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Original Article

Hyperoside inhibited the migration and invasion of lung cancer cells through the upregulation of PI3K/AKT and p38 MAPK pathways

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Abstract: Non-small cell lung cancer (NSCLC) is one of the most common human malignancies. Malignant tumor is characterized by the Invasion and metastasis of tumor. Based on previous research, human A549 cells could be induced to apoptosis by Hyperoside (Hyp). The aim of the present study was to investigate whether low concentrations of Hyp (1-10 μM) could inhibit the invasion and migration of lung cancer cells through regulation of phosphoinositide-3-kinase/serine threonine kinase (PI3K/AKT) and p38 mitogen-activated protein kinase (p38 MAPK) pathways. CCK8 assay was performed to identify the cell viability after human A549 cells treated with Hyp (1, 2 and 5 μM) 12, 24 and 48 h. Hyp (1, 2 and 5 μM) significantly suppressed the invasion and migration of A549 cells in a dose-dependant manner. Phosphorylation of AKT and P38 protein level were measured by western blot. By RT-PCR and western blot analysis, non-metastatic gene23-H1 (nm23-H1), metastasis-associated gene 1 (MTA1), tissue inhibitor of metalloproteinase-1 (TIMP-2) and matrix metalloproteinases-2/9 (MMP-2/9) expression was estimated. The results showed that the level of nm23-H1, MTA1, TIMP-2 and MMP-2/9 expression were all regulated dramatically in Hyp groups compared with the control group. The p-ATK and p-P38 were descended notably. These results suggested that Hyp significantly suppressed the invasion and migration of A549 cells by mediating the AKT/PI3K and p38 MAPK pathways and regulated the expressions of invasion and migration related genes.

Keywords: Hyperoside, invasion, migration, lung cancer, PI3K/AKT, p38 MAPK

Introduction

Lung cancer is a major cause of death among patients, and non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancers over many countries [1], so it also has become the No1 malignant tumor which was harmful to human health and life [2]. Meanwhile, tumor migration and invasion are not only the malignant marker and characteristics of lung cancer, but also the main cause of failure to cure and lose their life of the patients with lung cancer [3]. Therefore, inhibiting cancer cell migration and invasion is one of the strategies for the cancer therapy and research.

It is a complex biological consequence that involved in tumor metastatic gene and tumor metastatic suppressor gene, signal pathways, proteolytic degradation of the extracellular matrix (ECM) and basement membrane (BM) of normal surrounding tissues, et al [4, 5]. Non-metastatic gene23-H1(nm23-H1) is well known currently as a tumor metastasis suppressor gene in lung cancer [6]. At present, the over-expression of metastasis-associated gene 1 (MTA1) is closely related to tumor migration and invasion in lung cancer [7], but the relationship between nm23-H1 and MAT1 are scarcely reported in lung cancer. Matrix metalloproteinases (MMPs) belong to a family of zinc-dependent proteolytic enzymes [3]. Among all MMPs, MMP-2 and MMP-9 are known as key enzymes in the degradation of ECM proteins in BM [8]. It was reported that high levels of MMP-2 and MMP-9 expression are associated with cancer invasion and metastasis [9]. Tissue inhibitor of metalloproteinases (TIMP) is natural inhibitors of MMP, which dampens down activity of MMP, the balance of both have been recognized to play critical roles in process of invasion and

metastasis [10, 11]. Therefore, a set of genes associated with tumor migration and invasion are key to study cancer therapy. Amount of studies confirmed the hyper-active status of PI3K/AKT signaling pathway in multiple malignant tumors including cervical cancer, pancreatic carcinoma, gastric cancer, lung cancer and esophageal cancer, along with important roles in tumor invasion and metastasis [12, 13]. Mitogen-activated protein kinase (MAPK) is an important signal transduction pathways in mammalian cells, among which p38MAPK signal transduction pathway plays pivotal roles in tumor genesis, tumor invasion and metastasis [1, 14]. PI3K/AKT and p38MAPK signaling pathway involved in the expression and regulation of invasion and migration related genes.

