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Gene therapy for esophageal squamous cell carcinoma

Hideaki Shimada, Takenori Ochiai

Department of Frontier Surgery, Chiba University Graduate School of Medicine. 1-8-1 Inohana Chuo-ku Chiba-shi, Chiba, Japan

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1. ABSTRACT

Despite improvement of surgical treatment and application of multi-modality therapies to advanced esophageal cancer, the prognosis is extremely poor in patients with T4 tumors. Based on the genetic background of esophageal cancer, we have developed various gene therapy strategies against human esophageal cancer cells. In this article, we reviewed molecular events of esophageal cancer and gene therapy approaches for its treatment. First, we analyzed p53 genetic alterations and angiogenesis in esophageal cancer. Second, we evaluated an impact of p53 recombinant adenoviral vector (Ad5CMV-p53) on esophageal caner cells. Significant growth suppression was observed following infection with Ad5CMV-p53 in human esophageal squamous cell carcinoma cell lines. This observation suggests that Ad5CMV-p53 may be a potentially effective therapeutic agent for locally advanced esophageal cancer. Promising avenues for investigation include double gene therapy and adjuvant use of gene therapy with radiation therapy. Third, we have performed a clinical study for p53 gene therapy for un-resectable advanced esophageal cancer. This clinical trial was planned to evaluate vector tolerability and efficacy. A total of 10 patients were enrolled into this phase I/II trial.

2. INTRODUCTION

Epithelial tumors of the esophagus (i.e., squamous cell and adenocarcinoma) are responsible for more than 95 percent of all esophageal carcinomas, with an estimated 14,520 newly diagnosed cases and 13,570 deaths in 2005 (1). In more than 90% of all patients with esophageal cancer, the tumor is detected in an advanced stage (2). Conventional treatments are not adequate for the majority of esophageal cancer patients. Despite improvements in surgical techniques, rapid fatal recurrence is common in patients with advanced esophageal cancer (3). Because surgical resection alone rarely results in long-term survival for advanced cancer, efforts are now focused on combined multi-modality treatments in an attempt to improve local control and eliminate micro-metastasis present at the time of surgery (4-6). Recently, the use of neoadjuvant chemoradiotherapy followed by esophagectomy has become a widespread treatment owing to several favorable reports regarding this treatment (5, 6). Although combined treatment produced a lower rate of cancer-related deaths, it produced a higher rate of treatment-associated mortality. The prognosis is extremely poor in unresectable patients. The five-year survival rate in T4-stage (where cancer tissue infiltration affects other

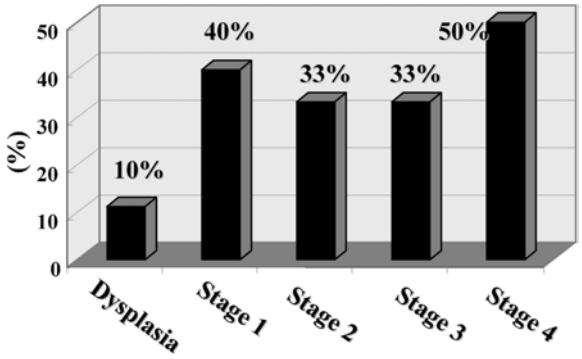

Figure 1. Frequency of p53 mutation according to pTNM/UICC stages in esophageal squamous cell carcinoma.

organs according to the esophageal cancer TNM classification system) patients treated with radiotherapy alone is 3.1% (7). The five-year survival rate after radical radiotherapy was 0% in Stage IV patients and T4 patients (8). Thus, no therapy that shows sufficient clinical efficacy has been established at present for these patients. In this context, it will be necessary to enhance the anti-tumor efficacy of chemotherapy and radiotherapy and reduce their systemic adverse effects in order to improve the outcome of multidisciplinary therapy.

3. MOLECULAR EVENTS IN ESOPHAGEAL CANCER

Abundant evidence exists that the process of malignant transformation is mediated by genetic alterations. The major lesions detected in cancer cells occur in dominant oncogenes and tumor suppressor genes (9). Oncogenes are the result of genetic alterations in a class of genes called proto-oncogenes, which participate in the critical functions of normal cells, including signal transduction and transcription. Oncogenes are typically dominant in their mode of action and, therefore, genetic alteration of both alleles is not necessary for cellular transformation. Tumor suppressor genes appear to require homozygous loss of function by mutation, deletion, or a combination of both, for transformation to occur. It is also possible that modification of the expression levels of dominant oncogenes and tumor suppressor genes may influence certain characteristics of cells that contribute to the malignant phenotype.

