Water extract of Frankincense and Myrrh inhibits liver cancer progression and epithelial mesenchymal transition (EMT) through Wnt/β-catenin signaling

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Research Article

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Abstract

Background

The Wnt/β-catenin signaling is closely related to epithelial mesenchymal transformation (EMT), which plays an important role in HCC invasion and metastasis. Frankincense and myrrh (FM) are anti-tumor agents commonly used in clinic. This study aimed to investigate the effect and mechanism of the water extract of FM on the progression of liver cancer cells.

Methods

Different concentrations of FM were applied to study cell proliferation of hepatocellular carcinoma cells. The ability of cell migration and invasion were detected by wound healing test and Transwell assay. Western blot was used to study the related protein levels of EMT and the Wnt/β-catenin signaling. The nuclear translocation of β-catenin was detected by the immunofluorescence assay.

Results

FM at a non-toxic dose significantly inhibited the invasion and metastasis of liver cancer cells. Furthermore, FM promotes EMT marker E-cadherin, while decreasing the expression of Vimentin and N-cadherin. Finally, the protein and the nuclear staining level of DVL2 and β-catenin were both suppressed by water extract of FM.

Conclusion

The water extract of FM inhibited the migration and invasion of liver cancer cells, and blocked the occurrence of EMT via suppressing the activation of Wnt/β-catenin signaling pathway.

Background

Hepatocellular carcinoma (HCC) remains one of the most lethal malignancies with the sixth highest incidence rate and the third leading cause of cancer death in the world[1]. While numerous treatment strategies exist for HCC, the overall survival rate for this disease is disappointingly low due to recurrence, aggressive growth, metastasis, chemoresistance[2–4].

Accumulating evidence has shown epithelial mesenchymal transformation (EMT) plays an important role in HCC invasion and metastasis, and EMT-related markers are closely related to HCC metastasis[5]. Among them, the Wnt/β-catenin signaling pathway is highly conserved in evolution and plays a vital role in tumor growth and metastasis[6, 7]. In brief, when Wnt ligands combine with the Frizzled receptors, Dvl/Dsh is phosphorylated and recruits AXIN1 and GSK3β, thus the formation of degradation complex is

inhibited. Finally, the activated β-catenin complex transports to the nucleus and promotes the transcription of genes regulating cell proliferation and metastasis[8]. Since the activation of the Wnt/β-catenin pathway induces HCC proliferation, migration, and invasion, targeting this signaling cascade hold promise in HCC treatment.

Frankincense and myrrh are traditional Chinese herbal medicine. Frankincense is the resin exudated from the bark of Boswellia, *Burseraceae* family, while myrrh is the dried resin of species of *Commiphora* family[9]. Both of them have been used as anti-inflammatory and anti-cancer drugs. Many pharmacological researches have studied the mechanisms underlying the anti-tumor function of frankincense and myrrh. β-elemene, one of the active components of frankincense myrrh, has been reported to treat colon cancer by inducing ferroptosis and inhibiting epithelial-mesenchymal transformation[10]. Gugulipid, a main extract of the *Commiphora mukul* tree, also induces apoptosis by targeting the β-catenin signaling pathway[11].

In our experiment, we generated a water-decocting extract of frankincense and myrrh.

Given the critical role of Wnt/β-catenin signaling in tumor progression, and the potent anti-tumor ability of frankincense and myrrh, we sought to investigate its underlying mechanisms involved in liver cancer progression and EMT by targeting the key EMT axis Wnt/β-catenin.

Materials And Methods

Cell culture

Human HCC cell line HCC-LM3 and Mice HCC cell line Hepa1-6 were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) containing 10% fatal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Life Technology, Grand Island, NY) and were maintained at 37℃ in humidified air containing 5% CO_2.

Reagents

Frankincense and myrrh were purchased from Jiangsu Province Hospital of Traditional Chinese Medicine (TCM). The anti-N-cadherin, E-cadherin, Vimentin, snail, slug, twist1 and ZEB1, DVL2, β-catenin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-GAPDH was purchased from Bioworld Technology, Inc.

