ORIGINAL ARTICLE

Sensitivity of ASPP and P-gp to neoadjuvant chemotherapy combined with gene therapy in locally advanced cervical cancer

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Summary

Purpose: To evaluate the sensitivity of apoptosis-stimulating protein of p53 (ASPP) and P-glycoprotein (P-gp) to the recombinant human adenovirus-p53 (rAd-p53) combined with neoadjuvant chemotherapy for locally advanced cervical cancer and their predictive value for efficacy.

Methods: 80 patients with histopathologically diagnosed locally advanced cervical cancer (stage Ib2-IIa2) and operative treatment from February 2009 to June 2013 were enrolled and randomly divided into 3 groups: radical hysterectomy group (RH group, n=30), simple intravenous chemotherapy with cisplatin paclitaxel group (TP group, n=30) and gene therapy + intravenous chemotherapy group with cisplatin+paclitaxel (rAd-p53 + TP group, n=20). Patients in the RH group were directly operated, those in the TP group were treated with TP chemotherapy for 2 courses, and those in the rAd-p53 + TP group were treated with the same chemotherapy regimen and the rAd-p53 agent (1×10¹² virus particles) was injected for the first time 3 days after chemotherapy for 3 times. The changes in tumor volume, adverse reactions and survival of patients in the TP group and rAd-p53 + TP group, and the levels of p53, ASPP2, inhibitory member of the ASPP family (iASPP) and P-gp in postoperative tumor tissues in the 3 groups were evaluated.

Results: The efficacy was evaluated for all patients in the TP group and rAd-p53 + TP group 3 weeks after chemotherapy.

The tumor was reduced by 10.90 ± 2.62 cm² after medication in the TP group and 15.25 ± 4.01 cm² after medication in rAd-p53 + TP group, and the difference was statistically significant. The response rate [complete response (CR) + partial response (PR)] was 76.7% in the TP group and 95% in the rAd-p53 + TP group, with statistically significant difference. The positive expression rate of p53 protein showed a gradual decreasing trend in the RH group, TP group and rAd-p53 + TP group, with statistically significant difference (p<0.05). The positive expression rate of ASPP2 displayed a gradual increasing trend in the RH group, TP group and rAd-p53 + TP group, without statistically significant difference (p>0.05). The positive expression rate of iASPP was different and gradually declined in the RH group, TP group and rAd-p53 + TP group (p<0.05). Moreover, the positive expression rate of P-gp was also different among the 3 groups, and it was increased in the TP group, while it was decreased in the RH and rAd-p53 + TP group (p<0.05).

Conclusion: The intratumor injection of rAd-p53 into patients with cervical cancer is safe and effective, showing a *new way in the gene therapy of cervical cancer. Both ASPP* and P-gp may be potential biomarkers in the treatment of cervical cancer.

Key words: cervical cancer, recombinant human p53 adenovirus injection, TP chemotherapy, ASPP2, iASPP, P-gp

Introduction

malignant tumors seriously threatening the health es every year around the world, and the new cases of females, which also shows a trend for younger in China account for one-third of the total. The

Cervical cancer is still one of the most common ages [1]. There are approximately 500,000 new cas-

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overall prognosis of cervical cancer patients with the clinical stage ≤IIa is good. However, in stage Ib2 and IIa2 disease (locally advanced cancer), the local tumor control is difficult and prone to recurrence and metastasis with a poor prognosis. Reports show that the 5-year overall survival rate of patients with stage Ib2 and IIa2 disease is reduced to 50-60% [2], so creating the need to explore new therapeutic methods. Neoadjuvant chemotherapy is able to reduce the lesion size and the intraoperative complications in locally advanced cervical cancer. Recombinant adenovirus-p53 (rAd-p53), as a gene agent, is safe and effective in the local treatment of head and neck squamous cell carcinoma [3]. Previous studies have demonstrated that the combined action of rAd-p53 and chemotherapeutic drugs can effectively reduce the tumor volume and lower the tumor stage [4]. In this paper, different methods were used to treat cervical cancer and different efficacy indexes were tested so as to possibly provide a new comprehensive regimen for the clinical treatment of locally advanced cervical cancer.

