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Original Article

The role of silymarin and MAPK pathway in the regulation of proliferation and invasion of non-small cell lung cancer cells

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Abstract

We aimed to explore the role of silymarin and mitogen-activated protein kinase (MAPK) pathway in the regulation of proliferation and invasion of non-small cell lung cancer cells. Non-small cell lung cancer cells were cultured and divided into groups and treated with drugs, and A blank control group was set up. The concentration of silymarin in the experimental group was 10 mg/L, 20 mg/L and 40 mg/L, respectively, which were recorded as groups A, B and C, and three repeated experiments were performed in each group. Absorbance (A value), survival rate and number of invasions were measured at 490 nm 24 h and 48 h after treatment, and the protein expression levels of MMP-2, MMP-9, p-p38, p-JNK and p-ERK 1/2 of cells in each group were detected. There were differences in the A value (control group > Group A > Group B > Group C), cell survival rate (control group < group A < group B < group C) and the number of cell invasions (control group > Group A > Group B > group C) at 24 h and 48 h among all groups ($P<0.05$). After 24 h of administration, the mRNA expression of MMP-2 and MMP-9, P-P38 and P-JNK protein expression were significantly different among groups, and the control group was > group A > Group B > group C ($P<0.05$). There were no significant differences in protein expression levels of p38, JNK, ERK 1/2 and P-ERK 1/2 among all groups ($P>0.05$). Silymarin may inhibit the proliferation and invasion of non-small cell lung cancer cells by inhibiting the activity of MAPK pathway, and the higher the concentration, the more obvious the inhibition effect, which provides a basis for further research and treatment of non-small cell lung cancer.

Keywords: Silymarin, Mitogen-activated protein kinase, Non-small cell lung cancer, Proliferation, Invade and attack.

1. Introduction

Lung cancer remains a global health concern, with a high incidence rate worldwide. Among the diverse forms of this disease, non-small-cell lung cancer (NSCLC) stands out as the most prevalent but unfortunately carries a poor prognosis for patients [1]. One critical factor contributing to this unfavorable outlook is the insensitivity of NSCLC patients to conventional chemotherapeutic agents [2]. Their resistance to these treatments significantly limits the effectiveness of current interventions, underscoring the pressing need to identify novel and precise therapeutic targets. These new targets could potentially revolutionize treatment strategies, offering hope for improved patient outcomes and prolonged survival rates. The ongoing quest for such innovative therapies is crucial in the battle against NSCLC and holds promise for enhancing the overall prognosis and quality of life for those affected by this challenging malignancy.

The mitogen-activated protein kinase (MAPK) pathway is involved in a variety of pathophysiological processes in the body, and members of the MAPK family mainly include p38, ERK, JNK, etc. [3,4]. Some studies have shown [5] that activation of the MAPK pathway can promote the release of kinds of inflammatory factors from the body and aggravate the condition of patients. It is hypothesized that MAPK may act as a target for certain diseases. Some studies have claimed that certain small molecule drugs and active ingredients of traditional Chinese medicine can target MAPK to achieve the pharmacological effect of inhibiting the expression of matrix metalloproteinase 2 and MMP9 in cancer cells, and inhibit the invasion and metastasis process of cancer cells [6,7].

Silymarin, a natural compound derived from milk thistle, has demonstrated potent anti-lipid peroxidation, free radical scavenging, and the ability to maintain cell membrane stability. Clinical studies have validated its hepatoprotective properties, making it a valuable candidate in the field of liver health and disease management [8]. A recent study found that silymarin inhibits the invasion of hepatocellular carcinoma cells and breast cancer cells, and inhibits the expression of MMP9 [9,10]. A few studies suggest that silymarin can inhibit the proliferation and migration process of NSCLC cells.

In order to find new therapeutic targets for NSCLC [11], the present study was conducted to investigate the

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role of silymarin and MAPK pathways in the regulation of NSCLC cell proliferation and invasion using NSCLC cells as experimental subjects.