Water decocting extract of frankincense and myrrh

The frankincense and myrrh were purchased from the Jiangsu Traditional Chinese Medical Hospital (Nanjing, China). The extract of frankincense and myrrh was prepared as previously described[12]. Briefly, the dry herb of frankincense (500 g) or myrrh (500 g) was extracted from boiling water (5 L) for twice and

filtered through gauze. Then the extracted solution was evaporated in a rotary evaporator under under vacuum at 55°C to obtain the powder of frankincense or myrrh.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyletrazolium bromide (MTT) assay

Cells were cultured in 96-well plates and treated with the indicated compounds. After a 12 h or 24 h of incubation, 20 μl of MTT reagent was added to each well and cells were incubated at 37℃ for 3 h. After that, the supernatant was removed and 100 μl of isopropanol was added into each well followed by shaking for 10 min. The absorbance at 570 nm was measured using the spectrophotometer.

Western blot

Cells were lysed on ice with radioimmunoprecipitation assay (RIPA) peptide lysis buffer (Beyotime Biotechnology, Shanghai, China). Equal amounts of protein were loaded and subjected to 10% SDS-PGAE. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland). The membrane was incubated with specific antibodies at 4℃ overnight after blocking with 5% nonfat milk. After incubating with the appropriate HRP-conjugated secondary antibodies, the signal was determined using an enhanced chemiluminescence reagent (Millipore, CA, USA) and visualized by a Tanon 5200 imaging system (Tanon, Shanghai, China).

Wound healing assay

HCC cells were seeded in 6-well plates and the wound were made using 200 μl tips to form a single confluent cell layer. At a determined time point, the width of the wound was photographed using a phase-contrast microscope.

Transwell invasion assay

To evaluate the cell invasion capacity, we used the Transwell chambers of pro size 8 μm (Corning Costar, Cambridge, MA, USA). Briefly, cells were seeded in the diluted Matrigel-coated (BD Biosciences, Bedford, MA, USA) upper chamber with 200 μl of serum-free DMEM medium, while the lower chamber was added with 10% DMEM medium of 10% serum as the chemoattractant. At the determined time points, cells in the upper chamber were removed and the migrated cells in the lower chamber were fixed with 4% paraformaldehyde followed by staining with 0.1% crystal violet.

Immunofluorescence

HCC cells were seeded on coverslips and were fixed with 4% paraformaldehyde at room temperature for 15 min. After washing with PBS, cells were incubated with specific antibodies at 4℃ overnight. The slides were then subjected to the matched secondary antibodies for 2 h at room temperature. Nuclei were stained with DAPI (Sigma) for 5 min at room temperature and images were visualized using a confocal microscope.

Statistical analysis

All data are shown as the means ± SD. Differences between groups were examed using *Student's t-test* or one-way *ANOVA* analysis. Graphs were performed with GraphPad software (Version 5.0). P-values less than 0.05 were considered as statistical significance.

Results

Cytotoxic Efficacy Of Fm On Hcc Cells

Firstly, we wanted to select a concentration of FM which had non cytotoxicity on HCC cells. For this purpose, we determined the growth inhibitory efficacy of FM against HCC-LM3 or Hepa1-6 cells. As shown in Fig. 1A&B, FM induced growth inhibitory effect on both human and murine cancer cells in a dose and time-dependent manner. Moreover, the FM showed moderate cytotoxicity effect on tumor cells with a concentration of less than 5 mg/ml and no growth inhibitory effect when the concentration of FM was lower than 1 mg/ml. Taken together, we determined the ex vivo dosage of FM as 0.5 mg/ml for our follow-up experiments.

