Original Article

ATRA and Genistein synergistically inhibit the metastatic potential of human lung adenocarcinoma cells

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Abstract: Objective: This study was to investigate the effects of all-trans retinoic acid (ATRA) in combination with Genistein on the proliferation, expression of apoptosis related proteins and adhesion molecules (MUC1 and ICAM-1) and invasiveness of A549 cells, aiming to investigate whether combined therapy of ATRA and Genistein is superior to monotherapy in suppressing metastasis of lung cancer cells. Methods: ATRA, Genistein and both were used to treat human lung adenocarcinoma cells (A549 cells). Immunohistochemistry was done for MUC1 expression, flow cytometry for ICAM-1 expression, fluorescence quantitative PCR for MUC1 expression and Western blot assay for the expressions of cell cycle related proteins (CDK4, Rb and p-ERK1/2) and apoptosis related proteins (Bax and Bcl-2). Cells were seeded into Matrigel pre-coated Transwell chambers, and the migrating cells were counted. Results: Combined treatment with ATRA and Genistein was able to reduce the expressions of Bcl-2, MUC1 and ICAM-1 and exerted synergistic effects to inhibit the invasion of A549 cells. Conclusion: ATRA and Genistein may synergistically inhibit MUC1 and ICAM-1 expressions and affect the expressions of cell cycle related proteins (CDK4, Rb and p-ERK1/2) and apoptosis related proteins (Bax and Bcl-2), inhibit the metastatic potential of lung cancer A549 cells.

Keywords: All-trans retinoic acid, Genistein, lung carcinoma, ICAM-1, MUC1, metastatic potential

Introduction

All-trans retinoic acid (ATRA) has been found to be able to effectively induce differentiation of cancer cells and inhibit their growth, and its therapeutic effect on lung cancer has been paid attention to [1]. However, the severe side effects of ATRA significantly limit its wide application in clinical practice. Genistein is a natural inhibitor of tyrosine kinase and increasing studies reveal its anti-tumor effect. Genistein at certain doses may not only induce the differentiation of cancer cells, but promote their apoptosis [2] and inhibit their invasion and metastasis [3]. Nevertheless, monotherapy with Genistein is insufficient to induce differentiation and facilitate apoptosis. Thus, it is important to investigate how to reduce the dose of anti-tumor drugs and attenuate their side effects without affecting their anti-tumor effects. Some investigators have applied combined therapy with anti-tumor drugs at low doses, which will be a new direction in the studies on the therapy of cancers [4]. In the present study, the effects of ATRA, Genistein and both on the proliferation, expression of apoptosis related proteins and adhesion molecules (MUC1 and ICAM-1) and invasiveness of human lung adenocarcinoma cells (A549 cells) were evaluated, aiming to investigate whether combined therapy is superior to monotherapy in suppressing the metastasis of lung cancer cells and to explore the potential mechanism underlying the inhibitory effects of combined ATRA and Genistein on the metastasis of cancer cells.
Materials and methods

Materials

Human lung adenocarcinoma cells (A549 cells) were maintained in RPMI 1640 containing 100 ml/L fetal bovine serum (FBS) at 37°C in an environment with 50 ml/L CO₂. RPMI 1640 and FBS were purchased from GIBCO, USA. ATRA, Genistein (Sigma, USA), rabbit anti-human monoclonal antibodies against MUC1 and ICAM-1 (Santa Cruz, USA), SYBRGREEN I (Shenzhen Dakewei Company), DAB, SP kit for immunohistochemistry (Zhangshan Golden Bridge Biotech Co., Ltd), Boyden chamber (Millipore, USA), Matrigel (BD, USA), reverse transcriptase, Taq polymerase, dNTP, RNAase inhibitor, Oligo(dT)15, 10 × PCR buffer, 100 bp DNA Ladder (Dingguo Biotech Co., Ltd) and primers (Shanghai Sangong Biotech Co., Ltd) were used in the present study.

Primers

Primers used for PCR were as follows: MUC1: 5'-AAT TGACTC TGG CCT TCC GA-3' (forward), 5'-TGC CACCAT TAC CTG CAG AA-3' (reverse), and anticipated size was 411 bp; β-actin: 5'-CCA TCA TGA AGT GTG ACG TGG-3' (forward), 5'-GTC CGC CTA GAA GCA TTT GCG-3' (reverse), and anticipated size was 293 bp.

