Kemp, B.; Khuri, F.R.; Kurie, J.M.; Liao, Z.; McDonnell, T.J.; Morice, R.; Morello, F.; Munden, R.; Papadimitrakopoulou, V.; Pisters, K.M.; Putnam, J.B., Jr.; Sarabia, A.J.; Shelton, T.; Stevens, C.; Shin, D.M.; Smythe, W.R.; Vaporciyan, A.A.; Walsh, G.L.; Yin, M. Induction of p53-regulated genes and tumor regression in lung cancer patients after intratumoral delivery of adenoviral p53 (INGN 201) and radiation therapy. *Clin. Cancer Res.,* **2003**, *9* (1), 93-101.

[12] Fujiwara, T.; Tanaka, N.; Kanazawa, S.; Ohtani, S.; Saijo, Y.; Nukiwa, T.; Yoshimura, K.; Sato, T.; Eto, Y.; Chada, S.; Nakamura, H.; Kato, H. Multicenter phase I study of repeated intratumoral delivery of adenoviral p53 in patients with advanced non-small-cell lung cancer. *J. Clin. Oncol.*, **2006**, *24* (11), 1689-1699.

[13] Hawkins, L.K.; Lemoine, N.R.; Kirn, D. Oncolytic biotherapy: a novel therapeutic plafform. *Lancet Oncol.,* **2002**, *3* (1), 17-26.

[14] Kawashima, T.; Kagawa, S.; Kobayashi, N.; Shirakiya, Y.; Umeoka, T.; Teraishi, F.; Taki, M.; Kyo, S.; Tanaka, N.; Fujiwara, T. Telomerase-specific replication-selective virotherapy for human cancer. *Clin. Cancer Res.,* **2004**, *10* (1 Pt 1), 285-292.

[15] Umeoka, T.; Kawashima, T.; Kagawa, S.; Teraishi, F.; Taki, M.; Nishizaki, M.; Kyo, S.; Nagai, K.; Urata, Y.; Tanaka, N.; Fujiwara, T. Visualization of intrathoracically disseminated solid tumors in mice with optical imaging by telomerase-specific amplification of a transferred green fluorescent protein gene. *Cancer Res.,* **2004**, *64* (17), 6259-6265.

[16] Taki, M.; Kagawa, S.; Nishizaki, M.; Mizuguchi, H.; Hayakawa, T.; Kyo, S.; Nagai, K.; Urata, Y.; Tanaka, N.; Fujiwara, T. Enhanced oncolysis by a tropism-modified telomerase-specific replication-selective adenoviral agent OBP-405 ('Telomelysin-RGD'). *Oncogene,* **2005**, *24* (19), 3130-3140.

[17] Shay, J.W.; Bacchetti, S. A survey of telomerase activity in human cancer. *Eur. J. Cancer,* **1997**, *33* (5), 787-791.

[18] Dong, C.K.; Masutomi, K.; Hahn, W.C. Telomerase: regulation, function and transformation. *Crit Rev. Oncol. Hematol.,* **2005**, *54* (2), 85-93.

[19] Hiyama, K.; Hirai, Y.; Kyoizumi, S.; Akiyama, M.; Hiyama, E.; Piatyszek, M.A.; Shay, J.W.; Ishioka, S.; Yamakido, M. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immunol.,* **1995**, *155* (8), 3711-3715.

[20] Tahara, H.; Yasui, W.; Tahara, E.; Fujimoto, J.; Ito, K.; Tamai, K.; Nakayama, J.; Ishikawa, F.; Tahara, E.; Ide, T. Immunohistochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections. *Oncogene,* **1999**, *18* (8), 1561-1567.

[21] Shay, J.W.; Wright, W.E. Telomerase: a target for cancer therapeutics. *Cancer Cell,* **2002**, *2* (4), 257-265.

[22] Feng, J.; Funk, W.D.; Wang, S.S.; Weinrich, S.L.; Avilion, A.A.; Chiu, C.P.; Adams, R.R.; Chang, E.; Allsopp, R.C.; Yu, J.; . The RNA component of human telomerase. *Science,* **1995**, *269* (5228), 1236-1241.

[23] Harrington, L.; McPhail, T.; Mar, V.; Zhou, W.; Oulton, R.; Bass, M.B.; Arruda, I.; Robinson, M.O. A mammalian telomerase-associated protein. *Science,* **1997**, *275* (5302), 973-977.

[24] Meyerson, M.; Counter, C.M.; Eaton, E.N.; Ellisen, L.W.; Steiner, P.; Caddle, S.D.; Ziaugra, L.; Beijersbergen, R.L.; Davidoff, M.J.; Liu, Q.; Bacchetti, S.; Haber, D.A.; Weinberg, R.A. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell,* **1997**, *90* (4), 785-795.

