malignant tumors [17], whereas telomerase is absent in most normal somatic tissues [18], with a few exceptions including peripheral blood leukocytes and certain stem cell population [19, 20]. There is also a gradient increase in telomerase activity between early and late stage tumors. The strong association between telomerase activity and malignant tissue suggests that telomerase can be a plausible target for the treatment of cancer [21].

The enzyme telomerase is a ribonucleoprotein complex responsible for the addition of TTAGGG repeats to the telomeric ends of chromosomes, and contains three components: the RNA subunit (known as hTR, hTER, or hTERC) [22], the telomerase-associated protein (hTEP1) [23], and the catalytic subunit (hTERT) [24, 25]. Both hTR and hTERT are required for the reconstitution of telomerase activity *in vitro* [26] and, therefore, represent the minimal catalytic core of telomerase in humans [27]. However, while hTR is widely expressed in embryonic and somatic tissues, hTERT is tightly regulated and is not detectable in most somatic cells. Thus, the hTERT promoter region can be used as a fine-tuning molecular switch that works exclusively in tumor cells.

TELOMERASE-SPECIFIC ONCOLYTIC ADENOVIRUS FOR CANCER THERAPEUTICS

Structure of hTERT Promoter-Driven Oncolytic Adenovirus

The use of modified adenoviruses that replicate and complete their lytic cycle preferentially in cancer cells is a promising strategy for treatment of cancer. One approach to achieve tumor specificity of viral replication is based on the transcriptional control of genes that are critical for virus replication such as *E1A* or *E4*. As described above, telomerase, especially its catalytic subunit hTERT, is expressed in the majority of human cancers and the hTERT promoter is preferentially activated in human cancer cells [17]. Thus, the broadly applicable hTERT promoter might be a suitable regulator of adenoviral replication. Indeed, it has been reported previously that the transcriptional control of E1A expression via the hTERT promoter could restrict adenoviral replication to telomerase-positive tumor cells and efficiently lyse tumor cells [28-31].

The adenovirus *E1B* gene is expressed early in viral infection and its gene product inhibits E1A-induced p53-dependent apoptosis, which in turn promotes the cytoplasmic accumulation of late viral mRNA, leading to a shut down of host cell protein synthesis. In most vectors that replicate under the transcriptional control of the *E1A* gene including hTERT-specific oncolytic adenoviruses, the *E1B* gene is driven by the endogenous adenovirus E1B promoter. However, Li *et al.* [32] have demonstrated that transcriptional control of both *E1A* and *E1B* genes by the α- fetoprotein (AFP) promoter with the use of IRES significantly improved the specificity and the therapeutic index in hepatocellular carcinoma cells. Based on the above information, we developed OBP-301 (Telomelysin), in which the tumor-specific hTERT promoter regulates both the *E1A* and *E1B* genes Fig. (**1**). OBP-301 is expected to control viral replication more stringently, thereby providing better therapeutic effects in tumor cells as well as attenuated toxicity in normal tissues [14].

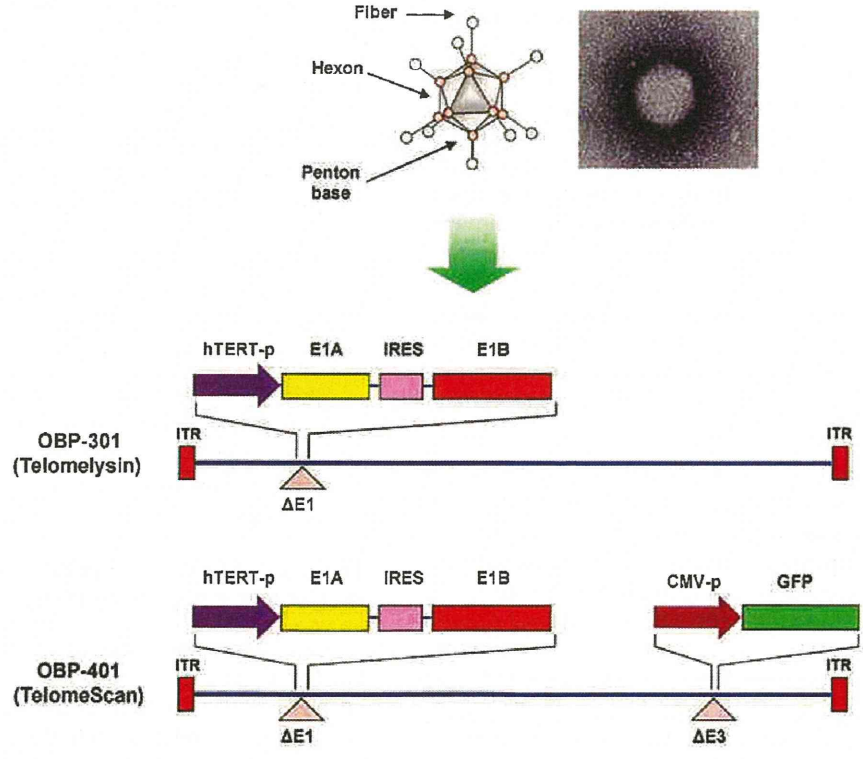

Fig. (1). Structures of telomerase-specific oncolytic adenovirus. OBP-301, in which the hTERT promoter element drives the expression of *E1A* and *E1B* genes linked with an IRES. OBP-401 (TelomeScan) is a telomerase-specific replication-competent adenovirus variant, in which *GFP* gene is inserted under cytomegalovirus (CMV) promoter into E3 region for monitoring viral replication. *Upper panel*, schematic representation depicting major structural components of OBP-301 (hexon, penton base, and fiber) and transmission electron microscopy image.

Preclinical Studies of hTERT Promoter-Driven Oncolytic Adenovirus

The majority of human cancer cells acquire immortality and unregulated proliferation by expression of the hTERT [17] and, therefore theoretically, hTERT-specific OBP-301 can possess a broad-spectrum antineoplastic activity against a variety of human tumors [14, 16]. OBP-301 induced selective E1A and E1B expression in cancer cells, which resulted in viral replication at 5-6 logs by 3 days after infection; on the other hand, OBP-301 replication was attenuated up to 2 logs in cultured normal cells [14, 16]. *In vitro* cytotoxicity assays demonstrated that OBP-301 could efficiently kill various types of human cancer cell lines including head and neck cancer, lung cancer, esophageal cancer, gastric cancer, colorectal cancer, breast cancer, pancreas cancer, hepatic cancer, prostate cancer, cervical cancer, melanoma, sarcoma, and mesothelioma in a dose-dependent manner [33]. These data clearly demonstrate that OBP-301 exhibits desirable features for use as an oncolytic therapeutic agent, as the proportion of cancers potentially treatable by OBP-301 is extremely high.

