In vitro and Clinical Studies of Gene Therapy with Recombinant Human Adenovirus-p53 Injection for Oral Leukoplakia

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Abstract

Purpose: Oral leukoplakia is a well-recognized precancerous lesion of squamous cell carcinoma. When accompanied with abnormal p53 expression, it suffered a higher risk of canceration. The present study was carried out to test whether the recombinant human adenovirus-p53 could introduce wild-type p53 gene to oral leukoplakia cellsand induce cell cycle arrest and apoptosis.
Experimental Design: We select p53(-) oral dysplastic keratinocyte POE-9n, to observe the growth inhibition, cell cycle change, apoptosis-induced effects, and elaborate the corresponding molecular mechanism of recombinant adenovirus-p53 on POE-9n cells. Meanwhile, we evaluate the feasibility, safety, and biological activity of multipoints intraepithelial injectionsof recombinant adenovirus-p53 in 22 patientswith dysplastic oral leukoplakia.
Results: Exogenous p53 could be successfully transduced into POE-9n cells by recombinant adenovirus-p53. The optimal infecting titer in thiss tudy wasmultiplicity of infection (MOI) = 100. Recombinant adenovirus-p53 could strongly inhibit cell proliferation, induce apoptosis, and arrest cell cycle in stage G1 in POE-9n cellsby inducing p21CIP/WAF and downregulating bcl-2 expression. In the posttreatment patients, p53 protein and p21CIP/WAF protein expression were significantly enhanced, yet bcl-2 protein presented low expression. Sixteen patients showed clinical response to the treatment, and 14 patients showed obvious histopathologic improvement. Conclusion: Intraepithelial injectionsof recombinant human adenovirus-p53 were safe, feasible, and biologically active for patients with dysplastic oral leukoplakia.

Oral leukoplakia (OLK) is defined as a white patch or plaque that cannot be clinically diagnosed as any other disease (1). Histologically, OLK is the epithelial hyperkeratosis accompanied with pure proliferation or epithelial dysplasia. It is the most common premalignant lesion of mucosa and 43% OLK develop into oral

squamous cell carcinoma (2, 3), which is the sixth common malignant carcinoma in the world (4). It has been shown by previous studies (5, 6) that abnormal p53 expression was detected in 33% to 76% of oral squamous cell carcinoma patients and 20% of OLK ones, and OLK accompanied with abnormal p53 expression suffered a higher risk of canceration.Therefore, the effective treatment toward OLK becomes one of the preventive methods against oral squamous cell carcinoma.

The wild-type human tumor suppressor p53 (wt-p53) gene is a primary mediator of cell cycle arrest, DNA repair, and apoptosis and is intimately involved in tumor development. The mutation and inactivation of p53 is a critical event in the formation and progression of head and neck carcinoma (7, 8). Transduction of the wt-p53 gene into OLK cells is expected to restore the tumor suppressor functions and prevent the canceration (9).

Reintroduction of wt-p53 has been accomplished with recombinant human adenovirus-p53 (rAd-p53), a replicationincompetent human type 5 adenovirus in which the E1 region has been replaced with an expression cassette containing the human wt-p53 cDNA (10). Adenovirus delivery of the wt-p53 gene results in strong p53 protein expression in tumor cells with minimal toxicity to the hematopoietic system (11–15). Clinical trials results showed that rAd-p53 is effective against a variety of malignancies, including colon, glioma, lung, ovarian, and head and neck tumors (11–17). In the clinical trials, the routes of administration typically used for rAd-p53 were intratumoral injection (17), perfusion (18), and i.v. infusion (19).

These in vitro and clinical studies evaluate for the first time a novel strategy to treat oral leukoplakia: gene therapy with recombinant human adenovirusp53 via the multipoint intraepithelial injection. The results show that exogenous p53 can be successfully transduced and inhibit cell proliferation, induce apoptosis, and arrest cell cycle in stage G1, and the clinical method was both safe and effective. We conclude that intraepithelial injection of recombinant adenovirus-p53 is an effective new strategy for treating oral leukoplakia. Meanwhile, the present study provided a feasible method to block the progression of the pre-malignant lesions.

In this article, we selected rAd-p53 as the intervention drug and POE-9n, an OLK cell line with negative p53 expression as the intervention object. Aiming to explore the molecular mechanism of rAd-p53, we observed changes of biological behaviors of preinfection and postinfection cells and analyzed gene expressions relating with the functional pathway of p53. Moreover, to evaluate the feasibility of rAd-p53 as a clinical therapy, 22 patients with dysplastic OLK received multipoint intraepithelial injections of rAd-p53. The results of in vitro and clinical studies are reported in this article.

Materials and Methods

Cell line

POE-9n cell line was purchased from the Institute of Medical Research, Harvard University. This p53 expression deletion cell line was derived from OLK lesion with severe epithelial dysplasia.POE-9n cells were cultured in D-keratinocyte serum–free medium supplemented with 5 ng/mL epidermal growth factor, 50 μg/mL bovine pituitary extractive (Invitrogen-Life Technologies), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were amplificated by using routine cell culture techniques at 37°C in 5% CO2.