4. P53 ALTERATIONS IN ESOPHAGEAL CANCER

The p53 gene is the most frequently mutated gene yet identified in human esophageal cancer. p53 abnormalities are observed in 40-60% of patients with esophageal cancer (Figure 1) (10-12). Indeed, p53 alteration is a good predictor for treatment response and survival in esophageal cancer (13-15). The five-year survival of our patient cohort demonstrated that the survival rate of the p53 mutation-negative group (n=28) is significantly higher than that of the p53 mutation-positive group (n=14) (51% vs 36%, respectively, $P<0.05$) (16). The p53 gene abnormalities usually consist of partial or total deletion of the short arm of chromosome 17, associated with the simultaneous occurrence of minimal mutations like point mutation in p53 alleles, leading to the inactivation of its tumor suppressor function.

5. ANGIOGENIC FACTOR IN ESOPHAGEAL CANCER

Angiogenic factors and growth factors also play an essential role in the process of growth and metastasis of esophageal carcinoma (17-20). Among several angiogenic factors, vascular endothelial growth factor (VEGF) and thymidine phosphorylase (dThdPase) have been shown to be vital for pathological angiogenesis. Immunohistochemical analyses of esophageal carcinomas have revealed that VEGF expression, dThdPase expression and angiogenesis are prognostic factors. Serum VEGF concentration as well as serum dThdPase concentration are significantly elevated in patients with primary esophageal cancer. High serum VEGF concentration as well as serum dThdPase concentration were associated with poor treatment response and poor survival (21, 22). Over-expression of VEGF protein is, therefore, partially responsible for the malignant potential of esophageal cancer and represents a useful prognostic marker. Based on the reports that indicate a significant association between VEGF and p53 expression (23), wild type p53 gene transfer into cancer cells is supposed to down regulate angiogenesis (24, 25). Because a significant association between dThdPase expression and p53 mutation was observed, wild type p53 gene transfer would also down regulate dThdPase expression (26).

6. P53 GENE THERAPY FOR HUMAN ESOPHAGEAL CANCER CELLS

Based on the concept of genetic alteration in carcinogenesis and angiogenesis, cancer gene therapy has rapidly developed as an alternative to conventional cancer therapy. We previously reported a growth inhibitory effect and an enhancement of the sensitivity to radiation and anti-cancer drugs after transduction of esophageal squamous carcinoma cell lines with retroviral vectors expressing wild-type p53 (27). Clinically, the recombinant adenoviral vector is expected to be a good agent for introducing genes of interest into cancer cells, due to its high transduction efficiency. Since few studies have been reported on esophageal cancer gene therapy, preclinical studies of adenoviral-mediated p53 gene delivery are required to confirm the anti-cancer effect on esophageal cancer (28).

7. *IN VITRO* CYTOTOXIC EFFECT OF AD.CMV-P53 INFECTION ON CELL GROWTH

The growth properties of human esophageal cancer cells (T.Tn), which revealed a p53 mutation at codon 258 (G to T, resulting in translational termination) in exon 7 of p53 gene, were examined at various MOIs of Ad5CMV-p53, ranging from 10 to 1000. AV1.0CMV-βgal infected cells revealed similar growth rates to those of non-infected cells at MOIs 0, 10, 30 and 100. However, Ad5CMV-p53 infected cells revealed partial growth

suppression at an MOI of 30, and complete growth suppression at an MOI of 100.

8. *IN VIVO* ANTI-TUMOR EFFECT OF AD5CMV-P53 INFECTION

The *in vivo* inhibition of tumorigenicity of T.Tn cells by injection of Ad5CMV-p53 or AV1.0CMV-βgal was examined. T.Tn cells inoculated with AV1.0CMV-β-gal at an MOI of 300 developed subcutaneous tumors in nude mice within 14 days post-inoculation (5-8 mm in diameter) in 5 of 5 mice (100%). T.Tn cells inoculated with Ad5CMV-p53 at an MOI of 30 developed tumors in 4 of 5 mice (80%) within 28 days post-inoculation. However, significant growth retardation was observed with this lower MOI. T.Tn cells inoculated with Ad5CMV-p53 at an MOI of 100 produced tumor in only one mouse (20%). T.Tn cells inoculated with Ad5CMV-p53 at an MOI of 300 developed no tumors. Tumor growth was thus significantly suppressed in the group treated with Ad5CMV-p53 inoculation.

