YiQi ChuTan Recipe Inhibits Epithelial Mesenchymal Transition of A549 Cells under Hypoxia

C-M. Chen, L-L. Sun, R-M. Fang, L-Z. Lin*

Abstract: This study aims to investigate mechanism of YiQi ChuTan Recipe (YCR) for inhibiting epithelial mesenchymal transition (EMT) of A549 cells under hypoxia. Flow cytometry was used to optimize YCR dosage by measuring A549 apoptosis, which were subjected to different treatments, including normal condition, hypoxia, hypoxia+YCR. Cell morphology and expression of EMT were measured with differential interference contrast microscopy, real-time PCR and western blot. Optimized condition of 4 mg/ml YCR and 2% O₂ for 72 h was used to establish hypoxia. Under hypoxic condition, morphology of A549 cells changed from oblate fusiform to elongated spindle. E-cadherin expression decreased while vimentin and fibronectin increased. EMT-related genes expression were significantly increased in hypoxia group compared to control group (P<0.05). After treatment with YCR, mesenchymal cells obviously decreased and EMT-related genes expression was significantly decreased (P<0.05). Changes of E-cadherin, vimentin and fibronectin were significantly attenuated by YCR when compared to hypoxia group. Expression of proteins GRP78, SRC, MAPK, smad2/3 were significantly increased in hypoxia group compared to control group, but was significantly inhibited by YCR treatment. In conclusion, A549 cells underwent EMT under hypoxia while YCR reversed the EMT through GRP78, smad2/3 and SRC/MAPK signal pathway.

Key words: Epithelial mesenchymal transition, YiQi ChuTan Recipe, A549, Hypoxia.

Introduction

The metastasis is a vital factor for the prognosis and a major factor for therapeutic failure and death in lung cancer which is a kind of malignant tumor with the highest mortality and morbidity in the world (1). Therefore, the prevention of lung cancer metastasis is a difficult problem. As a critical mechanism of tumor metastasis, epithelial-mesenchymal transition (EMT) has become the new hotspot for tumor study. EMT can increase the invasive and migrating capacities of tumor cells through multiple signals including mitogen-activated protein kinases (MAPK), tumor growth factor (TGF), and therefore contributes to tumor metastasis (2-4). Although there are large recent advancements in studying the mechanism of EMT, there is still no ideal therapeutic strategy for EMT and tumor metastasis.

Considering its bio-safety and mild effects, the Traditional Chinese Medicine (TCM) is receiving more and more attention in treating tumors (5,6). YiQi ChuTan Recipe (YCR) is an experiential TCM recipe, which could regulate the EMT pathway and the P4HB expression of A549 cells under hypoxia microenvironment (7). Previous study indicated that YCR could inhibit lung metastasis in implanted mouse model of Lewis lung carcinoma, down-regulated the expression of 18 proteins (particularly vimentin) in lung tissues with carcinoma (8). However, the detailed mechanism is not clear. In order to clarify the mechanism of YCR in treating metastatic lung carcinoma, the present study investigated the effects of YCR in EMT and related signaling pathway, particularly vimentin which is a key marker of EMT and plays critical role in the tumor metastasis (9).

Materials and Methods

Preparation of YCR

All herbal materials were purchased from the first Affiliated Hospital of Guangzhou University of Chinese Medicine, including pinellia ternate 15 g, American ginseng 30 g, pseudobulbus crambeae seu pleiones 30 g and bulbus fritillariae thunbergii 15 g. The herbal materials were ground and filtered with 30-mesh sieve, decocted with decoction machine according to the manual and concentrated under 60°C to make YCR containing 2.0 g/ml raw material. The YCR was aliquoted, stored at -20°C, and diluted and filtered with 0.22 μm membrane before using.

Cell culture and treatment

Human lung adenocarcinoma A549 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and were cultured in RPMI-1640 medium (Gibco, USA) supplied with 10% FBS (Gibco, USA) and 100 U/ml penicillin/streptomycin in 5% CO₂ incubator at 37°C. The medium was changed every 3 days. The cells at logarithmic phase were used for experiments using different concentrations of oxygen at 1%, 2%, 5% and 10%. The cell morphology was monitored daily under microscopy and the occurrence of obviously elongated fusiform mesenchymal cells was considered as establishment the hypoxia model. The condition was optimized as 2% O₂ and 72 h
for subsequent experiments.

The cells were treated differently, according to the following condition, including normal O\(_2\) (control group), 2\% O\(_2\) (hypoxia group), 2\% O\(_2\)+4 mg/ml YCR (YCR group).