Patients

A total of 22 patients with dysplastic OLK were enrolled in the clinical trial in West China Hospital of Stomatology, Sichuan University. All the cases were confirmed by clinical and pathologic diagnosis. The average area of the lesions was 4.1 cm2 with the largest of 7 cm2 and the smallest of 2 cm^2. All the cases were classified as mild (nine cases), moderate (eight cases), or severe dysplasia (five cases). The patients were excluded of systemic diseases and did not receive any treatments within 3 mo. The informed consents were signed by all patients before the therapy. This study has been approved by our Institutional Review Board. The Declaration of Helsinki protocols were followed during the whole study.

Treatment protocols

rAd-p53 (Shenzhen Sibiono Genetech Co. Ltd) was diluted by 0.9% saline solution to the concentration of 4 × 109 vp/mL before using. The whole course of treatment included 15 d, and multipoint intraepithelial injections were done on the 1st, 4th, 7th, 10th and 13th day under the local block anesthesia. With the identified best infection titer of rAd-p53 (MOI = 100) and the reported maximum single tolerant dose (20, 21), the injection specification was determined as 2 × 109 vp/cm2, i.e., one injection point per square centimeter and 0.5 mL rAd-p53 solution for one point. The injection needles should be pricked into mucosa at a 30- to 45-degree angle and the depths were 2 to 3 mm according to the thickness of the lesion, to inject the solution intraepithelially. On the 15th day of the course, samples were collected for biopsy under local anesthesia. Therapeutic response of all the patients was monitored during therapeutic course and 30 d after the therapy. All patients were followed up for 24 mo and the therapeutic effects were recorded. All of the patients did not receive any therapy during the follow-up except for recurrence and cancerization.

Infection efficiency of rAd-GFP. POE-9n cells in logarithmic growth phase were seeded onto six-well plate with the concentration of 1 × 105 per well. After 24h, rAd-GFP solution were added into the medium with the amount of MOI = 0, 25, 50, 100, 200, and 500. Cultured for another 72 h, fluorescence-activated cell sorting was done for quantitation of infection efficiency.

Cell morphology

POE-9n cells in logarithmic growth phase were seeded onto 25-mL culture flask.

After 24h of culture, rAd-p53 of MOI = 100 was added into medium, and then the cells were cultured for another 72 h. The POE-9n infected with rAd (Shenzhen Sibiono Genetech Co. Ltd, China) of MOI = 100, which does not harbor p53, was set as the blank control. The cell morphology was observed under an inverted phase-contrast microscopy.

Cell growth inhibition ratio

POE-9n cells were seeded onto wells of 96-well plates at 5 × 104 cells per well and cultured for 24h. rAd-p53 virus solution were added into wells in the amount of MOI = 0, 25, 50,100, 200, and 500. The control group was established with rAd of the same MOI. For each concentration, three paralleled holes were set up. Continue the cultivation for another 24, 48, 72, 96, and 120 h before the culture medium was abandoned. The proliferative activity was determined every 24h by MTT assay at 570 nm. Growth inhibition ratio was calculated by the formula of (ODcontrol - ODtreated)/ODcontrol × 100%.

Cell cycle and apoptosis assay

After rAd-p53 (MOI = 100) infection for 24 and 72 h, cell cycle and apoptosis was detected by flow cytometric analysis. The POE-9n infected with rAd (MOI = 100) for 72 h was set as the control. Quantification of apoptotic cells was done by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using a DeadEnd Fluorometric TUNEL System(Promega, Roche). In clinical samples, similar methods were used to evaluate cell apoptosis of pre-rAd-p53 and post-rAd-p53–treated patients. The correlation analysis was conducted between TUNEL staining and p53 protein staining.

Reverse transcription-PCR and Western blotting analysis

After rAdp53 and rAd infection (MOI = 100) for 24 and 72 h, POE-9n cells were detected at transcript and protein level. Total RNA was extracted with TRIzol reagent (Invitrogen). First-strand cDNA was prepared fromtotal RNA by reverse transcriptase using oligo (dT) primers, and β-actin was set as the internal control. The primers of wt-p53, p21CIP/WAF, bcl-2, and β-actin were synthesized for PCR as following:

p53: 620 bp, upstream: 5′-TACTCCCCTGCCCTCAACAAGA-3′; downstream: 5′-CTTAGCACCTGAAGGGTGAAATATTC-3′

p21CIP/WAF: 495 bp, upstream: 5′-TTAGGGCTTCCTCTTGGAGAAGAT-3′ downstream: 5′-ATGTCAGAACCGGCTGGGGATGTC-3′

Bcl-2: 318 bp, upstream: 5′-CGACGACTTCTCCCGCCGCTACCGC-3′ downstream: 5′-CCGCATGCTGGGGCCGTACAGTTCC-3′

β-actin: 548 bp, upstream: 5′-GTGGGGCGCCCCAGGCACCA-3′ downstream: 5′-CTCCTTAATGTCACGCACGATTC-3′

Messenger RNA expression of p53, p21CIP/WAF, and bcl-2 were investigated using reverse transcription-PCR. PCR products were separated with 1% agarose gel electrophoresis, and the results were recorded and analyzed by gel imaging system.