Fm Suppresses The Migration And Invasion Abilities Of Tumor Cells

To investigate the effect of FM on the motilities of cancer cells, Human HCC-LM3 cell line and murine Hepa1-6 cells were treated with 0.5 mg/ml FM. Since such dosage used in this experiment had non cytotoxicity on cancer cells, its antitumor metastasis ability could be studies unambiguously. As shown in the wound-healing assay, cell migration in the FM-treated group was significantly inhibited when compared with control group (Fig. 2A,B,C&D). Matrigel-coated transwell assay was used to detect the invasion inhibition of FM on the tumor cells. To this end, cells were pretreated with 0.5 mg/ml FM and were allowed to invade for 24 h. The results showed that the invasion ability was markedly inhibited by FM in both human and murine cancer cell lines (Fig. 2E,F,G&H). These results indicated that FM could inhibit both the migration and invasion capacity of cancer cells.

Fm Inhibits Emt In Liver Cancer Cells

Our next aim was to investigate how FM suppresses liver cancer migration and invasion. To this end, we detected the EMT markers of tumor cells using Western Blot assay. As illustrated in Fig. 3A&B, the epithelial marker E-cadherin increased while the expression of the mesenchymal marker vimentin decreased in the FM-treated group, compared with control group. Since EMT could be regulated by several transcription factors, such as Snail, Slug, Twist and ZEB1, we next determined whether they were involved in the FM induced EMT suppression. We found that the expression of Snail, Slug, Twist and ZEB1 all significantly decreased in FM-treated group when compared with control group (Fig. 3C&D). Based on these results, we concluded that FM suppressed EMT via down-regulating the transcription factors including Snail, Slug, Twist and ZEB1.

Fm Treatment Inhibits Emt By Disrupting Wnt/β-catenin Signaling In Hcc Cells

In order to determine the effect of FM on Wnt/β-catenin signaling, human or murine HCC cells were treated with or without FM. Our results demonstrated that FM treatment reduced the protein levels of DVL-2 and β-catenin (Fig. 4A&B). Moreover, immunofluorescence assays analyze the nuclear translocation of DVL-2 and β-catenin. In control group, DVL-2 protein was found in nuclear of nearly 80% of both the human and murine HCC cells. However, only 50%-60% of FM-treated cells showed nuclear staining with DVL-2 (Fig. 4C&D). In case of nuclear β-catenin, cells treated with FM also revealed a significantly reduction in the nuclear staining of β-catenin (Fig. 4E&F). These results support a possible Wnt/β-catenin signaling inhibition of FM in the HCC tumor cell lines.

Discussion

Frankincense and myrrh have been widely used in cancer treatment. However, its antitumor ability, especially in cancer progression has not been reported. In this study, we demonstrate that FM inhibited the invasion and metastasis in HCC cells. Moreover, we observed that FM-suppressed cancer progression was mediated by EMT. Finally, we clearly confirmed that FM inhibits the Wnt/β-catenin signaling pathway of EMT in HCC. Our findings show a novel antitumor/mechanism of this ancient herbal extract, which may provide a potential strategy for the clinical treatment of cancer.

The water extract of frankincense and myrrh have shown preliminary ability to inhibit cancer in vitro or in vivo studies. Peng Ren reported that frankincense could suppress tumor progress via regulating the AMPK/mTOR pathway[13]. Mengxue Sun also found that myrrh inhibits the proliferation and migration of cancer cells through the regulation of cyclooxygenase-2 expression[14]. Moreover, the Xihuang pill, mainly composed of frankincense and myrrh, has been applied for cancer therapy for more than 300 years[15]. In order to clarify the role of FM in tumor invasion and metastasis, the concentration of FM used in our study was non-toxic as 0.5mg/ml. Furthermore, by wound-healing and transwell assay, we found that FM markedly reduced the migration and invasion abilities of HCC cells. However, how FM suppresses the invasion and metastasis of HCC via the Wnt/β-catenin signaling of EMT is unclear.