The *in vivo* antitumor effect of OBP-301 was also investigated by using athymic mice carrying xenografts. Intratumoral injection of OBP-301 into human tumor xenografts resulted in a significant inhibition of tumor growth and enhancement of survival [14, 16]. Macroscopically, massive ulceration was noted on the tumor surface after injection of high-dose OBP-301, indicating that OBP-301 induced intratumoral necrosis due to direct lysis of tumor cells by virus replication *in vivo* [34]. Head and neck cancer is characterized by locoregional spread, and it is clinically accessible, making it an attractive target for intratumoral virotherapy. Thus, an orthotopic nude mouse model of human tongue squamous cell carcinoma was also used to explore the *in vivo* antitumor effect of OBP-301. Intratumoral injection of OBP-301 significantly shrunk the tongue tumor volumes, which in turn increased the body weight of mice by enabling oral ingestion [35]. Since the body weight loss due to a feeding problem in this orthotopic tongue cancer model resembles the disease progression in head and neck cancer patients, the finding that OBP-301 increased the body weight of mice suggests that telomerase-specific virotherapy could potentially improve the quality of life in advanced head and neck cancer patients [36].

Clinical Application of Telomerase-Specific Oncolytic Adenovirus

Preclinical models suggested that OBP-301 could selectively kill a variety of human cancer cells *in vitro* and *in vivo* via intracellular viral replication regulated by the hTERT transcriptional activity. Pharmacological and toxicological studies ranging from 10^6 to 10^{12} virus particles (VP) in mice and cotton rats demonstrated that none of the animals treated with OBP-301 showed signs of viral distress (e.g., ruffled fur, weight loss, lethargy, or agitation) or histopathological changes in any organs at autopsy. These promising data led us to design a phase I clinical trial of OBP-301 as a monotherapy.

The protocol "A phase I dose-escalation study of intratumoral injection with telomerase-specific replication-competent oncolytic adenovirus, Telomelysin (OBP-301) for various solid tumors" sponsored by Oncolys BioPharma, Inc. is an open-label, phase I, 3 cohort dose-escalation study [37]. The trial commenced following approval of the US Food and Drug Administration (FDA) in October, 2006. The study has completed to assess the safety, tolerability, and feasibility of intratumoral injection of the agent in patients with advanced solid cancer. The doses of OBP-301 were escalated from low to high VP in one log increment. Patients were treated with a single dose intratumoral injection of OBP-301 and then monitored over one month.

All patients received OBP-301 without dose-limiting toxicity. Additionally, it was demonstrated tat circulating OBP-301 viral genome became detectable in the plasma within 24 hours after injection in 13 of 16 patients. This dose-dependent initial peak in circulating virus was followed by a rapid decline; however, 3 patients showed a second peak of circulating viral DNA on days 7 and 14, suggesting OBP-301 replication in primary tumors. Clinical trials of CG7870, a replication-selective oncolytic adenovirus genetically engineered to replicate preferentially in prostate tissue, also demonstrated a second peak of the virus genome in the plasma [38,39], suggesting similar active viral replication and shedding into the bloodstream. One of the 3 "second peak" patients also had disappearance of the injected malignant lesion and loco-regional un-injected satellite nodules, fulfilling a definition of complete response at day 28. Seven patients fulfilled RECIST definition for stable disease day 56 after treatment. Thus, OBP-301 is well-tolerated and warrants further clinical studies for solid cancer.

SYNERGISTIC INTERACTION OF ONCOLYTIC VIROTHERAPY AND CHEMOTHERAPEUTIC AGENTS

Preclinical studies provided experimental evidence for effectively killing of cancer cells by oncolytic viruses [40-42]. In animal models, however, established xenograft tumors are rarely eliminated despite existence of persistently high viral titers within the tumor. Total elimination of solid tumor requires higher doses of oncolytic viruses, which might be toxic or lethal. The efficacy of virotherapy combined with anticancer drugs has been reported previously in preclinical studies. A replication-selective adenovirus, ONYX-015 combined with 5-fluorouracil or CDDP produced greater effect than each individual modality and prolonged survival [43, 44]. Furthermore, synergistic efficacy was also observed in the combination of a tumor-specific HSV mutant (HSV-1716) with chemotherapeutic agents in human non-small cell lung cancer [45].

Most of the clinical trials for oncolytic viruses have been also conducted in combination with chemotherapy or radiotherapy [46-49]. Disappointingly, a clinical trial of Onyx-015 showed no clinical benefit in the majority of patients, despite the encouraging biological activity [50]. Tumor progression was rapid in most patients, even though substantial necrosis was noted in the tumors after treatment [51, 52]. Therefore, multi-disciplinary therapy composed of oncolytic virotherapy combined with low-dose chemotherapeutic agent may provide capacity to enhance the antitumor efficacy. Moreover, the combination of two agents may allow the use of reduced dosage of each agent, and lessen the likelihood of adverse effects.

Enhanced Antitumor Efficacy of Telomerase-Selective Oncolytic Adenovirus with Docetaxel

Taxanes are novel antimicrotubule agents that promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network, which is essential for vital interphase and mitotic cellular functions, resulting in cell arrest in G2 and M phases [53, 54]. Apoptotic cell death is induced subsequently, but this does not inhibit DNA synthesis of host cells. Therefore, taxanes are promising for combination with virotherapy. It was reported previously that paclitaxel had a synergistic or an additive effect in several cancer models when combined with adenovirus vector-mediated p53 gene therapy [55]. It has been also demonstrated that CV787, a prostate cancer-specific adenovirus, exhibited synergistic antitumor effect when combined with taxanes [56].

Infection with OBP-401 (GFP-expressing OBP-301 was used as an alternative to OBP-301 in some experiments) alone or followed by treatment with docetaxel (Taxotere), a chemotherapeutic agent, resulted in a profound *in vitro* cytotoxicity in various human cancer cell lines originating from different organs (lung, colon, esophagus, stomach, liver, and prostate), although the magnitude of the antitumor effect varied among the cell types Fig. (**2**) [57]. Quantitative real-time PCR analysis demonstrated that docetaxel did not affect viral replication. For *in vivo* evaluation, mice xenografted with human lung tumor received intratumoral injection of OBP-301 and intraperitoneal administration of docetaxel. Analysis of growth of implanted tumors showed a significant, therapeutic synergism, while each treatment alone showed modest inhibition of tumor growth [57]. The antitumor effect of the combination therapy was likely additive *in vitro*; there might be, however, some particular interactions between OBP-301 and docetaxel to produce a synergistic effect *in vivo*. It has been reported that metronomic chemotherapy, which refers to long-term administration of comparatively low doses of cytotoxic drugs at close, regular intervals, has an antiangiogenic basis [58]. Like our approach, the potent antiangiogenic properties of drugs administered in a metronomic fashion find favor in a number of *in vivo* preclinical studies; to prove this efficacy by *in vitro* experiments is, however, technically difficult. There are some possible explanations for the superior *in vivo* antitumor activity in our experiments. Systemically administered docetaxel may attack the vascular endothelial cells at the tumor site, which in turn can block the escape of locally injected OBP-301 into the peripheral circulation. Another possibility is that OBP-301 itself may inhibit the vascular supply by killing endothelial cells.