2. Materials and Methods

2.1. Experimental apparatus and reagents

Non-small cell lung cancer cell lines were purchased from the Experimental Centre of the Chinese Academy of Sciences (Shanghai, China); silymarin was purchased from Shanghai Purely Excellent Bio-technology Co. (Shanghai, China). Under aseptic conditions, the drug was dissolved in DMSO, and diluted to the required concentration for each group using RPMI-1640 as the culture medium. The human lung adenocarcinoma A549 cell line was purchased from the cell bank of the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China).

2.2. Grouping and administration method

Cell culture was routinely performed, digestion was performed when the cell fusion reached 80%, cell passaging was routinely performed, and then the obtained cell suspension was inoculated in cell culture plates. No drug was added to the control group, and only medium containing 1% dimethyl sulfoxide was added. The experimental groups A, B and C were added with 10 mg/L, 20 mg/L and 40 mg/L concentration of thripsin drug solution respectively, and 3 compound wells were set in each group.

2.3. Observation indexes

Record the change of absorbance value (A-value) at 490 nm, cell survival rate, and number of invasions at 24 h and 48 h after drug administration, and record the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 mRNA in each group after 24 h of drug administration. Phosphorylated c-JunN-terminal kinase (p-JNK), phosphorylated extracellular signal-regulated kinase (p-ERK)1/2, and phosphorylated p38 (p-p38) protein expression levels were recorded in each group after 24 h of drug administration.

Cell viability was detected using MTT assay, 20 μl MMT working solution was added at 24 h of incubation, and the reaction was terminated by adding 150 μl DMSO after 4 h of incubation with sufficient shaking, and the A-value of each well at 490 nm wavelength was measured by enzyme labelling instrument, and the cell viability= measured A-value/blank control A-value×100%.

Transwell method to detect cell invasion: cells were collected after 24 h and 48 h of drug administration, and the cell density was adjusted to 1×10^5 /L using serum-free medium, 200 μl of the above cell suspension was inoculated in the upper chamber of the Transwell, and 500 μl of culture medium containing 10% serum was added to the lower chamber. After 24 h of culture, the upper chamber was removed, washed with PBS and then sequentially subjected to 4% paraformaldehyde fixation and crystal violet staining operation, and finally 5 fields of view were selected in each group under high magnification microscope, and the number of cells in the field of view was counted, which was the number of cell invasion.

Detection of MMP2 and MMP9 mRNA expression in cells: cells were collected at 24 h and 48 h of drug administration, and RNA was extracted by using the cellular RNA kit, and then RNA was reverse transcribed to cDNA. The reaction system was prepared by using the instructions of the fluorescence quantitative PCR kit, and the target genes were amplified in a PCR instrument, with β-actin as the internal reference. The cycling curve and cycling threshold (Ct) were obtained, and the mRNA expression levels of MMP2 and MMP9 were calculated according to Equation $2^{-\Delta\Delta}$Ct.

Detection of p-p38, p-JNK, p-ERK1/2 protein expression level: cells were collected after 24 h and 48 h of drug administration, protein was extracted by adding lysate, buffer was added and mixed with it, heated and boiled to deform it and then added into polyacrylamide gel for electrophoresis, and then transferred to membrane after electrophoresis. After electrophoresis, the membrane was transferred and then closed in 5% skimmed milk at room temperature for 1 h, and p-p38, p-JNK, p-ERK 1/2 primary antibody (1:1000) was added and incubated overnight at 4℃ on a shaker. On the following day, secondary antibody with a dilution ratio of 1:2000 was added and incubated for 1h at room temperature, after ECL colour development, and finally, it was placed into a gel analysis system for imaging, and protein bands were analysed semi-quantitatively to obtain the relative protein expression levels.

2.4. Statistical analysis

Statistic Package for Social Science (SPSS) 23.0 software (IBM, Armonk, NY, USA) was used for data processing, and the measurement data such as absorbance, cell survival rate, number of invasion, protein expression were expressed as ($\bar{x}$±s), independent samples *t*-test was performed between groups, and paired *t*-test was performed for intra-group comparisons. α=0.05 was the test level.

3. Results

3.1. Comparison of A-value and cell survival rate after drug administration in each group

The A-value of the cells at 24h and 48h (control group>Group A>Group B>Group C) and cell survival rate (Group A>Group B>Group C) between groups differed between the two comparisons (P<0.05). Table 1.

