Department of Otolaryngology – Head and Neck Surgery[1], Guangdong Provincial People's Hospital Zhuhai Hospital (Zhuhai Golden Bay Center Hospital), Zhuhai, China; Department of Stomatology[2], Guangdong Provincial People's Hospital Zhuhai Hospital (Zhuhai Golden Bay Center Hospital), Zhuhai, China; Department of Otolaryngology – Head and Neck Surgery[3], Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China

Inhibitory effect of icaritin on proliferation, migration, and invasion of human nasopharyngeal carcinoma cell CNE2 by regulating STAT3 activation

Xiangdong Li[1], Chunlei Li[3], Peiyu Zhou[1], Shaohua Chen[1,2,*]

Received June 18, 2019, accepted July 25, 2019

**Corresponding author: Shaohua Chen, Department of Otolaryngology-Head and Neck Surgery, Guangdong Provincial People's Hospital Zhuhai Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China chenshaohua12@aliyun.com*

Pharmazie 74: 685-687 (2019) *doi: 10.1691/ph.2019.9632*

The influence of icaritin on the proliferation, migration, and invasion of human nasopharyngeal carcinoma cell CNE2 and the influencing mechanisms were discussed. Results indicated that icaritin can realize dosage-dependent inhibition of CNE2 cell proliferation, migration, and invasion and inhibit the expression levels of VEGF, KDR, bFGF, MMP2, and MMP9. It can also inhibit STAT3 phosphorylation without affecting STAT3 expression. Icaritin exerts multiple bonding effects on the SH2 structural domain of STAT3. Therefore, icaritin can become a candidate drug for resisting nasopharyngeal carcinoma, and its mechanism is related to the blocking of STAT3 signaling pathway activation.

1. Introduction

About 15–50 in every 100,000 people are diagnosed with nasopharyngeal carcinoma yearly. As the principal active constituent of *Herba epimedii* (Chinese herb), icariin augments and promotes the hematopoietic function (Fang et al. 2017), is active in the nervous system (Jin et al. 2019), invigorates the kidney, and strengthens Yang (Shen et al. 2018), and shows anti-aging activity (Chen et al. 2019). Icaritin is an intestinal metabolite of icariin (Fig. 1), and research has shown that it exerts strong *in-vitro* inhibitory effects on hepatic carcinoma, lymphoma, and cervical carcinoma (Wu et al. 2015; Tan et al. 2016). Thus, comprehensive investigations of the anti-tumor effect of icaritin are continuously performed. However, no study has been conducted on the effect of icaritin on nasopharyngeal carcinoma. Through an analysis of the influence of icaritin on the proliferation, migration, and invasion of human nasopharyngeal carcinoma cell CNE2, the inhibitory mechanism of icaritin on nasopharyngeal carcinoma was preliminarily discussed in this work to provide a basis for further development and utilization of icaritin.

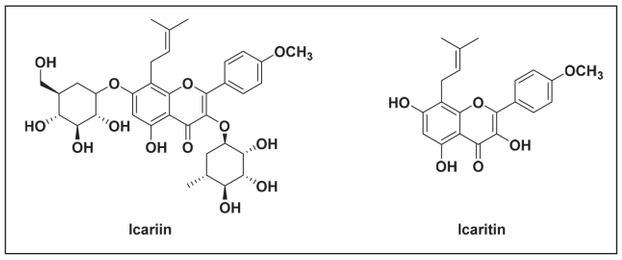

Fig. 1: Chemical structures of icariin and icaritin.

2. Investigations and results

2.1. Effect of icaritin on the proliferation of human nasopharyngeal carcinoma cell CNE2

The inhibitory effect of icaritin on human nasopharyngeal cell proliferation was evaluated through an MTT experiment. The results are shown in Fig. 2. After icaritin (concentration: 0, 1, 5, 10, 20, 40, and 80 mmol/L) reacted with CNE2 for 48 h, it exerted a dosage-dependent inhibitory effect on the proliferation of human nasopharyngeal carcinoma cell CNE2, and the IC_{50} value was 24.8±2.7 mmol/L.

2.2. Effect of icaritin on the invasion and migration of human nasopharyngeal carcinoma cell CNE2

An invasion and migration experiment was conducted to evaluate further the resisting effect of icaritin on nasopharyngeal carcinoma. Fig. 3 shows effects of icaritin on CNE2 cell invasion and migration. After CNE2 cells were treated using icaritin with concentrations of 20 and 40 mmol/L for 48 h, the invasion and migration capabilities of CNE2 cells were inhibited in a dosage-dependent manner. The analysis of related proteins influencing the invasion and migration of tumor cells showed that unlike in the blank control group, the expression levels of VEGF, KDR, bFGF, MMP2, and MMP9 in cell lines in all experimental groups declined with icaritin.

