Anticancer effect of myristicin on hepatic carcinoma and related molecular mechanism

Hailan Bao\textsuperscript{a} and Qi Muge\textsuperscript{b}

\textsuperscript{a}College of Traditional Mongolian Medicine, Inner Mongolia University for Nationalities, Tongliao, China; \textsuperscript{b}Mengxi Integrative Medicine Division of Respiratory and Critical Care Medicine, Affiliated Hospital of Inner Mongolia University for Nationalities, Tongliao, China

ABSTRACT

Context: Myristicin is a natural active compound that has inflammatory, antimicrobial and anti-proliferative properties. Yet, its effect on hepatic carcinoma has not been investigated.

Objective: To explore the role and related molecular mechanism of myristicin in hepatic carcinoma in vitro.

Materials and methods: Human hepatic carcinoma cell lines (Huh-7 and HCCLM3 cells) were treated with different concentrations of myristicin (0.5, 1 and 5 mM) for 24, 48 and 72 h. Then, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay (MTT), flow cytometer (FCM) analysis and transwell assay were performed to determine cell proliferation, apoptosis and migration/invasion, respectively. Protein levels of B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X (Bax), E-cadherin, N-cadherin and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signalling pathway-related proteins were detected using Western blot assay. Gene expression was determined using quantitative real-time polymerase chain reaction (qRT-PCR).

Results: Myristicin inhibited cell proliferation and induced apoptosis in Huh-7 and HCCLM3 cells; suppressed cell migration and invasion ability, and increased E-cadherin expression and decreased N-cadherin expression, thereby inhibiting epithelial-mesenchymal transition (EMT). Finally, the findings indicated that myristicin decreased phosphorylated (p)-mTOR and p-AKT expression at the protein level.

Discussion and conclusions: Myristicin exerts an efficient therapeutic effect on hepatic carcinoma by suppressing PI3K/Akt/mTOR signalling pathway; thus, it may be used as a new potential drug for hepatic carcinoma treatment.

Introduction

Hepatic carcinoma is one of the most common malignant tumours in China and the third most common cause of cancer death worldwide (Chedid et al. 2017; Hartke et al. 2017). It originates in liver cells or intrahepatic gallbladder epithelial cells, and there are obvious regional differences in its distribution. The main features of advanced hepatic carcinoma include liver pain, keratitis, jaundice, ascites and some other symptoms (El Jabbour et al. 2019). It is an aggressive type of tumour with a high rate of metastasis and recurrence that presents with no early symptoms (FaRazi and Depinho 2006). The main treatments include tumour resection, chemotherapy and radiotherapy. However, the five-year survival rate of hepatic carcinoma patients remains low, especially due to drug resistance.

Myristicin (1-allyl-5-methoxy-3,4-methylenedioxybenzene) is a natural alkenylbenzene compound found in nutmeg (Lee et al. 2005; Martins et al. 2011), and in many favoured foods and dietary supplements, such as fennel, cinnamon, cloves, fennel, coriander, star anise and dried celery (Hallström and Thuander 1997), and in some medicinal plants, such as Todaraa aurea (Sol.) Parl. (Apioaceae), Daucus glochidiatus (Labill.) Fisch. & C.A.Mey. (Apioaceae) and Pseudoraya pumila (L.) Grande (Apioaceae). Previous research showed that myristicin possesses anti-inflammatory, antimicrobial and anti-proliferative properties (Lee and Park 2011; Stefano et al. 2011). In traditional medicine, myristicin has been used to treat cholera, stomach cramps, nausea, diarrhoea and anxiety (Martins et al. 2011). The inhibitory effects of myristicin on tumorigenesis have also been reported (Zheng et al. 1992; Lee et al. 2005). In this study, we explored the anticancer effect of myristicin on hepatic carcinoma and analysed the underlying regulatory mechanism.

Materials and methods

Cells

Human hepatic carcinoma cell (HCC) line Huh7 and HCCLM3 were obtained from American Tissue Culture Collection (ATCC). Cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY) medium...
supplemented with 10% foetal bovine serum (FBS; Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Gibco, Grand Island, NY) in a humidified atmosphere containing 5% CO₂/95% air at 37°C.