On days 14 and 16, either an adenovirus vector or Dulbeco's Modified Eagle Medium was injected into two peritumoral sites at an MOI of 100. In this experiment, the tumors injected with medium, AV1.0CMV/293 or AV1.0CMV-βgal showed no growth retardation. The intra-tumoral injections of Ad5CMV-p53 significantly inhibited tumor growth and the tumor volumes remained stable for 10 days after treatment. Two injections of Ad5CMV-p53 suppressed tumor growth by 60-70% compared with the growth observed in tumors injected with AV1.0CMV/293, medium or AV1.0CMV-βgal.

In the above set of experiments, we demonstrated that adenovirus-mediated wild-type p53 gene transduction efficiently suppressed tumor growth of human esophageal cancer cell lines both in vitro and in vivo. The in vitro cytotoxic effects on normal esophageal epithelial cells were assessed and slight growth suppression was observed at an MOI of 300 but not at an MOI of 100 when using Ad5CMV-p53. The mechanism by which overexpression of wild-type p53 protein induces the above growth suppressive effect appears to be the induction of apoptosis. Precise determination of the mechanism underlying the growth suppressive effect of p53 will require further investigation. In vitro study showed that the therapeutic level of MOI was greater than 30 MOI and the transduction rate was approximately 60% at 100 MOI. Tumor cells inoculated with Ad5CMV-p53 demonstrated a significant suppression of tumorigenicity at an MOI of 100 and growth retardation at an MOI of 30. This strategy could be useful in a situation where relatively curative resection for locally advanced esophageal cancer has been performed and possible micro residual cancer cells are present.

9. CLINICAL STUDY OF P53 GENE THERAPY

Recently, clinical studies of p53 gene therapy for non-small cell lung cancer showed promising results and no severe side effects (29, 30). In vitro data regarding the efficacy of p53 gene therapy in the setting of esophageal cancer are similar to those obtained with lung and head and neck cancers. In clinical trials of lung cancer patients in the US, the protocol for the present study calls for administration twice or three times per cycle.

Basic studies of lung cancers showed that transduction with Ad5CMV-p53 reduced the proliferative capacity of cancer cells that had normal p53 gene status as well (31). This finding was corroborated by findings obtained in a Phase I clinical trial in which Ad5CMV-p53 showed good antitumor effects with only slight adverse reactions, independently of the p53 status of the patients' tumors. A phase I study of adenoviral p53 gene therapy, conducted by Swisher SG et al, demonstrated a partial response in two patients (8%) and disease stabilization in 16 patients (64%) (29). Therapeutic objective of the present trial was to inhibit the tumor cell growth and induce apoptosis by infecting the cancer cells with the p53-encoding adenovirus. Phase I/II clinical trials in the US conducted to date for head and neck cancer and non-small cell lung cancer confirmed good tolerability of Ad5CMV-p53 and showed no severe side effects.

10. CLINICAL PROTOCOL OF P53 ESOPHAGEAL CANCER GENE THERAPY

10.1. Patient eligibility (32)

Study patients included men and women between the ages of 20 and 80 with pathologically documented SCC of the esophagus. Subjects were eligible for the study if they were not candidates for radical surgery. Patients with non-resectable advanced esophageal SCC that was resistant to definitive chemoradiotherapy, more than 60Gy, were eligible. The tumors, revealed stable disease (SD) or progressive disease (PD) after completion of chemoradiation, were defined as chemoradiation-resistant tumor. The tumors, started to grow after partial response (PR) or complete response (CR), were also defined as chemoradiation-resistant tumor. Patients were judged to be able to survive for 12 weeks or more with performance status (PS) of 0-2 defined as follows: PS0, Normal activity, asymptomatic; PS1, Symptomatic, fully ambulatory; PS2, Symptomatic, in bed < 50% of time; PS3, Symptomatic, in bed >50% of time, not bedridden. Patients who refused previous treatment were not enrolled. Patients provided written informed consent. Eligibility criteria included ability to administer Ad5CMV-*p53* into tumors under endoscopy or ultrasonography. Tumors (<10 cm in its major axis) were detected and evaluated by physical examinations, endoscopy, ultrasonography, X-rays or computed tomography scanning. Normal bone marrow counts, liver enzymes, and renal function were maintained by adherence to the following criteria: neutrophil count >1,500m^3; platelet count >100,000/mm^3; hemoglobin >8 g/dl; total bilirubin <1.5 times the upper limit of normal; ALT and AST <2 times the upper limit of normal; alkaline phosphatase <5 times the upper limit of normal; creatinine <1.5 mg/dL; and PT and PTT within the normal range. The presence of a *p53* mutation in the tumor was not a requirement for study entry. Clinical classification of the