Table 1. Comparison of A-value and cell survival rate after drug administration in each group ($\bar{x}$±s).

Group	A value		Survival rate (%)	
	24 h	48 h	24 h	48 h
Control Group	1.23±0.11	1.01±0.08	——	——
Group A	0.98±0.12*	0.77±0.11*	79.67±5.98	76.24±5.12
Group B	0.75±0.08^{*+}	0.62±0.13^{*+}	60.99±4.46^{+}	61.33±3.44^{+}
Group C	0.61±0.09^{*+-}	0.45±0.09^{*+-}	49.59±3.45^{+-}	44.55±4.56^{+-}

Note: * is P<0.05 compared with control group; + is P<0.05 compared with group A; - is P<0.05 compared with group B.

3.2. Comparison of the number of cell invasion after drug administration in each group

At 24 h and 48 h, there was a difference in the number of cell invasions between the groups in a two-by-two comparison, control group > group A > group B > group C (P<0.05). Table 2, Figure 1.

3.3. Comparison of MMP-2 and MMP-9 mRNA expression between groups

After 24 h of drug administration, there was a difference in MMP-2, MMP-9 mRNA expression between groups in two-by-two comparisons, and control group > group A > group B > group C (P<0.05). Table 3, Figure 2.

Table 2. Comparison of the number of cell invasions after drug administration in each group ($\bar{x}$±s, pcs).

Group	24 h	48 h
Control Group	322.46±45.12	612.26±49.06
Group A	241.21±31.02*	465.23±55.15*
Group B	178.26±26.12*+	431.60±45.26*+
Group C	122.26±12.12*+-	116.49±37.38*+-

Note: * is P<0.05 compared with control group; + is P<0.05 compared with group A; - is P<0.05 compared with group B.

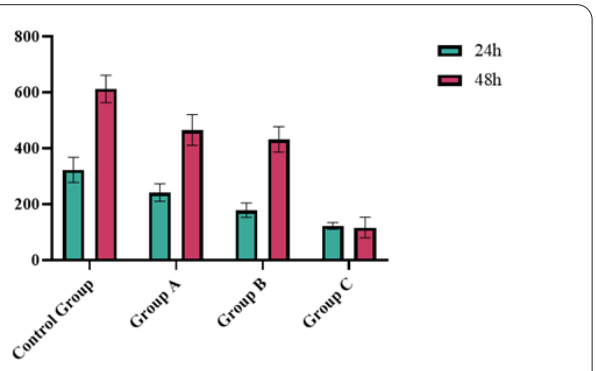

Fig. 1. Number of invasions at 24 h and 48 h after drug administration in each group.

Table 3. Comparison of MMP-2 and MMP-9 mRNA expression between groups ($\bar{x}$±s).

Group	MMP-2 mRNA	MMP-9 mRNA
Control Group	1.00±0.12	1.00±0.16
Group A	0.77±0.09*	0.88±0.15*
Group B	0.56±0.08*+	0.56±0.09*+
Group C	0.44±0.06*+-	0.33±0.08*+-

Note: * is P<0.05 compared with control group; + is P<0.05 compared with group A; - is P<0.05 compared with group B.

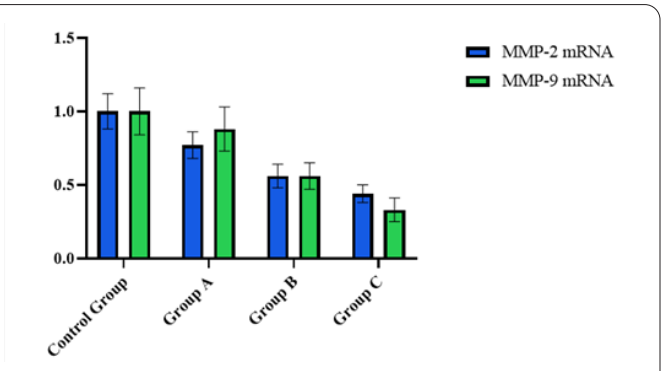

Fig. 2. Comparison of MMP-2 and MMP-9 mRNA expression between groups..

3.4. Comparison of p38, JNK, ERK 1/2 protein expression levels among groups

There was no statistically significant difference in the comparison of p38, JNK, and ERK 1/2 protein expression levels among the groups (P>0.05). Table 4, Figure 3.

