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Myricetin can control metastasis and invasion by suppressing ATF2-related signaling pathway in Rapamycin-resistant HepG2 hepatocellular cancer cells

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ABSTRACT: Hepatocellular carcinoma continues to be one of the major health problems. One of the most important challenges in the treatment of hepatocellular cancer is gaining resistance to chemotherapy. Therefore, there is an urgent need for discoveries of naturally occurring antitumor compounds in the diet and to elucidate the anticancer mechanisms of action of existing dietary origin compounds. Myricetin is a dietary flavonoid and its antitumor activity is reported. In this study, HepG2 cells were incubated with 100 nM rapamycin for 6 weeks to develop rapamycin resistance. 10, 100, 250 and 500 µM myricetin was applied to rapamycin resistant HepG2 human hepatocellular cancer cells (HepG2-RR) for 24 and 48 hours. Changes in ATF2, SMAD3, SMAD4 and Col1A1 expressions of HepG2-RR cells after myricetin application were evaluated by Western blot and qPCR analysis. In addition, the effect of myricetin treatment on the migration/invasion of HepG2-RR cells was specified using the BD 24-well Boyden chamber. The IC50 values of rapamycin and myricetin on HepG2 cells for 48 hours incubation was calculated as 89,03±6,14 nM and 126,25±7,32 µM, respectively. On the other hand, we found that HepG2-RR cells treated with myricetin decreased cell proliferation, invasion, and migration by reducing the expression of ATF2, SMAD3, SMAD4, and Col1A1. As a result, we were shown that myricetin administration inhibited cell growth and cell migration by suppressing ATF2 in hepatocellular carcinoma cells that gained drug resistance.

KEYWORDS: Myricetin; Rapamycin; Liver Cancer; Metastasis.

1. INTRODUCTION

According to the 2020 world cancer statistics, 905,677 new cancer cases have been diagnosed and 830,180 deaths have been reported worldwide. Liver cancer ranks sixth most common cancer and third leading cause of cancer-related death. Despite the development of new techniques in diagnosis and treatment, the incidence and mortality continues to increase. Therefore, there is an urgent need to elucidate the mechanisms of invasion and metastasis in hepatocellular cancer and to find more effective biomarkers for diagnosis [1-3]. Activating transcription factor 2 (ATF2) is a molecule belonging to the leucine zinc family of DNA-binding proteins. ATF2 regulates the transcription of genes involved in biochemical processes such as inflammation, proliferation, and apoptosis. Many researchers have reported that ATF2 plays a key role as a tumor promoter in many malignancies such as prostate cancer, skin cancer, non-small cell lung cancer, and hepatocellular cancer [4-6].

The mammalian target of rapamysin (mTOR) is a mechanism that controls the survival, growth and proliferation of mammalian cells [7]. mTOR protein kinase which is located at the center of the pathway, consists of two different complexes, mTOR complex1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 regulates cell growth effectors such as S6K1 and 4E-BP1, while mTORC2 phosphorylates the S473 residue of the prosurvival molecule akt/PKB [8]. Small molecules targeting mTOR are appealing increasing clinical interest, as disruptions in the mTOR pathway occur in various human diseases. One of the mTOR-targeted molecules is rapamycin, which is a drug that is frequently used in the clinic to prevent organ rejection after transplantation. Rapamycin blocks kinase activity by binding to the mTORC1 complex. Researchers have reported that Rapamycin has anti-cancer effects by slowing proliferation and promoting apoptosis in various cancer cell lines, as well as having antiangiogenic properties. Because of these features, scientific interest on rapamycin in cancer research have been increased [9,10].