Hyperoside (Hyp), which is a major pharmacologically active from *Prunella vulgaris* L, belong to compound of flavonoid, is one of the primary components of traditional patent medicines of China [15, 16]. It can exert properties of anti-inflammatory, analgesis, anti-oxidant, anti-hyperglycemic, anti-cancer, and cardiovascular protective effects [17-23]. Because of the components of medicinal herbs have different structures, various bioactivities, less toxic side effects, and a rich source, many investigators are interested in investigating the components of medicinal herbs used in complementary and alternative medicines [24], and now, the study found that Hyp induced apoptosis in human non-small cell lung cancer cells in vitro [25]. Based on these previous findings regarding the pharmacological action of Hyp were further assessed in the present study, according to effect of Hyp on tumor invasion and migration and the mechanisms involved for providing new thoughts for the treatment of NSCLC.

Materials and methods

Cells and reagents

Human A549 cells are human lung carcinoma cells, which were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cell were cultured in HyClone RPMI-1640 media (Gibco, USA) plus 10% fetal bovine serum and incubated at 37% in a 5% CO_2 atmosphere, Penicillin (100 U/ml) and streptomycin (100 U/ml) were added in the medium. Hyperoside was purchased from Zelang Biological Technology Co, Ltd. (Nanjing, China).

CCK8 assay

The cell proliferation status was assessed by Cell Counting Kit-8 (CCK)-8 Kit (Beyotime, China). In briefly, Human A549 cells were seeded into 96-well plates at a cell density of 4×10^3 cells/well with 100 μL culture medium. After cultured for 24 h, Hyp were added to the medium to the final concentrations (0, 1, 2 and 5 μM). The cells were then cultured for another 12, 24 and 48 h. Then, 10 μL of CCK8 assay solution was added to each well, and the culture was incubated for another 1 h at 37°C. The optical density (OD) values were read at 450 nm by a microplate reader (Thermo, USA). Experiments were repeated at least three times each time in triplicate.

Cell invasion assay

Cell invasion assay was performed by a 24-well Transwell chamber with a pore size of 8 μm (Sigma). The inserts were coated with 50 μl Matrigel (dilution at 1: 2; BD Bioscience, Franklin Lakes, NJ, USA). Cells treated with Hyp for 24 h and transferred to the upper Matrigel chamber in 100 μL of serum-free medium supplementing 1×10^5 cells and incubated for 24 hours. The lower chamber was filled with medium containing 10% FBS as chemo attractants. After incubation, the cells that passed through the filter were fixed and stained by 0.5% crystal violet. The numbers of invaded cells were counted in five randomly selected high power fields under a microscope (OLYMPUS).

Migration assay

Cells in logarithmic phase were digested by 0.25% trypsin (Gibco) and then suspended in RPMI-1640 (Hyclone) medium containing 10% fetal calf serum (Gibco). Cells were seeded in a 24-plate microplate at a density of 1×10^5 cells/mL and then incubated for 1 h. The supernatant was discarded and cells were washed 2 times by PBS (Gibco). 1 mL 4% paraformaldehyde (Zhongze biotech, Shanghai, China) was supplemented for 10 min and cells were stained by crystal violet (Zhongze) for 30 min. Then cells were washed three times and the optical density (OD) values were read at 570 nm by a microplate reader (Thermo, USA). Magration rate (%) = (OD1/OD0) × 100%, OD1: Hyp treated groups; OD0: control group.