Cells (1 × 107) were solubilized at 4°C in lysis buffer consisting of 0.125 mol/L Tris-Cl (pH 6.8), 2% SDS, 2.5%-mercaptoethanol, and 10% glycerol. The protein levels of p53, p21CIP/WAF, and bcl-2 were detected by Western blotting, and β-actin was used as inner control.

Immunohistochemical staining

POE-9n cells were cultured for 24h, and then rAd-p53 and rAd (MOI = 100) were added into the medium and cultured for another 48 h. Immunohisto- chemical staining was carried out to detect p53 expression according to the instruction of PV-9000 universal histostain TM-plus kits (ZYMED). The expressions of p53, p21CIP/WAF, and bcl-2 protein in samples of pre– and post–rAdp53 treatment were detected using the same method. The criterion of positive expression refers to Shimizu semiquantitative grade method (22). This scoring procedure included two parts: one was based on the staining intensity, in which no, mild, moderate, and strong staining were scored as 0, 1, 2, 3; the other part was based on the rate of positive cells, in which 0 to 5%, 6 to 30%, 31 to 70%, and 71 to 100% were scored as 0, 1, 2, and 3; and the total score of 0 to 2, 3 to 4, and ≥5 were labeled as negative (-), positive (+), and strong positive (++).

Statistical analysis

Statistical analyses were done using SPSS 15.0 statistics software (SPSS). The statistical differences between two groups were evaluated by t test. And a $\chi 2$/Fisher exact test was used for categorical variables. Spearman test was done to analyze the correlation of different groups. All statistical assessments were two sided and evaluated at the 0.05 level of significant difference.

Results

Infection efficiency of rAd-GFP. POE-9n cells were sensitive to adenovirus. Ten to 12 hours after rAd-GFP infection, green fluorescence could be detected under fluorescence microscope, and the fluorescence intensity reached its peak 72 hours after infection. Infection efficiencies were 36.1 ± 1.56%, 72.7 ±1.40%, and 95.6 ± 2.76%, respectively, when MOI were 25,50, and 100. A positive statistical correlations existed between efficiency and titer ($r = 0.849$; $P < 0.010$). When MOI were 200 and 500, the infection efficiencies were 97.1 ± 4.12% and 98.9 ± 1.19%, and the elevation of infection efficiency was less obvious ($r = 0.124$; $P > 0.05$).

Cell morphology. Under a phase-contrast microscope, POE-9n cells were

observed as adherent monolayer cells, with a tight cellular alignment and polygon shapes. After infection by rAd for 72 hours, there were not significant changes of cell morphology, the cell appearance was like cobblestone with unfolding cytoplasm, and the caryocinesia was easily observed (Fig. 1A). Twenty-four hours after rAd-p53 infection, changes of POE-9n cell morphology and growth pattern were detected. During 72 hours, POE-9n cells showed a loose arrangement, with round shrinking size, unclear outline, vacuole in the cytoplasm, karyopyknosis, partially incomplete cell membrane, and then they were departed from grouping cells and suspended in the culture solution (Fig. 1A).

Growth inhibition ratio. rAd-p53 could significantly interfere on POE-9n cell growth. At the same time point, significantly statistical difference was determined for the OD570 values between the experimental groups ($P < 0.05$; Table1). According to growth inhibition curve of POE-9n cell in each groups (Fig. 1B), the cell proliferative inhibition ratio gradually increased with the rAd-p53 titer. In groups with MOI of 100, 200, and 500, the inhibition effect on POE-9n cell proliferation was significant. With the time accumulation, the increase of inhibition effect in these three groups slowed down gradually ($P > 0.05$). At the time points of 96 and 120 hours, no significant difference in the inhibition effect was detected among the three groups with MOI of 100,200, and 500. The maximum inhibition efficiency of rAdp53 on POE-9n cell growth was 80%. In the control groups, when MOI of rAd was in the range of 100 to 500, no significant difference was detected in proliferative inhibition ratio of POE-9n cell, whereas in the MOI =500 group, significant cytotoxic effect was observed (Table 1).The above results indicated that rAd-p53 exerted inhibition effect on POE-9n cell growth, and MOI = 100 could be determined as the ideal titer for POE-9n cell rAd-p53 infection,which was able to maintain a relatively high infection efficiency as well as to avoid interference by the cell toxic action of the adenovirus.