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Flavonoids are phytochemicals commonly found in many plants. Myricetin is a natural type of flavonoid containing 6-hydroxyl groups. It can be found in abundance in vegetables, fruits, tea and aromatic plants. Myricetin has become a more important agent in the field of health sciences due to its iron-chelating capacity, antioxidant, antiviral and antimicrobial properties [11-12]. In the present study, we investigated whether myricetin suppresses the expression of ATF2, a molecule that provides tumor growth and metastasis, in HepG2 human hepatocellular cancer cells with rapamycin resistance. In addition, the changes in the expression of SMAD3, SMAD4 and Col1A1, which are molecules that allow the migration and invasion of cancer cells, were investigated. Myricetin inhibits metastasis by suppressing ATF2 in hepatocellular cancer cells.

2. RESULTS

2.1. Cytotoxic effects of rapamycin and myricetin on HepG2 cells

The cytotoxic effects of rapamycin and myricetin on HepG2 human hepatocellular carcinoma cells were investigated by MTT method. It was found that there was a dose and time-dependent decrease in cell viability in HepG2 cells treated with 10, 50, 100, 250 nM rapamycin (Figure 1b). A dose- and time-dependent decrease in cell viability was also detected in cells treated with 10, 100, 250, 500 µM myricetin (Figure 1a). Since the highest cytotoxic effect of both rapamycin and myricetin was obtained in 48 hours incubation, it was decided that the most appropriate incubation time for subsequent experiments was 48 hours. The IC_{50} values of rapamycin and myricetin on HepG2 cells for 48 hours incubation were calculated as 89,03±6,14 nM and 126,25±7,32 µM, respectively.

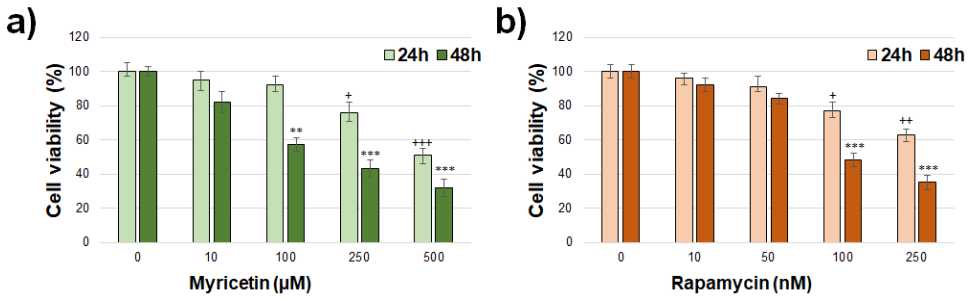

Figure 1 a) Cell viability graph of HepG2 cells treated with Myricetin for 24 and 48 hours. **b)** Cell viability plot of HepG2 cells treated with Rapamycin for 24 and 48 hours (**, p≤0.01; ***, p≤0.001, +, p≤0.05; ++, p≤0.01; +++, p≤0.001).

2.2. Induction of rapamycin resistance in HepG2 cells

HepG2 cells were incubated with 100 nM rapamycin for 6 weeks to acquire drug resistance. Morphological changes were not observed in HepG2 cells at the end of incubation (Figure 2a). HepG2 cells are known to overexpress mTOR and phosphorylated mTOR (P-mTOR). mTOR and P-mTOR protein levels were measured by Western blot in order to observe that the cells gained resistance (Figure 2b). At the end of 48 hours, while the amount of mTOR and P-mTOR decreased in cells treated with 100 nM rapamycin, it was observed that the expression of these proteins increased again when the cells acquired rapamycin resistance (HepG2-RR) (Figure 2c, 2d). In addition, it was determined that the gene expression of mTOR decreased after 100 nM rapamycin application and increased after HepG2-RR was developed (Figure 2e).