Methods

Experimental materials

The rAd-p53 injection (1×10¹² virus particles/ampule) was provided by Shenzhen SiBiono Gene Technology Co., Ltd., China, approved by the China Food and Drug Administration (CFDA), stored at -20°C prior to the experiment and thawed at room temperature before application. The rAd-p53 was injected in multiple points in the tumor. This material was diluted to 2 ml with normal saline before application for tumor with a diameter of less than 4 cm, while it was diluted to 4 ml with normal saline before application for tumor with a diameter of more than 4 cm.

Case selection and random grouping

Objects of observation

80 patients with stage Ib2 and IIa2 cervical squamous cell carcinoma treated in our hospital from February 2009 to June 2013 were selected. According to the clinical staging criteria of the International Federation of Gynecology and Obstetrics (FIGO), there were 41 cases in stage Ib2 and 39 cases in stage IIa2.

Inclusion criteria: (1) patients pathologically diagnosed with cervical squamous cell carcinoma in stage Ib2 and IIa2 for the first time; (2) patients whose gynecological biochemical and bone marrow examination results were normal; (3) patients with ECOG performance status score of 0-1; and (4) patients without lymph node metastasis and bilateral ureteral damage shown on imaging studies.

Exclusion criteria: (1) patients with a history of other malignant tumors or a history of radiotherapy or chemotherapy, or (2) pregnant patients or patients with Ltd., Wuhan, China and p53 and P-gp reagents were

mental disorders. The research scheme was approved by the Hospital's Ethics Committee, and patients signed informed consent.

Grouping

Patients were randomly divided into 3 groups: control group (n=30), TP group (n=30) and rAd-p53 + TP group (n=20).

In the RH group group, patients were 29-45 years old with an average age of 40, including 14 cases in stage Ib2 and 16 cases in stage IIa2.

In the TP group, patients were 28-45 years old with an average age of 40, including 16 cases in stage Ib2 and 14 cases in stage IIa2.

In rAd-p53 + TP group, patients were 32-45 years old with an average age of 42, including 7 cases in stage Ib2 and 13 cases in stage IIa2.

Therapeutic regimen and efficacy evaluation

1) In the RH, the operation was directly performed (Extensive uterine resection, pelvic lymph node excision, with or without paraaortic lymph node excision).

2) In the TP group, cisplatin + paclitaxel were adopted as the chemotherapy regimen, and the treatment lasted 3 days for a total of 2 courses with an interval of 3 weeks. Paclitaxel was infused intravenously for 4-5 h at 175 mg/m², and cisplatin 50 mg/m² was also infused intravenously in 3 days.

3) In the rAd-p53 + TP group, patients underwent intratumor injection of rAd-p53 (1×10¹² virus particles) under direct vision for the first time, 3 days after chemotherapy (once every 3 days for a total of 3 times). The rAd-p53 was injected through multiple points in the tumor. It was diluted to 2 ml of normal saline before application for tumors with a diameter of less than 4 cm, while it was diluted to 4 ml with normal saline before application for tumors with a diameter of more than 4 cm. The chemotherapy regimen in rAd-p53 + TP group was the same as that in TP group and the treatment lasted for 1 week.

Evaluation criteria for short-term efficacy

The efficacy in TP group and rAd-p53 + TP group was evaluated 3 weeks after drug withdrawal. Before and after treatment, patients in each group underwent gynecological and imaging examination (three-dimensional ultrasound or magnetic resonance imaging/MRI, the same imaging method was used for the same patient) to detect the dynamic changes in the tumor size. The efficacy was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST): complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). The short-term efficacy was analyzed (CR+PR: effective, SD+PD: ineffective).