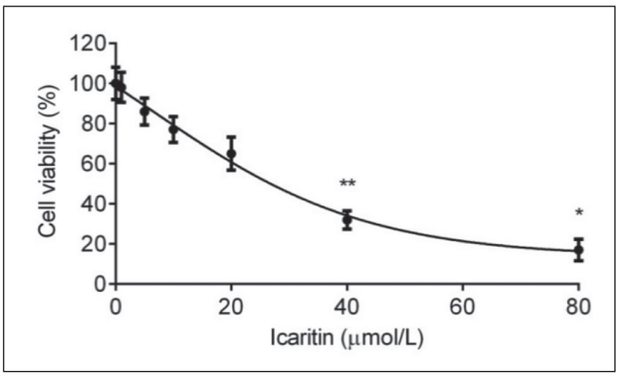

Fig. 2: Inhibitory effect of icaritin on CNE2 proliferation compared with the 0 mmol/L experimental group; * $p < 0.05$ and ** $p < 0.01$.

2.3. Inhibitory effect of icaritin on STAT3 phosphorylation

STAT3 plays a crucial role in the proliferation, migration, and angiogenesis of tumor cells (Bosch-Barrera et al. 2017). Studies

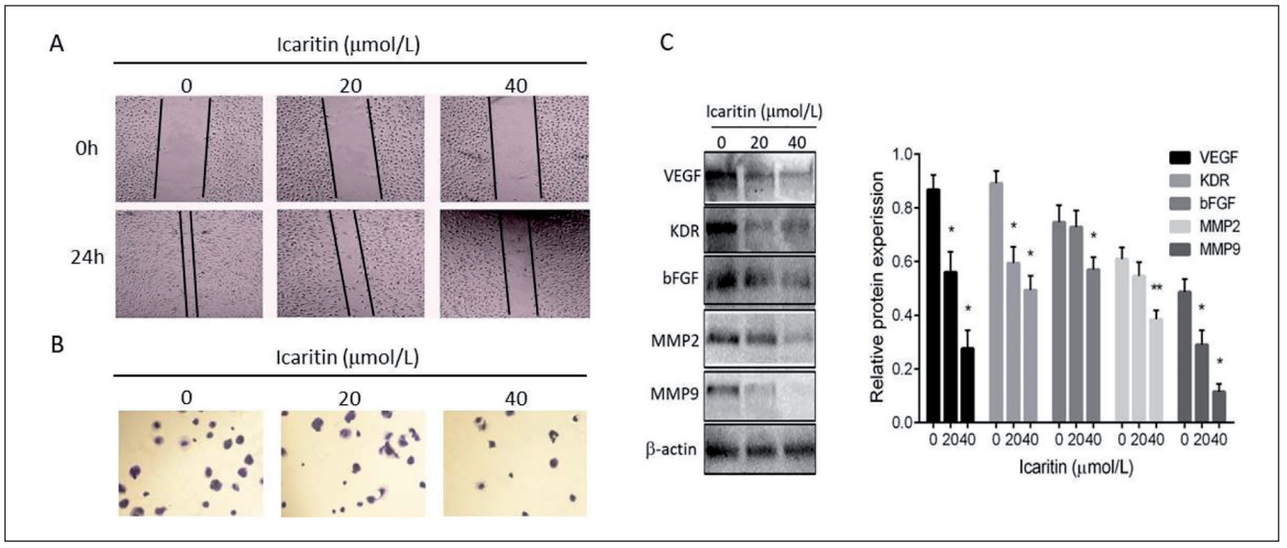

Fig. 3: Inhibitory effect of icaritin on the invasion and migration of CNE2 cells. (A, B) Migration (×100) and invasion (×200) of CNE2 cells after treatment with 20 and 40 mmol/L of icaritin for 48 h. (C) Detection of proteins related to invasion and migration in comparison with the 0 mmol/L experimental group, * $p < 0.05$, and ** $p < 0.01$.

have indicated that STAT3 activation regulates VEGF activation, and 705-site tyrosine phosphorylation (Tyr705) in STAT3 should be carried out to realize STAT3 activation (Furtek et al. 2016). The effects of icaritin on the expression and phosphorylation of STAT3 were investigated through Western blot analysis. The results are shown in Fig. 4. Icaritin exerted a dosage-dependent inhibitory effect on STAT3 phosphorylation without influencing STAT3 expression. This result reveals that icaritin may exert an anti-tumor effect by inhibiting STAT3 activation.

