disease. This model would be useful for evaluation of the therapeutic efficacy of antitumor drugs, and may be able to be utilized to study early events in the TME.

Acknowledgments

This paper is dedicated to the memory of A.R. Moossa, M.D.

References

1. White AC, Levy JA, McGrath CM (1982) Site-selective growth of a hormone-responsive human breast carcinoma in athymic mice. Cancer Res 42: 906–912
2. Rosenberg MP, Bortner D (1999) Why transgenic and knockout animal models should be used (for drug efficacy studies in cancer). Cancer Metastasis Rev 17: 295–299.
3. Kishimoto H, Kojima T, Watanabe Y, Kagawa S, Fujiwara T, et al. (2006) In vivo imaging of lymph node metastasis with telomerase-specific replication-selective adenovirus. Nat Med 12: 1213–1219.
4. Céspedes MV, Espina C, García-Cabezas MA, Trias M, Boluda A, et al. (2007) Orthotopic microinjection of human colon cancer cells in nude mice induces tumor foci in all clinically relevant metastatic sites. Am J Pathol 170: 1077–1085.
5. Hoffman RM (1999) Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. Invest. New Drugs 17: 343–359.
6. Furukawa T, Fu X, Kubota T, Watanabe M, Kitajima M, et al. (1993) Nude mouse metastatic models of human stomach cancer constructed using orthotopic implantation of histologically intact tissue. Cancer Res 53: 1204–1208.
7. Wang M, Bronte V, Chen PW, Gritz L, Panicali D, et al. (1995) Active immunotherapy of cancer with a non-replicating replicating recombinant fowlpox virus encoding a model tumor-associated antigen. J Immunol 154: 4685–4692.
8. Brattain MG, Fine WD, Khaled FM, Thompson J, Brattain DE (1981) Heterogeneity of malignant cells from a human colonic carcinoma. Cancer Res 41: 1751–1756.
9. Yamamoto N, Yang M, Jiang P, Xu M, Tsuchiya H, et al. (2003) Real-time imaging of individual fluorescent-protein color-coded metastatic colonies in vivo. Clin Exp Metastasis 20: 633–638.
10. Hoffman RM, Yang M (2006) Subcellular imaging in the live mouse. Nature Protocols 1: 775–782.
11. Hoffman RM, Yang M (2006) Color-coded fluorescence imaging of tumor-host interactions. Nature Protocols 1: 928–935.
12. Hoffman RM, Yang M (2006) Whole-body imaging with fluorescent proteins. Nature Protocols 1: 1429–1438.
13. Amoh Y, Yang M, Li L, Reynoso J, Bouvet M, et al. (2005) Nestin-linked green fluorescent protein transgenic nude mouse for imaging human tumor angiogenesis. Cancer Res 65: 5352–5357.
14. Yamauchi K, Yang M, Jiang P, Xu M, Yamamoto N, et al. (2006) Development of real-time subcellular dynamic multicolor imaging of cancer-cell trafficking in live mice with a variable-magnification whole-mouse imaging system. Cancer Res 66: 4208–4214.
15. Kimura H, Momiyama M, Tomita K, Tsuchiya H, Hoffman RM (2010) Long-working-distance fluorescence microscope with high-numerical-aperture objectives for variable-magnification imaging in live mice from macro- to subcellular. J Biomed Optics 15(6): 066029.
16. Tomayko MM, Reynolds CP (1989) Determination of subcutaneous tumor size in athymic (nude) mice. Cancer Chemother Pharmacol 24: 148–154.
17. Van den Broeck W, Derore A, Simoens P (2006) Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNCrl mice. J Immunol Methods 312: 12–19.
18. Gest TR, Carron MA (2003) Embryonic origin of the caudal mesenteric artery in the mouse. Anat Rec A Discov Mol Cell Evol Biol 271: 192–201.
19. Hamamoto T, Beppu H, Okada H, Kawabata M, Kitamura T, et al. (2002) Compound disruption of smad2 accelerates malignant progression of intestinal tumors in apc knockout mice. Cancer Res 62: 5955–5961.
20. Hoffman RM (2005) Orthotopic metastatic (MetaMouse) models for discovery and development of novel chemotherapy. Methods Mol Med 111: 297–322.
21. Fu X, Besterman JM, Monosov A, Hoffman RM (1991) Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically intact patient specimens. Proc Natl Acad Sci USA 88: 9345–9349.
22. Hoffman RM (2005) The multiple uses of fluorescent proteins to visualize cancer in vivo. Nat Rev Cancer 5: 796–806.
23. Winn B, Tavares R, Fanion J, Noble L, Gao J, et al. (2009) Differentiating the undifferentiated: immunohistochemical profile of medullary carcinoma of the colon with an emphasis on intestinal differentiation. Hum Pathol 40: 398–404.
24. Tan KK, Lopes Gde L Jr, Sim R (2009) How uncommon are isolated lung metastases in colorectal cancer? A review from database of 754 patients over 4 years. J Gastrointest Surg 13: 642–648.
25. Amoh Y, Li L, Yang M, Moossa AR, Katsuoka K, et al. (2004) Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells. Proc Natl Acad Sci USA 101: 13291–13295.

Author Contributions

Conceived and designed the experiments: H. Kishimoto. Performed the experiments: H. Kishimoto MM RA H. Kimura AS. Analyzed the data: H. Kishimoto MB TT RMH. Contributed reagents/materials/analysis tools: H. Kimura TF RMH. Wrote the paper: H. Kishimoto TF RMH.

EXPERT OPINION

Advances in adenovirus-mediated p53 cancer gene therapy

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Introduction: The tumor suppressor *p53* gene regulates diverse cellular processes, such as cell-cycle arrest, senescence, apoptosis and autophagy, and it is frequently inactivated by genetic alterations in ~ 50% of all types of human cancers. To restore wild-type p53 function in p53-inactivated tumors, adenovirus-mediated p53 gene therapy has been developed as a promising antitumor strategy in preclinical experiments and clinical studies.