**MTT assay**

Cells were cultured in a 96-well plate at a density of 1.5 × 10⁴ cells per well and treated with different concentrations of myristicin (0.5, 1 and 5 mM) (Lee et al. 2005) for 24, 48 and 72 h. Cells without myristicin treatment were considered as the control. At each time point, 20 µL of sterile MTT dye (5 mg/mL, Sigma, St. Louis, MO) was added to each well and incubated for another 4 h at 37°C. The absorbance was measured at 570 nm by a microplate reader (Bio-Rad, Hercules, CA). The data were analysed as the means ± standard deviation (S.D.) of three separate experiments.

**FCM assay**

Cell apoptosis was performed by using the annexin-V/propidium iodide (PI) Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ). Briefly, after cells were treated with different concentrations of myristicin (0.5, 1 and 5 mM) for 48 h. Cells without myristicin treatment were considered as the control. Cells were collected, centrifuged at low temperature at high speed, and resuspended in 100 µL of FITC-binding buffer. Subsequently, samples were mixed with 5 µL ready-to-use annexin V-FITC and 5 µL PI into the buffer for 30 min at room temperature in dark. Annexin V-FITC and PI fluorescence were assessed by BD FACSCalibur flow cytometer (BD Technologies, Franklin Lakes, NJ).

**Transwell assay**

We used a transwell experiment to detect the cell migration and invasion ability. The difference between migration and invasion experiments was whether Matrigel (BD Biosciences, Franklin Lakes, NJ) was presented. Huh-7 or HCCLM3 cells were plated in a 24-wells plate and treated with myristicin for 48 h. Cells without myristicin treatment were considered as the control. Cells were then digested and resuspended in a serum-free DMEM medium. Next, they were placed in an upper chamber, while a DMEM medium with 20% FBS was placed in a lower chamber. After 24 h, the cells in the lower chamber were washed, fixed and then stained with 0.1% crystal violet. Finally, the invasive and migratory cells were observed with a microscope.

**Caspase-3 activity detection**

We detect caspase-3 activity by using a caspase-3 activity detection kit (Beyotime Biotechnology, Shanghai, China). Briefly, cells were collected into an EP tube, centrifuged at 600×g for 5 min at 4°C, and lysed for 15 min in an ice bath. After centrifugation for 10–15 min, the supernatant was collected and placed in an ice-bath pre-cooled centrifuge tube, after which the caspase-3 enzyme activity was measured at 405 nm.

**qRT-PCR assay**

Total RNA was acquired using TRIzol (TaKara, Kusatsu, Japan) following the manufacturer’s instructions. The RNA was transformed into cDNA by reverse transcription using cDNA (Vazyme, Nanjing, China). Subsequently, cDNA was used for amplification. We performed qPCR with SYBR Green PCR kit (Vazyme, Nanjing, China) followed by reference manual. GAPDH was used as endogenous control. Primer sequences for PCR were listed as following:

| Gene       | Forward | Reverse |
|------------|---------|---------|
| GAPDH      | 5'-AC-3' | 5'-GGACGAACTGGACAGTAACATGG-3' |
| Bcl-2      | 5'-GTAGAGCCAGGGATGTTCTT-3' |
| Bax        | 5'-GACATGGAGATGGCGCAACGCTGGGAGA-3' |
| C-Myc      | 5'-GGATCCTCGAGATGGCGCAGCTGGGAGA-3' |
| E-cadherin | 5'-CTTTGGTATCGTGGAAGGACTC-3' |
| N-cadherin | 5'-CTTTGGTATCGTGGAAGGACTC-3' |

The 2^-ΔΔCT method (Livak and Schmittgen 2001) was used to quantify relative gene expression.