primary tumor, the degree of lymph node involvement, and organ metastasis were examined according to the TNM/UICC classification.

10.2. Study design and treatment plan

This was a single-center, phase I/II study of Ad5CMV-*p53* intra-tumoral injection. The primary objective was to determine the feasibility and safety of this therapy. The secondary objective was to observe biological responses and antitumor effects. The duration of efficacy, time to tumor progression, and survival period were also determined. The study vector was supplied by Introgen Therapeutics, Inc (Houston, TX). The protocol was approved by Chiba University Graduate School of Medicine Institutional Review Board and the Recombinant DNA Advisory Committee of the National Institutes of Health Office of Biotechnology Activities. Written informed consent was obtained from all participants.

The replication-defective adenoviral vector Ad5CMV-*p53* contains the cytomegalovirus promotor, wild-type human *p53* cDNA, and a SV40 polyadenylation signal in a minigene cassette inserted into the E1-deleted region of modified adenovirus-5. Individual doses were 10 to $25x10^{11}$ viral particles (VP) dependent on tumor size. The vector was suspended in phosphate-buffered saline, which was kept on ice until the time of administration. On a 28-day cycle, intra-tumoral injections of Ad5CMV-*p53* were administered on days 1 and 3 and treated for up to five cycles. The dose level for each patient was determined based on the estimated tumor size (sum of major axis of all measurable lesions). According to the results of clinical trials on head & neck cancer and lung cancer, dose levels were fixed as follows: <2cm, $5x10^{11}$VP; 2 to 4cm, $10x10^{11}$VP; 4 to 6cm, $15x10^{11}$VP; 6 to 8cm, $20x10^{11}$VP; and 8 to 10cm, $25x10^{11}$VP. The dosing volume was adjusted using a diluent to be 0.1-0.2 ml/cm^3. This dosing volume was approximately 20% (10-30%) of the estimated tumor volume. An endoscope was inserted under pharyngeal anesthesia, and a prepared Ad5CMV-*p53* solution was injected using a fine needle. In the case of lesions that allowed passage of the endoscope, injections were made at 1-cm intervals so that the entire lesion was covered. Biopsies were taken before installation from tumor and the subsequent intra-tumoral injections were performed through an endoscope when the treatment coincided with a follow-up endoscopic examination on day 3. All treatments were performed in a negative-pressure environment with biosafety precautions. Patients who were free of progression at day 56 were offered additional treatment up to a maximum of five cycles. In the case of lymph nodes, injections were made under ultrasonographic guidance.

10.3. Objective responses

A patient was regarded as a responder if complete response or partial response was obtained during the study, treatment period, or during the 12-month follow-up period and if responses lasted for at least 4 weeks. Evaluation was made to determine if the efficacy was attributable to Ad5CMV-*p53* and not due to adjuvant therapy prohibited by the protocol (surgery, chemotherapy, radiotherapy, etc.). "Guidelines for clinical and pathological studies on carcinoma of the esophagus, ninth edition" was applied to measurable tumors and inmeasurable tumors for tumor efficacy assessment (33).