Table 1. Primers used in Real-time PCR analysis

Gene	Primer sequence	Species
Nm23-H1	Forward: 5'-GCCTGGTGAAATACATGCAC-3' Reverse: 5'-AGAAGTCTCCACGGATGGTC-3'	Human
MTA1	Forward: 5'-TGGAAGACCACCGACAGATA-3' Reverse: 5'-TTGTTGACGCTGATTTGGTT-3'	Human
TIMP-2	Forward: 5'-ATCCCGTGCTACATCTCCTC-3' Reverse: 5'-ACAGGAGCCGTCACTTCTCT-3'	Human
MMP-2	Forward: 5'-TTGACGGTAAGGACGGACTC-3' Reverse: 5'-GGCGTTCCCATACTTCACAC-3'	Human
MMP-9	Forward: 5'-AAGGGCGTCGTGGTTCCAACTC-3' Reverse: 5'-AGCATTGCCGTCCTGGGTGTAG-3'	Human
GAPDH	Forward: 5'-CACCCACTCCTCCACCTTTG-3' Reverse: 5'-CCACCACCCTGTTGCTGTAG-3'	Human

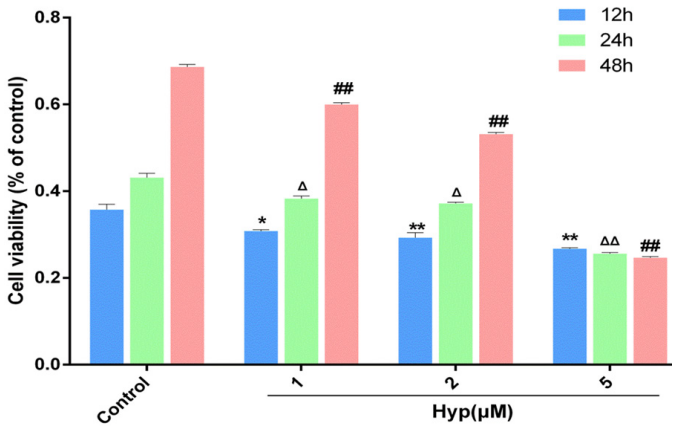

Figure 1. Effect of Hyp on human A549 cell viability. Cells were treated with different doses of Hyp (0, 1, 2 and 5 μM) for 12, 24 and 48 h, and CCK8 was performed to identify the proliferation. Data were presented as mean ± SD, n = 3, $^{*}P<0.05$, $^{\Delta}P<0.05$, $^{\#}P<0.05$, $^{**}P<0.01$, $^{\Delta\Delta}P<0.01$, $^{\#\#}P<0.01$.

Western blot

Human A549 cells were seeded at a density of 5 × 10^5 cells/well in 6-well plates, cultured overnight and then treated with Hyp (1, 2 and 5 μM) for 24 h. Each group of cells were harvest and washed twice with PBS and protein lysed in ice-cold radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) with freshly added 0.01% protease inhibitor PMSF (Amresco, Shanghai, China) and incubated on ice for 30 min. Cell lysis was centrifuged at 10,000 rcf for 5 min at 4°C and the supernatant (20-30 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a nitrocellulose nc membrane (Millipore, Shanghai, China), then detected with AKT, phosphorylated (p-) AKT, P38, p-P38, nm23-H1, MTA1, TIMP-2 and MMP-2/9 proteins. Protein loading was estimated using mouse anti-GAPDH monoclonal antibody. Blots were visualized using enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China). Antibodies were purchased from Cell Signaling Technology, Abcam, Santa Cruze.

Real time quantification PCR (RT-PCR)

Human A549 cells were seeded at a density of 5 × 10^5 cells/well in 6-well plates, cultured overnight and then treated with Hyp (1, 2 and 5 μM) for 24 h. Total RNA from Hyp treated cells was isolated using TRIzol reagent (Invitrogen). Reverse transcription reaction was performed using 2 μg of total RNA with a first strand cDNA kit (Thermo Scientific, Shanghai, China), according to the manufacturer's instructions. PCR amplification was performed for 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, annealing/extension at 60°C for 1 min in ABI 7300 Thermocycler (Applied Biosystems, Foster City, CA, USA), using the SYBR Premix Ex Taq kit (Thermo). The primer sequences for each gene were displayed in **Table 1**. Data analysis was done using the $2^{-\Delta\Delta CT}$ method for relative quantification, and all samples were normalized to GAPDH, which was used as an endogenous control.

Statistic analysis

The GraphPad Prism 5.0 software system was employed for statistical analysis. Data are expressed as the mean ± standard error. The differences between groups were analyzed using a Student t test when only 2 groups or 1-way analysis of variance when more than 2 groups were compared. All tests performed were 2-sided. $P<0.05$ was taken as statistical significance.