3.5. Comparison of p-p38, p-JNK, p-ERK 1/2 protein expression levels among groups

After 24 h of drug administration, there were differences in p-p38, p-JNK protein expression between groups in two-by-two comparisons between groups, control group>group A>group B>group C (P<0.05), and there was no statistical significance in comparing the expression level of p-ERK 1/2 proteins between groups (P>0.05). Table 5, Figure 4.

Table 4. Comparison of p38, JNK, ERK 1/2 protein expression levels among groups ($\bar{x}$±s)

Group	P38	JNK	ERK1/2
Control Group	0.75±0.06	0.56±0.11	0.58±0.08
Group A	0.74±0.11*	0.53±0.12*	0.56±0.13
Group B	0.76±0.13*+	0.52±0.08*+	0.52±0.15
Group C	0.71±0.09*+-	0.54±0.09*+-	0.53±0.14

Note: * is P<0.05 compared with control group; + is P<0.05 compared with group A; - is P<0.05 compared with group B.

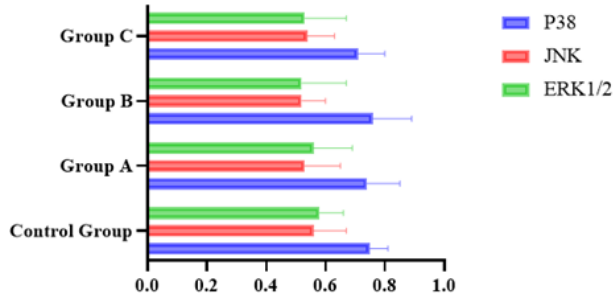

Fig. 3. Comparison of p38, JNK, ERK 1/2 protein expression levels among groups.

Table 5. Comparison of p-p38, p-JNK, p-ERK 1/2 protein expression levels among groups ($\bar{x}$±s).

Group	p-p38	p-JNK	P-ERK1/2
Control Group	0.88±0.13	0.94±0.11	0.82±0.11
Group A	0.72±0.11*	0.76±0.08*	0.78±0.12
Group B	0.55±0.13*+	0.66±0.12*+	0.81±0.15
Group C	0.35±0.07*+-	0.44±0.09*+-	0.79±0.13

Note: * is P<0.05 compared with control group; + is P<0.05 compared with group A; - is P<0.05 compared with group B.

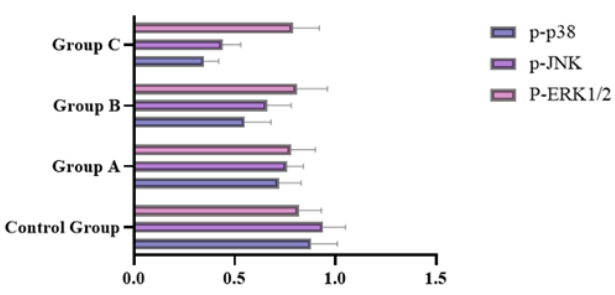

Fig. 4. Comparison of p-p38, p-JNK, p-ERK 1/2 protein expression levels among groups.

4. Discussion

In recent years, the antitumour effects of Chinese herbal medicines have received extensive clinical attention, and some Chinese herbal medicines and their active ingredients have both anticancer and chemo-sensitive enhancing effects. Silymarin is the active ingredient in silymarin, and its role in attenuating liver injury and inhibiting the development of cirrhosis has been clinically demonstrated [12]. MAPK is a class of intracellular serine/threonine protein kinase, which is an important signalling pathway for transmitting extracellular stimulus signals to the nucleus, where it can mediate cellular responses, and this pathway plays an important role in cell proliferation, differentiation and invasion [13]. There are fewer studies on the effects of silymarin on NSCLC cells, and this study aimed to investigate the role of silymarin and the MAPK pathway in the regulation of NSCLC cell proliferation and invasion.