2.4. Bonding effect of icaritin on the STAT3 SH2 structural domain through molecular docking

The molecular docking results are shown in Fig. 5. Icaritin occupied two sub-capsules of the STAT3 SH2 structural domain through which the solvent can enter and formed three intermolecular hydrogen bonds (ARG 595, PRO 639, and SER 636) in amino acid residues. This findings indicate that icaritin may inhibit STAT3 phosphorylation by bonding with the SH2 structural domain.

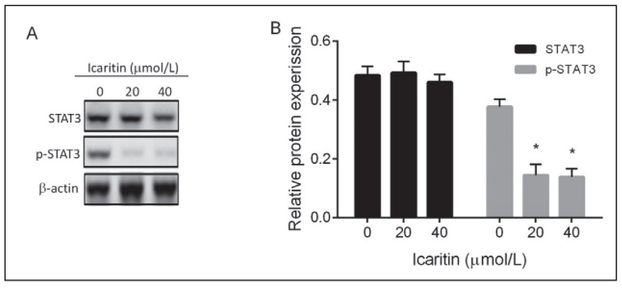

Fig. 4: Effect of icaritin on the expression levels of p-STAT3 and STAT3. (A) Protein bar graph and (B) relative expressions of p-STAT3 and STAT3 compared with the 0 mmol/L experimental group. * $p < 0.05$.

3. Discussion

Seeking for effective constituents in natural products is one of the important development directions for new drugs. As a flavonoid compound, icaritin is derived from a compound contained in traditional Chinese medicine, icariin. Icaritin has extensive anti-tumor effects, but its anti-tumor mechanism remains unclear. He et al. (2010) found that icaritin can inhibit the proliferation of hepatic carcinoma HepG2 by activating the JNK1 signaling pathway (He et al. 2010). Icaritin can inhibit the activation of JAK/STAT3, MAPK/ERK, and PI3K/AKT signaling pathways and induce apoptosis of

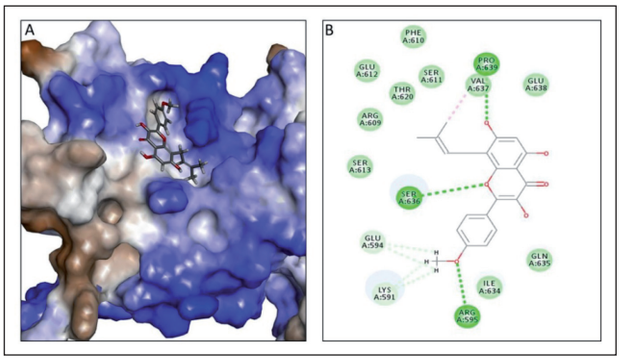

Fig. 5: Icaritin docking result for the STAT3 SH2 structural domain (PDB number: 1BG1)[4]. (A) 3D bonding mode pattern and (B) 2D bonding mode pattern. Green represents the formation of intermolecular hydrogen bonds, light green represents VDW force, and light blue represents the hydrophobic effect.

multiple lymphoma cells (Li et al. 2013; Wu et al. 2015). In this study, nasopharyngeal cells were selected as study objects, and the results showed that Icaritin can significantly inhibit the proliferation, migration, and invasion of CNE2 cells.

As a common pathway for signal transduction of multiple cytokines and growth factors among JAK/STAT signal transduction pathways, STAT3 plays a vital role in tumor invasion, migration, and angiogenesis (Mohr et al. 2012). During tumor invasion and migration, matrix metalloproteinase (MMP) can degrade most of the proteins of the basilar membrane and extracellular matrix (ECM), remodel the local microenvironment, and promote tumor neovascularization. Smooth transduction of the MMP signal can induce tumor genesis, and continuous activation of STAT3 can promote the development of the entire process (Liang et al. 2018). Tumor neovascularization is the basis for tumor cell invasion and migration. VEGF is the most direct acting factor that stimulates tumor angiogenesis at present, and continuously activated STAT3 can induce VEGF expression and cause tumor neovascularization. Through an analysis of the expression levels of VEGF, KDR, bFGF, MMP2, and MMP9, this study found that icaritin could significantly inhibit the invasion and migration of these tumor cells and the expression of related proteins. On the basis of this result, the effect of icaritin on the STAT3 signaling pathway was studied. The result showed that icaritin can inhibit STAT3 phosphorylation without affecting STAT3 expression, indicating that Icaritin inhibits the proliferation, migration, and invasion of CNE2 cells by inhibiting STAT3 activation.