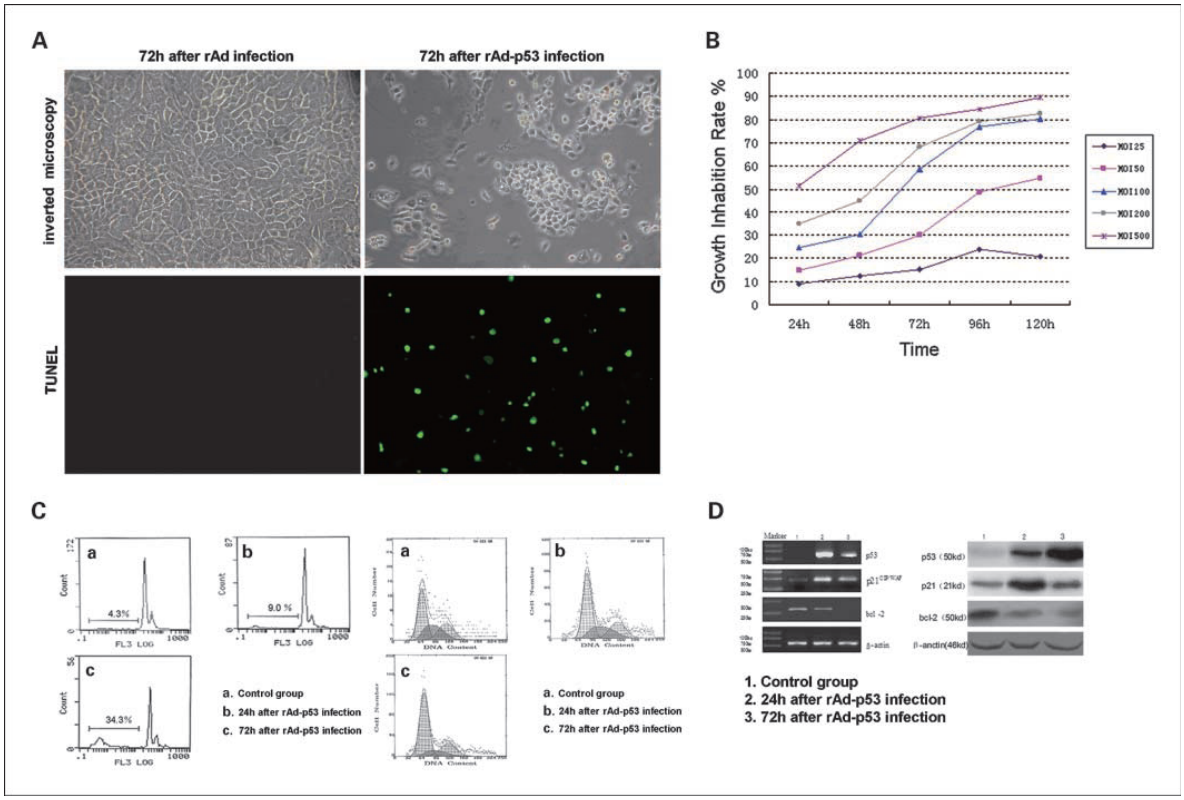

Fig. 1. Cell biology and biochemistry of POE-9n cells before and after rAd-p53 infection. A, cell morphology observed with inverted microscope (×200) and TUNEL results (×100). B, time-activity curve of growth inhibitory of POE-9n cells induced by rAd-p53 (MTT). C, flow cytometry results of POE-9n cells infected by rAd-p53. Left, cell apoptosis of POE-9n cells; right, cell cycle distribution of POE-9n cells. D, expression of mRNA by reverse transcription-PCR (left) and protein by West blotting (right) in POE-9n cells.

Table 1. Result of MTT assay (OD570 value; x ± S)

MOI	Infection time				
	24 h	48 h	72 h	96 h	120 h
0	0.9315 ± 0.0816	1.5292 ± 0.0486	1.9683 ± 0.1091	2.3458 ± 0.0756	2.6367 ± 0.0274
rAd-*p53*					
25	0.8467 ± 0.0565*	1.3347 ± 0.0165†	1.6660 ± 0.0140†	1.7828 ± 0.0262†	2.0856 ± 0.0617†
50	0.7923 ± 0.0172*	1.2020 ± 0.0249†	1.3759 ± 0.0928†	1.2013 ± 0.0476†	1.1918 ± 0.0429†
100	0.7026 ± 0.0704†	1.0669 ± 0.0503†	0.8194 ± 0.0100†	0.5442 ± 0.0640†	0.5260 ± 0.0581†
200	0.6054 ± 0.0460†	0.8456 ± 0.1240†	0.6273 ± 0.0190†	0.4914 ± 0.0346†	0.4625 ± 0.1002†
500	0.4538 ± 0.0140†	0.4499 ± 0.0125†	0.3848 ± 0.0042†	0.3688 ± 0.0190†	0.2824 ± 0.0496†
rAd					
25	0.9246 ± 0.0453	1.5185 ± 0.0562	1.9574 ± 0.0856	2.3264 ± 0.0658	2.6329 ± 0.0367
50	0.9193 ± 0.0657	1.5176 ± 0.0387	1.9452 ± 0.0695	2.3178 ± 0.0679	2.62867 ± 0.0569
100	0.9125 ± 0.0328	1.5059 ± 0.0795	1.9446 ± 0.0359	2.3076 ± 0.0756	2.6255 ± 0.0965
200	0.9018 ± 0.0475	1.4961 ± 0.0154	1.9227 ± 0.0759	2.2953 ± 0.0382	2.5067 ± 0.0859
500	0.8561 ± 0.0613*	1.4124 ± 0.1016*	1.8253 ± 0.0465*	1.9853 ± 0.0276†	2.2564 ± 0.0517†

*$P < 0.05$ compared with the control group.
†$P < 0.01$ compared with the control group.