Detection of p53, ASPP2, iASPP and P-gp in tumor tissues

The changes in p53, ASPP2, iASPP and P-gp in tumor tissues before and after treatment were immunohistochemically detected. ASPP2 and iASPP reagents were purchased from Boster Biological Technology Co., purchased from ZSGB Biotechnology Co. Ltd, Suzhou, China.

The score was given according to the number of positive cells and the intensity of staining: the number of positive cells <5% (0 point), 5-25% (1 point), 26-50% (2 points), 51-75% (3 points) and >76% (4 points). The intensity of staining: no staining (0 point), light yellow (1 point), brown yellow (2 points) and dark brown (3 points). The two scores were added up as the final result was: 0-1 point (-), 2-3 points (+), 4-5 points (++), and 6-7 points (+++).

Clinical routine examination and observation of adverse reactions

Blood routine, urine routine, stool routine and blood biochemical examinations, electrocardiography and chest radiography were performed regularly. Gastrointestinal reactions, liver function damage, bone marrow suppression and changes in body temperature were observed. Self-limiting fever generally occurred a few hours after the injection of rAd-p53, and antipyretics (indometacin suppositories) could be taken as appropriate.

Follow-up

The deadline of follow-up was March 2018, and patients were reviewed once every 3 months in the first year, once every 6 months in the second and third years, and once every year after the third year. The followup included gynecological examination, vagino-rectoabdominal examination, vaginal cytological examination, human papillomavirus detection, serum tumor marker SCC for squamous cell carcinoma detection, B ultrasound and X-ray examination. MRI was performed if necessary, and positron emission tomography (PET) was performed in case of suspected recurrence. Patients were followed up for 5-60 months on average (median 46).

Result analysis and statistical processing

SPSS 16.0 software package was used for the statistical processing of experimental data. T-test was used for the comparison of tumor size before and after treatment, and data were expressed as mean ± standard deviation (x±s).

Rank sum test was adopted for the comparison of efficacy, and one way analysis of variance (ANOVA) was used for adverse reactions and immunohistochemical results. A p value <0.05 was considered statistically significant.

Results

Comparison of efficacy between rAd-p53 + TP group and TP group

The efficacy was evaluated for all patients in TP group and rAd-p53 + TP group at 3 weeks after chemotherapy, and the randomized comparative analysis revealed that the combined application of rAd-p53 and chemotherapeutic drugs could reduce the tumor volume more effectively. The tumor was reduced to 10.90±2.62 cm² after medication in the TP group and 15.25±4.01 cm² after medication in the rAd-p53 + TP group (t=-4.652, p=0.000) (Table 1). In terms of short-term efficacy, the response rate (CR + PR) was 76.7% in the TP group and 95% in the rAd-p53 + TP group. It can be seen that the decline in the tumor volume in rAd-p53 + TP group was 1.39 times of that in the TP group, and the response rate in the rAd-p53 + TP group was 1.19 times of that in the TP group. The results of rank sum test of ranked data are shown in Table 2 (Z=2.137, p=0.033), and indicate that the shortterm efficacy had a significant difference between the two groups, and the efficacy in the rAd-p53 + TP group was superior to that in the TP group.

Detection of p53, ASPP2, iASPP and P-gp in tumor tissues

The positive staining of p53 was located in the nucleus of cervical cancer cells. The positive immunohistochemical staining of ASPP2 and IASPP was mainly expressed in the cytoplasm and/or nucleus. The positive staining of P-gp was mainly located in the cytoplasm and cell membrane. The

Table 1. Comparison of average reduction rate of tumor between the TP group and the rAd-p53+TP group before and after medication

Group	п	Difference in tumor size before and after medication
TP group	30	10.90±2.62
rAd-p53 + TP group	20	15.25±4.01
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(t=-4.652, p=0.000)