Enhanced Antitumor Efficacy of Telomerase-Selective Oncolytic Adenovirus with Histon Deacetylase Inhibitor

FR901228 (depsipeptide, FK228) is a novel anticancer agent isolated from the fermentation broth of *Chromobacterium violaceum*. FR901228 has been identified as a potent histone deacetylase (HDAC) inhibitor. Histone deacetylation is an important component of transcriptional control, and FR901228 increases Coxsackie's-adenovirus receptor (CAR) gene expression in various cancer cell lines [59-62]. Moreover, FR901228 is known to increase viral and transgene expression following adenovirus infection [59]. Indeed, FR901228 treatment upregulated CAR levels on target tumor cells, which in turn increased the amount of cellular OBP-301 replication, thereby promoting a synergistic antitumor effect [63]. These data indicate that FR901228 may be an appropriate partner for OBP-301 because it does not affect the virus life cycle. Two phase I clinical trials involving ad-

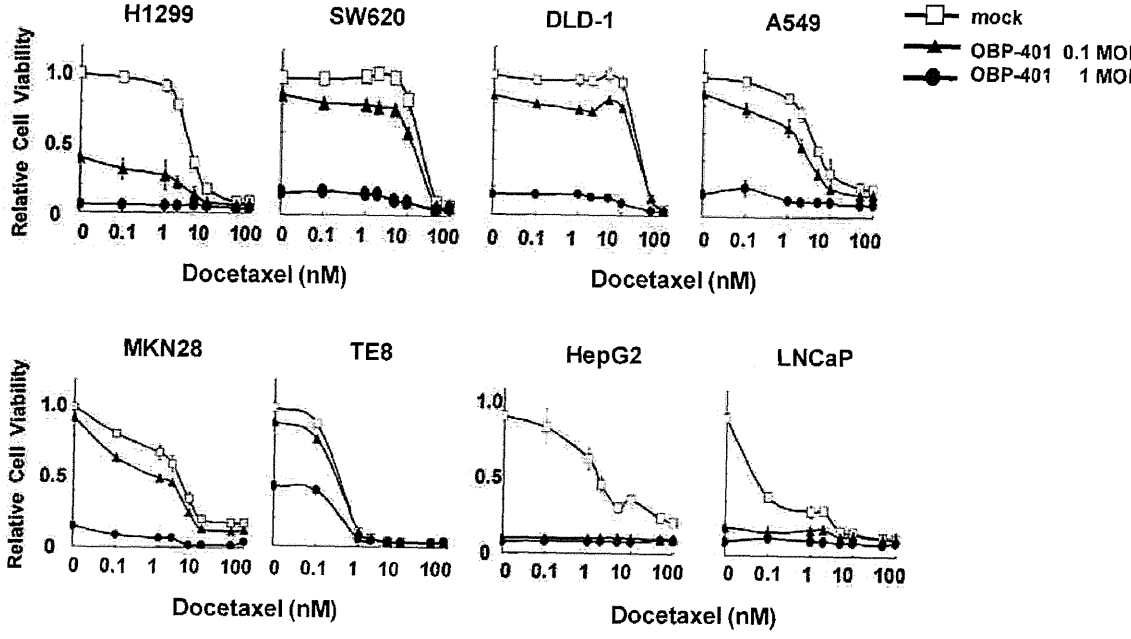

Fig. (2). Combination effect of OBP-401 and docetaxel on human cancer cell lines. Cells were infected with 0.1 or 1 MOI of OBP-401, and then exposed to docetaxel at the indicated concentrations at 24 hours after infection. Cell viability was assessed by XTT assay at 5 days after OBP-401 infection. The results of H1299 (lung), SW620 (colon), DLD-1 (colon), A549 (lung), MKN28 (stomach), TE8 (esophagus), HepG2 (liver), and LNCaP (prostate) cells were shown as representative of 10 cell lines. Bars, standard deviation (SD).

vanced cancer and leukemia have shown that FR901228 can be safely administered without life-threatening toxicity, including cardiac toxicity, although the appropriate administration schedule has to be further examined [64, 65].

Enhanced Antitumor Efficacy of Telomerase-Selective Oncolytic Adenovirus with Gemcitabine

Gemcitabine (2,2-difluorodeoxycytidine) is a third-generation agent that has been developed in the past decades. Gemcitabine is a deoxycytidine analogue that has shown efficacy as a treatment for many solid tumors and is now extensively used in the treatment of patients with various tumor types [66, 67], but inherent and acquired resistance has resulted in low response rates. Adenovirus therapy combined with gemcitabine has been reported in the treatment of pancreatic cancer. Halloran *et al.* reported that incubation of Panc-1 cells with either 5-FU or gemcitabine followed by adenovirus-mediated overexpression of $p16^{INK4A}$ resulted in a substantial reduction in cell viability under conditions where the drugs alone had minimal cytotoxicity [68]. It has been also reported that the type 5 adenoviral E1A sensitizes hepatocellular carcinoma cells to gemcitabine [69]. These observations support the notion that oncolytic adenoviruses combined with gemcitabine is a rational modality for the treatment of human cancer.

The antitumor efficacy of OBP-301 was found to be enhanced when combined with gemcitabine in human lung cancer cells *in vitro* and *in vivo* [70]. Gemcitabine is a deoxycytidine analogue and the incorporation of gemcitabine triphosphate into DNA causes chain termination, which is the major mechanism underlying the cytotoxicity of gemcitabine [71]. Although there was concern over whether gemcitabine would interrupt the viral replication of OBP-301, quantitative real-time PCR analysis showed that intracellular replication of OBP-301 was not affected by gemcitabine. The cytotoxic mechanisms of OBP-301 are distinct from those of gemcitabine and, therefore, combination effects could be observed provided that gemcitabine does not inhibit viral replication.