Apoptosis and cell cycle. The apoptosis of POE-9n cells after rAd-p53 infection

was detected with flow cytometry and TUNEL method. Results of flow cytometry showed (Fig. 1C) the following: no significant subdiploid apoptotic peak was observed in the control group (3.8 ± 1.46%); 24hours after rAd-p53 infection, the cell apoptosis percentage was increased, but with a relatively small increasing amplitude (9.0 ± 0.87%); 72 hours after rAd-p53 infection, significant apoptotic peak was observed (34.3 ± 2.01%). Meanwhile, TUNEL results showed (Fig. 1A) the following: almost no apoptotic cell in the control group was observed under microscope (1.3 ±0.15%); 24hours after rAd-p53 infection, only a few cell apoptosis was observed (16.9 ± 2.39%); apoptosis was observed for most of the cells under microscope 72 hours after rAd-p53 infection (65.3 ± 3.01%). There was a significant statistical difference when the 72-h group was compared with the control and 24-h groups ($P < 0.01$). Consistent results of flow cytometry and TUNEL were obtained with statistical analysis. In case samples, positive TUNEL staining was mainly located in the nucleus, with the appearance of densely arranged prunosus grains. No positive staining of individual cells was observed in the pretreatment samples of 22 cases. In post-treatment samples, abundant typical cell apoptosis was detected in 18 cases (81.8%; Table 2), especially in tissues with strong positive p53 expression ($P < 0.01$). But the TUNEL staining strap was not that of p53 staining, and no p53-positive staining was detected in TUNEL staining cells.

The distribution change of cell cycle of post-transduction POE-9n cells was analyzed with flow cytometry (Fig. 1C). In the control group, percentages of stage G1, S, and G2 were 42.3 ± 2.52%, 31.2 ± 1.56%, and 26.5 ± 1.53%, respectively. Twenty-four and 72 hours after rAd-p53 infection, the percentages of stage G1, S, and G2 were 51.7 ± 1.47% and 64.0 ±3.91%, 25.5 ± 4.10% and 12.9 ± 2.46%, and 22.8 ± 0.49% and 23.1 ± 1.83%, respectively. There were significantly statistical differences in the comparison among the three groups ($P <0.01$). It was shown that after rAd-p53 infection, POE-9n cell percentage of stage G1 increased significantly, percentage of stage S decreased significantly, and percentage of stage G2 showed no significant change.

Messenger RNA and protein expression of p53, p21CIP/WAF, and bcl-2. Messenger RNA and protein expressions of p53, p21CIP/WAF, and bcl-2 in POE-9n cells were examined by semi-quantitative reverse transcription-PCR and Western blotting, respectively (Fig. 1D). Consistent results were obtained with those two methods. In the control group, no change was detected for mRNA and protein expression of p53, whereas in rAd-p53 group, high expression of p53 mRNA and protein was observed 24hours after the transduction, and during 72 hours after transduction, p53 mRNA expression was reduced and p53 protein expression maintained a continuous increase. P21CIP/WAF mRNA and protein experienced a significant increase after rAd-p53 infection compared with the control group, whereas bcl-2 mRNA and protein experienced a significant decrease 72 hours after transduction (Fig. 1D).

Immunohistochemical staining. Almost all POE-9n cells infected by rAd-p53 displayed strong positive expression of p53 protein. The positive products were located in the nucleus and some of the kytoplasm, with the appearance of dark brown grains. However, in the control group, no p53 protein expression was observed (Fig. 2A).

According to the comparison of pretreatment and posttreatment pathologic samples, the H&E staining of post–rAd-p53 injection tissues displayed thinning of the cuticular layer, tight cellular arrangement, decrease or elimination of atypical cells (14 of 22, 63.6%), and mild to severe inflammatory cell infiltration in the proper layer (16 of 22, 72.7%; Fig. 2B). Results of immunohistochemical staining were shown in Table 2. Positive staining of p53 protein was mainly located in the nucleus, with the appearance of densely arranged dark yellow grains. In samples of pretreatment cases, 10 cases displayed negative expression (45.5%), 12 cases displayed low-level expression (55.5%), and the cells with positive expression were dispersed in different layers of epithelium (Fig. 2B). In samples of posttreatment cases, positive p53 protein expression was observed in all 22 cases (100%), and the positive staining was mainly located in the basal and spinous layer of epithelium, forming a significant dark yellow staining strap (Fig. 2B). No correlation was determined between the p53 statuses of pretreatment and post-treatment
tissues ($P > 0.05$).