Results

Effect of Hyp on human A549 cell viability

The effect of Hyp on human A549 cell viability measured by CCK assay are shown in **Figure 1**. Compared with the control group, low concen-

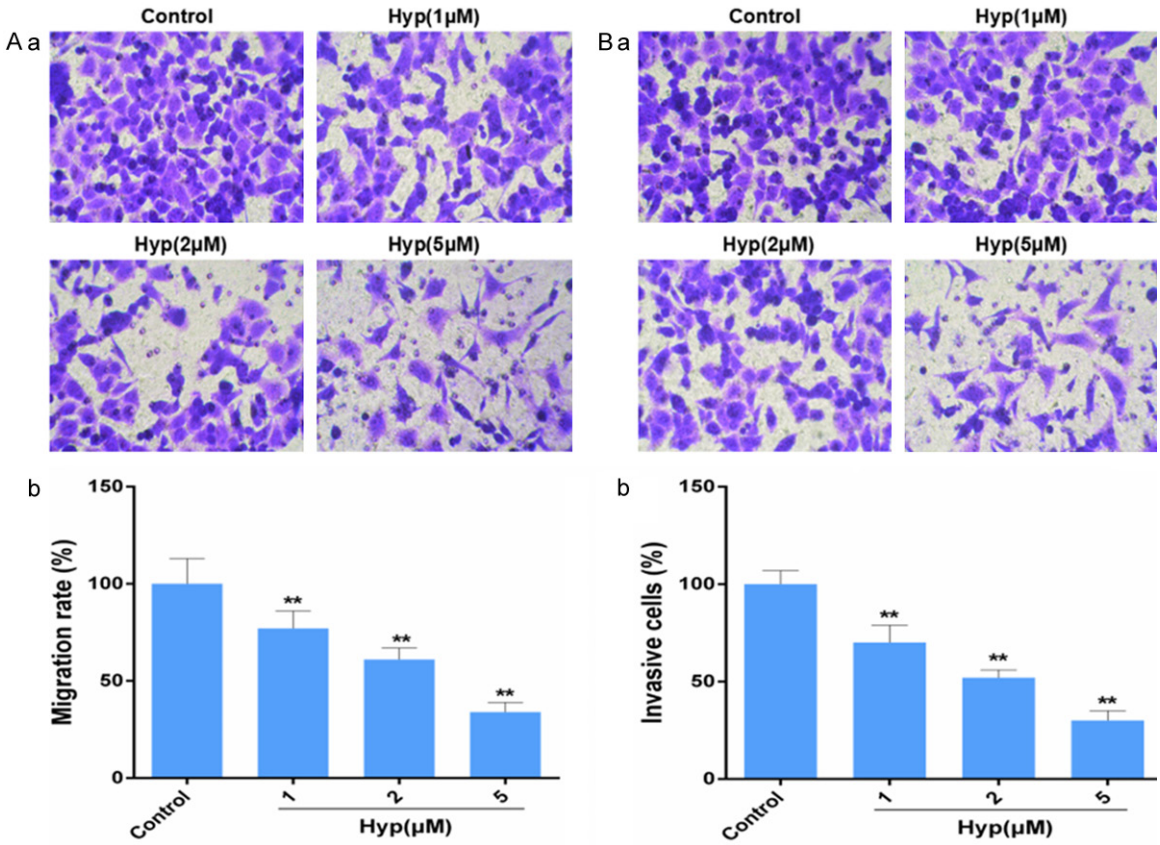

Figure 2. Effect of Hyp on migration and invasion of human A549 cell. Aa, Ab. Cells were treated with low concentration of Hyp (1, 2 and 5 μM) for 24 h, and cell migration was measured by crystal violet staining. Ba, Bb. Cells were treat with low concentration of Hyp (1, 2 and 5 μM) for 24 h, and cell invasion was identified by transwell assay. Data were presented as mean ± SD, n = 3, **P*<0.05, ***P*<0.01.

trations of Hyp (1, 2 and 5 μM) significantly decreased human A549 cell viability at 12, 24 and 48 h (*P*<0.05), and which have dose dependence on Hyp. Therefore, the doses of 1, 2 and 5 μM were used to carry out further migration and invasion investigations.