Epithelial-mesenchymal transition (EMT) was primarily mediated by a set of core EMT transcription factors (EMT-TFs), including the Snail family, the E-box bound Zinc Finger Proteins (Zeb) family, and the Twist family[16]. In tumor cells, EMT was activated, EMT-TFS and downstream regulated genes influence a large number of stages in cancer progression, including caner development and metastasis[17]. As a member of the Snail family, snail1 overexpression is usually negatively correlated with E-cadherin expression, and positively correlated with tumor cell migration, invasion and enhancement of metastasis, which also predicts a worse prognosis[18].

Similar to snali1, slug is also a major inducer of EMT and an important mediator of Twist-induced EMT and tumor metastasis[19]. As a member of the Twist family, Twist1 is a key factor inducing Vimentin

expression, which is also associated with poor prognosis and high metastasis rate[20]. The Zeb family consists of Zeb1 and Zeb2, which have similar functions and lead to the increased proliferation and malignancy via inhibiting E-cadherin[21]. In our study, we provided evidence that FM decreased the expression of mesenchymal markers (N-cadherin and Vimentin) and EMT-activating transcription factors (Snail, Slug, Twist1 and ZEB1) while increasing the expression of the epithelial marker E-cadherin.

The Wnt/β-catenin signaling is reported to promote the migration and invasion of cancer cells by mediating EMT mechanism[22]. Moreover, the Dishevelled protein(DVL) is critical for protecting the degradation of β-catenin in Wnt signaling, where the interaction of DVL and β-catenin promotes the transcription of Wnt signaling[23, 24]. Consistent with our assumptions, our study showed that FM could suppress the DVL2 nuclear translocation as well as its protein level, suggesting that instability of DVL2 play an important role in regulating the Wnt/β-catenin signaling pathway. Nevertheless, the detailed mechanisms deserve further intensive investigation.

Conclusion

This study preliminarily confirmed that FM can block the epithelial mesenchymal transformation process by inhibiting the Wnt/β-catenin signaling pathway, thus suppressing the occurrence and development of hepatocellular carcinoma, which will deepen the understanding of FM in the treatment of malignant tumors. Given that FM have already been used in clinics, our findings may provide a novel and practical strategy for HCC therapy.

Declarations

Ethics approval and consent to participate

The study was approved by the research ethics committee of Kunshan Affiliated Hospital of Nanjing University of Chinese Medicine. All experimental methods were carried out in accordance with the relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

Xian Lu: Conceptualization, Writing - review &editing. Xian Lu: Methodology, Validation, Writing - original draft. Jialei, Mao & Yaodong, Wang: Methodology, Writing - review & editing. Yonggang, Huang: Visualization, Investigation. Maolin, Gu: Data curation and resources.

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Not applicable.