[25] Nakamura, T.M.; Morin, G.B.; Chapman, K.B.; Weinrich, S.L.; Andrews, W.H.; Lingner, J.; Harley, C.B.; Cech, T.R. Telomerase catalytic subunit homologs from fission yeast and human. *Science,* **1997**, *277* (5328), 955-959.

[26] Nakayama, J.; Tahara, H.; Tahara, E.; Saito, M.; Ito, K.; Nakamura, H.; Nakanishi, T.; Tahara, E.; Ide, T.; Ishikawa, F. Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. *Nat. Genet.,* **1998**, *18* (1), 65-68.

[27] Beattie, T.L.; Zhou, W.; Robinson, M.O.; Harrington, L. Reconstitution of human telomerase activity in vitro. *Curr. Biol.,* **1998**, *8* (3), 177-180.

[28] Wirth, T.; Zender, L.; Schulte, B.; Mundt, B.; Plentz, R.; Rudolph, K.L.; Manns, M.; Kubicka, S.; Kuhnel, F. A telomerase-dependent conditionally replicating adenovirus for selective treatment of cancer. *Cancer Res.,* **2003**, *63* (12), 3181-3188.

[29] Lanson, N.A., Jr.; Friedlander, P.L.; Schwarzenberger, P.; Kolls, J.K.; Wang, G. Replication of an adenoviral vector controlled by the human telomerase reverse transcriptase promoter causes tumor-selective tumor lysis. *Cancer Res.,* **2003**, *63* (22), 7936-7941.

[30] Irving, J.; Wang, Z.; Powell, S.; O'Sullivan, C.; Mok, M.; Murphy, B.; Cardoza, L.; Lebkowski, J.S.; Majumdar, A.S. Conditionally replicative adenovirus driven by the human telomerase promoter provides broad-spectrum antitumor activity without liver toxicity. *Cancer Gene Ther.,* **2004**, *11* (3), 174-185.

[31] Kim, E.; Kim, J.H.; Shin, H.Y.; Lee, H.; Yang, J.M.; Kim, J.; Sohn, J.H.; Kim, H.; Yun, C.O. Ad-mTERT-delta19, a conditional replication-competent adenovirus driven by the human telomerase promoter, selectively replicates in and elicits cytopathic effect in a cancer cell-specific manner. *Hum. Gene Ther.,* **2003**, *14* (15), 1415-1428.

[32] Li, Y.; Yu, D.C.; Chen, Y.; Amin, P.; Zhang, H.; Nguyen, N.; Henderson, D.R. A hepatocellular carcinoma-specific adenovirus variant, CV890, eliminates distant human liver tumors in combination with doxorubicin. *Cancer Res.,* **2001**, *61* (17), 6428-6436.

[33] Hashimoto, Y.; Watanabe, Y.; Shirakiya, Y.; Uno, F.; Kagawa, S.; Kawamura, H.; Nagai, K.; Tanaka, N.; Kumon, H.; Urata, Y.; Fujiwara, T. Establishment of biological and pharmacokinetic assays of telomerase-specific replication-selective adenovirus. *Cancer Sci.,* **2008**, *99* (2), 385-390.

[34] Fujiwara, T.; Urata, Y.; Tanaka, N. Telomerase-specific oncolytic virotherapy for human cancer with the hTERT promoter. *Curr. Cancer Drug Targets,* **2007**, *7* (2), 191-201.

[35] Kurihara, Y.; Watanabe, Y.; Onimatsu, H.; Kojima, T.; Shirota, T.; Hatori, M.; Liu, D.; Kyo, S.; Mizuguchi, H.; Urata, Y.; Shintani, S.; Fujiwara, T. Telomerase-specific virotheranostics for human head and neck cancer. *Clin. Cancer Res.,* **2009**, *15* (7), 2335-2343.

[36] Fujiwara, T. Telomerase-specific virotherapy for human squamous cell carcinoma. *Expert. Opin. Biol. Ther.,* **2009**, *9* (3), 321-329.

[37] Nemunaitis, J.; Tong, A.W.; Nemunaitis, M.; Senzer, N.; Phadke, A.P.; Bedell, C.; Adams, N.; Zhang, Y.A.; Maples, P.B.; Chen, S.; Pappen, B.; Burke, J.; Ichimaru, D.; Urata, Y.; Fujiwara, T. A phase I study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. *Mol. Ther.,* **2010**, *18* (2), 429-434.