Areas covered: This review focuses on the clinical relevance of replication-deficient adenovirus vectors that carry the wild-type *p53* gene (Ad-p53; Advexin, Gendicine and SCH-58500) in clinical studies of patients with various cancers and the future perspectives regarding conditionally replicating adenovirus vectors expressing the wild-type *p53* gene (CRAd-p53; AdDelta24-p53, SG600-p53, OBP-702) in preclinical experiments. Moreover, the recent advances in our understanding of the molecular basis for the p53-mediated tumor suppression network induced by Ad-p53 and CRAd-p53 vectors and the combination therapies for promoting the therapeutic potential of adenovirus-mediated p53 gene therapy are discussed.

Expert opinion: Exploration of the molecular mechanism underlying the p53-mediated tumor suppression network and the effective strategy for enhancing the p53-mediated cell death signaling pathway would provide novel insights into the improvement of clinical outcome in p53-based cancer gene therapy.

Keywords: adenovirus, cancer, gene therapy, p53

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1. Introduction

Cancer gene therapy is expected to be a promising antitumor treatment for inducing cell death via introduction of a therapeutic tumor suppressor gene or the abrogation of an oncogene [1]. Among the potent therapeutic transgenes, the tumor suppressor gene *p53* encodes a multifunctional transcription factor that regulates diverse cellular processes, including cell-cycle arrest, senescence, apoptosis and autophagy, for tumor suppression [2]. Analyses of the IARC TP53 database (http://www-p53.iarc.fr/) [3] have shown that both epithelial and mesenchymal malignant tumors often harbor somatic mutations in the *p53* gene and that the types of *p53* mutations and the tumors that harbor them vary widely [4,5]. The *p53* gene is frequently inactivated by genetic alterations in ~ 50% of all types of human cancers. Patients with Li–Fraumeni syndrome, which is a cancer predisposition disorder, each carry a germline mutation in the *p53* gene, and they develop early onset tumors [6]. These findings suggest that the *p53* gene has potent and critical roles in the tumor suppression network. Moreover, tumor cells with impaired p53 function are often refractory to the genotoxic stresses induced by conventional chemoradiotherapy [7]. Thus, restoration of wild-type p53 function is a promising antitumor strategy because it could lead to suppression of tumor growth and progression.

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Article highlights.

- A replication-deficient p53-expressing adenovirus Ad-p53 vector has been recently evaluated in clinical studies for patients with various types of cancers.
- Intratumoral, intraperitoneal and intravesical administration of Ad-p53 vector were well tolerated as a monotherapy or in combination with conventional chemoradiotherapy.
- Suppression of p21/MDM2 expression enhances the p53-mediated cell death signaling pathway induced by Ad-p53 vector.
- A conditionally replicating p53-expressing adenovirus, CRAd-p53 vector, was developed to improve the therapeutic potential of p53-based cancer gene therapy.
- The E1A-dependent miRNA regulatory network is implicated in the fine-tuning of the p53-mediated cell death signaling pathway induced by CRAd-p53 vector.

This box summarizes key points contained in the article.

Gene replacement therapy to introduce the tumor suppressor *p53* gene is a promising antitumor strategy because ectopic expression of exogenous *p53* gene efficiently induces cell death in a variety of p53-inactivated tumor cells [8,9]. To induce exogenous expression of *p53* gene, a p53-expressing replication-deficient adenovirus vector (Ad-p53) is frequently used in preclinical *in vitro* and *in vivo* experiments for various cancers, including non-small-cell lung cancer (NSCLC) [10,11], head and neck cancer [12-14], malignant brain tumors [15-17], ovarian cancer [18,19], bladder cancer [20], cervical cancer [21], colorectal cancer [22] and esophageal cancer [23]. Recently, clinical studies of several Ad-p53 vectors, such as Advexin (INGN-201; Introgen Therapeutics, Inc.) [24], Gendicine (Shenzhen SiBiono GeneTech Co.) [25] and SCH-58500 (CANJI, Inc.) (**Figure 1A**) [26], have been conducted in patients with various cancers [27-29]. Gendicine is the first gene therapy product approved for clinical use in China [25]. In this review, we focus on the therapeutic potentials of Ad-p53 vectors in clinical studies and the future perspectives of a p53-expressing conditionally replicating adenovirus vector (CRAd-p53) in preclinical experiments. Additionally, we discuss the molecular mechanisms underlying the Ad-p53- and CRAd-p53-mediated tumor suppression networks to improve the clinical outcome of adenovirus-mediated p53 cancer gene therapy.

2. Clinical studies of replication-deficient Ad-p53 vector

Ad-p53-mediated gene therapy induces an extensive antitumor effect in tumor cells but has low cytotoxicity in normal cells in preclinical *in vitro* and *in vivo* experiments [12,13,30]. Ad-p53-mediated *p53* gene transfer also enhances the sensitivities to conventional chemotherapy and radiotherapy [11,31]. Recently, the Ad-p53 vectors, Advexin, Gendicine and SCH-58500 (**Figure 1A**) have been evaluated as a monotherapy or in combination with conventional chemotherapy or radiotherapy in clinical trials for patients with various cancers (**Table 1**).

2.1 Non-small-cell lung cancer

Lung cancer is the most common cause of cancer-related deaths worldwide [32]. The *p53* gene mutation occurs in ~ 40% of NSCLC tumors, and aberrant p53 expression correlates with poor prognosis in lung cancer patients [33]. Preclinical studies demonstrated that Ad-p53 vector efficiently induces p53 expression, the suppression of *in vivo* tumor growth and the enhancement of chemosensitivity in human NSCLC cells [10,11]. To determine the feasibility of Ad-p53 gene therapy in patients with NSCLC, Phase I clinical studies of two Ad-p53 vectors, SCH-58500 [34] and Advexin [35,36], have been conducted as a monotherapy (**Table 1**). A single intratumoral injection of SCH-58500 (1×10^7 to 1×10^{10} plaque-forming units [PFU]) was administered to 15 patients with advanced NSCLC [34]. In two other clinical studies, a total of 37 patients with advanced NSCLC received repeated intratumoral injections of Advexin (1×10^6 to 1×10^{11} PFU) [35,36]. Intratumoral injection of Ad-p53 was performed endobronchially by using a bronchoscope or percutaneously under computed tomography guidance. In the Ad-p53-treated tumors, the *p53* transgene expression was confirmed by quantitative reverse transcription-polymerase chain reaction (RT-PCR). There were no severe adverse events in Ad-p53-treated cancer patients, and intratumoral injection of Ad-p53 was found to be a safe and feasible treatment strategy for patients with NSCLC.