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Figures

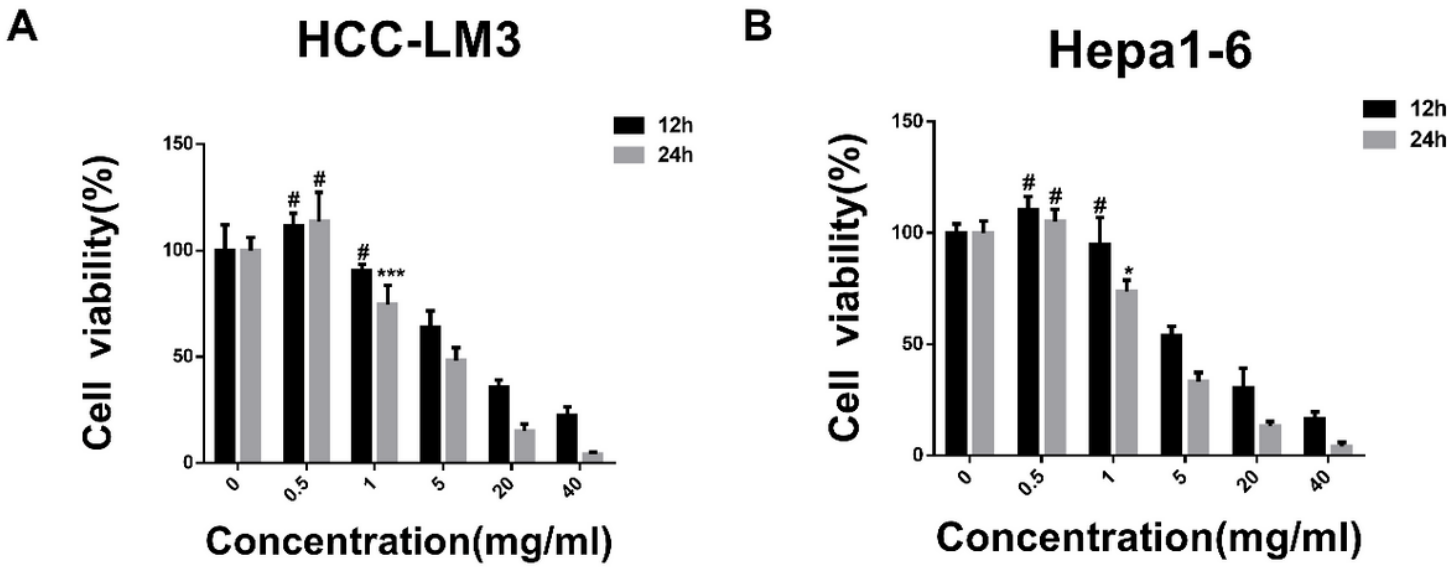

Figure 1

Growth inhibitory effect of FM on hepatocellular carcinoma cells. (A&B) HCC cell line-HCC-LM3 or Hepa1-6 cells were seeded in 96-well plates for overnight. Then different concentrations of FM were added into each well. After 12 h or 24 h, the cytotoxicity effect on tumor cells was determined by MTT assay. Untreated cells were considered as control. Data are shown as Means + SD. Statistical significance was determined as, #, not significant; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