Positive p21CIP/WAF protein staining was mainly located in the nucleus and cytoplasm, with the appearance of densely arranged dark yellow grains. In pretreatment tissues, only five cases displayed positive expression (22.7%), which was different from that of p53 protein staining, and the cells with positive expression were dispersed in different layers of epithelium (Fig. 2C). In post-treatment samples, 19 cases displayed high expression of p21CIP/WAF protein (86.4%), which was similar to that of p53 protein staining, and p21CIP/WAF protein was mainly located in the basal and spinous layer of epithelium(Fig. 2C). According to statistical analysis, p21CIP/WAF expression was significant increased in post-treatment tissues ($P < 0.01$), sharing positive correlation with the expression of p53 protein ($P < 0.01$).

Positive bcl-2 protein staining was mainly located in the kytoplasm, with the appearance of a densely arranged dark yellow grain ring. In pretreatment tissues, 12 cases displayed mild to strong positive expression (54.5%), which was different from that of p21CIP/WAF protein (Fig. 2D); in posttreatment tissues,only 4 cases displayed positive expression (18.2%), in which only a few positive basal cells and adjacent inflammatory cells were observed (Fig. 2D). It was shown by statistical analysis that bcl-2 expression was significantly decreased after local rAd-p53 injection ($P < 0.01$), sharing negative correlation with the expression of p53 protein ($P < 0.01$).

The initial clinical effect of rAd-p53. All of 22 patients with OLK completed the treatment course on schedule. Eighteen patients (81.8%) displayed mucous membrane

necrosis of different degree in the lesion regions after rAd-p53 treatment. After 24 months of follow-up, 5 cases experienced a complete regression of the disease (22.7%), 20% to 70% areas of the lesion disappeared in 11 cases (50%), 4 cases did not receive significant therapeutic effect (18.2%), and 2 cases were diagnosed as focal cancerization and received an extensive resection (9.1%). No severe dose-restriction toxicity and side effects was observed in all of the patients. The most common side effect was transient fever, which was encountered in 7 patients (31.8%) and generally emerged in 2 to 12 hours after first or secondary rAd-p53 injection, with the highest temperature of 38.9°C and duration of 3 to 5 hours. Besides, five patients (22.7%) complained about pain in the injection point, two patients (9.1%) experienced flu-like symptoms after the first injection, and four patients (18.2%) had a transient increase of WBC count in hematologic test after the therapy.

Discussion

Since the initial report on p53 gene in 1979 (23), the structural change and abnormal expression of p53 gene has been shown as a key procedure for tumor genesis and development (24). Wild-type p53 gene possesses a function of inhibiting carcinogenesis by maintaining genomic integrity. Therefore, p53 gene is also called as “housekeeping gene” (25). When p53 gene deletion or mutation occurs, cells could not enter into stage G1 and DNA reparation via p53-mediated pathway after DNA injury; therefore, cells with injured genetic information could enter into proliferation and finally induce malignant tumor (26, 27). rAd-p53 shared a high sensitivity toward tumor cells, and by intratumor injection, wild-type p53 could be successfully transduced and perform multiple antitumor actions (14, 27, 28). Introduction of rAd-p53 into eight different types of carcinoma of head and neck could induce significant cell growth inhibition in 14.5% to 47% cells (29). It has been shown by many preclinical trials that rAd-p53 treatment could induce cell apoptosis of different degrees in various kinds of malignant tumor cells (30–32), and this effect was not associated with p53 gene condition of the tumor cells (32).

Various cells have different sensitivities to adenovirus (33, 34), and the adenovirus cytotoxicity has significant relationships with the titer and infection time (35). The cytotoxicity was significant when MOI reached above 500, and there was no influence on cell growth when MOI was below 50 (36–38). In present research, relatively stable infecting efficiency (>95%) could be obtained when MOI of rAd was in the range of 100 to 500, and no significant difference was detected in proliferative inhibition ratio of POE-9n cell in MTT experiment, whereas in the MOI = 500 group, significant cytotoxic effect was observed. A significant positive staining of p53 protein in nucleus or kytoplasmwas observed in almost all cells after rAd-p53 infection by the titer of MOI = 100, indicating that when MOI was 100, rAd-p53 infection could effectively introduce exogenous p53 gene into POE-9n cells, and decrease the toxicity and side effects of adenovirus as far as possible.

Table 2. Biological analysis in biopsy specimens

Result	Specimens before treatment				Specimens after treatment			
	p53	*p21*	*bcl-2*	TUNEL	*p53*	*p21*	*bcl-2*	TUNEL
-	10	17	10	22	0	3	18	4
+	12	5	6	0	5	11	4	10
++	0	0	6	0	17	8	0	8
Positive rate	54.5%	22.7%	54.5%	0%	100%	86.4%	18.2%	81.8%