Combination therapy of Ad-p53 vectors (Advexin and SCH-58500) with chemotherapy or radiation has been assessed in Phase I/II clinical studies for patients with advanced NSCLC (**Table 1**). In Phase I clinical studies, intratumoral injection of Advexin (1×10^6 to 1×10^{11} PFU) in combination with intravenous administration of cisplatin (80 mg/m^2) was performed in 24 patients in the United States [37] and 6 patients in Japan [36]. In a Phase II clinical study, two chemotherapy protocols, (A) paclitaxel (175 mg/m^2) and carboplatin (targeted area under the curve of 6) or (B) cisplatin (100 mg/m^2) and vinorelbine (25 mg/m^2), were administered in combination with SCH-58500 (7.5×10^{12} virus particles [VP]) [38]. Thirteen patients with NSCLC received protocol (A) and 12 patients received protocol (B). Additionally, a Phase II clinical study of intratumoral injection of Advexin (1×10^{11} to 1×10^{12} VP) combined with radiation (60 Gy) was conducted in 19 patients with NSCLC [39]. In these clinical studies of combination therapies, the most common adverse events were only transient fevers, and some patients demonstrated tumor regression at the Ad-p53-treated tumors. Thus, combination therapy of Ad-p53 vector and conventional chemoradiotherapy is well tolerated and clinically beneficial in patients with advanced NSCLC.

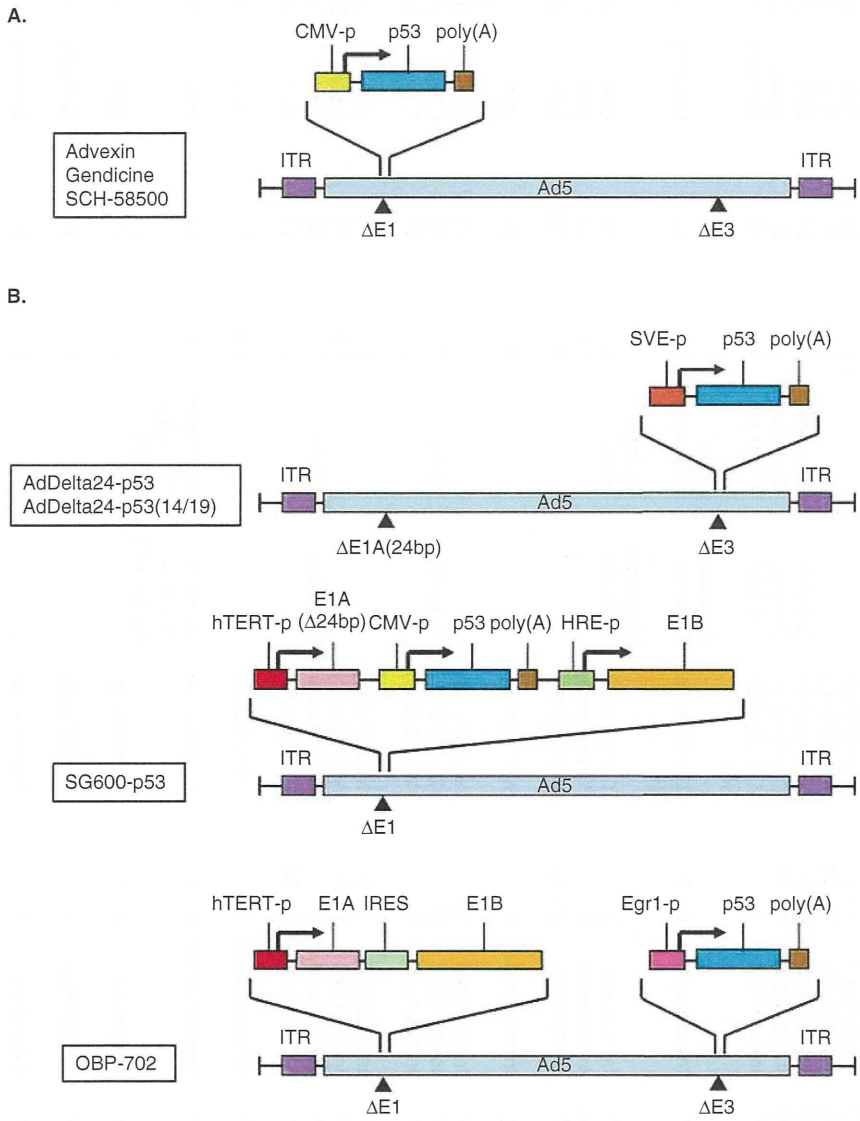

Figure 1. DNA structures of Ad-p53 and CRAd-p53 vectors. A. Ad-p53 vector (Advexin, Gendicine and SCH-58500) is a p53-expressing replication-deficient adenovirus; the *p53* gene expression cassette regulated under the cytomegalovirus promoter (CMV-p) is inserted into the E1 region and the E3 region is deleted. **B.** CRAd-p53 vector (AdDelta24-p53, AdDelta24-p53(14/19) and SG600-p53, OBP-702) is a p53-expressing conditionally replicating adenovirus. In AdDelta24-p53 and AdDelta24-p53(14/19), the RB protein-binding CR2 domain (24 base pairs) of the E1A region was deleted, and the p53 expression cassette under the regulation of simian virus 40 early promoter (SVE-p) was inserted into the E3 region. AdDelta24-p53 and AdDelta24-p53(14/19) vectors express wild-type p53 and MDM2-resistant p53 variant form, respectively. In SG600-p53, the *E1A* gene with a deletion of 24 nucleotides in the CR2 region is controlled under the hTERT-p and the *E1B* gene is regulated by the hypoxia response element promoter (HRE-p) and the *p53* gene cassette controlled by the CMV-p is inserted between the E1A and E1B regions. In OBP-702, the hTERT-p drives the expression of two adenoviral genes, *E1A* and *E1B*, that are linked to an internal ribosome entry site (IRES) and the *p53* gene cassette controlled by the Egr1 promoter (Egr1-p) is inserted into the E3 region.

Table 1. Clinical studies of p53 cancer gene therapy using Ad-p53 vector in patients with various types of cancers.