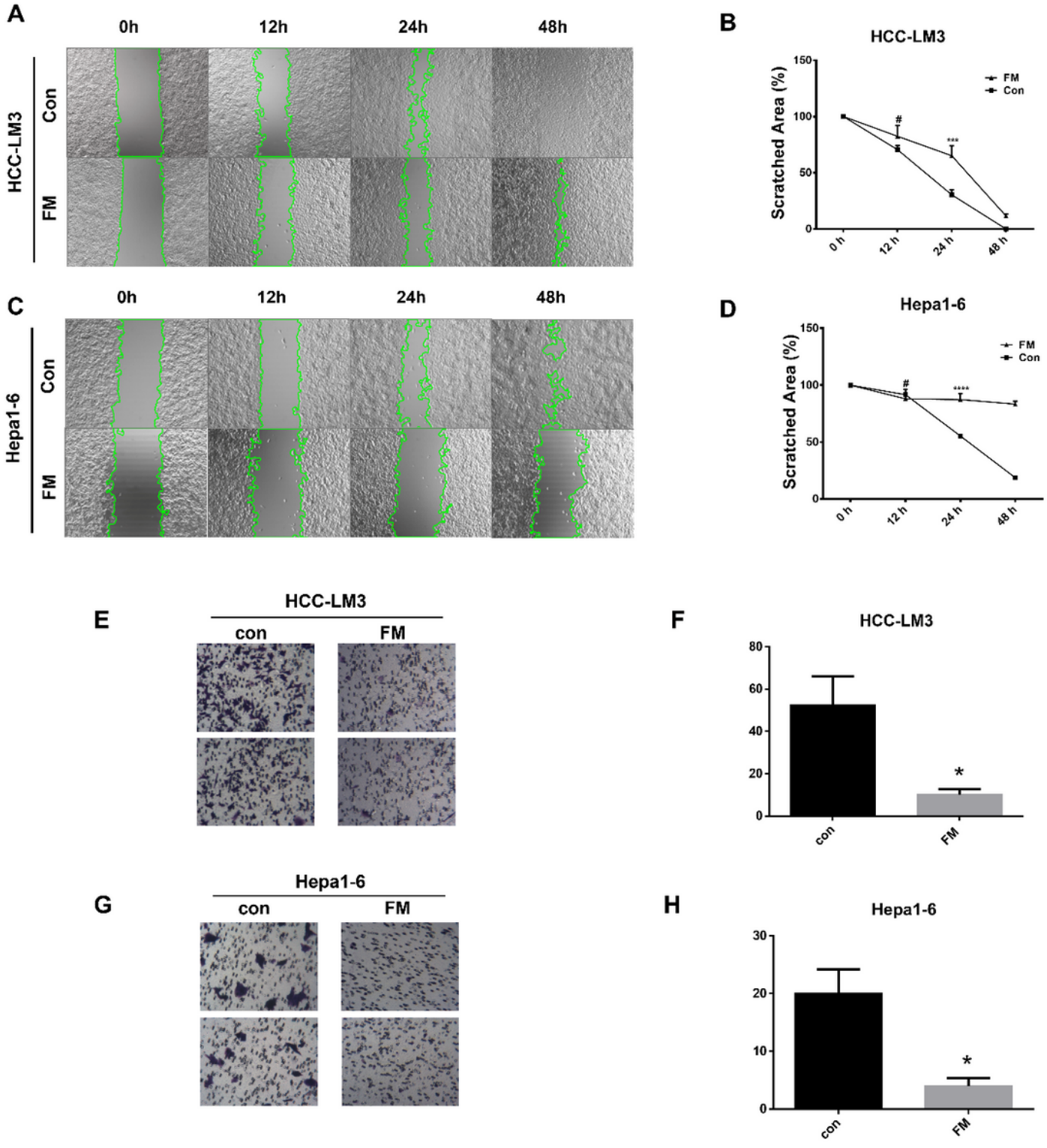

Figure 2

Wound-healing and transwell invasion assays after FM treatment. (A-D) The wound healing ability of HCC-LM3 and Hepa1-6 cells was inhibited by FM. Human HCC-LM3 or murine Hepa1-6 cells were treated with 0.5 mg/ml FM. At the determined time points, the wound area was photographed with phase contrast microscopy and the scratched area was quantified by Image J software. (E-H) The invasion assay of HCC cells treated with FM. HCC-LM3 or Hepa1-6 cells were pretreated with 0.5mg/ml FM for 24

h and the migrated cells were fixed and stained as described in the materials and methods. The quantification results are shown on the right. Statistical significance was determined as, #, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; and **** p < 0.0001.