Preparation of ATRA and Genistein solution

ATRA was dissolved in absolute alcohol into 10 mmol/L and stored at -30°C in dark. Before use, ATRA solution was diluted with RPMI1640, and cells were treated with 50 μmol/L ATRA. Genistein was dissolved in absolute alcohol into 10 mmol/L and stored in an environment containing nitrogen alone. Before use, Genistein solution was diluted with RPMI1640, and cells were treated with 40 μmol/L Genistein.

Grouping

ATRA group: ATRA was added to culture medium at a final concentration of 50 μmol/L; Genistein group: Genistein was added to culture medium at a final concentration of 40 μmol/L; ATRA+ Genistein group: Cells were treated with 50 μmol/L ATRA and 40 μmol/L Genistein; Control group: cells were treated with absolute alcohol alone (1.8 μl/well). In each group, the amount of alcohol in the medium was 1.8 μl/well. Incubation was done for 24 h.

Detection of MUC1 expression by immunocytochemistry

Two-step SP method was employed for immunocytochemistry. The pre-treated coverslips were placed in cell suspension. When cells were adherent to these coverslips, they were air-dried and treated with 30 ml/L H₂O₂ methanol at room temperature for 20 min, followed by incubation with 1 ml/L Triton X 100 for 10 min. Then, these cells were treated with primary antibody (1:200) at 4°C overnight. In negative control group, cells were treated with PBS instead of primary antibody. Subsequently, cells were incubated with secondary antibody (50 μl) at 37°C for 30 min, followed by visualization with DAB-H₂O₂.

Positive cells had yellow-brown granules in the cytoplasm and/or on cell membrane. The protein expression was determined with TJTY-300 image analysis system. Five fields were randomly selected, and 98 cells were randomly selected from each field. Mean absorbance (A) was measured at 550 nm.

Detection of ICAM-1 expression

Cells with good growth were seeded into 6-well plates (6 × 10⁵/well). When cells were adherent to the bottom of plates, cells were maintained overnight, and treated as abovementioned for 24 h. Cells were harvested and treated with primary antibody against ICAM-1 (CD54) at 4°C for 30 min. In negative control group, the primary antibody was replaced with PBS. After washing in PBS thrice, cells were incubated with FITC conjugated goat anti-mouse IgG at 4°C for 30 min. After washing in PBS thrice, the fluorescence intensity was determined by flow cytometry (FACstar; Becton Dickinson) in which a total of 10000 cells were detected.

Western blot assay

Total protein was extracted and quantified with Bradford method. Separating gel and stacking gel were prepared, and samples and protein marker were loaded, followed by electrophoresis at 20 mA. When the loading buffer reached the borderline between gels, further electrophoresis was performed at 120 V until the loading buffer reached 1 cm away from the bottom.
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of stacking gel. Then, the gel was taken out and the gel containing proteins was excised. Proteins were then transferred onto PVDF membrane at 0.65 mA/cm² for 1.5 h. After incubation with TNT buffer thrice (15 min for each), the membrane was blocked in blocking buffer at 4°C, followed by washing in TNT buffer thrice. The membrane was treated with primary antibody at room temperature for 1 h. Following washing in TNT buffer thrice (5 min for each), the membrane was incubated with secondary antibody for 1 h at room temperature. After washing in TNT buffer thrice (5 min for each), the membrane was incubated with Luminol Reagent A and B of equal volume for 1 min. After visualization, the optical density (OD) of each band was determined. The mean OD of target protein was normalized to that of β-actin.

Detection of MUC1 mRNA expression by fluorescence quantitative PCR

Total RNA was extracted and reverse transcribed into cDNA, followed by PCR. The PCR products were identified and retrieved. After DNA quantification and labeling of PCR products, SYBRGreen I was used to detect the copies of DNA. Two curves were delineated with the Ct values of MUC1 and β-actin (y = -0.19x+11.78 and y = -0.40x+14.11; correlation coefficient: 0.972 and 0.973). According to the curves, the sample concentration (copy/μl) was calculated, and following formula was used to calculate the relative mRNA expression of MUC1: relative mRNA expression = target gene/β-actin.

Detection of in vitro invasion

In brief, 200 μl of supernatant from NIH 3T3 cells was added to the lower chambers of Boyden chamber. The lower and upper chambers were separated via porous polycarbonate membrane (diameter: 12 mm; pore size: 12 μm). The member was pre-coated with 50 μl of Matrigel (50-μg Matrigel in DMEM). When the gel was dried, cells after different treatments were added to upper chambers (400 μl; 2 × 10⁵ cells), followed by incubation at 37°C in an environment with 50 ml/L CO₂ for 6 h. The membrane was taken out, and the gel and cells on the membrane were removed, and then fixed in methanol, followed by HE staining. The membrane was divided into 9 parts under a light microscope, and cells in each part were counted, followed by averaging. The mean number of cells was obtained in each group. Two samples were included in each detection, which was performed two times.

