INTRODUCTION

STAT3 is an important member of signal transducers and activators of transcription (STAT). Continual activation of JAK2/STAT3 (protein tyrosine kinase/signal transducer and activator 3) signal transduction pathway can lead to abnormal cell proliferation and malignant transformation, which is involved in occurrence and development of human malignancies.1,2 STAT3 signal transduction pathway is abnormally activated in a variety of tumor tissues like blood system tumors, breast cancer and prostate cancer, which blocks the pathway from becoming a new target for cancer therapy,3 while AG490 is the specific blocker of the pathway. 4 STAT signaling pathway is an important intracellular signal transduction pathway that regulates cell proliferation, differentiation and apoptosis, which is related to occurrence and development of human malignancies. Therefore, STAT3 is defined as an oncogene.5,6 So, is there any relationship between JAK2/STAT3 signal transduction pathway and biological behavior of nasopharyngeal carcinoma? To identify the role of signaling pathway in pathogenesis of nasopharyngeal carcinoma may find a new way to treat the disease.

The aim of this study was to determine the effect of AG490 on STAT3 signal transduction pathway and nasopharyngeal carcinoma cell proliferation and observe apoptosis to discuss the application value of AG490 in the treatment of nasopharyngeal carcinoma.

METHODOLOGY

This study was conducted at Xi’an Ninth Hospital, Xi’an, China, from May 2015 to December 2016. According to whether or not to add AG490, nasopharyngeal carcinoma C666-1 cells were divided into control group, AG490 drug group. AG490 was not added in the control group. Containing equal amount of DMSO, the two groups had DMSO volume fraction of not more than 0.1%.

Logarithmic growth phase cells were subjected to 0.25% trypsin digestion. The 5x10⁴/mL cell suspension was prepared with serum-containing medium, with 200 µL per well inoculated in 96-well culture plate. The culture plate was incubated at 37°C for 24 h in incubator containing 5% CO₂, followed by addition of AG490 drug dilution of varying concentrations, so that AG490 concentration in each group was 30, 60, 90 and 120 µmol/L, respectively. Blank group (no cell is added to culture medium, zero hole) and control group (RPMI 1640 medium is added), were set up, each group with six repeated wells. AG490 group were treated with MTT at 24, 48 and 72 hours after dosing. Before the detection, 20 µL of 5g/L MTT was added to each well and continually cultured for 4h. After liquid in well was absorbed, 100 µL of DMSO was added, followed by shaking bath incubation for 15 minutes at 37°C. After the MTT crystallization was sufficiently dissolved, OD value of the absorbance at each wavelength of 570 nm of each well was measured on a fully automatic enzyme-labeled spectrophotometer. The growth inhibition rate of AG490

Changes in cell cycle and cell apoptosis detected by flow cytometry

Add culture solution to stop the reaction, blow and beat, prepare single cell suspension, accurately count, adjust the cell density to 5×10⁵/mL, switch to 25 cm² culture flask, add 1mL cell suspension to each bottle, add serum-containing medium to 5mL, to be cultured at 37°C for 24h in incubator containing 5% CO₂. The experimental group was added with AG490 to a final concentration of 50 µmol/L, followed by continual culture, and the cells were digested at 0, 24, 48 and 72 hours, respectively and resuspended in 0.5 mL PBS. Then, the cells were fixed overnight with 70% ice ethanol. RNAaseA was added to the final mass concentration of 60 mg/L, followed by 1h water bath at 37°C constant temperature, addition of propidium iodide (PI) to the final mass concentration of 60 mg/L, dark staining for 1h at 4°C and detection by flow cytometry. After the test, the data were analyzed by ModFit software on a Macintosh computer for apoptosis detection and cell cycle analysis.

The expression of STAT3 and p-STAT3 protein in C666-1 cells after AG490 action: with reference to Santa Cruz protein extraction method, RIPA buffer was used to cleave nasopharyngeal carcinoma cells to obtain total protein. With bovine serum albumin (BSA) as a standard substance, draw the protein quantitative standard curve according to the protein quantitative kit instructions, measure absorbance value at 595nm by spectrophotometer, calculate the extract protein concentration. Protein samples were separated by electrophoresis of 7.5%-10.0% polyacrylamide gel, then transferred to nitrocellulose membrane. After sealing, the protein samples were added with primary antibodies STAT3, p-STAT3, with ratio of 1:400, and horseradish peroxidase-bound secondary antibody with ratio of 1:1000. The hybridization signal was detected by ECL chemiluminescence kit. The X-ray film was washed and scanned with a scanner after dried. The obtained image was analyzed by image processing software Scion Image, and total gray value of each protein band was obtained.