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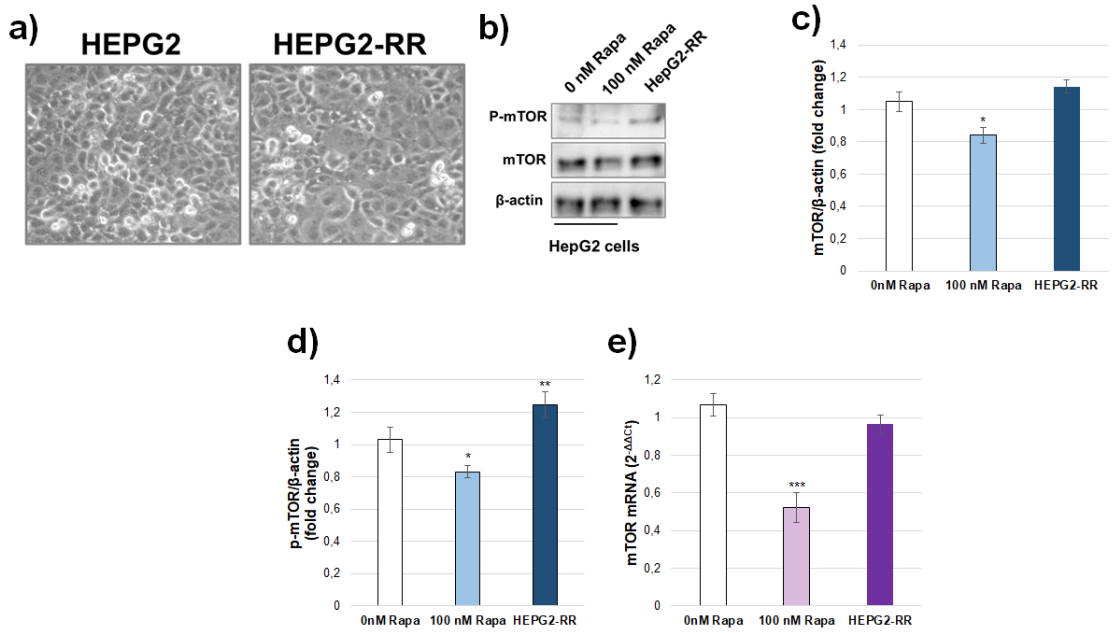

Figure 2. Rapamycin resistance studies **a)** Inverted microscope images of HepG2 and HepG2-RR cells under white light **b)** Western blot bands of Rapamycin treated HepG2 cells and HepG2-RR cells. **c)** Graphs of mTOR protein expressions 0 and 100 nM Rapamycin administered cells and HepG2-RR cells **d)** Graphs of P-mTOR protein expressions 0 and 100 nM Rapamycin administered cells and HepG2-RR cells **e)** Graphs of mTOR mRNA expressions 0 and 100 nM Rapamycin administered cells and HepG2-RR cells (*, p≤0.05; **, p≤0.01; ***, p≤0.001).

2.3. Molecular changes in HepG2-RR cells after myricetin administration

Since the expression of ATF2 is high in hepatocellular carcinoma cells, changes in ATF2 protein and gene expression levels were investigated by 100 µM myricetin administration in HepG2-RR cells for 48 hours. Figure 3a shows Western blot bands of ATF2 and β-Actin in HepG2-RR cells treated with myricetin. It was found that ATF2 protein and gene expressions of HepG2 cells decreased after myricetin administration (P<0.001).