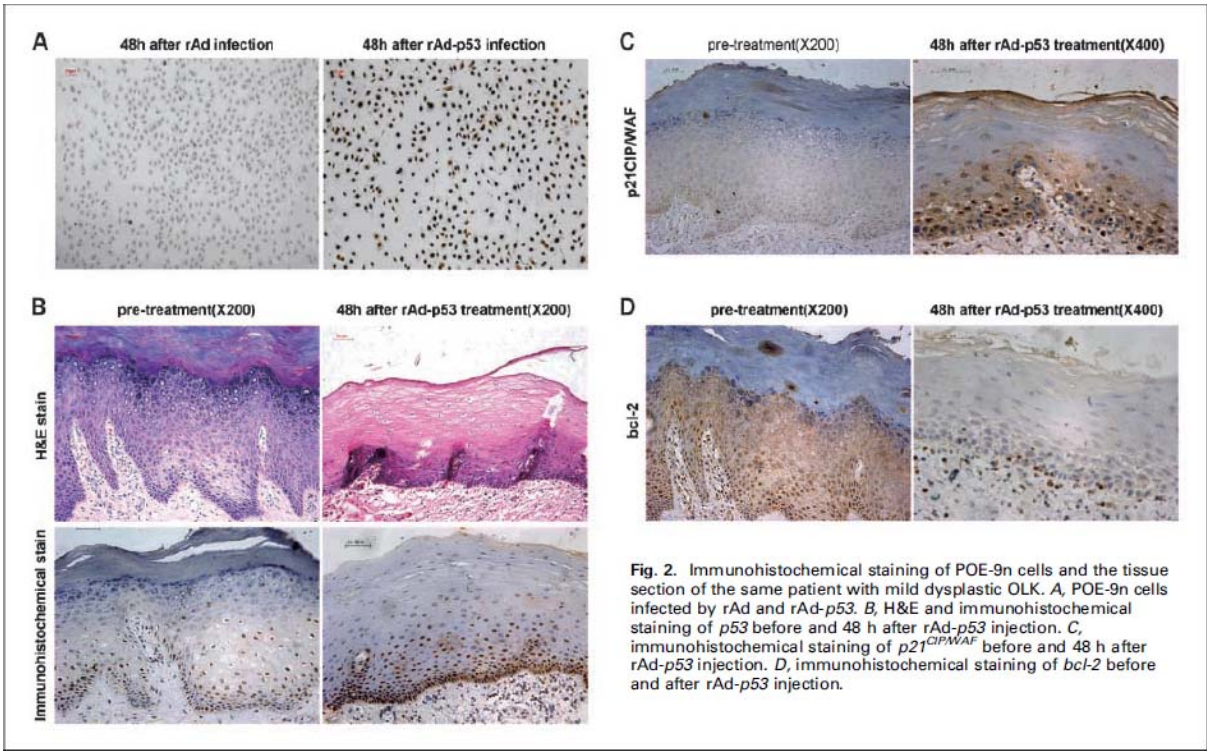

Fig. 2. Immunohistochemical staining of POE-9n cells and the tissue section of the same patient with mild dysplastic OLK. *A*, POE-9n cells infected by rAd and rAd-*p53*. *B*, H&E and immunohistochemical staining of *p53* before and 48 h after rAd-*p53* injection. *C*, immunohistochemical staining of $p21^{CIP/WAF}$ before and 48 h after rAd-*p53* injection. *D*, immunohistochemical staining of *bcl-2* before and after rAd-*p53* injection.

Cell morphologic change is one of the basic indexes for cell activity. Change of POE-9n cell morphology and growth characteristic was observed 24hours after rAd-p53 infection, and typical morphologic change was detected 72 hours after rAdp53 infection. These indicated that rAd-p53 could inhibit POE-9n cell proliferation by interfering with cell metabolism and changing the character of cell envelope. We also examined the apoptosis of POE-9n cells after rAd-p53 infection with flow cytometry and TUNEL, and similar results were obtained with the two methods. It was discovered that the apoptosis percentage of rAd-p53–treated groups was all higher than that of the control group, especially 72 hours after transduction, which indicated that rAd-p53 could induce apoptosis of oral hyperplastic cells and had a good application perspective for the inhibition of OLK malignant transformation. Cell cycle plays an important role in cell proliferation, differentiation, and tumor progression, which is regulated by intracellular and extracellular signal transduction pathway as well as feedback loop (39). The G1-S checkpoint is the key point in the regulation of cell proliferation (40, 41). One of the significant activities of wild-type p53 gene is to regulate G1-S checkpoint by coding normal p53 protein to stagnate cells with DNA damage at stage G1 for DNA reparation. It was discovered

by Von Gruenigen et al. (42) that introduction of Ad-CMV-p53 (MOI = 100) into ovarian cancer cells (OVCAR-3) could stagnate all cells at stage G1 (100%). According to the study of Wu et al. (43) on ovarian cancer cells (2774qw), significant accumulation of stage G1 cells was observed 4 hours after rAd-p53 infection, significant decrease of stage S cells was encountered 20 hours after infection, and stage G2 cells experienced no significant change. Similar to the above researches, in the present study, it was discovered that cells of stageG1 significantly increased, cells of stage S significantly decreased, and cells of stage G2 experienced no change in rAdp53–treated POE-9n cells 24hours after infection, and abundant cell apoptosis was observed 72 hours after infection, all of which indicated that rAd-p53 could successfully introduce exogenous p53 into POE-9n cells and induce stage G1 stagnation.