**Western blot assay**

The cells were lysed, and total protein was obtained by using RIPA buffer (Solarb, Beijing, China). A bicinchoninic acid assay kit (Pierce, Appleton, WI) was used to quantify the total protein. An equal number of proteins were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for 40 min and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked for 1.5 h with 5% non-fat milk to prevent non-specific binding and then incubated with primary antibodies including anti-B-cell lymphoma-2 (Bcl-2) (cat. no. ab32124; dilution: 1:1000; Abcam, Cambridge, UK), anti-E-cadherin (cat. no. ab76011; dilution: 1:1000; Abcam, Cambridge, UK), anti-mTOR (cat. no. ab134903; dilution: 1:1000; Abcam, Cambridge, UK), anti-N-cadherin (cat. no. ab76011; dilution: 1:1000; Abcam, Cambridge, UK), anti-internal control for normalization. Protein expression was quantified by ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD).

**Statistical analysis**

Results were analysed by GraphPad Prism 6.0 software (La Jolla, CA). Statistical significance between groups was determined by Student’s t-test or one-way analysis of variance (ANOVA) followed by Tukey’s post hoc tests. Data were from three
independent experiments. A $p < 0.05$ was considered to be statistically significant.

**Results**

**The effect of myristicin on HCC proliferation**

Myristicin is a natural alkenylbenzene compound. Its chemical formula is shown in Figure 1(A). MTT assay was used to explore the effect of myristicin on HCC viability. Our results showed that compared to the control group, myristicin significantly suppresses Huh-7 (Figure 1(B)) and HCCLM3 (Figure 1(C)) cell proliferation.

**The effect of myristicin on HCC apoptosis**

The effect of myristicin on early and late apoptosis was examined using a flow cytometer (FCM) assay to detect cell apoptosis. FCM assay showed that compared to the control group, myristicin significantly induced apoptosis and improved caspase-3 activity in Huh-7 cells (Figure 2(A–C)) and HCCLM3 cells (Figure 3(A–C)). Meanwhile, myristicin decreased Bcl-2 expression and increased Bax expression at protein and mRNA levels in Huh-7 cells (Figure 2(D–F)) and HCCLM3 cells (Figure 3(D–F)). Taken together, myristicin promoted cell apoptosis.

**The effect of myristicin on the migration and invasion of HCC**

Our results demonstrated that with the increase of myristicin concentration, migration (Figure 4(A,B)) and invasion (Figure 4(C,D)) ability of Huh-7 cells was significantly inhibited compared with the control group. The same results were observed in HCCLM3 cells (Figure 5(A–D)).

**The effect of myristicin on EMT of HCC**

Epithelial–mesenchymal transition (EMT) is an important sign of cancer metastasis. We detected EMT-related protein expression. Western blot assay indicated that compared to the control group, myristicin increased E-cadherin expression and decreased N-cadherin expression in HCC cells (Figures 6(A) and 7(A)). In addition, quantitative real-time polymerase chain reaction (qRT-PCR) assay showed that compared to the control group, E-cadherin was significantly up-regulated (Figures 6(B) and 7(B)) and N-cadherin was down-regulated (Figures 6(C) and 7(C)) in HCC cells.

**Myristicin suppressed PI3K/Akt/mTOR signalling pathway in HCC**

To explore the mechanism of myristicin in HCC, we detected some protein expression in phosphatidylinositol 3-kinase (PI3K)/
Akt/mTOR signalling pathway. Western blot assay showed that in Huh-7 and HCCLM3 cells, myristicin decreased p-mTOR and p-AKT expression (Figures 8(A) and 9(A)) and significantly decreased the ratio of p-mTOR/mTOR (Figures 8(B) and 9(B)) and p-AKT/AKT (Figures 8(C) and 9(C)) in HCC cells.

**Discussion**

The development of hepatic carcinoma has seriously affected people’s living standards. In recent years, although the medical level has been continuously improved, hepatic carcinoma has a high mortality rate due to the difficulty of early diagnosis of hepatic carcinoma patients, fewer therapeutic targets and poor post-operative recovery. After surgical resection of hepatic carcinoma patients, the number of patients who survived one year after surgery was more than 80%, and the number of patients who survived 5 years was more than 50% (Margarit et al. 2005). Therefore, it is very important to continuously explore new treatment strategies to treat hepatic carcinoma patients. In the present study, we provided some evidence that myristicin is a
natural compound that can be used for HCC treatment to a certain extent.