**Apoptosis measurement with Annexin V/PI**

A549 cells were inoculated in 6-well plates and YCR was added at different concentrations as 4, 8 and 12 mg/ml for another 72 h. Each group set 3 repetition wells. Then the cells were collected and digested. A total of 1×10\(^6\) cells were collected from each well and added with Annexin V and PI solutions. Then, the cells were incubated in dark for 10 min followed by measurement with flow cytometry. In this experiment, in order to investigate whether a concentration of YCR at 4 mg/ml has any significant effect on A549 cells apoptosis, the statistical analysis was performed at least three independent experiments.

**Real-time quantitative fluorescent PCR**

After treatments, the cells were collected to extract total RNA using Trizol method. The cDNA was synthesized with PrimeScript\textsuperscript{TM} RT Master Mix (RR036A; Takara, Japan) through reverse transcription and used as template to amplify target genes with real-time quantitative fluorescent PCR with SYBR\textsuperscript{®} Premix Ex TaqII (RR820A; Takara, Japan). The primers (Invitrogen, USA) were listed in Table 1. The reaction condition was 95°C for 30 s, followed by 95°C for 5 s and 60°C for 30 s with 40 cycles. The amplified productions were quantitatively analyzed with 2\(^{-\Delta\Delta C_t}\) method.

**Measurement of proteins with western blot**

After treatment, the total proteins were extracted with RIPA buffer and quantified with BCA assay (Beyotime, China). 50μg total proteins were separated with 10% SDS-PAGE (Shanghai Beyotime Biotechnology, China). The proteins were transferred to PVDF membrane (Millipore, USA), blocked with 5% BSA at room temperature for 1 h. The protein bands were cut according to the marker and added with primary antibodies, including rabbit anti-human p-ERK polyclonal antibody (sc-7345, 1:2000; Santa Cruz, CA, USA), mouse anti-human p-JNK monoclonal antibody (sc-7345, 1:2000; Santa Cruz, CA, USA), mouse anti-human p-P38 polyclonal antibody (sc-81502, 1:1000; Santa Cruz, CA, USA), mouse anti-human GAPDH monoclonal antibody (MB001; 1:1000; Bioworld Technology Inc., China), rabbit anti-human p-SRC polyclonal antibody (sc-32789, 1:1000; Santa Cruz, CA, USA), Rabbit anti-human p-SRC polyclonal antibody (catalogue 9211, 1: 2000; Cell Signaling Technology, MA, USA), rabbit anti-human p-P38 polyclonal antibody (catalogue 7291, 1: 800; Santa Cruz, CA, USA), rabbit anti-human p-AKT polyclonal antibody (catalogue 6591, 1: 800; Santa Cruz, CA, USA), mouse anti-human SRC monoclonal antibody (sc-377457, 1:1000; Santa Cruz, CA, USA), Rabbit anti-human p-SRC polyclonal antibody (catalogue 2979, 1:800; Cell Signaling Technology, MA, USA), rabbit anti-human P38 polyclonal antibody (catalogue 9212, 1: 800; Cell Signaling Technology, MA, USA), rabbit anti-human p-P38 polyclonal antibody (catalogue 9211, 1: 2000; Cell Signaling Technology, MA, USA), rabbit anti-human ERK polyclonal antibody (ab17942, 1:1000; Abcam, MA, USA), rabbit anti-human p-ERK polyclonal antibody (ab131438, 1:800; Abcam, MA, USA), mouse anti-human JNK monoclonal antibody (sc-7345, 1:2000; Santa Cruz, CA, USA), mouse anti-human p-JNK monoclonal antibody (sc-81502, 1:1000; Santa Cruz, CA, USA) and mouse anti-human GAPDH monoclonal antibody (MB001; 1:1000; Bioworld Technology Inc., China), respectively, and incubated in 4°C for overnight. The membranes were rinsed with TBST for 3 times, 10 min each time. Then secondary goat anti-rabbit or anti-mouse IgG-HRP antibodies (catalogue number AP201, AP503R, respectively; 1:5000; Bioworld Technology Inc., China) were added for incubation in room temperature for 2 h. The membranes were rinsed with TBST for 3 times, and 10 min per time. Then the membranes were developed with ECL (Beijing Kangwei Biotech, China) and taken photos to analyze the relative expression of proteins with GAPDH as internal referral.