10.4. Reverse transcription-PCR and real-time PCR

Tissue samples were either formalin-fixed or fresh-frozen at the time of resection. For RT-PCR, RNA was extracted from thawed, homogenized, and DNase-digested tissue. After reverse transcription, PCR was performed using primers specific to the Ad5CMV-*p53* vector or to glyceraldehyde phosphate dehydrogenase (GAPDH) as described previously. The esophageal cancer cell line T.Tn was transfected *in vitro* and used as a positive control. To determine the copy number of Ad5CMV-*p53* virus, viral DNA extracted from Ad5CMV-*p53* was used as an absolute standard, and the GAPDH gene was used a reference gene to count cell numbers. The vector-*p53* transcript contains viral sequences from the CMV promoter, which serve to distinguish it from endogenous *p5*3 mRNA. For RT-PCR detection of vector-*p5*3 gene expression, primers were constructed to bridge the viral and human transcribed sequences as follows: forward primer, 5'-TGGAGGAGCCGCAGTCAGAT-3'; reverse primer, 5'-ATATCGTCCGGGGACAGC-3'; and probe, 5'-TGCCGTCCCAAGCAATGGATGA-3'. For detection of *p21* gene expression, primers were constructed as follows: forward primer, 5'-CACTGGAGGGTGACTTCG-3'; reverse primer, 5'-CGTTTGGAGTGGTAGAAATC-3'; and probe, 5'-CCTTGGCCTGCCCAAGCTCT-3'. For detection of *MDM2* gene expression, primers were constructed as follows: forward primer, 5'-CTCACAGATTCCAGCTTCGG-3'; reverse primer, 5'-ACAGAGAAGCTTGGCACGC-3'; and probe, 5'-GGTTAGACCAAAGCCATTGCTTTTGAAG-3'.
Expression of mRNA was analyzed by quantitative real time PCR using the Light-Cycler system (Roche, Basel, Switzerland). The PCR products were detected by measuring the fluorescence of SYBR Green I, which selectively bound to double-stranded DNA and emitted greatly enhanced fluorescence. PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining.

10.5. Clinical monitoring

Patients were monitored for adverse events for a minimum of 56 days. A follow-up endoscopy was performed on days 3, 29, and 57 for all patients. Patients were seen for tumor assessment every month, which included endoscopic examination until there was evidence of progression. Hematology, serum chemistry (including electrolytes, ALT, AST, lactate dehydrogenase, total bilirubin, urea nitrogen, and creatinine), and urinalysis were performed before treatment and during follow-up. Follow-up information was collected from the time of study entry until patient death or through May 1, 2006. Toxicity was graded according to NCI common toxicity criteria (version 1.0). Toxicity not included in the toxicity scale was scored as grade 3 if hospitalization was required and grade 4 if toxicity was regarded as life threatening. Overall survival was defined as the interval between the first treatment and death or last follow-up visit. Time to progression was

Table 1. Characteristics of the patients with esophageal squamous cell carcinoma treated with Ad5CMV-p53

Patient No.	Age & Gender	Site of p53 Mutation	Viral Particles	Treatment Cycles	Tumor Size (mm)	Local & Overall Response[2]	Prognosis after GT
1	64M	Exon 5	15×10^{11}	2	43	SD & SD	19M dead
2	71M	Exon 7	10×10^{11}	5	25	SD & SD	15M dead
3	62M	Negative[1]	15×10^{11}	3	50	SD & PD	3M dead
4	78M	Exon 8	10×10^{11}	2	30	PD & PD	6M dead
5	66M	Exon 7	10×10^{11}	3	40	SD & SD	65M alive
6	60M	Exon 7	20×10^{11}	1	70	SD & PD	2M dead
7	67F	Exon 5	10×10^{11}	2	38	SD & SD	13M dead
8	58M	Exon 6	10×10^{11}	2	40	SD & SD	15M dead
9	48M	Exon 7	25×10^{11}	4	100	SD & SD	12M dead
10	77M	Negative[1]	20×10^{11}	2	68	SD & PD	2M dead

[1] No mutation among exon 5, 6, 7 and 8, [2] Treatment response was determined 4 weeks after completion of therapy by external review board.

idefined as the interval between first treatment and appearance of a new metastasis, increased size of an established metastasis, or increased size of an established esophageal tumor (25% or greater increase in the product or sum of axes of measurable lesions). Any changes in tumor dimension, radiographic appearance, or endoscopic appearance of an index lesion were noted.

The objective of this study was to evaluate the safety and therapeutic efficacy in patients with non-resectable esophageal cancer after injection of a normal *p53* gene expressing adenovirus vector into tumors. Safety was monitored for 28 days after completion of the final cycle of Ad5CMV-*p53* treatment. Long-term follow-up was conducted every two months until patient death, other treatments become necessary, or expiration of a one-year period after starting treatment.