Statistical analysis

Statistical analysis was performed with SPSS version 14.0 and data were expressed as mean ± standard deviation (x ± s). Comparisons were done with t test between two groups. A value of P < 0.05 was considered statistically significant.

Results

Effects of ATRA and Genistein on the MUC1 protein expression in A549 cells

Immunocytochemistry showed treatment with ATRA or Genistein could down-regulate the MUC1 expression, and the reduction in MUC1 expression was more obvious after combined treatment (P < 0.01 or P < 0.05 vs. control group). This suggests that ATRA and Genistein may synergistically inhibit the protein expression of MUC1 in A549 cells (Figure 1; Table 1).
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**Table 1.** Protein and mRNA expressions of MUC1 in A549 cells treated by ATRA and/or Genistein

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein (× 10^7)</th>
<th>mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>3.26 ± 0.11</td>
<td>1.069 ± 0.215</td>
</tr>
<tr>
<td>50 μmol/L ATRA</td>
<td>1.48 ± 0.12a</td>
<td>0.576 ± 0.134d</td>
</tr>
<tr>
<td>40 μmol/L Genistein</td>
<td>1.92 ± 0.17bcd</td>
<td>0.758 ± 0.167c</td>
</tr>
<tr>
<td>ATRA+ Genistein</td>
<td>0.99 ± 0.08a</td>
<td>0.087 ± 0.006a</td>
</tr>
</tbody>
</table>

Footnotes: n = 6; ^P < 0.01, vs. control group; ^P < 0.05, vs. control group; ^P < 0.01, vs. ATRA+ Genistein group; ^P < 0.05, vs. ATRA+ Genistein group.

**Table 2.** Proportion of ICAM-1 positive A549 cells after treatment with ATRA and/or Genistein

<table>
<thead>
<tr>
<th>Group</th>
<th>ICAM-1 Positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.12 ± 4.87</td>
</tr>
<tr>
<td>50 μmol/L ATRA</td>
<td>10.13 ± 1.68a</td>
</tr>
<tr>
<td>40 μmol/L Genistein</td>
<td>16.62 ± 7.37</td>
</tr>
<tr>
<td>ATRA+ Genistein</td>
<td>8.36 ± 1.31a</td>
</tr>
</tbody>
</table>

Footnotes: ^P < 0.05, vs. control group; ^P < 0.01, vs. control group.

**Effects of ATRA and Genistein on the MUC1 mRNA expression in A549 cells**

Fluorescence quantitative PCR showed ATRA or Genistein could down-regulate the mRNA expression of MUC1 although significant difference was not observed. However, combined treatment with ATRA and Genistein markedly inhibited the mRNA expression of MUC1 when compared with control group, ATRA group and Genistein group (P < 0.01, P < 0.05 and P < 0.01, respectively). This suggests that combined treatment ATRA and Genistein may synergistically inhibit the mRNA expression of MUC1 in A549 cells (Table 1).

**Effects of ATRA and Genistein on the ICAM-1 expression in A549 cells**

Flow cytometry showed, after ATRA treatment, the proportion of ICAM-1 positive cells was 10.13 ± 1.68%, which was significantly lower than that in control group (14.12 ± 4.87%) (P < 0.05); after Genistein treatment, the proportion of ICAM-1 positive cells was 16.62 ± 7.37%, which was comparable to that in control group (P > 0.05). However, after combined treatment, the proportion of ICAM-1 positive cells was 8.36 ± 1.31%, which was markedly lower than that in control group (P < 0.01) (Table 2).

**Effects of ATRA and Genistein on the protein expression of cell cycle related proteins (CDK4, Rb and p-ERK1/2) in A549 cells**

Results showed Genistein could significantly inhibit the protein expression CDK4 (P < 0.05 vs. control group), and the reduction in CDK4 expression was more obvious after combined treatment (P < 0.01 vs. control group; P < 0.01 vs. ATRA group). Genistein alone or in combination with ATRA could up-regulate the Rb protein expression in A549 cells, but the Rb protein expression remained unchanged after treatment with ATRA alone (P > 0.05 vs. control group). The p-ERK1/2 expression remained unchanged after Genistein treatment (P > 0.05 vs. control group). In addition, combined treatment with ATRA and Genistein dramatically reduced the p-ERK1/2 expression when compared with control group (P < 0.01 vs. control group), but this reduction was slightly lower than that in ATRA group (P > 0.05 vs. ATRA group; Table 3).