Hyp inhibited the migration and invasion of human A549 cell

Cell migration and invasion were studied by transwell assay. It is showed in **Figure 2**. The result showed the migration ability of human A549 cells treated with 1, 2 and 5 μM of Hyp were decreased notably in a concentration-dependent manner compared with that of the control group (**Figure 2Aa**). The migration rate of 1, 2 and 5 μM of Hyp treated groups were average 77.21 ± 8.11%, 61.33 ± 6.52% and 34.52 ± 7.55% respectively compared with control (**Figure 2Ab**). Low concentrations of Hyp (1, 2 and 5 μM) could also significantly inhibit the ability of invasion in human A549 cells in a concentration dependent manner in comparison with control group (**Figure 2Ba**). The invasion rate corresponding to 1, 2 and 5 μM of Hyp treated groups were average 70.14 ± 7.07%, 52.33 ± 6.26% and 30.1 ± 7.01% compared with control (**Figure 2Bb**).

Hyp regulated expression of nm23-H1, MTA1, TIMP-2 and MMP-2/9 in A549 cell

Nm23-H1, MTA1, TIMP-2 and MMP-2/9 are associated with tumor migration and invasion. Therefore, these genes were detected the expression by western blot and RT-PCR. MMP-2 and MTA1 expression were all significantly suppressed by Hyp compared with the control group (*P*<0.05) (**Figure 4**). It is shown in **Figure 4A-F**, there were the expression of nm23-H1 and TIMP-2 appeared significantly up-regulated in low concentrations of Hyp (1, 2 and 5 μM) groups (*P*<0.05), there were the expression of