Myristicin is the main active ingredient of nutmeg (Jana and Shekhawat 2010; Muchtaridi et al. 2010) and coriander leaf oil (Zheng et al. 1992; Wei and Shibamoto 2007), which is mainly used as a flavouring agent, but also as a spice in the cosmetics industry. Previous research showed that myristicin could produce excitement and hallucinogenic effects. It may also be potentially...
toxic, as overdose can cause hallucinations and, in some cases, even coma (Stein et al. 2001; Sivathanu et al. 2014). Lee and Park (2011) demonstrated that myristicin has anti-inflammatory effects by inhibiting inflammatory factor expression. Lee et al. (2005) further found that myristicin induces early apoptotic events in human neuroblastoma, including cytochrome c release, caspase-3 activation and PARP lysis, leading to the death of SK-N-SH cells. In our study, we investigated the effect of myristicin on hepatic carcinoma. Our results showed that myristicin suppressed cell proliferation and induced apoptosis in Huh-7 and HCCLM3 cells.

Hepatic carcinoma is an aggressive type of tumour with a high rate of metastasis. As the continuous metastasis of tumours leads to high incidence and mortality, it is necessary to effectively treat hepatic carcinoma from the perspective of inhibiting cancer metastasis (Wu et al. 2015). Tumour metastasis depends on the migration and invasion capabilities of cancer cells (Hua et al. 2018). In this study, myristicin inhibited cell migration and invasion.

EMT is a process that refers to the transformation of epithelial to mesenchymal cells, which gives cells the ability to transfer and invade (Lamouille et al. 2014). EMT is currently considered an important reminder of cancer metastasis (Ye et al. 2017). In addition, during the early stage of cancer metastasis, the adhesion of cancer cells can be separated by reducing EMT. The main markers of EMT include epithelial cell marker (E-cadherin) and mesenchymal cell markers (N-cadherin). In this study, we found that myristicin suppressed EMT by increasing E-cadherin expression and decreasing N-cadherin expression.

PI3K/Akt/mTOR signalling pathway is involved in development of many cancers (Costa et al. 2018; Alzahrani 2019; Ediriweera et al. 2019). Activation of this signalling pathway can promote cell growth, proliferation and participate in angiogenesis (Alzahrani 2019). It can also exert a crucial role in mediating cell movement, invasion and metastasis (Jeong et al. 2014; Ramadan et al. 2020). PI3K/Akt/mTOR signalling pathway inhibition has been shown to be used to inhibit HCC (Kudo 2011; Ye et al. 2019). Hua et al. (2018) indicated that ruscogenin inhibits HCC migration and invasion by regulating PI3K/Akt/mTOR signalling pathway. Furthermore, Song et al. (2020) indicated that miR-29-3p overexpression suppresses HCC growth by inhibition of Robo1 and inactivation of PI3K/Akt/mTOR signalling pathway. Also, Sun et al. (2019) showed that miR-1914 exerted the inhibiting effect through the PI3K/Akt/mTOR signalling pathway in HCC. Consistent with the previous research results, our data suggested that myristicin suppresses the activation of the PI3K/Akt/mTOR signalling pathway in hepatic carcinoma.

However, this study is a preliminary in vitro study of the effect of myristicin on hepatocellular carcinoma. In order to make myristicin’s effect on hepatocellular carcinoma more
convincing, more in-depth research is needed. For example, the role of myristicin in other types of hepatic carcinoma cell lines should be studied. Besides, the effect of more doses of myristicin (a minimum of five doses is required) on HCC cells should be studied. Moreover, additional in vivo studies are required to confirm these findings. Therefore, in our next study, we plan to examine the effect of more dose of myristicin in other types of hepatic carcinoma cell lines and in a mouse model bearing an HCC tumour.