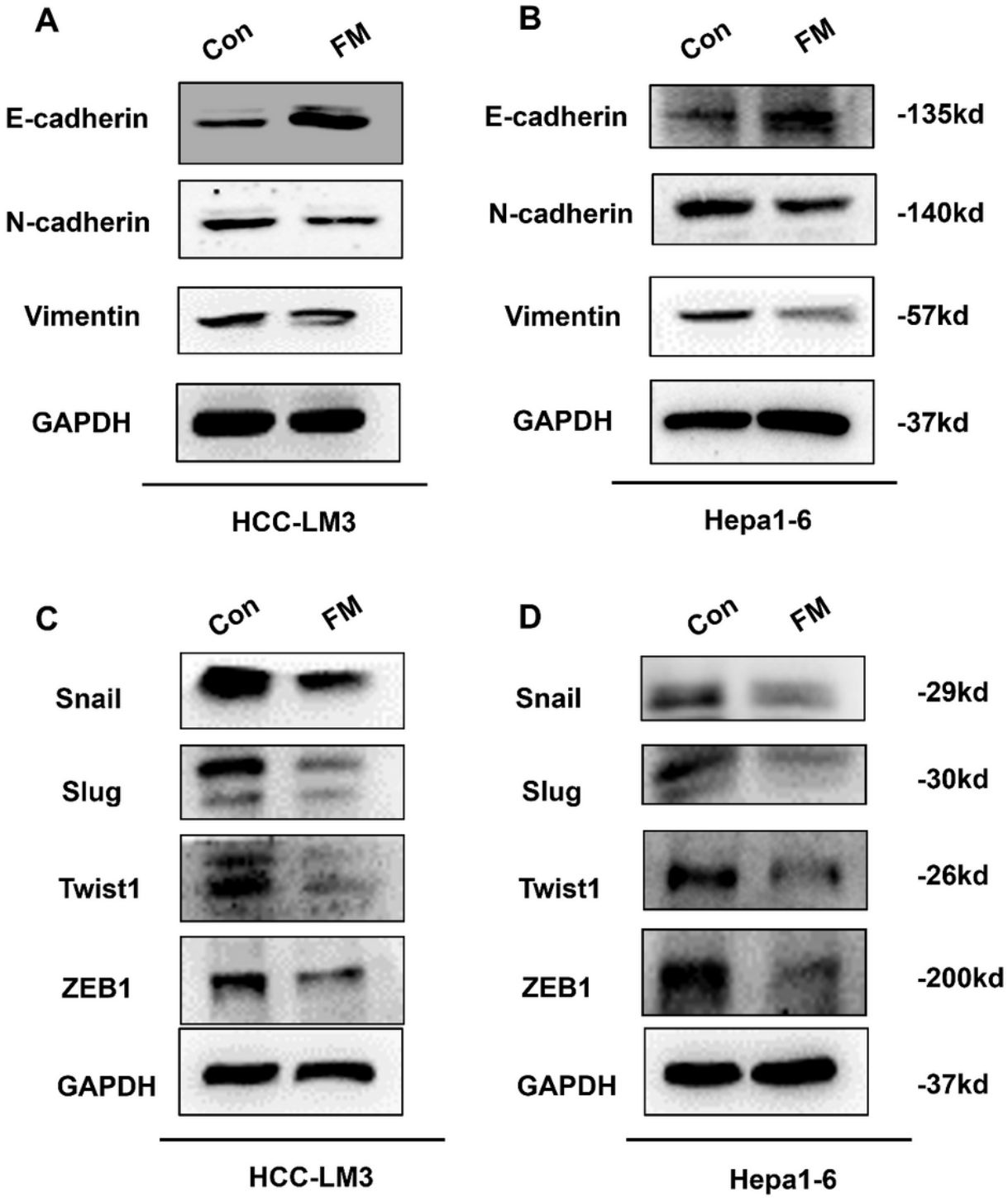

Figure 3

FM inhibits EMT by down-regulating Snail, Slug, Twist and ZEB1. (A&B) Expression of EMT markers in FM or control group. HCC-LM3 or Hepa1-6 cells were treated with or without 0.5 mg/ml FM for 24 h. Then EMT markers including E-cadherin, N-cadherin, Vimentin were measured by Western blot assay. (C&D) Expression of EMT transcription factors in liver cancer cells. Human or murine liver cancer cells were treated as described above. Expression of Snail, Slug, Twist and ZEB1 were determined with Western blot assay.