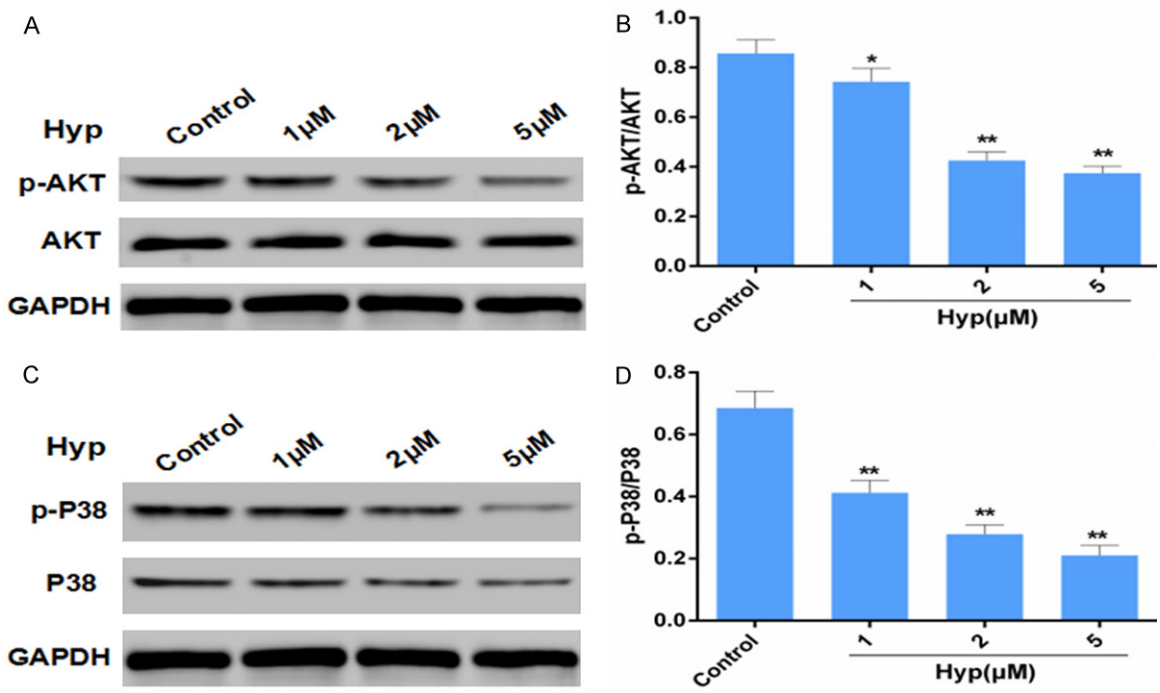

Figure 3. Effect of Hyp on phosphorylation of ATK and P38 of A549 cells. A, B. Cells were treated with different doses of Hyp (1, 2 and 5 μM) for 6 hours and lysed for western blot analysis using antibodies against the indicated AKT and p-AKT proteins. C, D. Cells were treated with different doses of Hyp (1, 2 and 5 μM) for 6 hours and lysed for western blot analysis using antibodies against the indicated P38 and p-P38 proteins. GAPDH was also detected as the control of sample loading. Data were presented as mean ± SD, n = 6, **P*<0.05, ***P*<0.01.

MTA1 and MMP-2 appeared significantly down-regulated in low concentrations of Hyp (1, 2 and 5 μM) groups (*P*<0.05). The expression of MMP-9 showed no significant difference between the Hyp treated groups and control group (**Figure 4E** and **4F**).

Hyperoside regulated the PI3K/AKT and p38 MAPK signaling pathways in A549 cell

PI3K/AKT signaling shows stimulative effect in the procession of tumor metastasis [26, 27]. p38 MAPK signaling involves in the occurrence, invasion and metastasis of many tumors by positive regulatory way [28]. The phosphorylation level of AKT and P38 were measured by western blot. As shown in **Figure 3A-D**, the p-AKT and p-P38 expression were both suppressed significantly in Hyp treated A549 cells in a dose-dependent manner in comparison with the control group.

Discussion

Malignant tumor is still difficult to cure, which is regarded as one of complicated diseases in hunman. It exhibits high morbidity and mortality, and data shows that 1 out of 4 cancer deaths from lung cancer [29, 30], and meanwhile NSCLC hold an un-neglectable status. Although effective chemotherapeutics and advanced treatment technology have increased the chances of long-term survival in lung cancer patients, the side effects, prevent recurrence and metastasis of cancer treatments remain a neglected problem. And so, more and more attention has been paid to the use of natural products isolated from Chinese medicinal herbs for cancer therapy because of combination of Chinese and Western medicine treatment can effectively reduce the toxicity and strengthen the immune function [24, 31]. Therefore, searching favorable product from Chinese medicinal herb for NSCLC therapy is of vital importance. Hyp was proved to have anti-tumor activity towards various human cancer cell lines and xenograft systems of human tumors, making it an excellent candidate for new anticancer agents [20, 32, 33].

Previous research found Hyp could induce apoptosis of human A549 cells [13, 25]. Given our results, Hyp (1, 2 and 5 μM) can inhibit

