rAd-p53 enhances the sensitivity of human gastric cancer cells to chemotherapy

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Abstract

AIM: To investigate potential antitumor effects of rAd-p53 by determining if it enhanced sensitivity of gastric cancer cells to chemotherapy.

METHODS: Three gastric cancer cell lines with distinct levels of differentiation were treated with various doses of rAd-p53 alone, oxaliplatin (OXA) alone, or a combination of both. Cell growth was assessed with a 3-(4,5)-dimethylthiazol-2-yl)-3,5-diphenyltetrazoliumbromide assay and the expression levels of p53, Bax and Bcl-2 were determined by immunohistochemistry. The presence of apoptosis and the expression of caspase-3 were determined using flow cytometry.

RESULTS: Treatment with rAd-p53 alone, OXA alone or combination treatment induced apoptosis of gastric cancer cells, which was accompanied by increased expression of caspase-3.

CONCLUSION: rAd-p53 enhances the sensitivity of gastric cancer cells to chemotherapy by promoting apoptosis. Thus, our results suggest that p53 gene therapy combined with chemotherapy represents a novel avenue for gastric cancer treatment.

Introduction

Gastric cancer is the most common malignant tumor of the digestive system. Currently, the major therapeutic methods for the treatment of gastric cancer are surgery, radiotherapy and chemotherapy. Despite recent improvements in these treatments, the 5-year survival rate for gastric cancer patients is only 45%. Thus, the development of new therapeutic approaches for gastric cancer, such as gene therapy, is urgently needed.

p53 is known as the “genome guard” and plays important roles in various cellular processes, including cell cycle regulation, DNA damage repair and apoptosis.
tosis. Genetic mutations in p53 are present in > 50% of human tumor tissues, and it is the most commonly detected genetic mutation in cancer[1]. Therefore, a gene therapy strategy has been developed that employs rAd-p53, a weakened adenovirus carrying the wild-type p53 gene. rAd-p53 has been shown to inhibit tumor growth, promote apoptosis by inducing the expression of Puma, Bax, Bak and Fas, and to sensitize tumor cells to radiotherapy and chemotherapy[2]. Clinical application of rAd-p53 has been used to treat lung cancer, breast cancer, oophoroma, liver cancer, and bladder carcinoma. However, few studies have investigated the therapeutic effects of rAd-p53 in gastric cancer.

Genetic mutation of p53 is found in > 60% of gastric cancer cases and has been shown to correlate not only with the onset and prognosis of gastric cancer, but also with the chemosensitivity of gastric cancer[3]. Thus, we speculated that rAd-p53 could be a potential treatment for gastric cancer. In this study, we investigated the effects of rAd-p53 treatment alone or in combination with oxaliplatin (OXA) on the growth and chemosensitivity of gastric cancer cells. Our results demonstrate that rAd-p53 has antitumor properties in gastric cancer.

MATERIALS AND METHODS

Reagents
rAd-p53 was purchased from Shenzhen Saibainuo Gene Technology Co. Ltd. (Shenzhen, China); OXA was purchased from Jiangsu Hengrui Medicine Co. Ltd. (Lianyungang, China). rAd-p53 was diluted to 5 × 10⁶ virus particles vp/mL or 5 × 10⁹ vp/mL in saline, and OXA was diluted to 2.5 mg/mL in 5% glucose and stored at -80 °C.

Cell culture
The human gastric cancer lines SGC-7901 (moderately differentiated), BGC-823 (poorly differentiated), and HGC-27 (undifferentiated) were purchased from the Chinese Academy of Sciences (Beijing, China). The cells were cultured in XX media containing 10% fetal bovine serum, 10⁵ U/L penicillin, and 100 ng/L streptomycin at 37 °C in 5% CO₂.

MTT assay
Cells were seeded in 96-well plates at 10⁴ cells/well and treated with rAd-p53 or OXA for 24, 48 or 72 h at 37 °C. Next, 150 μL MTT was added to each well and incubated for 4 h at 37 °C, followed by addition of 200 μL dimethyl sulfoxide to each well, and 10 min incubation to dissolve the formazan crystals. The absorbance was measured using an ELISA reader (EXL800; Bio-Tek, United States) at 450 nm. The data are presented as mean ± SD of tripli-
cating an ELISA reader (EXL800; Bio-Tek, United States) at

Immunohistochemistry
Cells were seeded in six-well plates at 10⁵ cells/well and then treated with rAd-p53 or OXA for 24 h. The cells were fixed with acetone for 20 min and then stained using an SP immunohistochemistry kit (Zhongshanqiao, Beijing, China) according to the manufacturer's protocol. In the gastric cancer cells examined, p53 expression was nuclear, whereas Bcl-2 and Bax expression were located in the cytoplasm.