[38] DeWeese, T.L.; van der, P.H.; Li, S.; Mikhak, B.; Drew, R.; Goemann, M.; Hamper, U.; DeJong, R.; Detorie, N.; Rodriguez, R.; Haulk, T.; DeMarzo, A.M.; Piantadosi, S.; Yu, D.C.; Chen, Y.; Henderson, D.R.; Carducci, M.A.; Nelson, W.G.; Simons, J.W. A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res.,* **2001**, *61* (20), 7464-7472.

[39] Small, E.J.; Carducci, M.A.; Burke, J.M.; Rodriguez, R.; Fong, L.; van, U.L.; Yu, D.C.; Aimi, J.; Ando, D.; Working, P.; Kirn, D.; Wilding, G. A Phase I Trial of Intravenous CG7870, a Replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. *Mol. Ther.,* **2006**.

[40] Bischoff, J.R.; Kirn, D.H.; Williams, A.; Heise, C.; Horn, S.; Muna, M.; Ng, L.; Nye, J.A.; Sampson-Johannes, A.; Fattaey, A.; McCormick, F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science,* **1996**, *274* (5286), 373-376.

[41] Rodriguez, R.; Schuur, E.R.; Lim, H.Y.; Henderson, G.A.; Simons, J.W.; Henderson, D.R. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.,* **1997**, *57* (13), 2559-2563.

[42] Kirn, D.; Martuza, R.L.; Zwiebel, J. Replication-selective virotherapy for cancer: Biological principles, risk management and future directions. Nat. Med., ***2001***, *7* (7), 781-787.

[43] Heise, C.; Sampson-Johannes, A.; Williams, A.; McCormick, F.; Von Hoff, D.D.; Kirn, D.H. ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nat. Med.,* **1997**, *3* (6), 639-645.

[44] You, L.; Yang, C.T.; Jablons, D.M. ONYX-015 works synergistically with chemotherapy in lung cancer cell lines and primary cultures freshly made from lung cancer patients. *Cancer Res.,* **2000**, *60* (4), 1009-1013.

[45] Toyoizumi, T.; Mick, R.; Abbas, A.E.; Kang, E.H.; Kaiser, L.R.; Molnar-Kimber, K.L. Combined therapy with chemotherapeutic agents and herpes simplex virus type 1 ICP34.5 mutant (HSV-1716) in human non-small cell lung cancer. *Hum. Gene Ther.,* **1999**, *10* (18), 3013-3029.

[46] Khuri, F.R.; Nemunaitis, J.; Ganly, I.; Arseneau, J.; Tannock, I.F.; Romel, L.; Gore, M.; Ironside, J.; MacDougall, R.H.; Heise, C.; Randlev, B.; Gillenwater, A.M.; Bruso, P.; Kaye, S.B.; Hong, W.K.; Kirn, D.H. a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin

and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat. Med.*, **2000**, *6* (8), 879-885.

[47] Reid, T.; Galanis, E.; Abbruzzese, J.; Sze, D.; Wein, L.M.; Andrews, J.; Randlev, B.; Heise, C.; Uprichard, M.; Hatfield, M.; Rome, L.; Rubin, J.; Kirn, D. Hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520): phase II viral, immunologic, and clinical endpoints. *Cancer Res.*, **2002**, *62* (21), 6070-6079.

[48] Hecht, J.R.; Bedford, R.; Abbruzzese, J.L.; Lahoti, S.; Reid, T.R.; Soetikno, R.M.; Kirn, D.H.; Freeman, S.M. A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. *Clin. Cancer Res.*, **2003**, *9* (2), 555-561.

[49] Galanis, E.; Okuno, S.H.; Nascimento, A.G.; Lewis, B.D.; Lee, R.A.; Oliveira, A.M.; Sloan, J.A.; Atherton, P.; Edmonson, J.H.; Erlichman, C.; Randlev, B.; Wang, Q.; Freeman, S.; Rubin, J. Phase I-II trial of ONYX-015 in combination with MAP chemotherapy in patients with advanced sarcomas. *Gene Ther.*, **2005**, *12* (5), 437-445.

[50] Nemunaitis, J.; Ganly, I.; Khuri, F.; Arseneau, J.; Kuhn, J.; McCarty, T.; Landers, S.; Maples, P.; Romel, L.; Randlev, B.; Reid, T.; Kaye, S.; Kirn, D. Selective replication and oncolysis in p53 mutant tumors with ONYX-015, an E1B-55kD gene-deleted adenovirus, in patients with advanced head and neck cancer: a phase II trial. *Cancer Res.*, **2000**, *60* (22), 6359-6366.