10.6. Clinical results

A total of 10 patients were enrolled into this trial. After providing informed consent, the first patient received injections of Ad5CMV-p53 on December 19, 2000 (Table 1). All patients had histologically confirmed SCC of the esophagus. Patients had been previously treated with definitive chemo-radiation therapy, which included more than 60Gy of radiation and concurrent 5-fluorouracil plus cisplatin. Initial response to chemoradiation revealed complete response in three, partial response in two and stable disease in four patients. The other one patient (#10), received chemoradiation because of positive margin in endoscopic resected specimen. At the time of starting gene therapy, five patients had tumor invasion into adjacent organs (Patient 3, 4, 7, 9, 10) and four had high risk factors for surgery (Patients 1, 2, 5, 8). The other one had T3 tumor with multiple lymph node metastases (Patient 6). Pretreatment tissue samples were obtained from all patients. Eight patients had tumors with *p53* mutations among exons 5 to 8.

Nine of 10 patients completed at least two cycles or four injections of treatment according to the protocol. Treatment cycles ranged from one to five cycles with the median number of cycles as two. Patient 6 did not undergo a second cycle of treatment because he showed rapid progression of distant metastases after the first cycle. Injected lesions were large and ranged from 25-100 mm (median=43 mm in its longest axis).

10.7. Adverse events in clinical study

Adverse events attributed to treatment with Ad.5CMV-*p53* generally were mild to moderate. The most common adverse events were fever and local pain (NCI grade 1 or 2). Fever was observed in all patients and pain in 30%**–**. Overall, drug administration was feasible and well tolerated. In one patient, the study had to be discontinued prematurely because of disease progression. This patient died 61 days after starting treatment (59 days after the second and last dose), but death was not related to treatment with Ad5CMV-*p53*. Three patients showed hyperglycemia, which was likely attributable to total parental nutrition. Two patients revealed hypocalcemia. One patient each experienced partial thromboplastin time elongation, an increase in serum amylase or creatinine. There were no other significant laboratory abnormalities detected on follow-up evaluations.

10.8. Detection of *p53* transgene, *p21* induction and *MDM2* induction in biopsy specimens

Tissue samples were obtained and fresh-frozen in liquid nitrogen before and after treatment. All patients demonstrated successful *p53* gene transfer by DNA-PCR on day 3 (Table 2), whereas all pre-treatment samples were negative. DNA levels were quantitiated in biopsies from patients 1-3 and demonstrated an average of 8.2×10^4 copies/10^5 gene copies. Post-treatment biopsies were obtained on day 3, day 29 and day 57. We found vector-specific *p53* expression in post-treatment esophageal biopsies from all patients. Expression levels increased in seven patients, but expression levels did not increase on day 3 in the other three patients (Patient 3, 7, 8) (Table 2). The expression level of *p53* on day 29 was higher in all but two patients. *P21* and *MDM2* expression levels were increased in six patients on day 3 (Patients 1, 4, 5, 6, 7, 10). Although the peaks of average mRNA levels of each gene were slightly different, all three levels increased after treatment. The ratio of expression between *p21* and *MDM2* increased on day 29. Biopsies taken on day 29 showed no evidence of disease in three patients (2, 3 and 5). Patients 2 and 3 also showed the highest levels of gene transfer.

Table 2. Detection of squamous cell carcinoma, Ad5CMV-p53 vector-specific DNA, and p53 mRNA in biopsy specimens during treatment

Patient No.	SCC[1] Day 28	p53 DNA Day 3	Quantity /10^5 gene copies	p53 mRNA Preinjection[4]	Log copies/μg total RNA[3] Postinjection[5]
1	Positive	Positive	$2.60x10^4$	4.93	5.88
2	Negative	Positive	$3.74x10^4$	4.88	5.05
3	Negative	Positive	$1.73x10^5$	4.33	4.07
4	Positive	Positive	NA[2]	4.21	5.77
5	Negative	Positive	NA	4.29	5.11
6	Positive	Positive	NA	5.06	5.62
7	Positive	Positive	NA	5.61	5.10
8	Positive	Positive	NA	5.07	4.51
9	Positive	Positive	NA	4.93	5.13
10	Positive	Positive	NA	4.53	6.68