It has been reported that many DNA viruses can drive quiescent cells through G1 into S phase by the expression of viral proteins [72-74]. During the early phase of the adenovirus infection, the host cell is transformed into an efficient producer of the viral genome. The first gene that is transcribed in the viral genome is E1A, which can bind to numerous cellular proteins and acts as a multi-functional protein. Our data demonstrated that OBP-301 infection increases the phosphorylation of Akt, as well as E2F-1 expression Fig. (**3**). These effects are thought to be due to adenoviral E1A protein expression, as the dl312 adenovirus lacking the E1 genes did not phosphorylate Akt. As cells progress into the cell cycle, cyclin-dependent kinases phosphorylate retinoblastoma (Rb), freeing E2F and allowing it to directly transactivate genes required for S phase entry [75]. In fact, replication-deficient adenovirus-mediated *E2F-1* gene transfer into human cancer cells resulted in accumulation of an S-phase cell population. Thus, OBP-301 infection expressed E1A protein, which in turn upregulated the expression of phosphorylated Akt and E2F-1, leading to cell cycle promotion and S phase entry presumably by the deactivation of Rb (Table **1**). The accumulation of the tumor cells in S phase increases the cytotoxicity of gemcitabine, which kills cells in S phase.

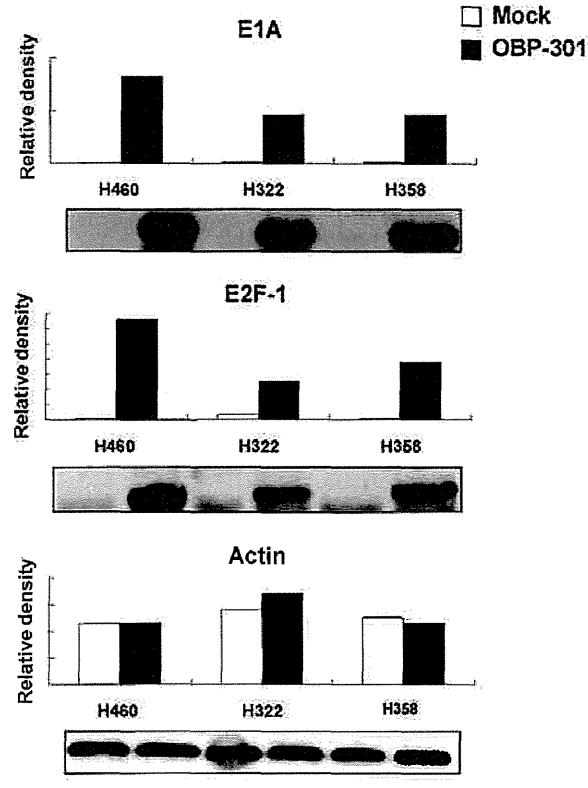

Fig. (3). Changes in cell cycle regulator protein expression following OBP-301 infection. H460, H322, and H358 cells were either mock-infected or infected with OBP-301 at an MOI of 40, 100 and 80 MOI, respectively. Following the removal of virus inocula, cells were collected at 24 h after infection, and were subjected to analysis. Equivalent amounts of protein obtained from whole cell lysates were loaded into each lane, probed with primary antibodies, and then visualized using an ECL detection system. Equal loading of samples was confirmed by reprobing with anti-actin antiserum. Protein expression was quantified by densitometric scanning using NIH Image software. Expression levels of adenoviral E1A and E2F1 greatly increased after OBP-301 infection as compared with the mock-infected controls in all three cell lines.

CONCLUSIONS AND PERSPECTIVES

There have been very impressive advances in our understanding of the molecular aspects of human cancer and in the development of technologies for genetic modification of viral genomes. Transcriptional targeting is a powerful tool for tumor selectivity in cancer therapy and diagnosis, and the hTERT-specific oncolytic adenovirus achieves a more strict targeting potential due to the amplified effect by viral replication. Several independent studies that used different regions of the hTERT promoter and different sites of adenoviral genome responsible for viral replication, have shown that the hTERT promoter allows adenoviral replication as a molecular switch and induces selective cytopathic effect in a variety of human tumor cells [14, 16, 28-30, 33]. Among these viral constructs, to the best of our knowledge, OBP-301 seems to be the first hTERT-dependent oncolytic adeno-

Table 1. Cell Cycle Analysis after OBP-301 Infection in Human Lung Cancer Cells

Cell Lines	Treatment	Cell Cycle		
		G1 (1%)	S (%)	G2 (%)
H460	Mock	43.54	43.85	8.61
	OBP-301	10.91	56.41	32.54
H322	Mock	40	46.72	10.85
	OBP-301	27.49	67.09	3.23
H358	Mock	45.89	38.22	14.29
	OBP-301	28.93	57.67	11.45

H460, H322 and H358 cell lines were treated with OBP-301 at 40 MOI, 100 MOI, and 80 MOI, respectively. Cells were then subjected to cell cycle analysis at 24 h after treatment by the FACS method. The percentages of cells in the G1, S and G2 phases are shown. The number of cells in S phase increased as compared with mock0infected cells after OBP-301 infection in all cell lines tested, although there was no increase in the sub-G0/G1 population indicating apoptotic cell death.

virus that has been used in a clinical trial based on preclinical pharmacological and toxicological studies. Thus, telomerase-specific targeted oncolytic adenovirus holds promise for the treatment of human cancer.

A future direction for OBP-301 includes combination therapy with conventional therapies such as chemotherapy, radiotherapy, surgery, immunotherapy, and new modalities such as antiangiogenic therapy. This review emphasized the synergistic interaction of OBP-301 with various types of chemotherapeutic agents. Since clinical activities observed by intratumoral injection of OBP-301 suggest that even partial elimination of the tumor could be clinically beneficial, the combination approaches may lead to the development of more advanced biological therapy for human cancer. We recently confirmed that OBP-301 infection and ionizing radiation mutually modulate their respective biological effects and thereby potentiate each other, profoundly enhancing *in vivo* antitumor activity [76]. Moreover, we demonstrated that preoperative delivery of OBP-301 into primary tumors prevented the exacerbation of lymph node metastasis by surgical procedures [77], suggesting that OBP-301 may be also valuable as adjuvant therapy in areas of microscopic disease to prevent recurrence or regrowth of tumors.

The field of targeted oncolytic virotherapy is progressing considerably and is rapidly gaining medical and scientific acceptance. Delineating specific virus/drug combinations tailored to be particularly effective in human cancer could potentially improve the already encouraging results seen in the field of oncolytic virotherapy.

CONFLICT OF INTEREST

None declared.