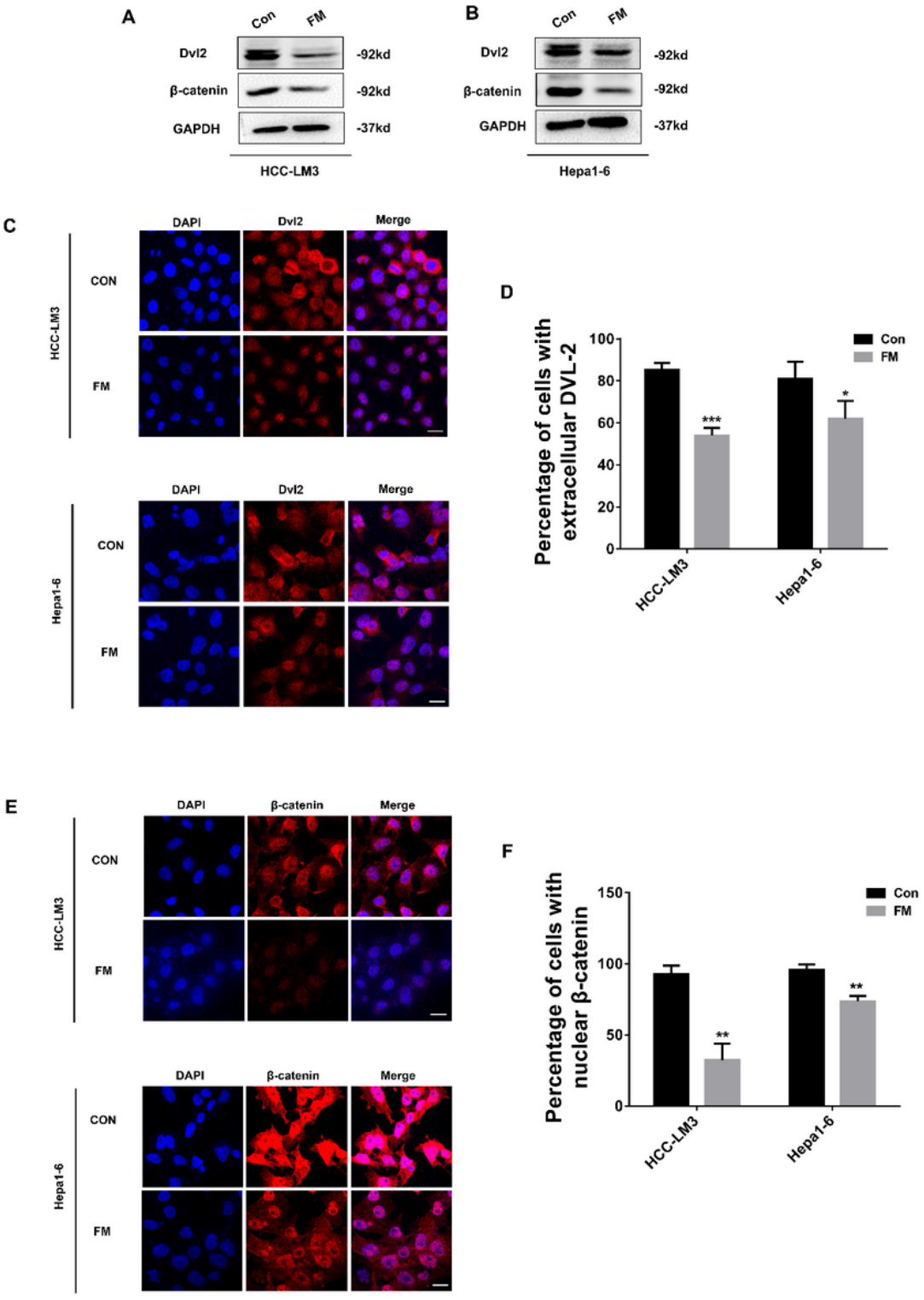

Figure 4

Effect of FM on the Wnt/β-catenin signaling pathway in liver cancer cells. (A&B) Proteins expression of the Wnt/β-catenin signaling pathway in control or FM group. HCC-LM3 or Hepa1-6 cells were treated with or without 0.5 mg/ml FM for 24 h. Then, cells were lysed for western blot to detect the specific protein. (C&E) DVL-2 or β-catenin immunostaining of HCC cells. HCC-LM3 or Hepa1-6 cells were treated as described in A&B, 24 h later, cells were harvested for the assay of immunofluorescence. Scale bars represent 20 μm. (D&F) Quantification of DVL-2 and β-catenin immunostaining in both human and murine cancer cells. Statistical significance was determined as, **#,** not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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