Flow cytometry analysis
Cells were seeded in six-well plates at 5 × 10⁵ cells/well and then treated with rAd-p53 or OXA for 24 h. Apoptotic cells were detected with an apoptosis detection kit (Invitrogen, Eugene, OR, United States).

RESULTS

Treatment with rAd-p53 or OXA inhibits the growth of gastric cancer cells in a time- and dose-dependent manner

The MTT assay results showed that rAd-p53 could inhibit the growth of the gastric cancer cell lines SGC-7901 (moderately differentiated), BGC-823 (poorly differentiated) and HGC-27 (undifferentiated) in a time- and dose-dependent manner (Figure 1A-C). A similar result was observed for OXA treatment (Figure 1D-F). Among the three cell lines, we found that the inhibitory effects of rAd-p53 and OXA were both strongest in SGC-7901 and weakest in HGC-27 when treatment dose and time were kept constant, suggesting that more differentiated gastric cancer cells are more sensitive to rAd-p53 and OXA treatments.

Combined treatment with rAd-p53 and OXA shows a synergistic effect on the inhibition of gastric cancer cell growth

We next used treated the three gastric cancer cell lines
with a combination of rAd-53 and OXA and found that the inhibition of cell growth was markedly stronger at a relatively low combined dose and with a short treatment time (Figure 2), compared to treatment with rAd-p53 or OXA alone (Figure 1). A q value > 1.15 indicated that rAd-p53 and OXA had synergistic effects on the inhibition of gastric cancer cell growth.

Expression of p53 in gastric cancer cells treated with rAd-p53 or OXA alone or with rAd-p53 in combination with OXA
As expected, when the gastric cancer cell lines were treated with rAd-p53 for 48 h, immunohistochemical staining showed that p53 expression increased gradually with respect to dose (Figure 3, Table 1). Moreover, when the same treatment doses were used, p53 expression was stronger in more differentiated gastric cancer cells. However, the combined use of OXA at 3.2 μg/mL and rAd-p53 had no obvious, additional effects on p53 expression, indicating that the antitumor effects of OXA were not related to the upregulation of p53 expression in tumor cells.

Expression of Bax and Bcl-2 in gastric cancer cells treated with rAd-p53 or OXA alone, or rAd-p53 in combination with OXA
Immunohistochemical staining also showed that the expression of the pro-apoptotic protein Bax increased...
gradually in gastric cancer cells treated with increasing doses of rAd-p53 for 48 h (Figure 4, Table 2), whereas the expression of the anti-apoptotic protein Bcl-2 decreased gradually (Figure 5, Table 3). Combination treatment with OXA at 3.2 μg/mL and rAd-p53 had modest effects on the levels of Bax and Bcl-2 expression, indicating that the antitumor effects of rAd-p53 and OXA were mediated by a mechanism that promoted gastric cancer cell apoptosis.

**Apoptotic ratio and expression of caspase-3 in gastric cancer cells treated with rAd-p53 or OXA alone or with rAd-p53 in combination with OXA**

To confirm that the antitumor effects of rAd-p53 and OXA were associated with induction of apoptosis in gastric cancer cells, we examined the expression of caspase-3 and the apoptotic rate in the three different gastric cancer cell lines by flow cytometric analysis. We found that caspase-3 expression was higher in treated gastric cancer cells compared to untreated cells (P < 0.05). Moreover, the combined treatment with rAd-p53 and OXA presented synergistic effects in the upregulation of caspase-3 expression and induction of apoptosis (P < 0.05) (Tables 4 and 5).

**DISCUSSION**

As the most important tumor suppressor gene, p53 plays an important role in the induction of apoptosis. However, the mutation rate of p53 gene is approximately 50% in human cancers[4], leading to the loss of p53 function, including its induction of apoptosis. Available data sug-
Figure 3  Detection of p53 expression in gastric cancer cells with immunohistochemistry. A: Untreated SGC-7901 cells; B: SGC-7901 cells treated with 5 × 10⁶ vp/mL rAd-p53 plus 3.2 μg/mL oxaliplatin (OXA); C: Untreated BGC-823 cells; D: BGC-823 cells treated with 5 × 10⁶ vp/mL rAd-p53 plus 3.2 μg/mL OXA; E: Untreated HGC-27 cells untreated; F: HGC-27 cells treated with 5 × 10⁷ vp/mL rAd-p53 plus 3.2 μg/mL OXA.