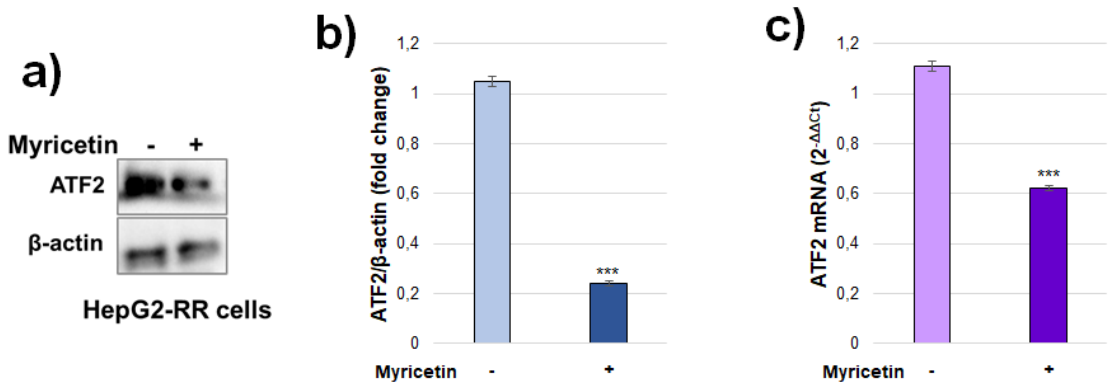

Figure 3 a) Western blot bands of ATF2 and β-Actin in HepG2-RR cells treated with Myricetin. **b)** ATF2 protein expression graph of HepG2-RR cells treated with Myricetin. **c)** ATF2 mRNA expression graph of HepG2-RR cells treated with Myricetin (***, p≤0.001).

Smad3/Smad4 are molecules that increase tumor growth, spread and metastasis other tissues in cancer. Figure 4a shows Western blot bands of Smad3, Smad4, Col1A1 and β-Actin in HepG2-RR cells treated with 100 µM myricetin for 48 hours. It was defined that the protein and gene expression level of Smad3 and Smad4 decreased in HepG2-RR cells treated with Myricetin compared to the control group (Figure 4 b, 4c, 4e, 4f). Col1A1 is not only a growth marker of hepatocytes, but also increases in liver damage. When compared

to the control group, it was stated that there was a decrease in the expressions of Col1A1 in HepG2-RR cells administered with Myricetin (Figure 4d, 4g).