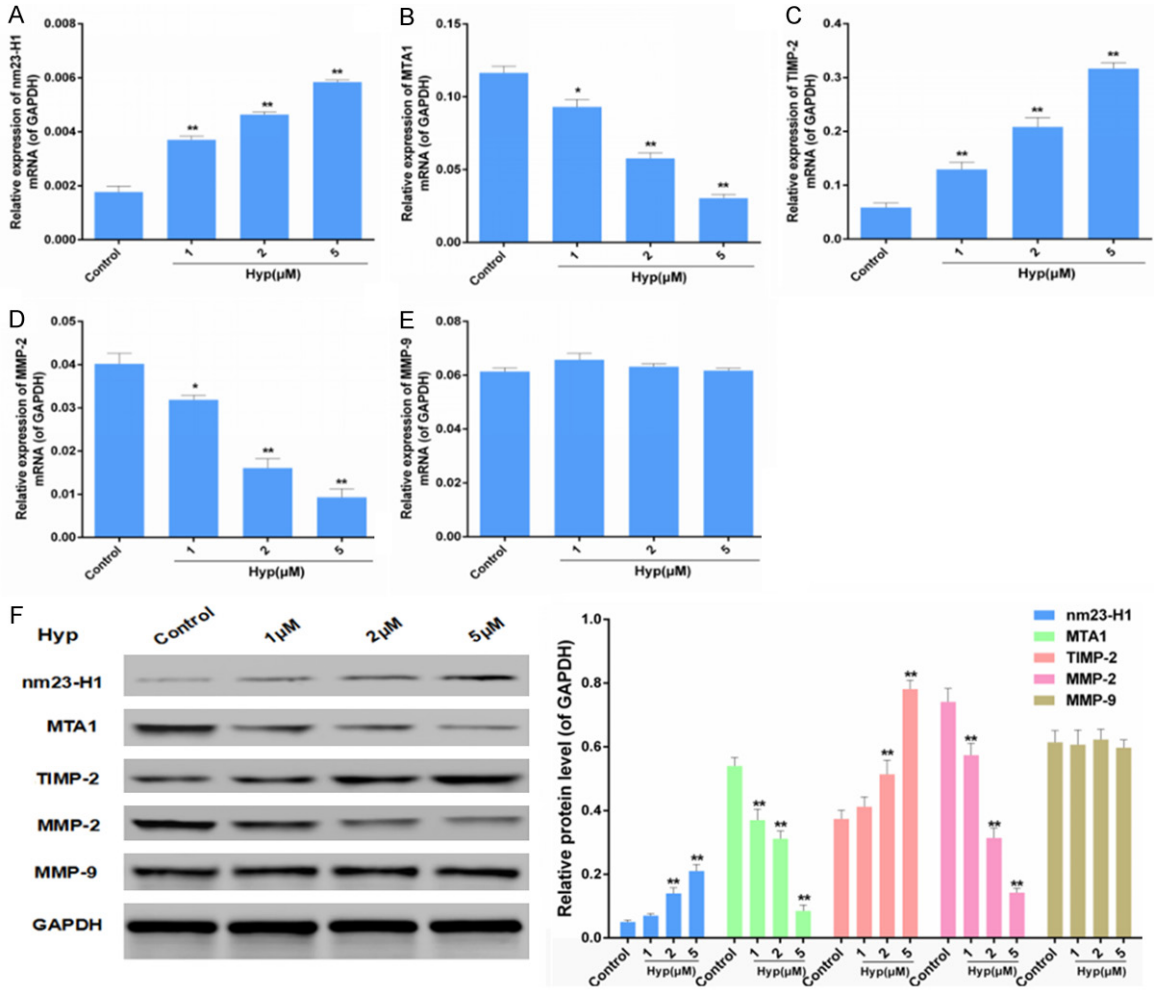

Figure 4. Effect of Hyp on expressions of nm23-H1, MTA1, TIMP-2 and MMP-2/9. A-E. Cells were treated with different doses of Hyp (1, 2 and 5 μM) for 12 hours and RT-PCR was performed for nm23-H1, MTA1, TIMP-2 and MMP-2/9 mRNA expression detection. F. Cells were treated with different doses of Hyp (1, 2 and 5 μM) for 24 hours and lysed for western blot analysis using antibodies against the nm23-H1, MTA1, TIMP-2 and MMP-2/9 proteins. GAPDH was also detected as the control of sample loading. Data were presented as mean ± SD, n = 6, $*P<0.05$, $**P<0.01$.

human A549 cells proliferation, migration and invasion in a concentration-dependent manner (**Figures 1** and **2**). Taken together, Hyp shows favorable potential of the NSCLC therapy. It also provided evidence that the mechanism underlying the above effects was related to the regulation of expression of nm23-H1, MTA1, TIMP-2 and MMP-2/9, which were regulated by the p-AKT and p-P38 signaling pathway [12-14, 26, 27, 34]. It was shed light on the correlation of nm23-H1, MTA1, TIMP-2 and MMP-2 (**Figure 4**). The expression of metastasis suppressor gene (nm23-H1 and TIMP-2) was up-regulated dramatically, and the expression of metastasis gene (MTA1 and MMP-2) was down-regulated dramatically in low concentrations of Hyp (1, 2 and 5 μM) groups.

