n=4). The experiments were performed in triplicate (p<0.05).

nexin V-FITC/PI dual staining assay revealed that with increasing molecule concentration there was a significant enhancement in the number of apoptotic cells (Figure 7). Thus AO/EB and annexin V-FITC/PI dual staining revealed that increasing drug concentration is associated with increasing miR-16 expression and the number of apoptotic cells.

Zingiberene treatment of HCC cells resulted in cell cycle arrest

Cell cycle analysis was performed after Zingiberene treatment of HCC cells through flow cytometry. The results revealed that there was a tremendous increase in G0/G1-phase cells as compared with S-phase and G2/M-phase cells. At drug concentrations of 0, 10, 25 and 100 μ M the number

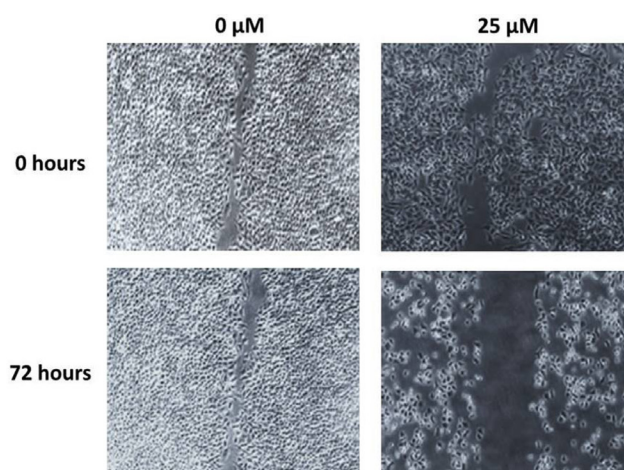


Figure 9. Wound healing assay for cell migration analysis after Zingiberene treatment and raising of miR-16 expression at indicated doses and indicated time interval. The experiments were performed in triplicate (p<0.05).

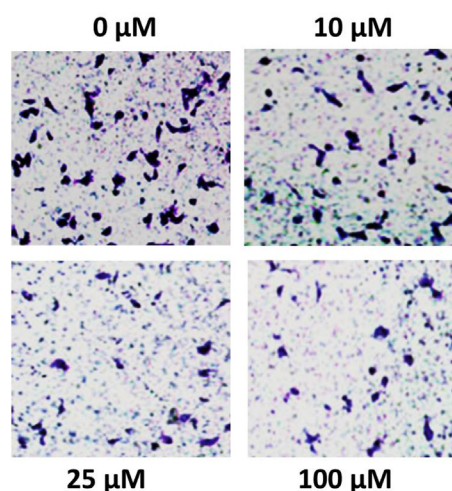


Figure 10. Analysis of cell invasion potential of HCC cells after Zingiberene treatment at indicated doses via transwell chambers. The experiments were performed in triplicate (p<0.05).

of G0/G1-phase cells was 25, 45, 73 and 80, the S-phase cells were 40, 28, 22 and 12 and the G2/M-phase cells were 35, 28, 5 and 9, respectively, thus indicating G0/G1-phase cell cycle arrest. Furthermore, western blotting analysis revealed significant and dose-dependent suppression of cyclin B1 expression, which is a cell cycle associated protein (Figure 8).

Zingiberene targets the miR-16/cyclin-B1 axis to regulate cell migration and invasion in HCC cells

The effect of Zingiberene on HCC cells resulted in increasing the expression of miR-16 as evidenced by real-time quantitative PCR analysis. The effect on cell migration and invasion potential of HCC cells was measured via wound healing and transwell chambers assay. The results of wound healing assay revealed dose-dependent inhibition by the molecule on cell migration (Figure 9). Transwell chambers with Matrigel was used for the determination of cell invasion capability of HCC cells, revealing significant concentration-dependent decrease in the number of invaded cells (Figure 10). Thus it may be concluded that increasing Zingiberene concentrations miR-16/cyclin-B1 axis is associated with induction of potential inhibitory effects on cell migration and invasion.

Discussion

Liver cancer is a lethal malignancy and remains a challenge for researchers due to poor prognosis, lower survival rate, limited treatment options and lack of effective therapeutic agents [24,25] so the hunting for new therapeutic targets and effective therapeutic agents continues. MiRs serve as novel therapeutic targets in cancer treatment as some of them downregulate the expressions of oncogene and induce tumour suppression. Herein, regarding Zingiberene targeting miR-16/cyclin-B1 axis in HCC cells was found to increase the expression of miR-16, which was found to be associated with suppression of induction of cell growth inhibition, cell cycle arrest, cell migration and invasion. Real-time quantitative PCR analysis was performed to find out the impact on Zingiberene of miR-16 expression, revealing tremendous dose-dependent increase. In addition, MTT cell viability assay was performed to unveil the effects of Zingiberene on

cell viability and the results revealed that it resulted in dose as well as time-dependent inhibition of cell growth and proliferation. Cell morphology was studied through phase-contrast microscopy, revealing condensed disintegrating and retracted cells from surrounding cells floating over culture media and their number was found to increase on higher drug concentrations as compared to the controls. Next, increased miR-16 expression was associated with induction of apoptotic cell death as evidenced by AO/EB and annexin V-FITC/PI dual staining. The number of apoptotic cells was found to be increasing tremendously with increasing Zingiberene concentrations (increased miR-16 expressions). Flow cytometric analysis of Zingiberene treated HCC cells suggested that it induces G0/G1-phase cell cycle arrest, which was also evidenced by western blotting analysis showing downregulation of cyclin B1 expression levels. Finally, the effect of Zingiberene targeting miR-16/cyclin-B1 axis was found to be linked with cell invasion and migration inhibition. Cell migration and invasion was evaluated through wound healing assay and transwell chambers respectively, both revealing dose-dependent inhibition by Zingiberene treatment.

Conclusions

In conclusion, all the above results evidenced that Zingiberene treatment of HCC cells resulted in enhancing the expression of miR-16 thus targeting miR-16/cyclin-B1 axis. Increased miR-16 expression was associated with cell growth inhibition via induction of apoptosis, cell cycle arrest and inhibition of cell migration and invasion. Thus Zingiberene is a potential miR-16 enhancer thereby induces anticancer effects in HCC cells and can prove lead molecule in liver cancer treatment.

Funding support

This study was supported by Hubei Municipal Health and Family Planning Commission (No: WJ2017Q034), and Wuhan Municipal Health and Family Planning Commission (No:WX16E11).

Conflict of interests

The authors declare no conflict of interests.

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