No.	Cancer type	Recombinant adenovirus	Viral dose	Unit	Method of injection	Number of injection	Therapy type	Combination therapy	Dose	Phase of study	Number of patient	Country	Years	Refs.
1	NSCLC	SCH-58500	$1 \times 10^7 - 1 \times 10^{10}$	PFU	Intratumoral	1	Monotherapy			I	15	Germany	1998	[34]
2	NSCLC	Advexin	$1 \times 10^6 - 1 \times 10^{11}$	PFU	Intratumoral	1 – 6	Monotherapy			I	28	USA	1999	[35]
3	NSCLC	Advexin	$1 \times 10^9 - 1 \times 10^{11}$	PFU	Intratumoral	1 – 14	Monotherapy			I	9	Japan	2006	[36]
4	NSCLC	Advexin	$1 \times 10^6 - 1 \times 10^{11}$	PFU	Intratumoral	1 – 6	Combination	Cisplatin	80 mg/m^2	I	24	USA	2000	[37]
5	NSCLC	Advexin	$1 \times 10^9 - 1 \times 10^{10}$	PFU	Intratumoral	1 – 10	Combination	Cisplatin	80 mg/m^2	I	6	Japan	2006	[36]
6	NSCLC	SCH-58500	7.5×10^{12}	VP	Intratumoral	1 – 3	Combination (A)	Paclitaxel Carboplatin	175 mg/m^2 6 AUC	II	13	Germany	2001	[38]
7	NSCLC	SCH-58500	7.5×10^{12}	VP	Intratumoral	1 – 3	Combination (B)	Cisplatin Vinorelbine	100 mg/m^2 25 mg/m^2	II	12	Germany	2001	[38]
8	NSCLC	Advexin	$1 \times 10^{11} - 1 \times 10^{12}$	VP	Intratumoral	3	Combination	Radiation	60 Gy	II	19	USA	2003	[39]
9	SCCHN	Advexin	$1 \times 10^6 - 1 \times 10^{11}$	PFU	Intratumoral	3	Monotherapy			I, II	33	USA	1998	[42,43]
10	SCCHN	Advexin	$5 \times 10^{10} - 2.5 \times 10^{12}$	VP	Intratumoral	3 – 12	Monotherapy			II	106	USA	2009, 2011	[44,45]
11	SCCHN	Advexin	2×10^{12}	VP	Intratumoral	6	Monotherapy			III	35	USA	2009, 2011	[44,45]
12	SCCHN	Gendicine	$1 \times 10^{10} - 1 \times 10^{12}$	VP	Intratumoral	10	Monotherapy			I	12	China	2005	[46]
13	SCCHN	Gendicine	1×10^{12}	VP	Intratumoral	24	Combination	Radiation	70 Gy	II	63	China	2005	[46]
14	Dysplastic OLK	Gendicine	1×10^8	VP	Intratumoral	5	Monotherapy			I	18	China	2009	[47]
15	Glioma	Advexin	$3 \times 10^{10} - 3 \times 10^{12}$	VP	Intratumoral	1 – 2	Monotherapy			I	15	USA	2003	[49]
16	ESCC	Advexin	$1 \times 10^{12} - 2.5 \times 10^{12}$	VP	Intratumoral	1 – 5	Monotherapy			I, II	10	Japan	2006	[52]
15	HCC	Gendicine	$1 \times 10^{12} - 3 \times 10^{12}$	VP	Intratumoral	2	Combination	Radiation	50 Gy	I	40	China	2010	[57]
17	Ovarian cancer	Advexin	$3 \times 10^{10} - 3 \times 10^{12}$	VP	Intraperitoneal	1 – 30	Monotherapy			I	17	USA	2004	[63]
18	Ovarian cancer	SCH-58500	$7.5 \times 10^{10} - 7.5 \times 10^{12}$	VP	Intraperitoneal	1	Monotherapy			I, II	17	USA	2002	[64,65]
19	Ovarian cancer	SCH-58500	$7.5 \times 10^{12} - 7.5 \times 10^{13}$	VP	Intraperitoneal	6 – 15	Combination	Cisplatin Paclitaxel Carboplatin	100 mg/m^2 175 mg/m^2 6 AUC	I, II	24	USA	2002	[64,65]
20	Bladder cancer	Advexin	$1 \times 10^{10} - 1 \times 10^{12}$	VP	Intravesical	2 – 8	Monotherapy			I	13	USA	2003	[68]
21	Bladder cancer	SCH-58500	7.5×10^{11}	VP	Intratumoral	1	Monotherapy			I	3	Germany	2002	[69]
22	Bladder cancer	SCH-58500	$7.5 \times 10^{11} - 7.5 \times 10^{13}$	VP	Intravesical	1	Monotherapy			I	9	Germany	2002	[69]

ESCC: Esophageal squamous cell carcinoma; HCC: Hepatocellular carcinoma; NSCLC: Non-small-cell lung cancer; OLK: Oral leukoplakia; PFU: Plaque-forming unit; SCCHN: Squamous cell carcinoma of the head and neck; VP: Virus particle.

2.2 Head and neck cancer

There are 139,000 new cases of head and neck cancer per year in European countries, and > 90% of head and neck cancers are squamous cell carcinoma (SCC) [40]. Despite surgical resection and postoperative radiation, SCC of the head and neck (SCCHN) often shows recurrence and distant metastasis [41]. Although postoperative chemotherapy in combination with radiotherapy improves the local recurrence rate, the frequencies of severe adverse effects (grades 4 and 5) are increased [41], thus indicating that novel antitumor therapies without severe adverse effects are needed for patients with SCCHN. In preclinical experiments, Ad-p53 vector induced profound apoptotic cell death in human SCCHN tumor cells [12-14]. Phase I/II/III clinical studies of monotherapy with two Ad-p53 vectors, Advexin and Gendicine, have been conducted in patients with advanced SCCHN (**Table 1**). In Phase I/II clinical studies, 33 patients with SCCHN received repeated intratumoral injections of Advexin (1×10^6 to 1×10^{11} PFU), and the safety and overall patient tolerance of Advexin were demonstrated [42,43]. In a Phase II clinical study, 106 patients with recurrent SCCHN received repeated intratumoral injections of Advexin (5×10^{10} to 2.5×10^{12} VP) [44,45]. In a Phase III randomized clinical study, 28 and 35 patients with SCCHN received intravenous methotrexate and Advexin, respectively. The *p53* gene sequence and p53 protein expression of tumor tissues were examined after treatment with Advexin or methotrexate, and there was a significant increase in time to progression and survival following Ad-p53 therapy in patients with favorable p53 profiles. In a Phase I clinical study, 10 patients with advanced SCCNH received repeated intratumoral injections of Gendicine (1×10^{10} to 1×10^{12} VP) and only transient fever was observed in one patient. In a Phase II clinical study, 63 SCCNH patients received Gendicine (1×10^{12} VP) combined with radiation (70 Gy), whereas 72 patients received radiation therapy alone [46]. The response rates (complete and partial responses) were significantly greater in the combination therapy group as compared to the radiation group. These findings suggest that intratumoral injection of Ad-p53 is a well-tolerated and effective antitumor therapy and that p53 genetic status in tumor tissues is a predictive biomarker of the response to Ad-p53 therapy in patients with advanced SCCHN. In a Phase I clinical study, repeated intraepithelial injection of Gendicine was well tolerated in patients with dysplastic oral leukoplakia, the most common precursor of oral SCC (**Table 1**) [47]; thus, premalignant tumors with the wild-type *p53* gene may be more sensitive to Ad-p53 therapy than p53-inactivated malignant tumors in SCCHN patients.