When tumor cells acquire the capacity to move around and invade other tissues, there is a risk of metastases and cancer treatment becomes more difficult. Tumor metastasis involves in cells adhesion, migration and invasion, cell movement, degradation of extracellular matrix and multiplication dropout, etc [35]. The cancer cells with higher metastasis ability tend to show active the ability of cell movement [36], but cell movement is necessary for healing wounds and fighting off diseases, stopping it completely is not an option. Therefore, interruption of one or more of adhesion, migration and invasion is considered as a serviceable strategy. In our study, the result indicated that Hyp showed obvious inhibition of migration and invasion in human A549 cell in a dose-dependent manner.

Cellular functions are regulated by multiple signal pathways, such as PI3K/AKT and p38 MAPK signaling pathways.

PI3K with better catalytic activity can regulate phosphorylation level of Akt and develop PI3K/AKT signaling pathway. It plays an important role in migration and invasion of cell [37, 38], p38 MAPK signal transduction pathway is closely associated with tumor invasion and metastasis [14, 34], studies have also shown that there is interaction between PI3K/AKT and p38 MAPK signaling pathways on function [39]. AKT and p38 are downstream gene of PI3K/AKT and p38 MAPK signaling pathways, the phosphorylation of AKT and p38 can promote the activation of PI3K/AKT and p38 MAPK signaling pathways, and then tumor invasion and metastasis is exacerbated. Our results show that Hyp significantly suppresses phosphorylation levels of AKT and p38 proteins in a dose-dependent manner (**Figure 3**), which indicates Hyp impact on migration and invasion of human A549 cell via inhibiting activation of PI3K/AKT and p38 MAPK signaling pathways.

From the data analysis and results, we found that nm23-H1, MTA1, TIMP-2 and MMP-2 were significantly regulated by low concentrations of Hyp. However, no changes of expression level of MMP-9 mRNA and protein in each group (**Figure 4E** and **4F**). According to the results, we can speculate that there are two ways it could be. One possibility is that PI3K/AKT and p38 MAPK signaling pathways are inhibited by Hyp, which can up-regulate the expression of nm23-H1 and TIMP-2 and down-regulate the expression of MTA1 and MMP-2, Perhaps because structure and function are different between MMP-2 and MMP-9, which cannot be regulated. But the concrete reasons and mechanism remain unclear. Another possibility is that PI3K/AKT and p38 MAPK signaling pathways are suppressed by Hyp, which can promote the expression of nm23-H1 and TIMP-2 to reducing the expression of MTA1 and MMP-2. Based on the analysis results and related report, the expression of MTA1 and MMP-2 can be inhibited by nm23-H1 and TIMP-2, respectively [7-11]. It is reported that the ratio of MMP-9/TIMP-1 was closely correlated with the degree of renal interstitial lesions [40, 41]. Therefore, we demonstrated MMP-9/TIMP-1 was subject to no regulation via regulating PI3K/AKT and p38 MAPK signaling pathways. However, further research is needed to determine whether the associations are causal. Down regulation of MTA1 and MMP-2 expressions are mediated by multiple signaling cascades, especially by the PI3K/AKT and p38 MAPK. In general, PI3K/AKT and p38 MAPK signaling suppressed by Hyp possibly leds to the elevation of nm23-H1 and TIMP-2.

In conclusion, we demonstrate herein that Hyp inhibits A549 cell migration and invasion properties by suppressing the regulation and expression of nm23-H1, MTA1, TIMP-2 and MMP-2, which are very important factor for lung cancer cells. PI3K/AKT and p38 MAPK signaling in A549 cells contributes to the underlying mechanism of invasive potential of Hyp. Our results may also be relevant for lung carcinoma therapy.

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Disclosure of conflict of interest

None.

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