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Review

Ataxia-Telangiectasia Mutated and the Mre11-Rad50-NBS1 Complex: Promising Targets for Radiosensitization

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Radiotherapy plays a central part in cancer treatment, and use of radiosensitizing agents can greatly enhance this modality. Although studies have shown that several chemotherapeutic agents have the potential to increase the radiosensitivity of tumor cells, investigators have also studied a number of molecularly targeted agents as radiosensitizers in clinical trials based on reasonably promising preclinical data. Recent intense research into the DNA damage-signaling pathway revealed that ataxia-telangiectasia mutated (ATM) and the Mre11-Rad50-NBS1 (MRN) complex play central roles in DNA repair and cell cycle checkpoints and that these molecules are promising targets for radiosensitization. Researchers recently developed three ATM inhibitors (KU-55933, CGK733, and CP466722) and an MRN complex inhibitor (mirin) and showed that they have great potential as radiosensitizers of tumors in preclinical studies. Additionally, we showed that a telomerase-dependent oncolytic adenovirus that we developed (OBP-301 [telomelysin]) produces profound radiosensitizing effects by inhibiting the MRN complex via the adenoviral E1B55kDa protein. A recent Phase I trial in the United States determined that telomelysin was safe and well tolerated in humans, and this agent is about to be tested in combination with radiotherapy in a clinical trial based on intriguing preclinical data demonstrating that telomelysin and ionizing radiation can potentiate each other. In this review, we highlight the great potential of ATM and MRN complex inhibitors, including telomelysin, as radiosensitizing agents.

Key words: ATM (ataxia-telangiectasia mutated), MRN (Mre11-Rad50-NBS1) complex, radiosensitization, adenovirus, E1B55kDa

Radiotherapy is one of the standard treatment options for various malignant cancers and is often combined with surgical resection and/or chemotherapy as a part of multidisciplinary treatment. More than 50% of patients with cancer receive radiotherapy at some point during their treatment process [1]. Like surgical resection, radiotherapy is a local treatment, and it often targets not only primary tumors but also regional lymph nodes. One of the advantages of radiotherapy over surgical resection is that it is less invasive; for that reason, radiotherapy contributes significantly to treatment of cancers in areas of the body in which resection could greatly impair quality of life, such as the esophagus and the head and neck. Although the systemic side effects of radiotherapy are much less severe than those of chemotherapy, radiotherapy sometimes causes severe local adverse effects such as radiodermatitis, because normal tissues adjacent to tumors are usually included in the radiation

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fields. Although both stereotactic and fractionated radiotherapy have contributed to the improvement of irradiation methods in clinical practice, radiotherapy still has plenty of room for improvement [2, 3].

Hypoxia is one of the major limitations of radiotherapy, and researchers have made many attempts to improve it, such as through oxygenation, blood transfusion, and treatment with erythropoietin [4-6]. Although the oxygen level in a tumor is one of the most important factors in its response to radiotherapy, improving the local tumor control and survival rates for radiotherapy using pretreatment oxygenation is controversial. In one study, correction of tumor hypoxia significantly improved the locoregional tumor control and overall survival rates after radiotherapy for head and neck cancer, but was less effective for other types of cancer [7]. Although the rationale for intratumoral oxygenation before radiotherapy appears to be convincing, oxygenation alone does not improve radiotherapy sufficiently.

Many studies have been conducted in an attempt to improve radiotherapy, with much of the work being based on either of 2 hypotheses (Fig. 1). The first is that radiosensitizing agents should increase the cytotoxic effects of radiation on cancer cells by increasing the cells' radiosensitivity. The second is that radioprotective agents should decrease the adverse effects of radiation on normal cells by increasing their radioresistance. In this review, we describe several chemotherapeutic and molecularly targeted agents that have displayed radiosensitizing effects in preclinical and/or clinical studies and then focus on the potential of inhibitors of ataxia-telangiectasia (A-T) mutated (ATM) and the Mre11-Rad50-Nijmegen breakage syndrome (NBS) 1 (MRN) complex as radiosensitizing agents. Furthermore, we highlight the great potential of OBP-301 (telomelysin), a telomerase-dependent oncolytic adenovirus that we developed, as an MRN complex inhibitor.

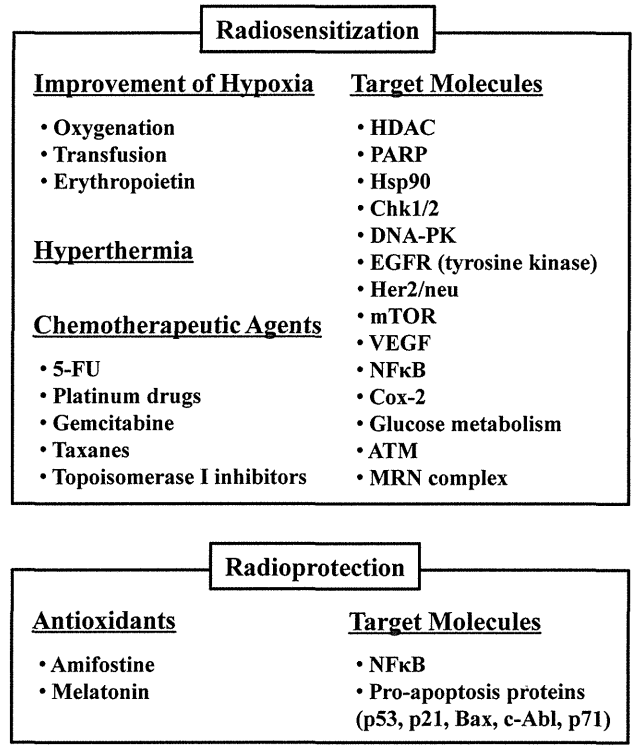

Fig. 1 Approaches to improvement of radiotherapy. Radiosensitizing agents are designed to increase the cytotoxic effects of radiation on cancer cells, and radioprotective agents are designed to decrease the adverse effects of radiation on normal cells. Hsp90, heat shock protein 90; NF-κB, nuclear factor-κB; COX-2, cyclooxygenase-2.

DNA Double-Strand Break Response: DNA Repair and Cell Cycle Checkpoints

Following DNA double strand-breaks (DSBs) induced by ionizing radiation, DNA repair and cell cycle checkpoints are the main mechanisms of maintenance of genomic stability [8]. Cells have several checkpoints that function at various phases of the cell cycle. Specifically, the G1/S and intra-S checkpoints prevent inappropriate DNA replication, whereas the G2/M checkpoint prevents cells with DNA damage from entering mitosis. When these checkpoints detect DNA damage at each phase, they induce cell cycle arrest and make time for repair of DNA damage. ATM plays a central role in the DNA damage response pathway by controlling the checkpoints via effector proteins such as Chk1, Chk2, p53 and BRCA1.