2.3 Glioma

Among malignant brain tumors, glioblastoma multiforme is the most common glioma in adults, and the frequency of *p53* gene mutation is 28% in primary glioblastomas and 65% in secondary glioblastomas [48]. In preclinical experiments, Ad-p53 vector efficiently suppressed the *in vitro* cell proliferation and *in vivo* tumor growth of human glioma cells through exogenous p53 activation and subsequent apoptosis induction [16,17]. In a Phase I clinical study of Advexin as a monotherapy (**Table 1**), 15 patients with recurrent gliomas received Advexin (3×10^{10} to 3×10^{12} VP) administered intratumorally or into the postresection cavity [49]. Although clinical toxicity was minimal, the distribution of Advexin in tumor cells was limited to the areas near the injection site. These findings suggest that intratumoral injection of Ad-p53 is well tolerated in glioma patients, but the widespread distribution of Ad-p53 vector is necessary for inducing profound antitumor effects in brain tumors.

2.4 Esophageal cancer

Esophageal cancer affects > 450,000 people worldwide, and esophageal SCC (ESCC) is a more frequent histological type than adenocarcinoma [50]. Despite advances in the treatment of esophageal cancer, the overall 5-year survival rate remains 15 to 25%. Approximately 50% of ESCCs possess the *p53* gene mutation [51], and a preclinical experiment demonstrated that Ad-p53 vector efficiently induces p53 expression and subsequent apoptotic cell death in human esophageal cancer cells [23]. A Phase I/II clinical study of Advexin as a monotherapy has been conducted in patients with chemoradiation-resistant advanced ESCC (**Table 1**) [52]. Ten patients received repeated intratumoral injection of Advexin (1×10^{12} to 2.5×10^{12} VP), and the Ad-p53 therapy was well tolerated in these patients. Expression of exogenous p53 gene transfer was confirmed in tumor tissues from all patients by PCR analysis. Of the 10 patients who received intratumoral injection of Ad-p53, 9 patients had stable disease and one patient had progressive disease. These findings suggest that intratumoral injection of Ad-p53 is safe and feasible in patients with advanced chemoradioresistant ESCC. Since Ad-p53 vector increases the radiosensitivity of human tumor cells via p53-mediated suppression of DNA repair machinery [53], combination therapy of Ad-p53 vector and radiation may be a more effective strategy than monotherapy with Ad-p53 in patients with advanced ESCC.

2.5 Liver cancer

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer, and the incidence of HCC is increasing due to the dissemination of hepatitis virus infection throughout the world [54]. The *p53* gene mutation was found in ~ 30 to 50% of HCC and was associated with poorly differentiated tumors [55,56]. Intratumoral injection of Ad-p53 (Gendicine) (1×10^{12} to 3×10^{12} VP) combined with fractionated stereotactic radiotherapy (50 Gy) was assessed in 40 HCC patients in a Phase I clinical study (**Table 1**) [57]. There were no severe adverse effects, and the intratumoral injection of Ad-p53 was well tolerated. However, since HCC patients frequently have liver dysfunction based on virus-induced hepatitis, caution is needed for the possibility of liver toxicity associated with Ad-p53-based gene therapy. The

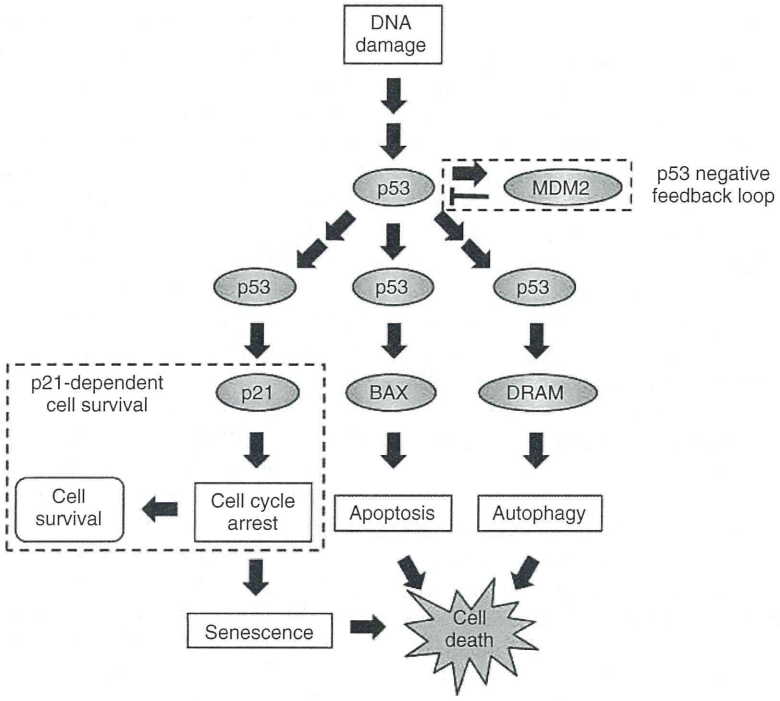

Figure 2. A schematic representation for p53-mediated tumor suppression network. DNA damage induces senescence, apoptosis or autophagy through the activation of three p53-target genes, p21, BAX or DRAM, respectively. Mild DNA damage induces a small amount of p53 activation, and therefore p21-dependent cell-cycle arrest, contributing to cell survival. In contrast, severe DNA damage induces a large amount of p53 accumulation and activation of three distinct cell death pathways – senescence, apoptosis and autophagy – resulting in cell death. In contrast, p53-induced MDM2 activation functions as a p53-negative feedback loop via ubiquitin-mediated p53 degradation.

incorporation of normal cell-specific microRNA (miRNA)-targeted sequences is a useful strategy for reducing the cytotoxic activity of adenovirus in normal cells including hepatocytes [58-60]. Therefore, novel Ad-p53 vector with miRNA-targeted sequences may be a safer approach for the treatment of patients with advanced HCC.