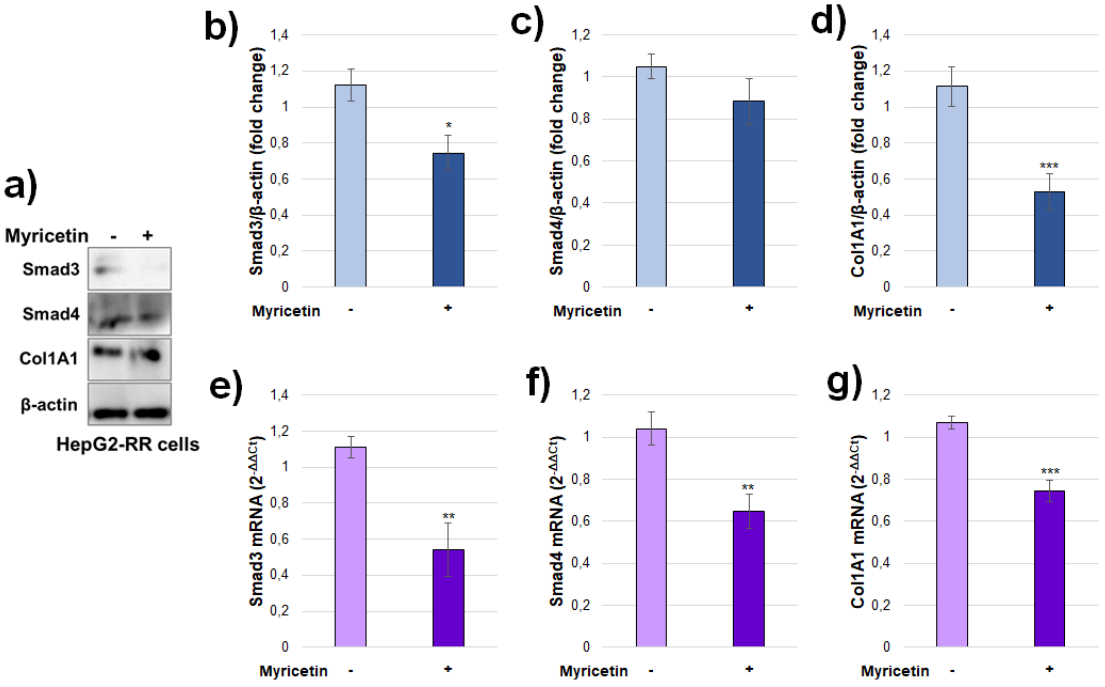

Figure 4 a) Western blot bands of Smad3, Smad4, Col1A1 and β-Actin in HepG2-RR cells treated with Myricetin. **b)** Smad3 protein expression graph of HepG2-RR cells treated with Myricetin. **c)** Smad4 protein expression graph of HepG2-RR cells treated with Myricetin. **d)** Col1A1 protein expression graph of HepG2-RR cells treated with Myricetin. **e)** Smad3 mRNA expression graph of HepG2-RR cells treated with Myricetin. **f)** Smad4 mRNA expression graph of HepG2-RR cells treated with Myricetin. **g)** Col1A1 mRNA expression graph of HepG2-RR cells treated with Myricetin (*, p≤0.05; **, p≤0.01; ***, p≤0.001).

2.4. Effects of Myricetin on HepG2-RR Cell Migration/Invasion

Migration/Invasion assay is an in-vitro test that determines the ability of tumor cells to metastasize to distant tissues and organs. According to the experiment results, it was determined that the 100 µM Myricetin treatment decreased the migration and invasion ability of HepG2-RR cells (Figure 5a, 5c). Also, the percentage of the migrative and invasive HepG2 cells were decreased after treatment 100 µM Myricetin (Figure 5b, 5d).