**Statistical analysis**

The data were expressed as mean±SD and analyzed with software of SPSS17.0. Comparison between 2 groups was performed with t-test for independent samples and comparison for multiple groups was performed with one-way ANOVA following LSD (equal variances) or Dunnett’s t-test (unequal variances). P<0.05 was set as significant level.

**Results**

**Effects of different dosages of YCR on the apoptosis of A549 cells**

The results of flow cytometry indicated that YCR induced apoptosis of A549 cells with a concentration-dependent manner from 0 to 12 mg/ml (Figure 1A-D). The apoptosis rate of A549 at 0, 4, 8 and 12 mg/ml YCR was 0.7%, 4.6%, 17.85 and 28.7%, respectively. YCR at 4 mg/ml had no obvious effect on the cell apoptosis and was chosen for subsequent experiments.

**Effects of hypoxia and YCR on the cell morphology**

Under microscopy, the A549 cells changed the morphology from oblate fusiform-shaped epithelial cells (Figure 2A) to elongated spindle-shaped mesenchymal cells after 72 h treatment with 2\% O\(_2\) (Figure 2B) and YCR (Figure 2C). The results indicated that YCR could inhibit the process of epithelial cells to mesenchymal cells by changing the expression of EMT-related genes.
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The present study indicated that hypoxia induced human lung carcinoma cell A549 to transit from oblate fusiform-shaped epithelial cells to elongated spindle-shaped mesenchymal cells. Real-time fluorescence quantitative PCR indicated that the EMT-related genes were increased under hypoxia. Western blot results indicated that E-cadherin protein representing epithelial phenotype was increased while vimentin and fibronectin proteins representing mesenchymal phenotype was increased and there was significant difference when compared to that in control group (p<0.05; Figure 3, Table 3). Meanwhile, the expression of vimentin and fibronectin proteins representing mesenchymal phenotype was increased and there was significant difference when compared to that in control group (p<0.05; Figure 3, Table 3). The treatment with YCR attenuated the changes induced by hypoxia. The expression of protein E-cadherin in hypoxia+YCR group (4 mg/ml, 2% O2 72 h) was increased while the expression of vimentin and fibronectin decreased when compared to that in hypoxia group (2% O2 72 h), and there was significant difference between these 2 groups (p<0.05; Figure 3, Table 3).

In addition, the expression of proteins GRP78, smad2/3, p-smad2/3, SRC, p-SRC, P38, p-P38, ERK, p-ERK, JNK and p-JNK in A549 cells were also increased in hypoxic condition (2% O2 72h), and there was significant difference when compared to control group (p<0.05; Table 3). However, the expression of proteins PI3K, p-PI3K, AKT and p-AKT were not increased by hypoxia (2% O2 72h) when compared to that in control group (p>0.05; Figure 3, Table 3). In hypoxia+YCR group (4 mg/ml, 2% O2 72 h), the expression of proteins GRP78, smad2/3, p-smad2/3, SRC, p-SRC, PI3K, p-PI3K, AKT, p-AKT, P38, p-P38, ERK, p-ERK, JNK and p-JNK was decreased and there was significant difference when compared to that in hypoxia group (2% O2 72 h) (p<0.05; Figure 3, Table 3).

**Discussion**

The present study indicated that hypoxia induced human lung carcinoma cell A549 to transit from oblate fusiform-shaped epithelial cells to elongated spindle-shaped mesenchymal cells. Real-time fluorescence quantitative PCR indicated that the EMT-related genes were increased under hypoxia. Western blot results indicated that E-cadherin protein representing epithelial phenotype was increased while vimentin and fibronectin proteins representing mesenchymal phenotype was increased and there was significant difference when compared to that in control group (p<0.05; Figure 3, Table 3). Meanwhile, the expression of vimentin and fibronectin proteins representing mesenchymal phenotype was increased and there was significant difference when compared to that in control group (p<0.05; Figure 3, Table 3). The treatment with YCR attenuated the changes induced by hypoxia. The expression of protein E-cadherin in hypoxia+YCR group (4 mg/ml, 2% O2 72 h) was increased while the expression of vimentin and fibronectin was decreased when compared to that in hypoxia group (2% O2 72 h), and there was significant difference between these 2 groups (p<0.05; Figure 3, Table 3).