**Effects of ATRA and Genistein on the protein expression of apoptosis related proteins (Bax and Bcl-2) in A549 cells**

Results showed Bcl-2 protein expression reduced after treatment with ATRA and/or Genistein, suggesting that Bcl-2 play an important role in the ATRA and/or Genistein induced apoptosis of A549 cells and Bcl-2 may be a major regulatory gene. Bax protein expression increased markedly after treatment with ATRA and/or Genistein (P < 0.01 vs. control group), and the increase in Genistein group was markedly lower than that in ATRA group and combined treatment group (P < 0.01, vs. ATRA+ Genistein). Bax/Bcl-2 increased significantly after different treatments, especially in ATRA+ Genistein group (P < 0.01 vs. ATRA; P < 0.05 vs. Genistein; Table 4).

**Effect of ATRA and Genistein on the invasiveness in vitro**

The number of A549 cells migrating across the membrane was used to evaluate the invasiveness of A549 cells. Our results showed the invasiveness of A549 cells was significantly compromised after ATRA treatment when compared with control group (P < 0.05). Although the invasiveness of A549 cells was also com-
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Discussion

Apoptosis is a complex process and has involvement of multiple genes including Bcl-2 and Bax. Bcl-2 is an important gene in the regulation of apoptosis. Bcl-2 expression and its change at different levels may directly affect the whole process of apoptosis. Studies have shown that to inhibit Bcl-2 expression may induce the apoptosis of a variety types of cancer cells [5]. In prostate cancer, colon cancer, neuroblastoma and NSCLC, studies have confirmed that Bcl-2 over-expression predicts a poor prognosis [6-9]. Currently, the specific molecular mechanism of how Bcl-2 combats apoptosis to prolong cell survival is still unclear. There is evidence showing that Bcl-2 may regulate the complex signaling pathways controlling cell death and may serve as a transit point in these pathways [10, 11]. Bcl-2 can inhibit the release of Cytochrome C from the mitochondria into cytoplasm, which blocks the activation of caspases in the cell apoptosis [10, 12]. Bcl-2 protein, a product of Bcl-2 gene, may bind to Bax protein, and the resultant complex plays a key regulatory role in the anti-apoptosis [13]. Over-expression of Bax may counteract the anti-apoptotic effect of Bcl-2. The ratio of Bax to Bcl-2 determines that whether cells receive apoptosis related signals, and thus Bax and Bcl-2 are regarded apoptosis related proteins.
which can negative and positively regulate cell apoptosis, respectively. The increased apoptosis is ascribed to both down-regulation of Bcl-2 and up-regulation of Bax, and the Bax/Bcl-2 ratio determines the responses of cells to apoptosis related signals. Our previous findings [14] showed treatment with 50 μM ATRA or 40 μM Genistein for 24 h and 48 h could induce the apoptosis of A549 cells, and the apoptosis was more obvious after combined treatment. This implies that ATRA and Genistein may synergistically promote the apoptosis of A549 cells. In the present study, results showed, after treatment with ATRA and/or Genistein, Bcl-2 expression increased significantly, but Bax expression increased markedly after ATRA treatment and combined treatment, and only slightly elevated after Genistein. Of note, the Bax/Bcl-2 in different groups increased markedly, especially in combined treatment group. This reminds us that ATRA in combination with Genistein may synergistically promote the apoptosis of A549 cells, which may be ascribed to the regulation of Bax/Bcl-2 balance.

CDK is a key protein regulating the transition between G1 phase and S phase and between G2 phase and M phase, and plays important roles in the DNA replication and division. Persistent CKD activation may promote the cell cycle progression, and facilitate the progression from G1 phase to S phase and from G2 phase to M phase. As a result, cells in S phase increase, DNA replication is promoted, replication of oncogene increases and cancer cells proliferate actively. Genistein may competitively inhibit ATP binding site and suppress the phosphorylation of receptors including Grb-2, to exert effects. In the present study, combined treatment with ATRA and Genistein significantly reduced CDK4 protein expression when compared with control group and ATRA group, suggesting that ATRA and Genistein may synergistically inhibit CDK4 expression. In addition, combined treatment with ATRA and Genistein arrested cells in G2/M phase [14]. Above findings imply that combined treatment with ATRA and Genistein may affect the cell cycle progression of A549 cells at different levels, of which inhibition of mitosis is key one.