Conclusions

Myristicin prevents the malignant biological behaviour of hepatic carcinoma cells by inhibiting the PI3K/Akt/mTOR signalling pathway. Thus, myristicin is a potential therapeutic agent for hepatic carcinoma.

Disclosure statement

The authors declare no conflict of interest.

Funding

This research was supported by the Natural Science Foundation of Inner Mongolia Autonomous Region (Grant No. 2020LH08012).

ORCID

Qi Muge http://orcid.org/0000-0003-4988-7514

References

Alzahrani AS. 2019. PI3K/Akt/mTOR inhibitors in cancer: at the bench and bedside. Semin Cancer Biol. 59:125–132.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jamal A. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 68(6):394–424.

Chedd MF, Kruel CRP, Pinto MA, Grezza-Filho JTM, Leinpitz I, Kruel CDP, Scafferio LA, Chedd AD. 2017. Hepatocellular carcinoma: diagnosis and operative management. Arq Bras Cir Dig. 30(4):272–278.

Costa RLB, Han HS, Gradishar WJ. 2018. Targeting the PI3K/AKT/mTOR pathway in triple-negative breast cancer: a review. Breast Cancer Res Treat. 169(3):397–406.

Ediriweera MK, Tannekoon KH, Samarakoon SR. 2019. Role of the PI3K/AKT/mTOR signaling pathway in ovarian cancer: biological and therapeutic significance. Semin Cancer Biol. 59:147–160.

El Jabbour T, Lagana SM, Lee H. 2019. Update on hepatocellular carcinoma. Ediriweera MK, Tennekoon KH, Samarakoon SR. 2019. Role of the PI3K/AKT/mTOR pathway in triple-negative breast cancer: a review. Breast Cancer Res Treat. 169(3):397–406.

Hartke J, Johnson M, Ghabril M. 2017. The diagnosis and treatment of hepatocellular carcinoma. Semin Diagn Pathol. 34(2):153–159.

Hua H, Zhu Y, Song YH. 2018. Ruscogenin suppressed the hepatocellular carcinoma metastasis via PI3K/Akt/mTOR signaling pathway. Biomed Pharmacother. 101:115–122.

Jana S, Shekhawat GS. 2010. Anethum graveolens: an Indian traditional medicinal herb and spice. Pharmacogn Rev. 4(8):179–184.

Jeng SY, Cho YJ, Shin JM, Kang JH, Park KK, Choe JY, Baes YS, Han SM, Kim CH, et al. 2014. Melittin suppresses EGFR-induced cell motility and invasion by inhibiting PI3K/Akt/mTOR signaling pathway in breast cancer cells. Food Chem Toxicol. 68:218–225.

Kudo M. 2011. mTOR inhibitor for the treatment of hepatocellular carcinoma. Dig Dis. 29(3):310–315.

Lamouille S, Xu J, Derynick R. 2014. Molecular mechanisms of epithelial–mesenchymal transition. Nat Rev Mol Cell Biol. 15(3):178–196.

Lee BK, Kim JH, Jung JW, Choi JW, Han ES, Lee SH, Ko KH, Ryu JH. 2005. Myristicin-induced neurotoxicity in human neuroblastoma SK-N-SH cells. Toxicol Lett. 157(1):49–56.

Lee JY, Park W. 2011. Anti-inflammatory effect of myristicin on RAW 264.7 macrophages stimulated with polyinosinic–polycytidylic acid. Molecules. 16(8):712–7142.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 25(4):402–408.

Margarit C, Escartín A, Castells L, Vargas V, Allende E, Bilbao I. 2005. Resection for hepatocellular carcinoma is a good option in Child-Turcotte-Pugh class A patients with cirrhosis who are eligible for liver transplantation. Liver Transpl. 11(10):1242–1251.

Martins C, Duran C, Laires A, Ruff J, Rodrigues AS. 2011. Genotoxic and apoptotic activities of the food flavourings myristicin and eugenol in Aa8 and XRCc1 deficient EM9 cells. Food Chem Toxicol. 49(2):385–392.