The SH2 structural domain is located at the top end of the "clamp-shaped" STAT3 protein. In the JAK/STAT3 signal transduction pathway, activated JAKs catalyze the tyrosine phosphorylation of the receptor itself and form corresponding STAT3 docking sites so that STAT3 is bonded with the receptor through the SH2 structural domain and realizes phosphorylation under the effect of JAKs. Afterward, STAT3 forms homodimers/heterodimers, which are transported to the nucleus and bond with the target gene promoter to activate corresponding genetic transcription and expression (Li et al. 2018). Molecular docking revealed that icaritin occupied two sub-capsules of the SH2 structural domain through which the solvent could enter and bind with the SH2 structural domain through multiple actions. This result indicates that icaritin may inhibit STAT3 phosphorylation by bonding with the SH2 structural domain to inhibit the formation of STAT3 dimer and its transportation to the nucleus and block the activation of the STAT3 signaling pathway. However, whether icaritin can exert this effect still requires experimental verification.

In summary, the study results indicate that icaritin can inhibit the proliferation, migration, and invasion of nasopharyngeal carcinoma cell CNE2, and its mechanism may be related to the activation of the STAT3 signaling pathway. This result provides an additional theoretical bases for icaritin to become a lead compound for anti-cancer drugs.

4. Experimental

4.1. Materials

Icaritin was purchased from Guangzhou Zhongshan Enantiotech Co., Ltd. 3-(4,5-dimethyl-2-thiazolyl)-2,5-dyphenyl-2H-tetrazolium bromide (MTT) was purchased from Shanghai Crystal Pure Reagents Co., Ltd. Anti-VEGF, anti-bFGF, anti-MMP2, anti-MMP9, anti-KDR, anti-STAT3, anti-p-STAT3 (Tyr705), and anti-b-actin antibodies were purchased from US Cell Signaling Technology Corporation.

4.2. Cytotoxicity experiment

CNE2 cells in the logarithmic phase were inoculated on a 96-pore plate (density was 5,000 cells/pore). After 24 h of culturing, icaritin (concentration: 0, 1, 5, 10, 20, 40, and 80 mmol/L) was added for another 48 h of culturing. Then, 20 mL of 5 mg/mL MTT was added to each pore after the culture medium was absorbed. The mixture was continuously incubated for 4 h at 37 °C, and 100 mL of DMSO was added in each pore. After blending, absorbance was detected at a wavelength of 490 nm. All experiments were repeated three times.

4.3. Scratch test

CNE2 cells in the logarithmic phase were inoculated on a six-pore plate. When the cells grew to 80–90 %, the culture medium was absorbed, and a line was drawn along the middle axis of the cell pore by using a sterile spearhead. The cells were scratched away, and the scratched cells were rinsed with PBS and added with a serum-free medium with concentrations of 0, 20, and 40 mmol/L of icaritin for continuous 48 h of culturing. The mixture was then placed under an inverted microscope to observe cell growth at the scratches, and photos were obtained.

4.4. Transwell experiment

CNE2 cells in the logarithmic phase were inoculated on a 24-pore plate. They were cultured continuously for 24 h by using a serum-free medium. Cells were collected, and cell density was adjusted to 1.5×10^5. Matrigel-coated Transwell pores were placed on the 24-pore plate, and a 200 mL cell suspension was added to the upper chamber. Meanwhile, a 20 % fetal calf serum culture medium containing icaritin (final concentration: 0, 20, and 40 mmol/L) was added to the lower chamber. Culturing for 24 h was implemented in a 37 °C incubator, and the chamber was subsequently removed and immobilized using ethanol. After dyeing, it was placed under an inverted microscope, followed by observation and photo taking.