Table 1  p53 expression in gastric cancer cells 48 h after treatment with rAd-p53, oxaliplatin or rAd-p53 plus oxaliplatin

| Treatment        | Gastric cancer cell line |
|------------------|--------------------------|
|                  | SGC-7901          | BGC-823          | HGC-27           |
| OXA              | 11.83 ± 1.02⁸       | 8.67 ± 1.35⁹     | 6.36 ± 1.62⁹     |
| rAd-p53          | 36.65 ± 1.04⁸       | 25.13 ± 2.73⁹    | 21.26 ± 1.07⁹    |
| 5 × 10⁶ OXA      | 40.32 ± 1.03⁹       | 32.45 ± 2.39⁹    | 25.35 ± 1.28⁹    |
| 5 × 10⁷ OXA      | 48.86 ± 1.26⁹       | 38.25 ± 2.16⁹    | 29.67 ± 1.31⁹    |
| 5 × 10⁸ OXA      | 60.38 ± 1.14⁹       | 49.37 ± 1.07⁹    | 33.25 ± 2.03⁹    |
| rAd-p53 + OXA    | 37.23 ± 1.07⁸       | 26.54 ± 1.53⁹    | 22.17 ± 1.13⁹    |
| 5 × 10⁶ OXA      | 39.83 ± 1.32⁹       | 34.17 ± 1.26⁹    | 24.83 ± 1.07⁹    |
| 5 × 10⁷ OXA      | 49.03 ± 1.26⁹       | 40.28 ± 1.43⁹    | 30.45 ± 1.32⁹    |
| 5 × 10⁸ OXA      | 61.54 ± 1.18⁹       | 50.37 ± 1.27⁹    | 35.21 ± 2.11⁹    |
| Control          | 12.55 ± 1.15        | 8.23 ± 1.13      | 6.15 ± 1.36      |

Table 2  Bax expression in gastric cancer cells 48 h after treatment with rAd-p53, oxaliplatin or rAd-p53 plus oxaliplatin

| Treatment        | Gastric cancer cell line |
|------------------|--------------------------|
|                  | SGC-7901          | BGC-823          | HGC-27           |
| OXA              | 73.52 ± 0.83⁸       | 56.43 ± 0.74⁹    | 36.47 ± 1.21⁹    |
| rAd-p53          | 63.25 ± 1.32⁹       | 53.86 ± 1.54⁹    | 33.71 ± 1.41⁹    |
| 5 × 10⁶ OXA      | 76.14 ± 0.73⁹       | 59.32 ± 1.45⁹    | 39.47 ± 1.03⁹    |
| 5 × 10⁷ OXA      | 79.62 ± 1.46⁹       | 64.74 ± 1.08⁹    | 41.35 ± 1.15⁹    |
| 5 × 10⁸ OXA      | 82.54 ± 1.28⁹       | 69.53 ± 1.02⁹    | 43.75 ± 1.11⁹    |
| rAd-p53 + OXA    | 78.22 ± 0.88⁹       | 56.64 ± 1.07⁹    | 49.15 ± 1.04⁹    |
| 5 × 10⁶ OXA      | 84.32 ± 1.02⁹       | 62.74 ± 1.19⁹    | 52.9 ± 1.31⁹     |
| 5 × 10⁷ OXA      | 87.41 ± 1.03⁹       | 67.38 ± 1.14⁹    | 55.25 ± 1.06⁹    |
| 5 × 10⁸ OXA      | 89.71 ± 0.36⁹       | 75.14 ± 1.65⁹    | 58.67 ± 1.12⁹    |
| Control          | 26.32 ± 1.04        | 19.91 ± 0.87     | 16.74 ± 1.23     |

*P < 0.05 vs control; **P > 0.05 vs control; †P > 0.05, rAd-p53 vs rAd-p53 + oxaliplatin (OXA) with the same dose of rAd-p53; ‡P < 0.05, OXA vs rAd-p53 + OXA with the same dose of OXA.
Figure 4 Detection of bax expression in gastric cancer cells with immunohistochemistry. A: Untreated SGC-7901 cells; B: SGC-7901 cells treated with $5 \times 10^9$ vp/mL rAd-p53 plus 3.2 $\mu$g/mL oxaliplatin (OXA); C: Untreated BGC-823 cells; D: BGC-823 cells treated with $5 \times 10^9$ vp/mL rAd-p53 plus 3.2 $\mu$g/mL OXA; E: Untreated HGC-27 cells; F: HGC-27 cells treated with $5 \times 10^9$ vp/mL rAd-p53 plus 3.2 $\mu$g/mL OXA.