Homologous recombination (HR) and nonhomologous end joining (NHEJ) are major DNA DSB repair pathways, and cells use them according to the phase of the cell cycle and condition of the DSB ends [9, 10]. HR provides accurate genetic recombination using a sister chromatid as a template, which is essential for maintenance of genomic stability. Although HR is a desirable method of DNA DSB

repair, it is limited in cells during the S and G2 phases because of the need for a sister chromatid. NHEJ is a simple method of directly connecting the DSB ends. Although NHEJ is not as accurate as HR, it plays an important role in minimizing DNA damage, especially in cells in the G0 and G1 phases, in which HR is not available. Ku70/80, the DNA-dependent protein kinase, catalytic subunit, and DNA ligase IV are major contributors to NHEJ.

DNA repair and cell cycle checkpoints must cooperate closely to repair DNA damage and maintain genomic stability. Defects in this network produce dysfunction in the repair of DNA damage induced by ionizing radiation, which results in enhancement of the cytotoxic activity of radiation. Thus, molecules involved in these mechanisms can be suitable targets for radiosensitization.

Chemotherapeutic Agents as Radiosensitizers

As described above, radiotherapy is often combined with chemotherapy, and several chemotherapeutic agents are known to enhance the radiosensitivity of cancer cells [11, 12]. 5-Fluorouracil (5-FU), one of the most commonly used chemotherapeutic agents, is a member of the thymidylate synthase inhibitor family; these inhibitors produce cytotoxic effects by interfering with DNA synthesis [13]. Researchers have tested the combination of 5-FU and ionizing radiation and shown it to be effective against various types of cancers. This combination is a central component of current chemoradiation regimens [14].

Cisplatin, another commonly used chemotherapeutic agent, causes cytotoxicity by cross-linking DNA and interfering with cell division. Although cisplatin use is often combined with radiotherapy, oxaliplatin, another platinum derivative, has displayed more profound radiosensitizing effects [14, 15].

Gemcitabine, which is a nucleoside analogue that produces cytotoxic activity by blocking DNA replication, is another chemotherapeutic agent that is considered to be a radiosensitizer [16]. In preclinical studies, gemcitabine produced radiosensitization by interfering with Rad51 function and HR repair [17] as well as by redistributing cells into S phase by correlating with Chk1 and Chk2 [18]. Gemcitabine and radiotherapy have been shown to exert synergistic effects against cancers of the lung, pancreas, and head and neck in several clinical trials [19-21].

Taxanes such as paclitaxel and docetaxel produce cytotoxic activity by disrupting the function of microtubules that lead to cell division. A remarkable point is that taxanes arrest cells at the G2/M phase, which is the phase at which ionizing radiation is most effective [22]. Not only preclinical studies but also several clinical trials of regimens including taxanes and ionizing radiation used to treat cancers of the head and neck, esophagus, and lung have shown that taxanes are effective radiosensitizers [23-27].

Topoisomerase I inhibitors such as irinotecan, topotecan, and camptothecin interfere with topoisomerases, which are enzymes that are essential for winding and unwinding the DNA double helix during DNA replication and repair. Considering that ionizing radiation targets DNA and causes DNA DSBs, the combination of a topoisomerase I inhibitor and ionizing radiation may produce synergistic effects. Many preclinical studies using cultured cells and animal models have supported the synergy of this combination, although the specific mechanism of the synergistic effects remains unclear [28]. Also, many clinical trials have shown that these combinations are effective against various solid tumors, including head and neck, esophageal, lung, and brain tumors [29-32].

Molecularly Targeted Therapy for Radiosensitization

Although traditional chemotherapeutic agents that target rapidly dividing cells are still central to current cancer therapy, the attention of scientists is moving toward targeted therapy, which is expected to increase the effectiveness of treatment against cancer cells while reducing its harmfulness to normal cells [33]. Several small molecules and monoclonal antibodies that target epidermal growth factor receptor (EGFR), Her2/neu receptor, and vascular endothelial growth factor (VEGF) are currently in clinical use, and investigators have developed various types of molecularly targeted agents and are currently testing them in clinical trials [34, 35]. Some examples of molecularly targeted agents that are undergoing testing in clinical trials and expected to be used as radiosensitizers of tumors are described below.

Histone deacetylases (HDACs) are enzymes that control histone acetylation in coordination with the

opposing actions of histone acetyltransferases and play important roles in the regulation of gene expression. Physicians have long employed HDAC inhibitors such as valproic acid as anticonvulsants and mood-stabilizing drugs in the clinic, and use of these agents recently has generated a great deal of interest in their potential as antitumor drugs [36]. HDAC inhibitors have induced tumor-selective apoptosis and growth arrest in preclinical studies and exhibited effectiveness against tumors alone or in combination with chemotherapy in many clinical trials [37, 38]. To date, two HDAC inhibitors approved by the U.S. Food and Drug Administration—vorinostat and romidepsin—are in clinical use for treatment of T-cell lymphoma. Regarding the potential radiosensitizing effect of HDAC inhibitors, histone hyperacetylation induced by HDAC inhibitors appears to increase the cytotoxic activity of ionizing radiation [39, 40], and several clinical trials are testing these inhibitors in combination with radiotherapy for many types of cancer [41, 42].

Poly (ADP-ribose) polymerase (PARP) enzymes are proteins that play critical roles in DNA repair and replication. PARP1, which is the most abundant PARP and accounts for most PARP activities in cancer cells, binds to both DNA single-strand breaks (SSBs) and DSBs, but its role in SSB repair is better established. Although PARP inhibitors mainly contribute to SSB repair and often do not directly contribute to DSB repair, which is more critical for cell survival, defects in HR brought about by PARP inhibitors appear to increase the cytotoxic activity of ionizing radiation, especially in cells that are defective in DSB repair or NHEJ function [43-46]. Many PARP inhibitors are currently in clinical trials as single agents or in combination with DNA damage-inducing chemotherapeutic agents, and the PARP inhibitor ABK-888 administered in combination with radiotherapy recently entered clinical trials [47].

In addition, inhibitors of heat shock protein 90 or Chk1/2, some of which are currently in clinical trials as monotherapy or in combination with chemotherapeutic agents, have exhibited potential as radiosensitizers in preclinical studies, although combinations of them with radiotherapy have yet to be tested in clinical trials as far as we know [48-50]. Some EGFR tyrosine kinase inhibitors such as erlotinib and gefitinib and VEGF inhibitors such as bevacizumab, which are currently in clinical use for cancer therapy, also have displayed radiosensitizing effects in many preclinical studies and clinical trials [51].