Table 2. Comparison of short-term encacy between the two grou	i snort-term emcacy between the two gro	nort-term emcacy	arison of s	2. Comp	ble 2	Ta
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Group	п	CR	PR	SD	PD	Response rate (%)
TP group	30	0	23	6	1	76.7
rAd-p53 + TP group	20	2	17	1	0	95
(7 2177 002()						

(Z=-2.137, p=0.026)

positive expression rate of p53 protein showed a gradual decreasing trend in the RH group [83.3% (25/30)], the TP group [60.0% (18/30)] and the rAdp53 + TP group [25.0% (5/20)], with statistically significant differences (p<0.05) (Figures 1-3).

played a gradual increasing trend in the RH group group [70.0% (21/30)] and the rAd-p53 + TP group

[60.0% (18/30)], the TP group [63.0% (19/30)] and the rAd-p53 + TP group [65.0% (13/20)], showing no statistically significant differences (p>0.05) (Figures 4-6).

The positive expression rate of iASPP gradual-The positive expression rate of ASPP2 dis- ly declined in the RH group [90.0% (27/30)], the TP



Figure 1. Positive expression of p53 in the RH group (×400).



Figure 3. Negative expression of p53 in the rAd-p53 + TP group (×400).



Figure 2. Weakly positive expression of p53 in the TP group (×400).



Figure 4. Weakly positive expression of ASPP2 in the RH group (×400).



Figure 5. Weakly positive expression of ASPP2 in the TP group (×400).



Figure 6. Weakly positive expression of ASPP2 in the rAdp53+TP group (×400).

[60.0% (12/20)], and the differences were statisti- Adverse reactions cally significant (p<0.05) (Figures 7-9).

The positive expression rate of P-gp was 40.0% (12/30) in the RH group, 90.0% (27/30) in the TP group and 60.0% (12/20) in the rAd-p53 + TP group, displaying statistically significant differences (p<0.05) (Figures 10-12, Table 3).



Figure 7. Strongly positive expression of iASPP in the RH group (×400).



Figure 9. Weakly positive expression of iASPP in the rAdp53+TP group (×400).



Figure 11. Positive expression of P-gp in the TP group (×400).

The main adverse reactions in both groups were gastrointestinal, bone marrow suppression, liver function damage and fever. In the TP group, there were 15 cases of gastrointestinal discomfort manifested as nausea and vomiting, 3 cases of



Figure 8. Positive expression of iASPP in the TP group (×400).



Figure 10. Weakly positive expression of P-gp in the RH group (×400).



Figure 12. Weakly positive expression of P-gp in the rAdp53+TP group (×400).

Group	Total number of cases	Positive expression of p53	Positive expression of ASPP2	Positive expression of iASPP	Positive expression of P-gp
		n/positive rate	n/positive rate	n/positive rate	n/positive rate
RH group	30	18/83.3%	18/60.0%	27/90.0%	12/40%
TP group	30	18/60.0%	19/63.0%	21/70.0%	27/90.0%
rAd-p53 + TP group	20	13/25.0%	13/65.0%	12/60.0%	12/60.0%

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 Table 4. Comparisons of toxic and side effects between TP group and rAd-p53 + TP group

Group	Bone marrow suppression	Liver function damage	Gastrointestinal reactions	Fever
TP group	4	3	15	10
rAd-p53+TP group	1	2	13	15
(p>0.05)				

grade II bone marrow suppression and 1 case of grade III bone marrow suppression after chemotherapy. The symptoms were improved after supportive treatment with recombinant human granulocyte colony-stimulating factor without infection symptoms. The liver enzymes were increased in 3 cases and improved after i.v.infusion of glutathione and oral administration of bifendate and the fever occurred in 10 cases and improved after symptomatic treatment. In the rAd-p53 + TP group, there were 13 cases of gastrointestinal reactions which were tolerable. Bone marrow suppression occurred in 1 case, and the symptoms were improved after supportive treatment with recombinant human granulocyte colony-stimulating factor. The liver enzymes were increased in 2 cases and improved after liver protecting treatment described above. Fever occurred in 15 cases on the day of injection of rAd-p53 (38°C) and in 10 cases the temperature returned to normal 12-24 h after patients drank much water. The body temperature was 39°C in 5 cases and returned to normal 12-24 h after symptomatic treatment with indometacin, and all of them were accompanied with signs and symptoms of upper respiratory infection. Fever, gastrointestinal reactions, bone marrow suppression and liver function damage had no statistically significant differences between the two groups of patients (Table 4).