Muchtaridi A, Subarana A, Apriyantono R. 2010. Identification of compounds in the essential oil of nutmeg seeds (Myristica fragrans Houtt.) that inhibit locomotor activity in mice. Int J Mol Sci. 11:4771–4781.

Ramadan F, Fahs A, Ghayad SE, Saab R. 2020. Signaling pathways in rhadomyosarcoma invasion and metastasis. Cancer Metastasis Rev. 39(1):287–301.

Siegel RL, Miller KD, Jemal A. 2019. Cancer statistics, 2019. CA Cancer J Clin. 69(1):7–34.

Sivathanu S, Sampath S, David HS, Rajavelpu KK. 2014. Myristicin and phenylthioctin toxicity in an infant. BMJ Case Rep. 2014:bcr2013203000.

Song Q, Zhang H, He J, Kong H, Tao R, Huang Y, Yu H, Zhang Z, Huang Z, Wei L, et al. 2020. Long non-coding RNA LINCO0473 acts as a microRNA-29a-3p sponge to promote hepatocellular carcinoma development by activating Rob1-dependent PI3K/AKT/mTOR signaling pathway. Ther Adv Med Oncol. 12:1758835920937890.

Stefano VD, Pitzonzo R, Schillaci D. 2011. Antimicrobial and antiproliferative activity of Athamanta sicula L. (Apiaceae). Pharmacogn Mag. 7(25):31–34.

Stein U, Greyer H, Hentschel H. 2001. Nutmeg (myristicin) poisoning report on a fatal case and a series of cases recorded by a poison information centre. Forensic Sci Int. 118(1):87–90.

Sun L, Wang L, Chen T, Yao B, Wang Y, Li Q, Wang Y, Liu Z. 2019. microRNA-194, which is regulated by IncRNA DUXAP10, inhibits cell proliferation by targeting the GPR39-mediated PI3K/AKT/mTOR pathway in HCC. J Cell Mol Med. 23(12):8292–8304.

Wei A, Shibamoto T. 2007. Antioxidant activities and volatile constituents of the cinal herb and spice. Pharmacogn Rev. 4(8):179–184.

Xie Z, Wei L, et al. 2020. Long non-coding RNA LINC00473 acts as a ceRNA sponge to promote hepatocellular carcinoma development by activating Rob1-dependent PI3K/AKT/mTOR signaling pathway. Ther Adv Med Oncol. 12:1758835920937890.

Yi S, Hua H, Zhu Y, Song YH. 2018. Ruscogenin suppressed the hepatocellular carcinoma metastasis via PI3K/Akt/mTOR signaling pathway. Biomed Pharmacother. 101:115–122.

Zhang L, Wang L, Yang H, Zhao Y, Han J, et al. 2007. Recurrently deregulated lncRNAs in hepatocellular carcinoma. Nat Commun. 8:14421.

Ye L, Mayerle J, Ziesch A, Reiter FP, Gerbes AL, De Toni EN. 2019. The anti-inflammatory activity of Athamanta Sicula L. ( Apiaceae). Pharmacogn Mag. 7(25):31–34.

Ye L, Mayerle J, Ziesch A, Reiter FP, Gerbes AL, De Toni EN. 2019. The anti-inflammatory activity of Athamanta Sicula L. ( Apiaceae). Pharmacogn Mag. 7(25):31–34.

Ye L, Mayerle J, Ziesch A, Reiter FP, Gerbes AL, De Toni EN. 2019. The anti-inflammatory activity of Athamanta Sicula L. ( Apiaceae). Pharmacogn Mag. 7(25):31–34.

Ye X, Brablitz T, Kang Y, Longmore GD, Nieto MA, Stanger BZ, Yang J, Weinberg RA. 2017. Upholding a role for EMT in breast cancer metastasis. Nature. 547(7661):E1–E3.

Zheng GQ, Kenney PM, Zhang J, Lam LKT. 1992. Inhibition of benzo[a]pyrene-induced tumorigenesis by myristicin, a volatile aroma constituent of parsley leaf oil. Carcinogenesis. 13(10):1921–1923.