Table I: Inhibitory effects of AG490 on C666-1 cell growth (x ± s, %).

<table>
<thead>
<tr>
<th>Concentration (µmol/L)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>7.13 ±0.62</td>
<td>10.32 ±0.95</td>
<td>27.56 ±1.07</td>
<td>0.003</td>
</tr>
<tr>
<td>60</td>
<td>16.05 ±1.41</td>
<td>34.96 ±1.67</td>
<td>45.21 ±1.55</td>
<td>0.005</td>
</tr>
<tr>
<td>90</td>
<td>47.78 ±1.93</td>
<td>56.03 ±2.54</td>
<td>64.95 ±2.43</td>
<td>0.008</td>
</tr>
<tr>
<td>120</td>
<td>56.46 ±2.88</td>
<td>64.82 ±2.91</td>
<td>75.78 ±2.62</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

There was no apoptotic peak or only low apoptotic peak in C666-1 cells by flow cytometry. AG490 group revealed high and low apoptotic peaks. The apoptosis rate was 2.17 ±0.22% after treatment with AG490 for 24 hours, cell apoptosis was unobvious. The apoptotic cells were increased from 1.86 ±0.13% to 10.53 ±1.01% after treatment for 48h, and cell apoptosis significantly increased; apoptotic cells increased to 19.61 ±1.95% after treatment with AG490 for 72h, the difference was statistically significant (p <0.0001, Table II). AG490 affected growth cycle of C666-1 cells. The proportion of G1 phase in C666-1 cells was the highest under the normal growth condition, followed by S phase, while G2/M phase had the least proportion. AG490 caused cell cycle changes. After treatment with 60 µmol/L AG490 for 24 h, G1 phase cells began to increase from 47.42 ±1.06% to 54.65 ±1.83%; S phase cells began to decrease from 38.67 ±1.72% to 30.76 ±1.45%; after 48 h, G1 phase cells increased to 59.72 ±1.54% and S phase cells decreased to 23.25 ±1.34%; after 72 hours, number of G1 phase cells increased further to 84.09 ±1.05%, while S phase cells decreased further to 11.48 ±0.66%.

The expression of STAT3 and p-STAT3 protein was not statistically different from the control group after treatment with AG490 (60 µmol/L) (%, x ± s, n=5).

Table II: Changes in cell cycle and cell apoptosis detected by flow cytometry with AG490 (60 µmol/L) (% , x ± s , n=5).

<table>
<thead>
<tr>
<th>Cell cycle</th>
<th>0 h (control group)</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>47.42 ±1.06</td>
<td>54.65 ±1.83</td>
<td>59.72 ±1.54</td>
<td>84.09 ±1.05</td>
<td>0.686</td>
</tr>
<tr>
<td>S</td>
<td>38.67 ±1.72</td>
<td>30.76 ±1.45</td>
<td>23.25 ±1.34</td>
<td>11.48 ±0.66</td>
<td>0.541</td>
</tr>
<tr>
<td>G2/M</td>
<td>11.81 ±1.96</td>
<td>12.13 ±1.12</td>
<td>13.63 ±0.91</td>
<td>11.42 ±0.85</td>
<td>0.735</td>
</tr>
<tr>
<td>AP</td>
<td>1.86 ±0.13</td>
<td>2.17 ±0.22</td>
<td>10.53 ±1.01</td>
<td>19.61 ±1.95</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

RESULTS

AG490 inhibited growth of C666-1 cells in vitro, and the inhibitory effect was characterized by time and concentration dependence. At the same concentration, rate of AG490 inhibition on C666-1 cell growth increased with the passage of time; at the same time, increased AG490 concentration increased inhibition rate of C666-1 cell growth, as shown in Table I. Comparing growth inhibition rate of each drug concentration group with the control group, there was statistical significance in the difference in inhibition rate of C666-1 cells between different drug concentration groups and at different time (p<0.0003, 0.005, 0.008, <0.0001 respectively, Table I).
treatment with AG490 for 24 hours (p=0.741,0.692). The expression of STAT3 and p-STAT3 protein began to decrease after treatment with AG490 for 48 hours, and decreased further after 72 hours. The difference was statistically significant (all p<0.0001), as shown in Table III and Figure 1.