Follow-up of survival in the three groups of patients

During follow-up, there were 6 cases of recurrence and 3 cases of death in the RH group, 6 cases of recurrence and 2 cases of death in the TP group, and 5 cases of recurrence and 4 cases of death in the rAd-p53+TP group. The recurrence and death rates had no statistically significant differences. The 3-year tumor-free survival rate was 80.0% (24/30) in the RH group, 80.0% (24/30) in the TP group and 75.0% (15/20) in the rAd-p53+TP group, while the 3-year overall survival rate was 90.0% (27/30) in the RH group, 93.3% (28/30) in the TP group and 80.0% (16/20) in the rAd-p53+TP group, respectively. No statistically significant differences were noted in the 3-year tumor-free survival and overall survival rate among the three groups.

Discussion

According to the clinical staging criteria of FIGO (2009) for cervical cancer, stage Ib2 signifies that the cancer is confined in the cervix, and the largest lesion is >4 cm. Stage IIa2 signifies that the cancer breaks through the cervix and involves the vagina (the lower 1/3 part of vagina is not involved) without parauterine involvement, and the cancer largest diameter is >4 cm. Both of them are customarily referred to as "locally advanced cervical cancer" in a narrow sense [5]. Due to larger local lesions, such patients have unique clinicopathological features and poor prognosis, and the therapeutic regimens remain controversial.

Neoadjuvant chemotherapy refers to the systemic intravenous chemotherapy or arterial chemotherapy for patients with cervical cancer before operation or radiotherapy, an especially therapeutic strategy proposed for young and middle-aged patients with locally advanced cervical cancer [6,7]. According to previous studies, the total response rate of neoadjuvant chemotherapy was 60-80%. In this study, the total response rate after chemotherapy in the TP group was 76.7%, suggesting that the regimen had a better efficacy in the preoperative neoadjuvant chemotherapy, and the adverse reactions were milder, without increasing the incidence of intraoperative and postoperative complications.

The rAd-p53 agent is a kind of replicationdefective live virus carrying the p53 gene, which is composed of the vector and human p53 gene in structure. The p53 gene can be transferred into cancer cells and highly expressed in the nucleus, thereby arresting the cell cycle, activating or inhibiting other tumor-related genes, inhibiting the tumor cell growth and promoting apoptosis [8]. Studies have demonstrated that the p53 gene therapy combined with chemotherapy has a good therapeutic effect on head and neck squamous cell carcinoma, lung cancer and gastric cancer [9-11]. In previous studies, the gene therapy combined with i.v. cisplatin+vincristine+bleomycin (PVB) chemotherapy was applied in the treatment of locally advanced cervical cancer, and it was found that the therapeutic effect of this approach is superior to chemotherapy alone. In this study, the gene therapy combined with TP chemotherapy was used in the treatment of locally advanced cervical cancer.

According to statistical analysis there were no differences in the age and the clinical stage of tumor between the two groups of patients (p>0.05), indicating that the subjects in both groups had good homogeneity. Before treatment the tumor size in the rAD-p53 group+TP group was larger than in the TP group and t-test showed that the difference was not significant. It was also found that the tumor volume could be reduced in both the TP group and the rAd-p53+TP group, the decline in the tumor volume in the rAd-p53+TP group was 1.39 times of that in the TP group, and the response rate in rAd-p53+TP group was 1.19 times of that in the TP group, indicating that rAd-p53 has a significant synergistic effect with chemotherapy in cervical cancer.