There are some parallel signalling pathways in the MAPKs family, ERK1/2, p38, JNK3 have been the focus of research in recent years, their susceptibility to extracellular stimuli differ, and there are certain characteristics of their own functional role. The results of this study showed that after the administration of silymarin, the expression of MMP-2, MMP-9 mRNA, p-p38, and p-JNK protein expression between the groups differed in two-by-two comparisons, and the control group>Group A>Group B>Group C (P<0.05). It is suggested that silymarin can have some effects on the activation of ERK1/2, p38, JNK3 pathways and the expression of MMP2 and MMP9 in MAPK family. The three signalling molecules, ERK1/2, p38 and JNK, can be phosphorylated and activated, and the phosphorylated forms of p-p38, p-JNK and p-ERK 1/2 enter the cells to regulate the expression of MMP2 and MMP9, and modulate the biological effects of the cells [14, 15]. In this study, we found that after 24h of silymarin action on NSCLC cells, the expression of p-p38 and p-JNK in the cells showed a trend of decreasing, and the change of p-ERK1/2 level was not obvious, which indicated that silymarin could activate the p38 pathway and JNK pathway of MAPK family in NSCLC cells, and phosphorylated them, which could inhibit the biological behaviours of cancer cells. Combined with the results of this study, the p38 pathway and JNK activate and move to the nucleus after cell stimulation, and control the transcription process of many genes after phosphorylation, which then affects cell proliferation, differentiation, migration and other processes, and silymarin plays a role as a stimulus for this pathway.

In order to further explore the mechanism of silymarin-induced proliferation and invasion of A549 cells, we chose the MAPK signal transduction pathway for our study because MAPK is a key signalling pathway involved in differentiation, proliferation, malignant transformation and apoptotic effects of cells [16]. The results of this study showed that the A-value of cells at 24 h and 48 h (control group>group A >group B>group C), cell survival (group A>group B>group C), and the number of cell invasion (control group>group A>group B>group C) differed in two-by-two comparisons between groups (P<0.05). The analysis is that silymarin may inhibit the activity of MAPK pathway and inhibit the proliferation and invasion process of A549 cells, and the higher the concentration the more obvious inhibition. Some studies have shown [17] that the activation of the MAPK signalling pathway releases a large number of inflammatory factors and aggravates the inflammatory response. After administration of silymarin, p-p38 and p-JNK protein expression in the MAPK signalling pathway was inhibited, which in turn alleviated lung invasion and inhibited the biological behaviour of cancer cells.

By detecting cell invasion, it is known that silymarin inhibits NSCLC cell invasion in a dose-dependent manner.MMP2 and MMP9 hydrolyse the extracellular matrix and are well-recognised proteases that promote malignant tumour invasion and metastasis [18]. In the pathogenesis of NSCLC, increased expression of the above factors is closely associated with both tumour infiltration and lymph node metastasis [19]. In this study, after different doses of silymarin acted on NSCLC cells, it could inhibit the mRNA expression levels of MMP2 and MMP9 in cells in a dose-dependent manner. After drug action, silymarin reduced the number of cancer cell survival and invasion, activated the p38 pathway of the MAPK family, the JNK pathway and inhibited the expression of MMP2 and MMP9. The analysis may be that the inhibition of NSCLC cell proliferation, invasion and MMP2 and MMP9 expression by silymarin is partly related to the activation of MAPK-related pathways, and that both pathways, the p38 pathway and the JNK pathway, are related to the biological effects of silymarin. The results of this study are consistent with the inhibitory effect of silymarin on NSCLC cell proliferation and invasion [20]. This study further confirms that silymarin can effectively inhibit the proliferation and invasion of NSCLC cells.

This study was limited by the experimental conditions and only one NSCLC cell line was used, which is a shortcoming of this study. In conclusion, this study found that the effect of silymarin on NSCLC cells was related to the activation of p38 and JNK pathways, which provides a basis for the clinical discovery of new therapeutic targets.

In conclusion, silymarin may inhibit the proliferation and invasion of NSCLC cells by inhibiting the activity of MAPK pathway, and the higher the concentration, the more obvious the inhibitory effect.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions

Jing Zhang, Kaige Zhang: Conceptualization, methodology, writing original draft preparation. Jing Li, Jia Zhang: Investigation, software, statistical analysis. Xiaohai Cui: Reviewing and editing, funding acquisition, supervision.

All authors read and approved the final manuscript.

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