Based on the study of rAd-p53 influence on apoptosis and cell cycle of POE-9n cell, the expression of the key molecule of p53 passage (p21CIP/WAF, bcl-2) was further detected, in an attempt to explore the associated molecular mechanism of rAd-p53 on POE-9n cells. p21CIP/WAF gene is the first cyclin-dependent kinase–inhibiting factor discovered in mammalian recently (44), which could bind with almost all cyclin-dependent kinase compound, and induce stage Gl stagnation (45). As the main downstream molecule of p53 gene in cell cycle regulation, the binding site of wild-type p53 gene has been detected at upstream2. 4 Kbm of p21CIP/WAF gene, and p53 could upregulate p21CIP/WAF expression at transcriptional level, whereas mutant p53 has not such function (46). In this study, the POE-9n cells were p53 gene deletion cells, and p21CIP/WAF was unactivated; therefore, the basic mRNA level was quite low. After introduction of exogenous p53 into POE-9n cells by rAd-p53, mRNA and protein expression of p21CIP/WAF was significantly elevated, which was positively correlated with p53 protein by the statistical analysis, indicating that rAd-p53 could up-regulate p21CIP/WAF expression to regulate cell cycle.

Exogenous passage of p53-dependent cell apoptosis network is usually activated during DNA damage, which is regulated by bcl-2 family (47). P53 gene could precisely regulate cell apoptosis via activating the apoptosis promoter of bcl-2 family such as bax, BH3, and bid, or inhibiting the anti-apoptosis molecules such as bcl-2, bcl-xl, and survivin. It was discovered in this study that relatively high expression of bcl-2 mRNA and protein existed in normal POE-9n cells, which significantly decreased 72 hours after rAd-p53 infection. The statistical analysis showed the negative correlation between protein expressions of bcl-2 and p53, indicating that exogenous p53 inhibited bcl-2 expression and induced the apoptosis.

The biological reaction of rAd-p53 treatment in OLK was observed by immunohistochemical staining of exogenous p53 protein and its function proteins like p21CIP/WAF and bcl-2, as well as TUNEL staining of apoptotic cells. High p53 protein expression was observed in tissues after local rAd-p53 injection. The positive

staining was mainly located in basal and spinous layer of epitheliumand formed a significant dark yellow staining strap, which was significantly different from the dispersed arrangement of p53 protein in pretreated tissues. Meanwhile, the primary p53-negative staining tissue displayed high p53 expression after rAd-p53 therapy with no exception, further indicating the successful transduction of exogenous p53 gene, which also confirmed that exogenous p53 gene transduction was not associated with the primary p53 gene condition of the disease (36). And we deemed that the clinical responses were only correlated with the rAd-p53 transduction, but not with the primary p53 status. Therefore, it was speculated that after successful transduction, p53 gene exerted the action of transcription factor via activating p21CIP/WAF and inhibiting bcl-2, and therefore performed the activities of influencing cell cycle, inducing cell apoptosis and inhibiting cell growth of abnormal hyperplasia.

Besides, the biological function of exogenous p53 was shown by TUNEL staining. Typical TUNEL staining of apoptotic cell was detected in 83.3% rAd-p53 post-treatment tissues, and a significant correlation was determined between TUNEL and p53 staining. However, we discovered that TUNEL staining strap was located in the upper layer of epithelial tissue, not as the p53 staining strap, which was in the basal layer and spinous layer. This phenomenon might be associated with the time effect of p53-inducing cell apoptosis (12). Namely, cells of the upper layer were first infected by rAd-p53, which induced in situ apoptosis with clinical manifestation of surface layer necrosis of mucous membrane.

Although the involved case number and follow-up time was rather limited in this study, we observed the therapeutic effect of rAd-p53 injection in OLK after 24 months. Significant clinical effect was detected in 16 patients, and meanwhile, 63.6% patients displayed pathologic improvement. Compared with traditional therapy (21), the result was exciting. The common side effects after rAd-p53 injection included transient fever, flu-like symptoms, and transient increase of WBC number, which were the inflammatory reaction of patients toward viral vector. We also observed a large amount of local inflammatory cell infiltration in H&E sections of the post-treatment tissues. Compared with other clinical reports (14, 28), the toxic and side effects induced by rAd-p53 injection in this study were slighter, which might be owed to the relatively low dose as a result of the special lamellar lesion of OLK. However, we have to admit that as a result of the limited case number and follow-up time in this study, the evaluation of therapeutic effects of intraepithelial rAd-p53 injection in the treatment of OLK requires further expansion of the sample size and extension of the follow-up period for confirmation.

In conclusion, with short term clinical research of this study, it was shown that rAd-p53 could successfully introduce exogenous wild-type p53 gene into the cells of OLK and exert biological effects. Multipoint intraepithelial rAd-p53 injection in the treatment of hyper-plastic OLK was safe and feasible. The results of this research provided a new way for the further clinical application.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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