Table 3 Bcl-2 expression in gastric cancer cells 48 h after treatment with rAd-p53, oxaliplatin or rAd-p53 plus oxaliplatin

| Treatment          | Gastric cancer cell line | SGC-7901 | BGC-823 | HGC-27 |
|--------------------|--------------------------|----------|---------|--------|
| OXA                |                          | 26.32 ± 1.21 ‡‡‡‡ | 47.53 ± 1.13 ‡‡‡‡ | 56.64 ± 1.33 ‡‡‡‡ |
| rAd-p53            |                          | 28.62 ± 1.07 ‡‡‡‡ | 58.23 ± 1.04 ‡‡‡‡ | 61.23 ± 1.07 ‡‡‡‡ |
| rAd-p53 + OXA      |                          | 24.34 ± 1.05 ‡‡‡‡ | 46.26 ± 1.31 ‡‡‡‡ | 49.54 ± 1.14 ‡‡‡‡ |
| rAd-p53 + OXA      |                          | 18.62 ± 1.32 ‡‡‡‡ | 40.81 ± 1.15 ‡‡‡‡ | 47.34 ± 1.06 ‡‡‡‡ |
| rAd-p53 + OXA      |                          | 15.37 ± 1.51 ‡‡‡‡ | 38.37 ± 1.08 ‡‡‡‡ | 44.31 ± 1.03 ‡‡‡‡ |
| Control            |                          | 38.97 ± 1.06     | 73.71 ± 2.02     | 84.03 ± 1.02     |

Note: $P < 0.05$ vs control; $P < 0.05$, rAd-p53 vs rAd-p53 + oxaliplatin (OXA) with the same dose of rAd-p53; $P < 0.05$, OXA vs rAd-p53 + OXA with the same dose of OXA.

Table 4 Caspase-3 expression in gastric cancer cells 48 h after treatment with rAd-p53, oxaliplatin or rAd-p53 plus oxaliplatin

| Treatment          | Gastric cancer cell line | SGC-7901 | BGC-823 | HGC-27 |
|--------------------|--------------------------|----------|---------|--------|
| OXA                |                          | 12.32 ± 0.8 ‡†¨†† †††‡ | 11.21 ± 1.05 ‡†¨†† †††‡ | 8.86 ± 1.01 ‡†¨†† †††‡ |
| rAd-p53            |                          | 7.89 ± 1.13 ‡††‡ †††† | 6.07 ± 0.97 ‡††‡ †††† | 4.32 ± 1.03 ‡††‡ †††† |
| rAd-p53 + OXA      |                          | 10.03 ± 1.03 ‡††‡ †††† | 8.38 ± 1.04 ‡††‡ †††† | 6.03 ± 0.99 ‡††‡ †††† |
| rAd-p53 + OXA      |                          | 12.34 ± 1.05 ‡††‡ †††† | 10.52 ± 0.89 ‡††‡ †††† | 8.31 ± 1.02 ‡††‡ †††† |
| Control            |                          | 13.04 ± 1.03 ‡‡‡‡ †††† | 11.34 ± 0.85 ‡‡‡‡ †††† | 10.12 ± 1.05 ‡‡‡‡ †††† |

Note: $P < 0.05$ vs control; $P < 0.05$, rAd-p53 vs rAd-p53 + oxaliplatin (OXA) with the same dose of rAd-p53; $P < 0.05$, OXA vs rAd-p53 + OXA with the same dose of OXA.
gest that p53 mutations are linked to the development of multiple malignant tumors, such as liver cancer, breast cancer, bladder carcinoma, gastric cancer, colon carcinoma, prostatic carcinoma, ovarian cancer, brain cancer, esophageal cancer, lung cancer, lymphocyte tumor, soft tissue sarcoma, and osteogenic sarcoma [5-20].

rAd-p53, which is an adenovirus carrier containing the p53 tumor suppressor gene, is the first gene therapy drug. In this therapy, the adenovirus is used to deliver the p53 gene to target cells; restoration of p53 expression in the targeted cells results in antitumor effects. The mechanisms of p53 action include: (1) inhibition of cell cycle progression and induction of apoptosis in tumor cells through the modulation of the expression of apoptosis- and cell-cycle-related genes; (2) sensitization of tumor cells to radiotherapy and chemotherapy; and (3) stimulation of antitumor immunity through the bystander effect. Clinical application studies have demonstrated that rAd-p53 not only strengthens tumor cell sensitivity to radiotherapy and chemotherapy, but also reduces side effects of chemotherapy. For these reasons, a combination of p53 gene therapy and chemotherapy has been successfully applied to cure a variety of cancers, including lung adenocarcinoma, liver cancer and oophoroma [21,22].