P53 gene is an important tumor suppressor, which negatively regulates the cell growth. The expression product of the wild-type p53 gene is difficult to detect, and the configuration of protein product will be changed and become more stable after p53 mutation. It is believed that the positive p53 protein in immunohistochemical detection indicates the presence of mutant-type p53 gene. The p53 gene mutation is consistent with the expression of p53 protein in tissues [12]. In this study, the expression of mutant-type p53 protein in the TP group and the rAd-p53+TP group was decreased compared with that in the RH group, and declined more significantly in the rAd-p53+TP group than in the RH and the TP group, suggesting that the rAdp53 agent can effectively reconstruct the mutant p53 gene in tumor cells and inhibit the abnormal expression of mutant-type p53 protein.

The ASPP family is a kind of new tumor suppressor genes, including three members (ASPP1, reconstruct the mutant p53 gene in tumor cells af-

ASPP2 and iASPP) [13-15]. The mechanism of action between ASPP family and p53 is as follows: The ASPP family members can bind to p53 to form the ASPP-p53 complex, and then act on the apoptotic gene promoter. A little change in ASPP can easily alter the binding ability of p53 to DNA, thus affecting the p53-induced apoptosis. iASPP may compete with ASPP1 and ASPP2 for the binding site of p53, thereby affecting the binding ability of ASPP-p53 complex to DNA and preventing the apoptotic process of p53. Studies have revealed that the decline in ASPP2 content in tumor tissues can lead to the tolerance of tumor cells to chemotherapeutic drugs, and inhibiting iASPP expression can dramatically increase the sensitivity of tumor to chemotherapy and promote apoptosis of tumor cells. In this study, the expression of ASPP2 showed an increasing trend in the control, TP and rAd-p53+TP groups, but without statistically significant differences due to the small sample size. The above results may indicate to some extent that the rAd-p53 may indirectly promote the p53-induced cell cycle arrest and apoptosis through increasing the ASPP2 content in tumor cells. The sample size should be expanded for further analyses. The expression of iASPP declined successively in the control, TP and rAd-p53+TP groups, with statistically significant differences, suggesting that chemotherapy and rAd-p53 can also inhibit the expression of iASPP to some extent, and the p53 protein will bind to ASPP1/ASPP2 after iASPP is blocked, promoting the apoptosis of DNA-damaged cells. RAd-p53 can not only effectively reconstruct the mutant p53 gene in tumor cells and inhibit the abnormal expression of mutant-type p53 protein, but also alter the binding ability of wild-type p53 to DNA through changes in ASPP2 and iASPP levels, thus affecting the p53-induced apoptotic process.

P-gp, as an energy-dependent membrane efflux pump, can increase the extracellular transport of drugs and reduce the effective drug concentration in cells. P-gp can protect normal tissues and produce resistance for tumor tissues to chemotherapy [16]. In this study, the expression of P-gp in the TP group was increased compared with the RH group, and it was decreased in the rAd-p53+TP group compared with the TP group, indicating that chemotherapy leads to resistance of tumor cells, and the expression level of P-gp is positively correlated with the degree of resistance of malignant cells. Moreover, the rAd-p53 can effectively inhibit the resistance of tumor to chemotherapy, and the relevant mechanism remains to be clarified via further research.

In conclusion, it is believed that rAd-p53 can

ter entering tumor cells, and indirectly enhance the Acknowledgments antitumor activity of wild-type p53 gene through affecting the expression levels of ASPP family and P-gp. Besides, rAd-p53 exerts a sensitizing effect on neoadjuvant chemotherapy, thus reducing the chemotherapy resistance and creating operative treatment conditions for more patients with middle-advanced cervical cancer. Of course, these conclusions need to be verified via multicenter larger-sample prospective studies.

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Conflict of interests

The authors declare no conflict of interests.

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