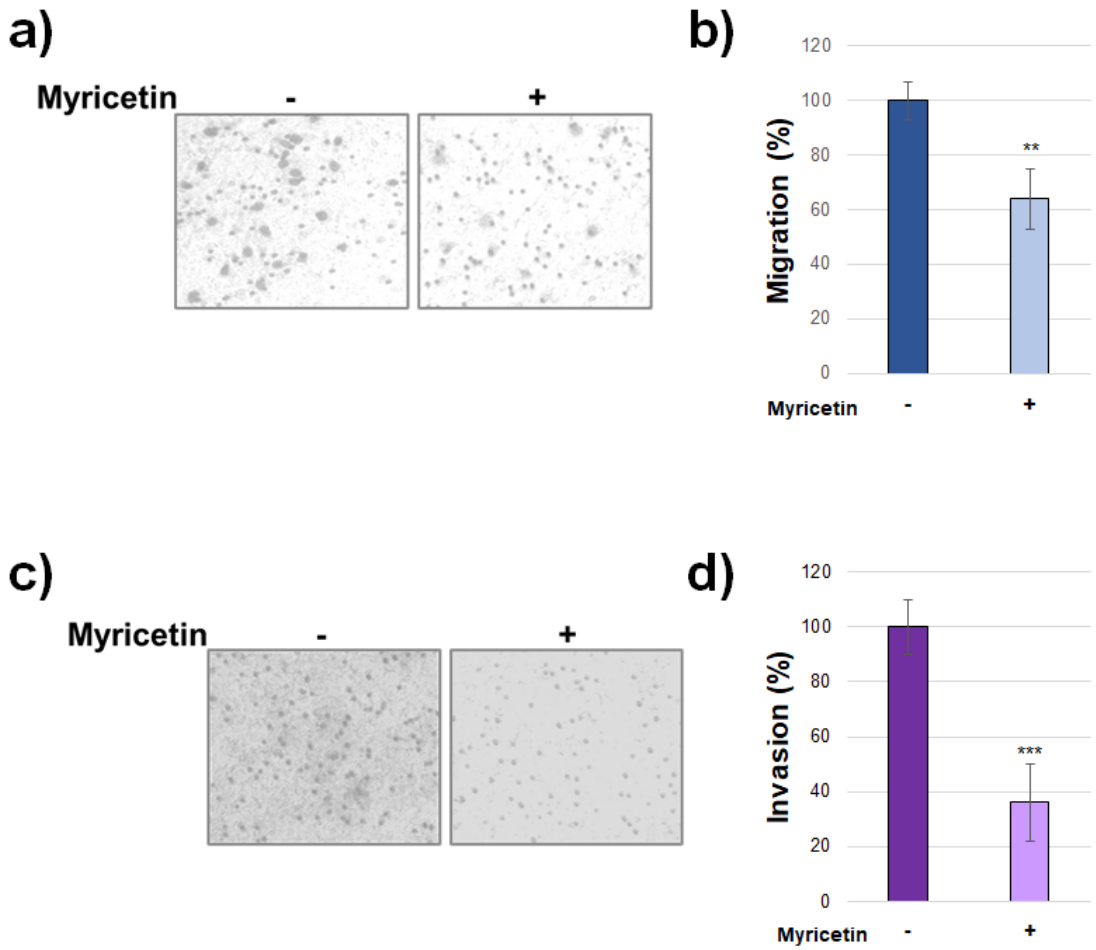

Figure 5 a) The morphological images of migrative HepG2-RR cells treatment with Myricetin**. b)** The percentages of migrative HepG2-RR cells treatment with Myricetin. **c)** The morphological images of invasive HepG2-RR cells treatment with Myricetin. **d)** The percentages of invasive HepG2-RR cells treatment with Myricetin.

3. DISCUSSION

Cancer is a serious health problem which is characterized by cells starting to grow abnormally and then spreading or invading to other tissues and organs. According to Globocan 2020 data, 19,292,789 new cancer cases have been detected worldwide [1]. One of the biggest problems in cancer treatment is drug resistance. Researchers have remarked that many cells have developed resistance to rapamycin in cancer treatment [13,14]. Tian et al. found that the oncoprotein MDM2 expression was increased in rapamycin-resistant A492 renal cancer cells. In both in vitro and in vivo models, the combination of rapamycin with MI-319 improved anti-tumoral activity by reducing MDM2 expression and increasing rapamycin susceptibility [15]. Li et al. reported that sorafenib administration increased the antitumor activity of rapamycin in rapamycin resistant HepG2, HCCLM3 and MH3924A hepatocellular cancer cells. Also, they stated that activation of PI3K/AKT and MAPK pathways was induced by increasing PDGFRb expression after sorafenib administration [16]. In addition to developing new strategies to overcome rapamycin resistance, especially intensive research continues on dietary compounds [17]. Myricetin is a flavonoid that is abundant in plant-derived the diet. Researchers reported that myricetin shows an anticancer effect against different types of cancers in various pathways. Myricetin has an important role in regulating cancer cell proliferation in many types of human cancer. For example, in hepatocellular carcinoma, it has been confirmed that myricetin regulates a G2/M cell cycle inhibitory mechanism in HepG2 cells. Myricetin inhibits the functioning of the Cyclin B/CDK1 complex, which promotes tumorigenesis by increasing the expression of tumor suppressors and CDK inhibitors p53, p21 and p27 [18]. Another mechanism by which myricetin acts in anticancer treatment is the MAPK (mitogen-activated protein kinase) pathway. MAPK is a signaling protein that plays an significant role in tumor growth, tumor invasion and metastasis. Molecular studies have reported that

myricetin administration downregulates the MAPK pathway in cancer cells and inhibits cell proliferation and invasion [19, 20].

Cancer development and progression is not only dependent on the characteristics of cancer cells, but also the tumor microenvironment has important contributions. Transforming growth factor beta (TGF-β) suppresses carcinogenesis in normal cells, while its overexpression in cancer cells contributes to tumor growth. Cancer cell-derived TGF-β may trigger to tumor development by contributing extracellular matrix degradation, angiogenesis and epithelial-mesenchymal transition Smad3 is a molecule that is a mediator of the TGF-β signaling pathway and is a significant agent in the transcriptional regulation of TGF-β [21]. Tang et al. reported that SMAD3 gene deletion suppressed tumor growth, invasion, and metastasis in an in-vivo tumor model [22]. In our study, it was found that SMAD3 protein expression decreased in HepG2-RR cells after myricetin administration. Like SMAD3, SMAD4 is an overexpressed molecule in cancer cells that regulates TGF-β expression. Alhopuro et al., in their study on 75 Dukes C colorectal cancer patients, stated that patients with low SMAD4 expression responded better to treatment and had a longer survival time than patients with high SMAD4 expression [23]. In our study, it was determined that there was a decrease in SMAD4 expression after myricetin administration.