4.5. Western blot

Cells were collected after icaritin (0, 20, and 40 mmol/L) was incubated with CNE2 cells for 48 h or after independent cell culturing. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was implemented to separate the protein transmembrane after protein quantification, which was then sealed in a buffer solution containing 5 % defatted milk and prepared using TBS-T. An hour later, the membrane was rinsed three times by using a TBS-T buffer solution, and primary antibodies with a dilution ratio of 1:1000 (anti-VEGF, anti-bFGF, anti-MMP2, anti-MMP9, anti-KDR, anti-STAT3, anti-p-STAT3 (Tyr705), and anti-b-actin antibodies) were added for overnight incubation at 4 °C. After the membrane recovered to room temperature, it was rinsed three times using a TBS-T buffer solution. Secondary antibodies diluted at 1:3000 were added for 1 h of incubation, and after the membrane was taken out, it was rinsed three times using a TBS-T buffer solution and sucked dry. A chemical luminescence reagent was added for gel-imaging detection. Optical density was calculated, and the results were analyzed.

4.6. Molecular docking

The target protein was STAT3 (PDB number: 1BG1). The Libdock program of Discovery Studio 2017 software was used to dock the SH2 structural domain of STAT3. 3D and 2D combined mode patterns were acquired, and the bonding effect between small molecules and the ligand was analyzed. The protein crystal structure was derived from Protein Data Bank.

4.7. Statistical analysis

Statistical analysis was performed using SPSS 19.0 software, and data results were represented by $\bar{x} \pm s$. A t test was conducted for intergroup comparison, and analysis of variance (ANOVA) was adopted for a comparison of multiple mean values. $P<0.05$ indicated statistical significance.

Acknowledgments: This work was supported by the Zhuhai Medical Research Project (20181117A010044).

Conflicts of interest: The authors declare that there are no conflicts of interest.

References

Bosch-Barrera J, Queralt B, Menendez JA (2017) Targeting STAT3 with silibinin to improve cancer therapeutics. Cancer Treat Rev 58: 61-69.

Chen FJ, Liu B, Wu Q, Liu J, Xu YY, Zhou SY, Shi JS (2019) Icariin delays brain aging in senescence-accelerated mouse prone 8 (SAMP8) model via inhibiting autophagy. J Pharmacol Exp Ther 369: 121-128.

Fang J, Zhang Y. (2017) Icariin, an anti-atherosclerotic drug from Chinese medicinal herb horny goat weed. Front Pharmacol 8: 734.

Furtek SL, Backos DS, Matheson CJ, Reigan P (2016) Strategies and approaches of targeting STAT3 for cancer treatment. ACS Chem Biol 11: 308-318.

He J, Wang Y, Duan F, Jiang H, Chen MF, Tang SY (2010) Icaritin induces apoptosis of HepG2 cells via the JNK1 signaling pathway independent of the estrogen receptor. Planta Med 76: 1834-1839.

Jin J, Wang H, Hua X, Chen D, Huang C, Chen Z (2019) An outline for the pharmacological effect of icariin in the nervous system. Eur J Pharmacol 842: 20-32.

Li S, Priceman SJ, Xin H, Zhang W, Deng J, Liu Y, Huang J, Zhu W, Chen M, Hu W, Deng X, Zhang J, Yu H, He G (2013) Icaritin inhibits JAK/STAT3 signaling and growth of renal cell carcinoma. PLoS One 8: e81657.

Li X, Ma H, Li L, Chen Y, Sun X, Dong Z, Liu JY, Zhu W, Zhang JT (2018) Novel synthetic bisindolylmaleimide alkaloids inhibit STAT3 activation by binding to the SH2 domain and suppress breast xenograft tumor growth. Oncogene 37: 2469-2480.

Liang Y, Yang N, Pan G, Jin B, Wang S, Ji W (2018) Elevated IL-33 promotes expression of MMP2 and MMP9 via activating STAT3 in alveolar macrophages during LPS-induced acute lung injury. Cell Mol Biol Lett 23: 52.

Mohr A, Chatain N, Domoszlai T, Rinis N, Sommerauer M, Vogt M, Müller-Newen G (2012) Dynamics and non-canonical aspects of JAK/STAT signaling. Eur J Cell Biol 91: 524-532.

Shen R, Wang JH (2018) The effect of icariin on immunity and its potential application. Am J Clin Exp Immunol 7: 50-56.

Tan HL, Chan KG, Pusparajah P, Saokaew S, Duangjai A, Lee LH, Goh BH (2016) Anti-cancer properties of the naturally occurring aphrodisiacs: icariin and its derivatives. Front Pharmacol 7: 191.

Wu T, Wang S, Wu J, Lin Z, Sui X, Xu X, Shimizu N, Chen B, Wang X (2015) Icaritin induces lytic cytotoxicity in extranodal NK/T-cell lymphoma. J Exp Clin Cancer Res 34: 17.