Rb involves in the activation of transcription factors and regulation of cell cycle, and its deficiency and inactivation are closely related to the occurrence of cancers [15]. Rb gene deficiency may result in absence or functional alteration of Rb protein, and the ability of Rb protein to bind to transcription factors reduces, and subsequently cell proliferation is loss of control, resulting in tumorigenesis. ATRA may inhibit the Rb phosphorylation (pRb) which is essential for the progression into S phase [16]. In the present study, results showed Rb protein expression remained unchanged after ATRA treatment. This may be explained as that the Rb activity is not reflected by Rb protein, but more importantly by its phosphorylation. On the basis of findings from detection of cell cycle, ATRA arrested cells in G0/G1 phase [14], which may be ascribed to the inhibition of Rb phosphorylation. In the present study, Genistein increased Rb protein expression in A549 cells. The increased Rb may reduce the free nuclear transcription factor E2F, inhibiting the expression of intranuclear genes (such as c-myc, b-myb and cdc2) and cell proliferation. The regulation of Rb protein expression may be one of mechanisms underlying the inhibitory effects of Genistein on cell proliferation. There is evidence showing that low dose Genistein may increase cyclin D1 expression, activate CDK2 and stimulate the Rb phosphorylation, which promote the cell cycle progression; but high dose Genistein is able to inhibit Rb phosphorylation, and prolong the cell cycle [17]. Thus, the relationship between Genistein induced inhibition of cell growth and RB protein expression is required to be further studied.

ERK1/2 signaling pathway play an important role in the cyclin D1 expression, which may be ascribed to the nuclear translocation of ERK, subsequent activation of transcription factors and their bindings to cyclin D1 promoter, resulting in the initiation of gene transcription and promoted cell proliferation [18]. After Genistein treatment, p-ERK1/2 expression remained

<table>
<thead>
<tr>
<th>Table 5. Number of migrating cells after different treatments</th>
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<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>Control group</td>
</tr>
<tr>
<td>50 μmol/L ATRA</td>
</tr>
<tr>
<td>40 μmol/L Genistein</td>
</tr>
<tr>
<td>ATRA+ Genistein</td>
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Footnotes: aP < 0.05, vs. control group; bP < 0.01, vs. control group.
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unchanged; after ATRA treatment, p-ERK1/2 expression reduced; after combined treatment, p-ERK1/2 expression reduced significantly when compared with control group, but was still slightly higher than that in ATRA group.

Adhesion is an important step in the invasion and metastasis of cancer cells. Adhesion molecules mediate the interactions of cancer cells with extracellular matrix, vascular endothelial cells and targeted organs. This, adhesion is closely related to the invasion and metastasis of cancers [19]. Studies have revealed that cancer cells with ICAM-1 over-expression have more potent invasion and metastatic capabilities [20], which may be attributed to the LFA-1 mediated heterotypic binding of cancer cells to tumor infiltrating lymphocytes (TIL) with metastatic and invasive capabilities, resulting in reduced adhesion of cancer cells, and the subsequent migration of cancer cells along lymphocytes. Cancer cells binding to TIL cells or lymphocytes are free of damage during crossing the vascular wall and are easy to form thrombi in the vessels. Thus, these cancer cells usually escape from the killing of immune system and are susceptible to stay in capillaries and lymphatic sinus to form metastatic foci. MUC1 is an anti-adhesion molecule and expressed in multiple cancers and can inhibit the intercellular adhesion [21]. MUC1 may down-regulate E-cadherin expression to elevate cellular invasiveness [22]. It has been reported that the elevated invasiveness of a variety of types of cells is related to the high MUC1 expression [23]. In lung cancer, high MUC1 expression has been reported [24], MUC1 may serve as a marker for the differentiation of lung cancer cells, and MUC1 expression reduces with the differentiation of lung cancer cells [25]. In our study, the effects of ATRA and Genistein on the expression of ICAM-1 and MUC1 were investigated. Results showed the proportion of ICAM-1 positive cells reduced significantly after ATRA treatment when compared with control group; the ICAM-1 positive cells remained unchanged after Genistein treatment; combined treatment with ATRA and Genistein also dramatically reduced the proportion of ICAM-1 positive cells as compared to control group (P < 0.01). In addition, ATRA and/or Genistein could down-regulate the mRNA and protein expression of MUC1, especially in combined treatment group. On the above findings, ATRA and Genistein may synergistically inhibit the expression of ICAM-1 and MUC1 in A549 cells, which compromises the invasive and metastatic potentials of lung cancer cells.

Taken together, ATRA in combination with Genistein may significantly inhibit the invasiveness of A549 cells in vitro, which may be attributed to the down-regulation of MUC1 and ICAM-1 expression, altered expression of cell cycle related proteins (CDK4, Rb and p-ERK1/2) and apoptosis related proteins (Bax and Bcl-2).

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Disclosure of conflict of interest

None.

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