In addition to being the most important component of the tumor microenvironment, the extracellular matrix also plays a role in tumor growth, invasion and metastasis. Collagen type I alpha 1 (COL1A1) is reported to be associated with the development of many human diseases, especially cancer [24]. Liu et al. reported that the expression of COL1A1 up regulation at mRNA and protein levels from grade I to III in cervical cancer tissues, in contrast to normal cervical tissues [25]. In another study Zhao et al. after COL1A1 knockdown stated decreases in the expression of the cell proliferation factor cyclin D1 and the apoptosis marker protein BCL-2, along with an increase in the expression levels of the apoptosis regulator Bax [26]. In our study, we found that Col1A1 expression of HepG2-RR cells was downregulated after myricetin application. The decrease in the percentage of invasive and migrative cells after myricetin application in our study supports the SMAD3, SMAD4 and Col1A1 expression results.

4. CONCLUSION

Hepatocellular cancer continues to be one of the biggest health problems today. With the results of this study, it was shown that myricetin administration inhibited cell growth and cell migration by suppressing ATF2 in hepatocellular carcinoma cells that gained drug resistance. The results of this study will guide the mechanistic studies in hepatocellular cancer studies and will shed light on the studies especially on the research of diet-derived molecules.

5. MATERIALS AND METHODS

5.1. Cell Culture

HepG2 human hepatocellular carcinoma cell line was purchased from American Type Culture Collection (ATCC HB-8065, Rockville, MD, USA) Cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. The cultures were incubated in a humidified incubator with 5% CO_2 in air at 37°C. The culture medium was replaced with fresh medium every 2 days until reaching suitable confluency of about 90%. All experiments were repeated three times.

5.2. Cell Viability Test (MTT Assay)

The toxic effects of rapamycin and myricetin on HepG2 cells were examined using a tetrazolium-based microplate assay with MTT. Briefly, The HepG2 cells were seeded into a 96-well plate at a density of 1×10^4 cells/well with the 100 µL DMEM medium. After 24h incubation to allow cell attachment, different concentrations of rapamycin (10, 50, 100 and 250 nM) and myricetin (10, 100, 250, 500 µM) were incubated for 24h. After the incubation, cells were incubated with 10 µL MTT dye (0.5 mg/mL, ODC Inc) in each well in the 100 µL medium for 4h at 37°C. After removing all the culture medium, 100 µL DMSO was added in each well. The percentage of cell viability was measured on ELISA reader (BiotekCo., USA) at a wavelength of 570 nm. The % cell viability was calculated using the formula given below in the Eq. (1). Besides, the IC_{50} values of rapamycin and myricetin on the HepG2 cells were calculated using Graphpad Prism [27].

% Cell Viability= (OD test sample/OD control) X 100 Eq. (1)