2.6 Ovarian cancer

Ovarian cancer is a major cause of gynecologic cancer in the United States; approximately 22,300 new cases and 15,500 deaths were predicted for 2012 [32]. The survival rate of patients with ovarian cancer has changed little, despite the development of platinum-based chemotherapy [61]; therefore, novel antitumor therapies to improve the clinical outcome of patients with advanced ovarian cancer are required. Approximately 80% of ovarian cancers possess *p53* gene mutation [62], and Ad-p53 vector induced an antitumor effect through p53 overexpression in human ovarian cancer cells in preclinical experiments [18,19]. Phase I clinical studies of two Ad-p53 vectors, Advexin and SCH-58500, have been conducted as a monotherapy in patients with chemotherapy-resistant and recurrent ovarian cancers, respectively (**Table 1**) [63-65]. Intraperitoneal injection of Advexin (3×10^{10} to 3×10^{12} VP) or SCH-58500 (7.5×10^{10} to 7.5×10^{12} VP) was performed in 17 patients each. In a Phase I/II clinical study, intraperitoneal injection of SCH-58500 (7.5×10^{12} to 7.5×10^{13} VP) was combined with chemotherapeutic drugs, such as intraperitoneally administered cisplatin (100 mg/m^2) or intravenously administered paclitaxel (175 mg/m^2) and carboplatin (targeted area under the curve of 6), in 24 patients with recurrent ovarian cancers (**Table 1**). No severe adverse effects occurred in patients treated with monotherapy or combination therapy. Thus, intraperitoneally administered Ad-p53 vector is well tolerated as a monotherapy and in combination with chemotherapy in patients with advanced ovarian cancers.

2.7 Bladder cancer

Bladder cancer is a major cancer of the urinary system with 73,510 new cases and 14,880 deaths estimated for 2012 in the United States [32]. Patients with noninvasive and invasive bladder cancers have a high recurrence rate (~ 80%); therefore, a novel strategy for efficiently curing malignant bladder tumors is required [66]. Patients with p53 abnormalities have a much higher probability of disease progression, and the p53 status is a predictive biomarker for tumor recurrence [67], suggesting the therapeutic potential of p53 restoration for suppression of

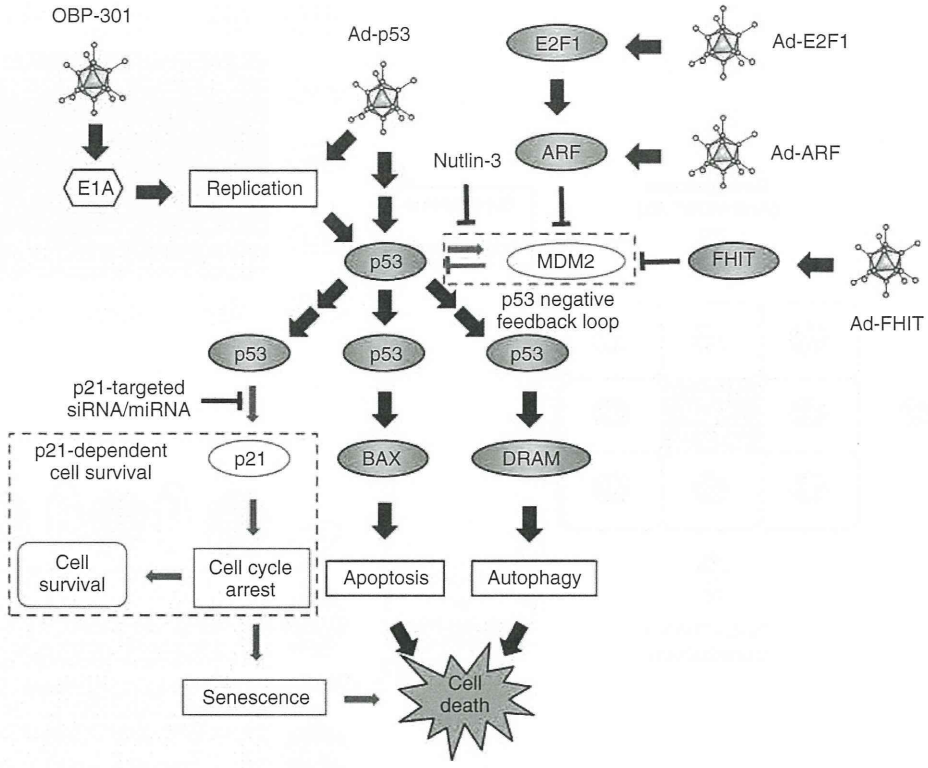

Figure 3. A schematic representation of Ad-p53-mediated induction of programmed cell death pathways and combination strategies for enhancing the therapeutic potential of Ad-p53 vector. Ad-p53 vector induces BAX- and DRAM-mediated apoptosis and autophagy, respectively, resulting in cell death, rather than p21-dependent cell-cycle arrest and cell survival, when combined with replication competent OBP-301, replication-deficient adenovirus vectors (Ad-E2F1, Ad-ARF and Ad-FHIT), chemical compound (Nutlin-3) or p21-targeted siRNA/miRNA.

tumor progression. In a preclinical experiment, Ad-p53 vector efficiently induced the p53 and p53-downstream target genes, the suppression of cell viability and the enhancement of chemosensitivity in human bladder cancer cells [20]. Phase I clinical studies of two Ad-p53 vectors, Advexin and SCH-58500, have been conducted as a monotherapy in patients with advanced bladder cancers [68,69]. Thirteen patients received intravesical injections of Advexin (1×10^{10} to 1×10^{12} VP), nine patients received intravesical injections of SCH-58500 (7.5×10^{11} to 7.5×10^{13} VP) and three patients received intratumoral injections of SCH-58500 (7.5×10^{11} VP). No dose-limiting toxicity was observed in these patients. Thus, in addition to intratumoral and intraperitoneal injections, intravesical injection is also a safe approach for the administration of Ad-p53 vector to cancer patients.