In the present study, we treated three different gastric cancer cell lines with a combination of rAd-p53 and OXA and found that these agents had significant inhibitory effects on cancer cell growth that were dependent on treatment time and dose. In addition, we observed that more differentiated cells were more sensitive to rAd-p53 and OXA treatment. To investigate whether

### Table 5  Apoptotic rate in gastric cancer cells 48 h after treatment with rAd-p53, oxaliplatin or rAd-p53 plus oxaliplatin

| Treatment                  | rAd-p53 (vp/mL) | OXA (μg/mL) | SGC-7901 | BGC-823 | HGC-27 |
|----------------------------|-----------------|-------------|----------|---------|--------|
| OXA                        | 0               | 3.2         | 33.52 ± 1.6** | 23.28 ± 1.35** | 18.72 ± 1.61** |
| rAd-p53                    | 5 × 10⁶         | 0           | 7.89 ± 1.33** | 6.51 ± 0.97**  | 4.07 ± 0.83**  |
|                            | 5 × 10⁷         | 0           | 12.47 ± 1.43** | 8.78 ± 1.34**  | 6.43 ± 0.79**  |
|                            | 5 × 10⁸         | 0           | 21.84 ± 1.05** | 14.24 ± 0.89** | 11.72 ± 1.12** |
|                            | 5 × 10⁹         | 0           | 36.73 ± 1.03** | 28.64 ± 1.75** | 21.82 ± 1.81** |
| rAd-p53 + OXA              | 5 × 10⁶         | 3.2         | 42.38 ± 1.51** | 35.72 ± 1.13** | 28.84 ± 1.21** |
|                            | 5 × 10⁷         | 3.2         | 54.84 ± 1.26** | 48.63 ± 1.62** | 34.51 ± 1.47** |
|                            | 5 × 10⁸         | 3.2         | 58.41 ± 1.13** | 51.71 ± 1.41** | 38.5 ± 1.16**  |
|                            | 5 × 10⁹         | 3.2         | 63.91 ± 1.23** | 55.73 ± 1.35** | 42.92 ± 1.33** |
| Control                    | 0               | 0           | 4.67 ± 1.32  | 1.74 ± 0.67  | 1.15 ± 0.58  |

*P* < 0.05 vs control; †*P* < 0.05, rAd-p53 vs rAd-p53 + oxaliplatin (OXA) with the same dose of rAd-p53; ‡*P* < 0.05, OXA vs rAd-p53 + OXA at the same dose of OXA.

**Figure 5** Detection of Bcl-2 expression in gastric cancer cells with immunohistochemistry. A: Untreated SGC-7901 cells; B: SGC-7901 cells treated with 5 × 10⁶ vp/mL rAd-p53 plus 3.2 μg/mL oxaliplatin (OXA); C: Untreated BGC-823 cells; D: BGC-823 cells treated with 5 × 10⁷ vp/mL rAd-p53 plus 3.2 μg/mL OXA; E: Untreated HGC-27 cells; F: HGC-27 cells treated with 5 × 10⁸ vp/mL rAd-p53 plus 3.2 μg/mL OXA.
the antitumor effects of rAd-p53 and OXA are related to the induction of apoptosis in gastric cancer cells, we examined the expression of apoptosis-related proteins. Bcl-2 is the most important anti-apoptotic protein\cite{23,24}, whereas Bax is a pro-apoptotic protein\cite{25}. Furthermore, it is well known that caspase-3 is critical in chemotherapyclinduced apoptosis of cancer cells\cite{26-30}. Therefore, we examined the expression of Bel-2, Bax and caspase-3 in gastric cancer cells treated with rAd-p53. As expected, our results demonstrated that the expression Bax and caspase-3 was increased, whereas the expression of Bcl-2 was decreased in a dose-dependent manner. Consistent with these data, we found that the apoptosis of gastric cancer cells was increased.

In conclusion, in the present study, we demonstrated that rAd-p53 inhibited gastric cancer cell growth and sensitized these cells to the chemotherapeutic agent OXA. The underlying mechanisms of these effects involved the induction of apoptosis, which was achieved via downregulation of Bel-2 and upregulation of Bax and caspase-3. Our results suggest that the combination of p53 gene therapy and chemotherapy represents a novel avenue for gastric cancer treatment.

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