5.3. Protein Expression Analysis (Western Blotting)

HepG2 cells were seeded in 6-well plates at $1x10^6$ cells per well. The cells were incubated in a carbon dioxide oven for 24 hours to adhere to the bottom of the plate. In drug resistance induction experiments, HepG2 cells were incubated with 100 nM rapamycin. The other protein expression analysis, cells were incubated with IC_{50} doses of myricetin for 24 hours. After 24 hours of incubation, the cells were removed and and the cells were lysed by adding 350 µL of 2X sample loading buffer. Using solutions of pH 6.8 0.5 M Tris, pH 8.8 1.5 M Tris, 30% Acrylamide-Bisacrylamide, 10% Ammonium persulfate, 10% sodium dodecylsulfate and TEMED, 4% stacking and 12% separation gel were poured. The samples containing 5-10 µg of protein were loaded onto a gel and run with a running buffer (5 mM Tris, 38.4 mM Glycine, 1% SDS) at 100V constant current for 1-2 hours. After electrophoresis, immunoblotting was applied with a semi-dry system to transfer the protein bands in the gel to the PVDF membrane. The gel was carefully placed on the membrane, and a nitrocellulose layer wetted with transfer buffer was placed on it again. In a semi-dry transfer device (Biorad Transblot Turbo), blotting was performed at 25 volts and 1 Amp constant current for 30 minutes. After transferring the proteins to the PVDF membrane, the membrane was washed 3 times with TBST (20 mM Tris, 154 mM NaCl, 0.1% Tween 20) and blocked with 2.5% BSA for 2 hours. After washing 3 times with TBST, the PVDF membrane was incubated with primary antibodies (ATF2 (Santa Cruz SC242), SMAD3 (Santa Cruz SC101154), SMAD4 (Santa Cruz SC7966), Col1A1 (Santa Cruz SC25974), B-Actin (Santa Cruz SC47778)) at +4 ^oC overnight on an orbital shaker. After incubation, the membrane was washed 3 times with TBST for 10 minutes. After the membrane was incubated with HRP-conjugated secondary antibody for two hours at room temperature, the membrane was incubated with chemiluminescence reagent (ECL, Santa Cruz) for 1 minute in the dark. The images of the bands were taken with the imaging system (Syngene GBOX). Densitometric analysis of the bands was done with ImageJ program [28].

5.4. Gene Expression Analysis

The total RNA content was isolated from $5x10^6$ HePG2 cells as previously described [29]. 1 µg total RNA was reverse transcribed as the template for cDNA synthesis using high-capacity cDNA Reverse Transcription Kit (Applied Biosytems). Quantitative real-time PCR was carried out Bcl-2, Bax, LC3B, Beclin-1 and GAPDH primers. The primers sequence was: ATF2: Forward 5′-ATCCCAGGTCCTTCCTCTCC-3′, Reverse 5′-GGAGGATGTTGCTGGGTCAA-3′; SMAD3: Forward 5′-TAGACTTGGGATGGGGAGGG-3′, Reverse 5′-GGCGCTCATCGATTTTTCCC-3′; SMAD4: Forward 5′-CACAAGTCAGCCTGCCAGTA-3′, Reverse 5′-CAGTCCAGGTGGTAGTGCTG-3′; COL1A1: Forward 5′-CCTGGGGCAAGACAGTGATT-3′, Reverse 5′-AACGTCGAAGCCGAATTCCT-3′; mTOR: Forward 5′-TTTAAACATAGCTCCCGGCTTAGA-3′, Reverse 5′-TGAGGTTCGCGCGGCTT-3′ GAPDH: Forward 5′-AGGGCTGCTTTTAACTCTGGT-3′, Reverse 5′-CCCCACTTGATTTTGGAGGGA-3′). 100 nanograms of cDNA were amplified by Sybr Green PCR Master Mix (Applied Biosytem) on the ABI StepOne Plus detection system. The programme parameter for amplification was 95 °C for 10 min, then 40 cycles of: 95 °C for 15 s, 59 °C for 1 min, 72 °C for 30 sec. The qPCR datas were resolved using StepOne Software v2.3 (Applied Biosystems, Foster City, CA) and the genes of interest were normalized to the corresponding GAPDH results. Data plotted as fold change versus control.

5.5. Migration/Invasion Experiments

Migration/invasion assays were carried out using BD 24-well Boyden chambers (8- µM pore size) as described [30]. Shortly, $2x10^5$ cells were seeded to the top insert, and 750 µl of 5% FBS-including medium was added to the bottom chamber. After a 16-h incubation, cells that migrated to the lower surface of the membrane were fixed, stained with a Diff-Quick stain kit (Dade Behring, Inc., Westwood, MA), and counted.

5.6. Statistical Analysis

Data from three independent experiments are presented as mean ± SD. Variations between groups were stated by One Way Anova test. Statistical analysis was carried out GraphPad Prism version 7.0 software. Statistical importance is described as follows: *, p≤0.05; **, p≤0.01; ***, p≤0.001.

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