3. Molecular mechanism of antitumor effect induced by Ad-p53 vector

Administration of Ad-p53 vector efficiently induces exogenous expression of the *p53* gene and a subsequent antitumor effect in preclinical *in vitro* and *in vivo* experiments [8,9]. In the Ad-p53-mediated tumor suppression system, there are three cell death pathways: senescence, apoptosis and autophagy [2]. These cell death pathways are determined by the induction of p53-downstream target genes, such as $p21^{WAF1}$ (p21) [70], BAX [71] or DRAM (**Figure 2**) [72]. To promote the Ad-p53-mediated cell death pathways, there are combination strategies for enhancing viral replication and p53 expression in the Ad-p53-infected tumor cells (**Figure 3**). Moreover, Ad-p53 transduction has an antitumor effect in not only Ad-p53-infected cells but also uninfected tumor cells, because the exogenous p53 expression induces bystander effects, such as the inhibition of tumor angiogenesis and the infiltration of neutrophils, in tumor microenvironments (**Figure 4**). Next, we discuss the molecular mechanism underlying the Ad-p53-mediated antitumor effect via p53 overexpression.

3.1 p53-mediated cell death signaling pathway

When tumor cells with intact p53 function are exposed to genotoxic stresses including chemotherapy and therapeutic radiation, many p53-downstream target genes, such as

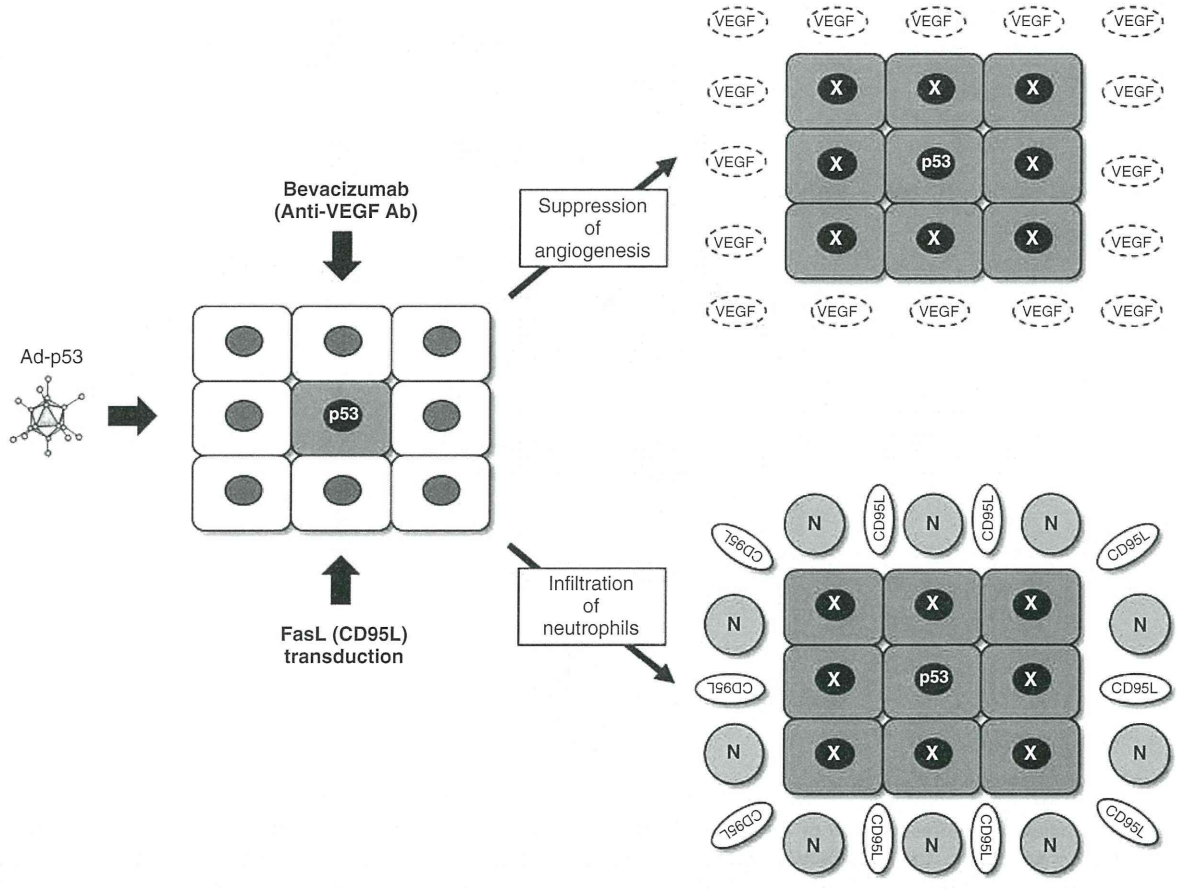

Figure 4. A schematic representation of Ad-p53-mediated bystander effects on neighboring, uninfected tumor cells. When tumor cells are infected with Ad-p53 vector, p53 overexpression induces cell death in the Ad-p53-infected tumor cells. Moreover, surrounding uninfected tumor cells are also eradicated due to bystander effects, which include suppression of angiogenesis by VEGF downregulation and massive infiltration of neutrophils by CD95 ligand (CD95L) upregulation in the tumor microenvironment. Combination therapy of bevacizumab (anti-VEGF antibody) and FasL (CD95L) transduction may enhance the Ad-p53-mediated bystander effects.

p21 [70], BAX [71] and DRAM [72], are transcriptionally induced by activated p53, and these p53 targets cooperatively regulate cellular processes that curb or reverse tumor progression [2]. In response to mild DNA damage, p53 mainly activates p21 expression for the induction of cell-cycle arrest that allows for the repair of DNA damage and consequently contributes to cell survival or senescence. In contrast, severe DNA damage induces more p53 accumulation, which activates BAX- and DRAM-related signaling pathways that lead to apoptosis and autophagy, respectively, and subsequently induces cell death (**Figure 2**). However, when the p53-downstream target gene MDM2 [73], which negatively regulates p53 via the ubiquitin–proteasome pathway, is upregulated following p53 activation, MDM2 activation inhibits the p53-mediated signaling pathway as a p53-negative feedback loop (**Figure 2**). Thus, suppression of the p21-dependent cell survival pathway and/or MDM2-dependent p53-negative feedback loop would be an effective strategy for the enhancement of apoptosis- and autophagy-related cell death pathways in p53-activated tumor cells.