ATM as a Target for Radiosensitization

As described above, molecules involved in DNA repair or cell cycle checkpoints can be targets to enhance tumor radiosensitivity. Interest in molecularly targeted therapy has deepened our understanding of the signaling pathways for DNA repair and cell cycle checkpoints, and ATM has been revealed to play a central in these signaling pathways. Studies originally identified the *ATM* gene in A-T, a disease that causes several severe disabilities, such as cerebellar degeneration, immunodeficiency, hypersensitivity to radiation and genomic instability, and increased incidence of malignancies [52, 53]. All patients with A-T have mutations in the *ATM* gene, and intensive investigation of such patients and A-T cells has contributed to the elucidation of ATM function. The construction of the ATM protein is similar to that of ATM- and RAD3-related (ATR), the DNA-dependent protein kinase, catalytic subunit, and mammalian target of rapamycin (mTOR), and ATM belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase family.

Following DNA damage, ATM immediately activates signaling pathways for DNA repair and cell cycle checkpoints. Although recent studies have shown that downstream signaling of ATM is becoming increasingly complicated, p53 and Chk2 are undoubtedly the main targets of ATM and control the G1/S and G2/M checkpoints while interacting with each other. Also, inhibition of these checkpoints allows damaged cells to move to the mitotic phase without undergoing proper DNA repair, leading to mitotic catastrophe, which is currently considered a main cause of cell death induced by radiotherapy [54-56]. Moreover, ATM is known to affect HR repair by directly or indirectly phosphorylating at least 12 targets, such as BRCA1/2 and NBS1, and defects in ATM function lead to dysfunction in HR repair [57, 58]. These findings indicate that targeted ATM inhibition is an attractive approach to enhancing tumor radiosensitivity.

Caffeine and wortmannin, which are nonspecific PI3K inhibitors, have been widely used in studies related to ATM/ATR functions [59, 60]. However,

some of the effects of caffeine and wortmannin in cells, such as apoptosis and checkpoint abrogation, are caused not only by ATM/ATR inhibition but also by other factors in the PI3K family [60, 61]. Recently, researchers developed several more specific ATM and ATM/ATR inhibitors—KU-55933, CGK733, and CP466722—and tested their potential as radiosensitizers in preclinical studies. KU-55933 was found to exhibit a specific inhibitory effect on ATM but not on other PI3K-family proteins, such as PI3K, DNA-PK, ATR, and mTOR, and sensitized cells to ionizing radiation by blocking phosphorylation of γH2AX, NBS1, and Chk1 [62]. CGK733 demonstrated selective inhibition of ATM and ATR, which led to blockage of the checkpoint signaling pathways, and researchers showed that its inhibitory effects were more beneficial than its small interfering RNA-mediated inhibition [63]. CP466722 exhibited inhibition of ATM and its downstream signaling pathways in the same way that KU-55933 did, and investigators emphasized that transient (4h or less) inhibition of ATM expression was sufficient to increase the radiosensitivity of tumor cells [64]. Small interfering RNAs and antisense DNA for ATM also exhibited potent radiosensitizing effects [65, 66]. Based on this preclinical evidence, ATM inhibitors are expected to be promising candidate radiosensitizers.

The MRN Complex as a Target for Radiosensitization

Although the importance of the ATM signaling pathway in DNA repair and cell cycle checkpoints has been established, the MRN complex has emerged as an essential factor in ATM activation. Mre11 and Rad50 were originally isolated from the yeast *Saccharomyces cerevisiae* in genetic screens in which an Mre11 mutant was defective in meiotic recombination [67] and a Rad50 mutant was sensitive to DNA damage [68]. NBS1 was isolated as a member of the complex that binds with Mre11 and Rad50, and mutations in this gene cause NBS, which is characterized by high cancer incidence, cell-cycle-checkpoint defects, and radiosensitivity [69]. Mutations in the *Mre11* gene have been reported to cause A-T like disorder [70], and deficiency of the *Rad50* gene causes NBS-like disorder [71]. The indispensability of the MRN complex to cells is emphasized by the fact that null mutations of either of these genes cause embryonic lethality in mice [72]. The Mre11 protein is uniformly distributed in the nucleus under undamaged conditions, but it migrates to sites of damage within 30 minutes after DNA DSB induction and forms a complex with Rad50 and NBS1, which is visualized as nuclear foci [73].

The MRN complex plays important roles in signal transduction related to DNA repair and cell cycle checkpoints [10]. One of these roles is activation of the ATM/ATR signaling pathway. Dysfunction of the MRN complex results in impairment of the ATM signaling pathway, which leads to hypersensitivity to DNA-damaging agents. The MRN complex has also been reported to contribute to the DNA DSB-repair pathway directly or indirectly via ATM activation [9]. In the HR repair process, the MRN complex serves as a primary damage sensor and is involved in the early steps of HR repair, which include processing of the broken DNA ends: in other words, removal of the 5' strand to uncover the 3' single strand [74]. Whereas Ku70/80 and DNA-PK are well known to be the main components in NHEJ, the importance of the MRN complex to NHEJ has only recently been demonstrated, and whether the MRN complex is correlated with Ku70/80 and DNA-PK in NHEJ remains unclear [10, 75].

As might be expected from the fact that mutations in members of the MRN complex are hypersensitive to DNA DSBs, inhibitors of the MRN complex enhance the cytotoxic activity of ionizing radiation. Although disruptions of the MRN complex by gene therapy have been reported to be effective in combination with radiotherapy, researchers recently isolated a novel small-molecule inhibitor of the MRN complex called mirin from a chemical genetic screen [76, 77]. Mirin inhibited MRN complex-dependent ATM activation and Mre11-associated exonuclease activity, leading to abolishment of the G2/M checkpoint and impairment of HR repair. These results are consistent with the known and anticipated functions of the MRN complex. Considering the importance of the MRN complex in DNA repair and cell cycle checkpoints, MRN complex inhibitors appear to be very promising as radiosensitizers.

The Radiosensitizing Effect of the Adenoviral E1B55kDa Protein

We recently demonstrated that telomelysin sensitizes cancer cells to the cytotoxic activity of ionizing radiation [78]. Telomelysin is a telomerase-dependent oncolytic adenoviral agent whose replication is controlled by the human telomerase reverse transcriptase (hTERT) promoter. Telomelysin can thus induce cell death via oncolysis by replicating only in cancer cells whose hTERT activity is high [79–81]. An American Phase I clinical trial of single-agent telomelysin evaluated the clinical safety and pharmacokinetics of the agent in the human body following its approval by the U.S. Food and Drug Administration in 2006. When injected intratumorally in patients with various solid tumors such as melanoma, sarcoma, lung cancer, breast cancer, and head and neck cancer, telomelysin proved to be effective and well-tolerated without any severe adverse events [82].