[1] SCC, squamous cell carcinoma, [2] NA, not applicable, [3] Values represent the mean of duplicate samples, [4] Preinjection samples were obtained just before first injection, [5] Postinjection samples were obtained on day 3 just before second injection

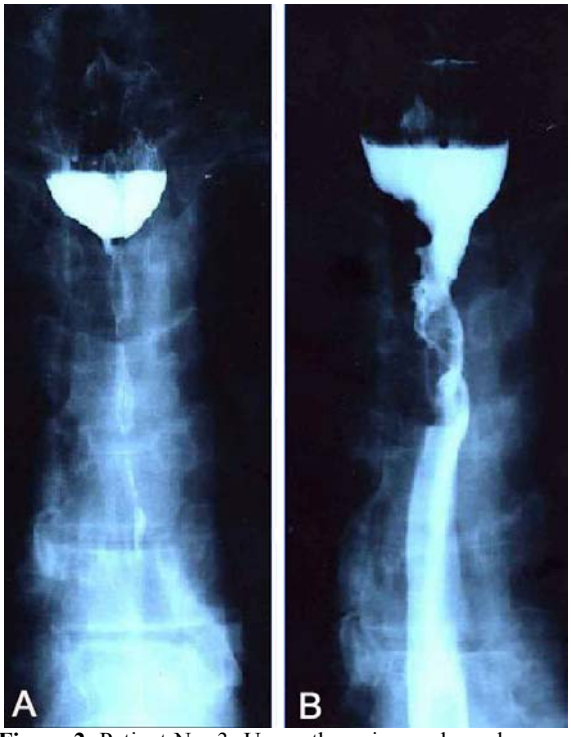

Figure 2. Patient No. 3: Upper thoracic esophageal cancer unable to undergo surgery because of tracheal invasion. (A) Esophagography shows complete obstruction before p53 gene transfer. (B) Esophageal obstruction was reduced after treatment.

10.9. Long-term follow up

Nine of the 10 eligible patients have died of disease. One patient had local tumor progression and four patients had systemic progression during the initial 56 days. Six of 10 patients were alive for more than one year (Table 1). Three patients showed no evidence of tumor on multiple biopsies after treatment (Patients 2, 3, and 5). Patient 2 had a tumor with deep ulcer before treatment. After three or four cycles, ulcer was changed to mild shape. No viable cancer cells were observed in biopsy specimens. Stable local tumor and no residual SCC were found on esophageal biopsies during the five cycles of treatment. Patient 3 had a large 50 mm mass and was unable to swallow at the initiation of gene therapy (Figure 2A). Analysis of biopsies after the first cycles of treatment showed mild dysplasia and necrotic tissue. After two injections, he was able to swallow liquid and meals (Figure 2B). Although the endoscope could not pass through the stenotic portion of the lesion before treatment, it passed through after treatment. Although the local tumor was controlled during treatment, distant metastases occurred and the patient died at 3 months. Patient 5 did not show tumor progression for 24 months after initial gene therapy. Although slight viable cancer cells were observed in biopsy specimens at 24 months, the tumor was well controlled by argon-plasma coagulation. He remained progression-free and alive 50 months after completion of treatment as of today. He received a total of three courses with 1011 viral particles on days 1 and 3 of each course. With an intent to treat analysis, overall survival rate was 60% at one year. Four patients have developed clinically diagnosed distant metastases (two bone metastasis, one liver and lymph node, and one lung). Three of these four patients, with development of distant metastases, died of disease within three months after initial viral treatment. Median time to local as well as distant progression was 6 months.

11. FUTURE CLINICAL TRIAL OF P53 GENE THERAPY COMBINED WITH CHEMORADIOTHERAPY

According to recent cancer gene therapy reports, adenovirus-mediated p53 gene transfer is frequently used, together with cis-dichloro-diammineplatinum (II) administration (34) or ionizing radiation (35). We herein report the findings of a preclinical study that reveal the efficacy of Ad5CMV-p53 treatment on human esophageal squamous cell carcinomas both in vitro and *in vivo*. Although the transduction efficiency was relatively low on esophageal carcinoma, an MOI of 100 was sufficient to show efficacy in clinical trials. Since an MOI of 100 may represent a relatively high viral concentration for clinical use, combination therapy using CDDP or other apoptotic agents should also be investigated. Further experimentation is required to