Ad-p53-mediated exogenous wild-type p53 introduction also induces the expression of p53-downstream targets p21, BAX, DRAM and MDM2 in a variety of p53-inactivated tumor cells (**Figure 3**). There are several potential approaches by which Ad-p53-mediated p53 expression and cell death signaling pathway could be enhanced (**Figure 3**). First, Ad-p53-mediated p53 expression is enhanced if combined with E1A-expressing oncolytic adenovirus because Ad-p53 is an E1A-deleted replication-deficient adenovirus vector. We previously developed a telomerase-specific replication-competent oncolytic adenovirus, OBP-301 (Telomelysin), which induces tumor-selective oncolytic cell death in a telomerase-dependent manner [74-76]. A combination therapy of Ad-p53 and OBP-301 enhanced p53 expression; this combination

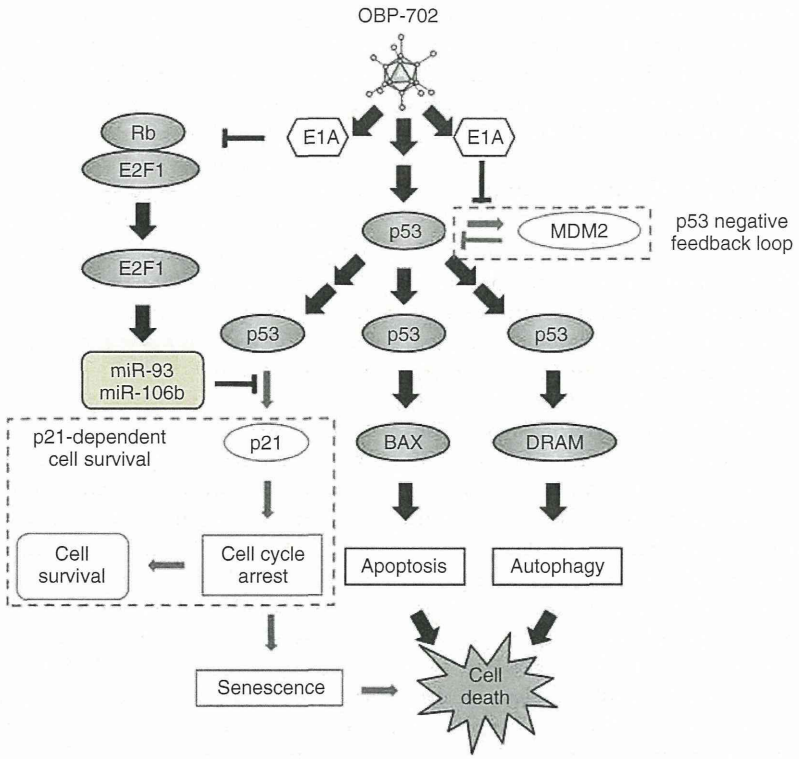

Figure 5. A schematic representation of molecular mechanism in the OBP-702-mediated induction of programmed cell death pathways. OBP-702 induces apoptosis and autophagy, resulting in cell death; these effects are dependent on p53-mediated BAX/DRAM activation and E1A-mediated suppression of p21/MDM2 expression via E2F1-inducible miRNA activation.

therapy resulted in a more profound antitumor effect and enhanced apoptotic cell death when compared to monotherapy with Ad-p53 [77]. Adenoviral E1A expression induced by OBP-301 infection would support the replication of Ad-p53 and subsequently enhance the Ad-p53-mediated p53 expression. Second, the Ad-p53-mediated cell death signaling pathway is enhanced if combined with a MDM2 inhibitor because MDM2 functions as a negative regulator of p53 via ubiquitin-mediated p53 degradation, and MDM2 suppression can stabilize exogenous p53 expression. Treatment with a small molecule compound, Nutlin-3 [78], or infection with the tumor suppressor *FHIT* gene [31] enhances Ad-p53-mediated p53 expression and apoptotic cell death through MDM2 suppression in human cancer cells. Moreover, overexpression of the *ARF* gene introduced via a recombinant adenovirus vector, Ad-ARF [79] or Ad-E2F1 [80], also induces enhanced p53 expression and antitumor effects induced by Ad-p53 through ARF-induced MDM2 suppression. Third, Ad-p53-mediated cell death in tumor cells could be enhanced via p21 suppression. Suppression of p21 expression by genetic deletion [81] or an exogenous p21-targeted small interfering RNA (siRNA) [82] enhances Ad-p53-induced apoptosis. More interestingly, a combination of p21-targeted miRNAs, *miR-93* and *miR-106b*, also enhances Ad-p53-mediated apoptosis and autophagy [83] because p21 suppresses apoptosis [81] and autophagy [84]. Each of these three strategies for enhancing the Ad-p53-mediated cell death pathway should improve the clinical outcomes of adenoviral p53 cancer gene therapy.

3.2 Ad-p53-mediated bystander effect

Ad-p53-mediated *p53* gene transfer has been shown to induce bystander effects to neighboring tumor cells via multiple mechanisms in preclinical *in vivo* settings (**Figure 4**). For example, Ad-p53 infection markedly inhibited the expression of an angiogenic factor and vascular endothelial growth factor (VEGF) and increased the expression of an antiangiogenic factor; together, the effects of p53 expression suppress neovascularization in tumor tissues [85,86]. Additionally, Ad-p53-mediated *p53* transfer induced overexpression of CD95 ligand (CD95L, FasL) in tumor cells, which resulted in the massive infiltration of neutrophils into tumor tissues [87]. Overexpression of CD95L was also partially responsible for the Ad-p53-induced apoptosis that was mediated by the Fas receptor/ligand system [88]. These findings suggest that Ad-p53-mediated p53 overexpression is a promising antitumor therapy that has antitumor effects because angiogenesis is suppressed and immune responses are involved within