The adenoviral E1B55kDa protein has been reported to play an important role in creating the optimal intracellular environment for adenoviral protein synthesis by inhibiting the function of the MRN complex and p53 in cooperation with the adenoviral E4 protein [83]. Inhibition of the MRN complex is also considered to be a self-defense response to concatemer formation of the double-strand DNA genome of adenovirus by the MRN complex [84–86]. We showed that expression of the MRN complex in cancer cells began to decrease about 24 h after telomelysin treatment, when the E1B55kDa protein began to be expressed, which led to inhibition of ATM phosphorylation by ionizing radiation and inhibition of DNA repair. We determined the importance of the presence of E1B55kDa in regard to this inhibitory effect by comparing telomelysin with the E1B-defective oncolytic adenovirus dl1520 (onyx-015), which has been used in many clinical trials [87].

We demonstrated that inhibition of the MRN complex by telomelysin via the E1B55kDa protein produced a profound radiosensitizing effect *in vitro*; interestingly, on the other hand, ionizing radiation increased the cytotoxic activity of telomelysin, presumably by increasing viral uptake into cancer cells, which means that telomelysin and ionizing radiation potentiate each other. Furthermore, combined therapy with telomelysin and ionizing radiation exhibited a strong synergistic antitumor effect in animal studies [78]. A clinical study of the combination of telomelysin and ionizing radiation against cancers of the head and neck and esophagus is currently under consideration in Japan, and additional telomelysin-based treatment is expected to contribute to improvement of the survival rates and quality of life in patients with these cancers. Moreover, this inhibitory effect on the MRN complex via the E1B55kDa protein may apply to not only telomelysin but also all of the other oncolytic adenoviruses that produce this protein, which may provide new clues to clinical applications of oncolytic adenovirotherapy (Fig. 2).

Perspectives on ATM and MRN Complex Inhibitors

Precise cellular responses to DNA DSBs require efficient recognition of the damaged DNA sites and organized activation of the signaling pathways leading to DNA repair and cell cycle checkpoints. Numerous preclinical studies have shown that ATM and the MRN complex play critical roles in this response, which indicates that these molecules are promising targets for radiosensitization. In fact, the ATM and MRN complex inhibitors described above have exhibited profound radiosensitizing effects in preclinical studies. The next step should be to test these inhibitors toward clinical application is to be tested in clinical settings, but to our knowledge, none of them have entered clinical trials.

One of the factors that could impede the success of ATM and MRN complex inhibitors in clinical trials is tumor selectivity. The expression and functions of ATM and the MRN complex do not appear to differ much in cancer cells and normal cells, which means that unless these inhibitors are delivered to tumors selectively, severe adverse events may occur when they are combined with radiotherapy. Recent developments in the field of drug delivery could have remarkable outcomes when combined with developments in the field of drug discovery. For example, nanomedicine has revolutionized drug delivery, and nanosized carriers such as liposomes, polymers, and micelles increase the stability of therapeutic drugs in the bloodstream [88]. Moreover, these carriers can acquire tumor-targeting potential by being equipped with antibodies or peptides that target biomarkers that are overex-

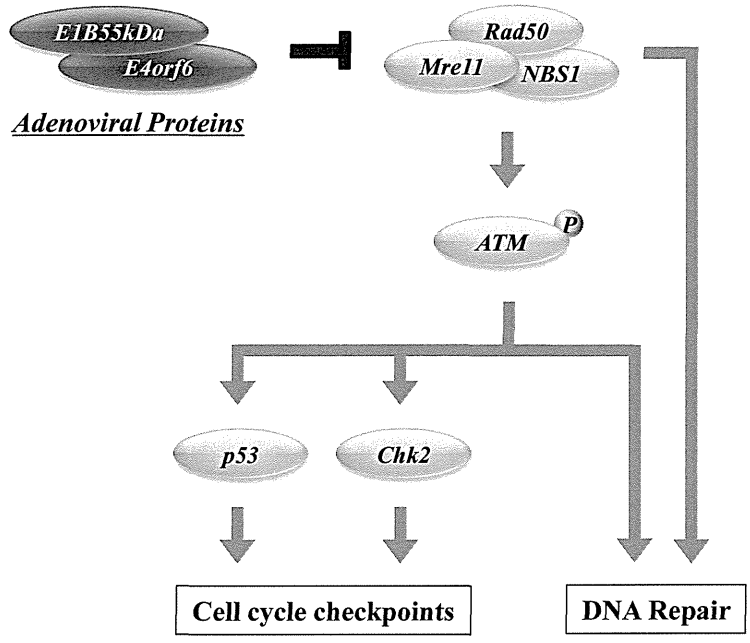

Fig. 2 The molecular mechanism of radiosensitization via the adenoviral E1B55kDa protein. E1B55kDa inhibits the function of the MRN complex in cooperation with the adenoviral E4orf6 protein, which inhibits the ATM signaling pathway and leads to cell-cycle-checkpoint abrogation and DNA-repair dysfunction.

pressed in tumors [89]. This type of improvement in drug delivery may be necessary for the use of ATM or MRN complex inhibitors before they enter clinical trials.

Regarding tumor-targeting potential, telomelysin may be a step ahead of these ATM or MRN complex inhibitors because its effect is strictly limited to cancer cells with high telomerase activity levels. Moreover, Phase I clinical trials in the United States have already determined the safety of monotherapy with telomelysin, and this agent is about to undergo testing in combination with ionizing radiation in a clinical trial in Japan.

However, telomelysin also has some challenging drawbacks that must be overcome in order to increase its attractiveness and its application as a cancer therapeutic agent. One of these issues is that telomelysin currently can only be administered via local injection and not systemically. The majority of intravenously administered adenoviruses become trapped in the liver, and thus they are not present at sufficient levels at the tumor sites [90]. In addition, most people have neutralizing antibodies against adenovirus type 5, which is one of the common cold viruses. Therefore, telomelysin, which consists of this adenovirus, is removed by the immune system immediately after systemic administration. For this reason, application of telomelysin is currently limited to cancers confined within locoregional areas, and improvements in telomelysin that would facilitate its systemic delivery will be needed before the drug can be used in the treatment of distant metastases.

In summary, the field of targeted radiosensitization of tumors is developing rapidly and drawing much attention. ATM and the MRN complex play central roles in the DNA DSB-response pathways, and inhibitors of these molecules are promising candidate radiosensitizing agents. An upcoming clinical trial of telomelysin combined with ionizing radiation will test this agent's function as an MRN complex inhibitor, and the outcome of this trial is expected to open new opportunities for other oncolytic adenoviruses that produce the E1B55kDa protein as promising radiosensitizers.

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