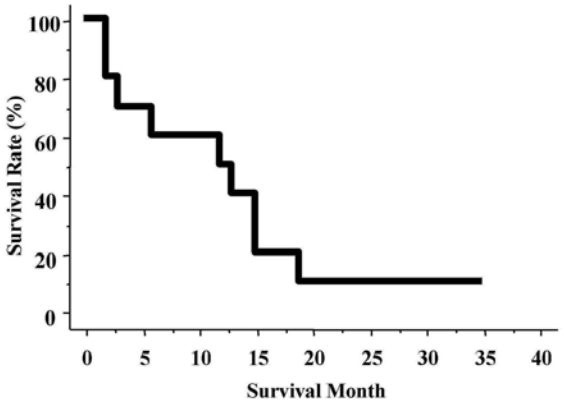

Figure 3. Overall survival of all patients-.

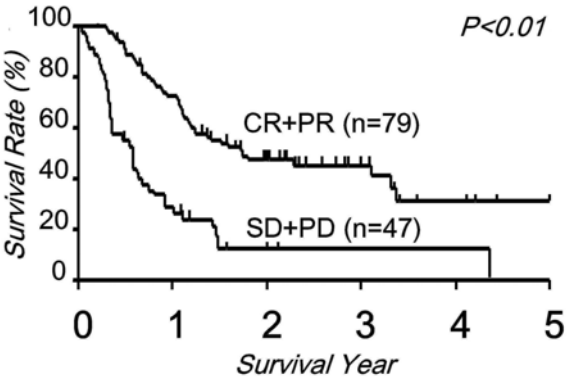

Figure 4. Treatment response and survival of patients with thoracic esophageal carcinoma. A total of 126 patients were treated with chemoradiation between 1997 and 2001.

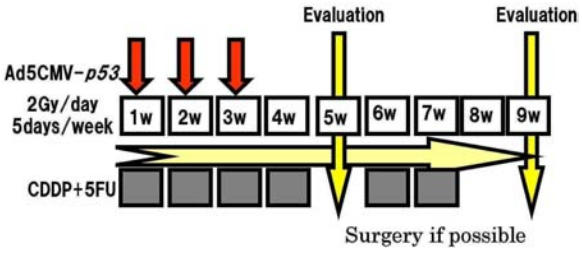

Figure 5. Protocol of p53 gene therapy combined with chemoradiation for T4 esophageal carcinoma.

develop a new combination therapy with a low dose CDDP and/or 5-FU and Ad5CMV-p53 at a low MOI and also a new combination therapy with concurrent chemoradiotherapy. A phase II trial of adenoviral-mediated p53 gene transfer in conjunction with radiation therapy for lung cancer patients was safe and effectiveness (35). We also confirmed a synergistic anti-tumor effect of Ad5CMV-p53 and radiation therapy for human esophageal cancer (36). Because treatment response is strongly associated with survival (Figure 4), combination treatment with standard chemoradiation and Ad5CMV-p53 may be an attractive modality in the near future (Figure 5).

12. DOUBLE GENE TRANSFER TO ENHANCE P53 GENE THERAPY

Several reports have shown that double gene transfer enhanced anti-tumor activity of single gene transfer (37-39). Some reports suggested that p14, p16 and p33 genes were able to enhance apoptotic tumor cell death of p53 gene therapy (38-40). We suggested a role of Mdm2 in the synergistic effect of p33 gene transfer in combination with p53 gene transfer (40). Although stable expression of both genes was claimed for double gene therapy, this mode of gene therapy should undergo clinical study in the near future. We herein reviewed the findings of a preclinical study of esophageal cancer gene therapy and reviewed a protocol of clinical p53 gene therapy for unresectable advanced esophageal squamous cell carcinomas.

13. ACKNOWLEDGEMENT

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Abbreviations: VEGF: vascular endothelial growth factor; dThdPase: thymidine phosphorylase; PS: performance status; VP: viral particles; GAPDH: glyceraldehyde phosphate dehydrogenase

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Send correspondence to : Dr Hideaki Shimada, Department of Frontier Surgery, Chiba, University Graduate School of Medicine, 1-8-1 Inohana Chuo-ku Chiba-shi, Chiba, 260-8670, Japan, Tel: 81-43-226-2110, Fax: 81-43-226-2113, E-mail: hshimada@faculty.chiba-u.jp

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