**DISCUSSION**

The abnormal activation of STAT3 pathway is associated with abnormal regulation of upstream kinase JAK.7 Opdam et al. found that tyrosine kinase JAK played an important role in invasion and metastasis of malignant lymphoma.8 Blocking JAK kinase with its inhibitor could inhibit STAT3 activity in highly invasive malignant lymphoma cell line, demonstrating anti-tumor effect. Inhibitors for upstream tyrosine kinases such as JAK, Src, EGFR in the STAT3 signaling pathway have been successfully developed; some have entered clinical trials, and AG490 is one of them. AG490 is a synthetic lipid derivative of benzylidene malononitrile, which is structurally similar to tyrosine and competes with receptor tyrosine kinases for binding sites.9,10 AG490 is a JAK2 inhibitor that is a signal molecule that inhibits cytokine receptor-mediated signaling pathways.11,12 There is a gradually increasing anti-tumor research on AG490, which was mainly leukemia-related in the early days, but now reports on its application to gastric and liver cancer and other malignancies, too, gradually appear.13,14 However, application of AG490 in nasopharyngeal carcinoma has not been reported so far. The results showed that AG490 inhibited growth and proliferative activity of C666-1 cells in a time and dose-dependent manner. AG490 affected the growth cycle of C666-1 cells, and with the prolongation of AG490 action, G1 phase cells gradually increased, S phase cells gradually decreased. It suggests that AG490 can prevent G1 phase transformation to S phase of C666-1 cells, and the cells are mainly blocked in G1 phase. Savell et al.15 treated mouse fibroblast cell line BALB/c-3T3 cells with AG490, and found that cell G1 ~ S transformation was blocked. This study found that after treating nasopharyngeal carcinoma cell line C666-1 cells with AG490 to block STAT3 signal transduction pathway, the cells were blocked in G1 phase.

The results of this study show that AG490 can induce apoptosis of C666-1 cells at the same time, and it is time-dependent. After C666-1 cells were treated by AG490, the cell proliferation level decreased with time while apoptosis rate increased, which proved that intervention treatment of nasopharyngeal carcinoma with JAK / STAT3 as a target is feasible. The molecular mechanism of STAT3 activation suggests that STAT3 activation is characterized by phosphorylation of tyrosine residues.16,17 In this study, STAT3 and p-STAT3 antibodies were used, of which, the former was used to detect the overall expression of STAT3 protein, that is, active and inactive states of STAT3; the latter reacted with p-STAT3 tyrosine 705 site, suggesting detection of STAT3 activity status. Western blot results suggest that STAT3 protein is significantly expressed in C666-1 cells. The expression of STAT3 protein decreased after treatment of C666-1 cells with AG490 for 48 hours, and STAT3 protein decreased further after 72 hours, which was significantly different from that of the control group. In this study, the authors also used anti-p-STAT3 antibody in Western blot test, finding that p-STAT3 protein expression also decreased after treatment with AG490, which indicated that AG490 not only blocked STAT3 expression, but also reduced its activity and suggested that AG490 could effectively inhibit expression of STAT3 gene in nasopharyngeal carcinoma.18,19 The results showed that AG490, as STAT3 activated upstream kinase inhibitor, induced apoptosis of nasopharyngeal carcinoma by inhibiting STAT3 signaling pathway. The results provide a new way of thinking for the treatment of nasopharyngeal cancer. This study is helpful to elucidate the mechanism of signal transduction pathway related to proliferation and apoptosis of nasopharyngeal carcinoma cells, and provide experimental and theoretical basis for establishing new therapeutic mode of nasopharyngeal carcinoma – signaling pathway block therapy.20,21

**CONCLUSION**

AG490 can down-regulate expression of STAT3 and p-STAT3 protein in C666-1 cells, inhibit proliferation of

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**Table III:** STAT3 and p-STAT3 protein expression results with AG490 (60 µmol/L) (¯x±s).

<table>
<thead>
<tr>
<th>Protein</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>Experience group</td>
<td>p-value</td>
</tr>
<tr>
<td>STAT3</td>
<td>0.43±0.021</td>
<td>0.43±0.043</td>
<td>0.741</td>
</tr>
<tr>
<td>p-STAT3</td>
<td>0.36±0.008</td>
<td>0.39±0.032</td>
<td>0.692</td>
</tr>
</tbody>
</table>

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![Figure 1: Western blot result of STAT3, p-STAT 3, β-actin. A (Control group), B (Experience group).](image-url)
nasopharyngeal carcinoma cells and promote apoptosis of nasopharyngeal carcinoma cells.

REFERENCES