**Table 2.** Expression of EMT-related genes in different groups (mean ±SD).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control group</th>
<th>Hypoxia group</th>
<th>Hypoxia+YCR</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail1</td>
<td>1.00±0.01</td>
<td>4.83±0.03*</td>
<td>1.37±0.04*</td>
<td>14869.99</td>
<td>0.000</td>
</tr>
<tr>
<td>Snail2</td>
<td>1.00±0.00</td>
<td>4.80±0.11*</td>
<td>1.50±0.16*</td>
<td>1020.707</td>
<td>0.000</td>
</tr>
<tr>
<td>Twist</td>
<td>1.00±0.00</td>
<td>4.81±0.16*</td>
<td>1.46±0.05*</td>
<td>1365.563</td>
<td>0.000</td>
</tr>
<tr>
<td>ZEB1</td>
<td>1.00±0.01</td>
<td>4.77±0.19*</td>
<td>1.29±0.23*</td>
<td>457.314</td>
<td>0.000</td>
</tr>
<tr>
<td>ZEB2</td>
<td>1.00±0.01</td>
<td>4.76±0.06*</td>
<td>1.46±0.09*</td>
<td>2990.93</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Note: * P<0.01 vs control group; ^ P<0.01 vs hypoxia group.
EMT is a biological process that epithelial cells transform to cells with mesenchymal phenotype through special mechanism (3). EMT can result in loss of the polarity of tumor cells, weaken the intercellular junction and make the cells incompact, which attributes to stronger invasive and migrating capacities for tumor cells and therefore provides favorable conditions for tumor metastasis (10). The inductive factors for EMT derive from extracellular stimulation, i.e., EMT is determined by the surrounding signaling factors such as cytokines, hypoxia and ischemia. Hypoxia is currently considered as an important factor for EMT and tumor metastasis (4,11). Consistently, the present study indicated that human lung carcinoma cell A549 demonstrated EMT in morphology under hypoxia.

MAPK members including JNK, P38 and ERK can upregulate snail, decrease the expression of E-cadherin and promote EMT and metastasis (12,13). Previous studies indicated that the signal pathways involved in hypoxia-induced EMT including p38MAPK in prostate cancer (14) and ERK in breast cancer (15), which are consistent with the present study that the expression of p38, ERK and p-ERK were increased in A549 cells under hypoxia. GRP78 is one member of the hot shock protein 70 family, plays key role in endoplasmic reticulum (ER), is significantly increased during ER stress and, therefore, is often used as biomarker for ER stress (16). Furthermore, the ER stress in tumor cells is closely correlated with EMT and the microenvironment of tumor can stimulate the ER stress and mediate the occurrence of EMT (17,18). Previous studies (19-21) indicated that smad2/3 and SRC/MAPK signals are involved in EMT through translocation to cellular nuclei to correlate with transcriptional factor snail and to inhibit the expression of E-cadherin. SRC can be involved in EMT through phosphorylation of cytoskeleton and adhesive structure to induce cell migration, activation of MAPK signal to produce reduction of intercellular junction, promotion of matrix degradation and increase of tumor invasive.

Table 3. Expression of EMT related proteins in different groups (mean ±SD).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control group</th>
<th>Hypoxia group</th>
<th>Hypoxia+YCR</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>0.40±0.18</td>
<td>0.89±0.05*</td>
<td>0.31±0.04*</td>
<td>24.03</td>
<td>0.001</td>
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<tr>
<td>E-cadherin</td>
<td>0.77±0.12</td>
<td>0.26±0.04*</td>
<td>0.45±0.07*</td>
<td>29.767</td>
<td>0.001</td>
</tr>
<tr>
<td>Vimentin</td>
<td>0.31±0.04</td>
<td>0.77±0.12*</td>
<td>0.29±0.16*</td>
<td>15.837</td>
<td>0.004</td>
</tr>
<tr>
<td>GRP78</td>
<td>0.55±0.07</td>
<td>1.14±0.14*</td>
<td>0.23±0.13*</td>
<td>47.187</td>
<td>0.000</td>
</tr>
<tr>
<td>smad2/3</td>
<td>0.32±0.08</td>
<td>0.67±0.11*</td>
<td>0.35±0.11*</td>
<td>10.950</td>
<td>0.010</td>
</tr>
<tr>
<td>p-Smad2/3</td>
<td>0.17±0.07</td>
<td>0.47±0.11*</td>
<td>0.20±0.07*</td>
<td>11.888</td>
<td>0.008</td>
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<tr>
<td>PI3K</td>
<td>0.51±0.15</td>
<td>0.68±0.11*</td>
<td>0.31±0.05*</td>
<td>8.202</td>
<td>0.019</td>
</tr>
<tr>
<td>p-PI3K</td>
<td>0.31±0.06</td>
<td>0.36±0.10*</td>
<td>0.17±0.06*</td>
<td>5.413</td>
<td>0.045</td>
</tr>
<tr>
<td>AKT</td>
<td>0.77±0.10</td>
<td>0.83±0.12*</td>
<td>0.54±0.07*</td>
<td>7.064</td>
<td>0.026</td>
</tr>
<tr>
<td>p-AKT</td>
<td>0.37±0.17</td>
<td>0.59±0.07*</td>
<td>0.21±0.06*</td>
<td>8.369</td>
<td>0.180</td>
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<tr>
<td>SRC</td>
<td>0.29±0.04</td>
<td>0.51±0.12*</td>
<td>0.27±0.09*</td>
<td>6.984</td>
<td>0.027</td>
</tr>
<tr>
<td>p-SRC</td>
<td>0.19±0.06</td>
<td>0.43±0.13*</td>
<td>0.13±0.03*</td>
<td>10.311</td>
<td>0.011</td>
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<tr>
<td>P38</td>
<td>0.29±0.13</td>
<td>0.98±0.19*</td>
<td>0.31±0.08*</td>
<td>22.670</td>
<td>0.002</td>
</tr>
<tr>
<td>p-P38</td>
<td>0.14±0.06</td>
<td>0.48±0.14*</td>
<td>0.17±0.05*</td>
<td>12.126</td>
<td>0.008</td>
</tr>
<tr>
<td>ERK</td>
<td>0.29±0.05</td>
<td>0.59±0.11*</td>
<td>0.28±0.09*</td>
<td>10.809</td>
<td>0.010</td>
</tr>
<tr>
<td>p-ERK</td>
<td>0.17±0.05</td>
<td>0.35±0.10*</td>
<td>0.18±0.05*</td>
<td>5.976</td>
<td>0.037</td>
</tr>
<tr>
<td>JNK</td>
<td>0.33±0.15</td>
<td>0.71±0.05*</td>
<td>0.33±0.12*</td>
<td>11.131</td>
<td>0.010</td>
</tr>
<tr>
<td>p-JNK</td>
<td>0.23±0.13</td>
<td>0.53±0.11*</td>
<td>0.21±0.02*</td>
<td>9.510</td>
<td>0.014</td>
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</table>

Note: *P<0.05 vs control group; †P<0.05 vs control group; ‡P<0.05 vs hypoxia group.
capacity (22). Consistently, we found that the expression of GRP78, smad2/3, p-smad2/3, SRC, p-SRC, JNK and p-JNK was increased by hypoxia, suggesting hypoxia activates these signaling to be involved in the EMT in human lung tumor A549 cells. However, the expression of PI3K, p-PI3K, AKT and p-AKT was not significantly changed by hypoxia, suggesting these signals play no important role in the EMT of lung carcinoma under hypoxia.

Modulating the tumor microenvironment is one advantage of TCM in antitumor mechanism and increasing studies indicated that TCM is a potential approach to treat tumors by inhibiting EMT through snail/E-cadherin signal (23-25). YCR is an experiential recipe used in the Department of Tumor in the hospital to treat lung carcinoma and showed good clinical effects (2), however the mechanism is not clear. In the present study, we found that YCR inhibited the phenotype change of A549 cells from epithelial morphology to mesenchymal morphology and significantly attenuated the changes of E-cadherin, vimentin and fibronectin induced by hypoxia, suggesting that YCR can inhibit the EMT of A549 cells induced by hypoxia. Furthermore, we found that YCR blocked the change of EMT-related genes and proteins expression in A549 cells induced by hypoxia. These results suggest that YCR may play its anti-tumor effect in human lung carcinoma through modulating these signals including GRP78, SRC, MAPK, Smad and Snail pathways, and, therefore, provides evidence for the potential application of YCR in clinic treating tumors.

The pervious study (7) also proved that the YCR could inhibits the hypoxia induced EMT by down-regulating the P4HB mRNA expression in A549 cells. Though this study (7) discovered the inhibition effects of YCR on the hypoxia induced EMT, however, this study only clarified by activating the P4HB mRNA expression. In the present study, we proved for the first time that YCR inhibits the EMT by activating GRP78, Smad2/3 and SRC/MAPK signaling pathway. Therefore, we think that our study added some novel data for the function of YCR in the EMT.

However, the detailed mechanism is unclear and remains to be further studied. In the future study, we will apply gene silencing techniques and specific protein inhibitors to study the specific signal involved in the inhibition of EMT by YCR in hypoxic condition.

Acknowledgements
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References