[51] Jacobs, C.; Lyman, G.; Velez-Garcia, E.; Sridhar, K.S.; Knight, W.; Hochster, H.; Goodnough, L.T.; Mortimer, J.E.; Einhorn, L.H.; Schacter, L. A phase III randomized study comparing cisplatin and fluorouracil as single agents and in combination for advanced squamous cell carcinoma of the head and neck. *J. Clin. Oncol.*, **1992**, *10* (2), 257-263.

[52] Vokes, E.E. Chemotherapy and integrated treatment approaches in head and neck cancer. *Curr. Opin. Oncol.*, **1991**, *3* (3), 529-534.

[53] Jordan, M.A.; Toso, R.J.; Thrower, D.; Wilson, L. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc. Natl. Acad. Sci. USA*, **1993**, *90* (20), 9552-9556.

[54] Dumontet, C.; Sikic, B.I. Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J. Clin. Oncol.*, **1999**, *17* (3), 1061-1070.

[55] Nielsen, L.L.; Lipari, P.; Dell, J.; Gurnani, M.; Hajian, G. Adenovirus-mediated p53 gene therapy and paclitaxel have synergistic efficacy in models of human head and neck, ovarian, prostate, and breast cancer. *Clin. Cancer Res.*, **1998**, *4* (4), 835-846.

[56] Yu, D.C.; Chen, Y.; Dilley, J.; Li, Y.; Embry, M.; Zhang, H.; Nguyen, N.; Amin, P.; Oh, J.; Henderson, D.R. Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel. *Cancer Res.*, **2001**, *61* (2), 517-525.

[57] Fujiwara, T.; Kagawa, S.; Kishimoto, H.; Endo, Y.; Hioki, M.; Ikeda, Y.; Sakai, R.; Urata, Y.; Tanaka, N.; Fujiwara, T. Enhanced antitumor efficacy of telomerase-selective oncolytic adenoviral agent OBP-401 with docetaxel: Preclinical evaluation of chemovirotherapy. *Int. J. Cancer*, **2006**, *119* (2), 432-440.

[58] Shaked, Y.; Emmenegger, U.; Francia, G.; Chen, L.; Lee, C.R.; Man, S.; Paraghamian, A.; Ben-David, Y.; Kerbel, R.S. Low-dose metronomic combined with intermittent bolus-dose cyclophosphamide is an effective long-term chemotherapy treatment strategy. *Cancer Res.*, **2005**, *65* (16), 7045-7051.

[59] Kitazono, M.; Goldsmith, M.E.; Aikou, T.; Bates, S.; Fojo, T. Enhanced adenovirus transgene expression in malignant cells treated with the histone deacetylase inhibitor FR901228. *Cancer Res.*, **2001**, *61* (17), 6328-6330.

[60] Goldsmith, M.E.; Kitazono, M.; Fok, P.; Aikou, T.; Bates, S.; Fojo, T. The histone deacetylase inhibitor FK228 preferentially enhances adenovirus transgene expression in malignant cells. Clin. *Cancer Res.*, **2003**, *9* (14), 5394-5401.

[61] Pong, R.C.; Lai, Y.J.; Chen, H.; Okegawa, T.; Frenkel, E.; Sagalowsky, A.; Hsieh, J.T. Epigenetic regulation of coxsackie and adenovirus receptor (CAR) gene promoter in urogenital cancer cells. *Cancer Res.*, **2003**, *63* (24), 8680-8686.

[62] Hemminki, A.; Kanerva, A.; Liu, B.; Wang, M.; Alvarez, R.D.; Siegal, G.P.; Curiel, D.T. Modulation of coxsackie-adenovirus receptor expression for increased adenoviral transgene expression. *Cancer Res.*, **2003**, *63* (4), 847-853.

[63] Watanabe, T.; Hioki, M.; Fujiwara, T.; Nishizaki, M.; Kagawa, S.; Taki, M.; Kishimoto, H.; Endo, Y.; Urata, Y.; Tanaka, N.; Fujiwara, T. Histone deacetylase inhibitor FR901228 enhances the antitumor effect of telomerase-specific replication-selective adenoviral agent OBP-301 in human lung cancer cells. *Exp. Cell Res.*, **2006**, *312* (3), 256-265.

[64] Marshall, J.L.; Rizvi, N.; Kauh, J.; Dahut, W.; Figuera, M.; Kang, M.H.; Figg, W.D.; Wainer, I.; Chaissang, C.; Li, M.Z.; Hawkins, M.J. A phase I trial of depsipeptide (FR901228) in patients with advanced cancer. *J. Exp. Ther. Oncol.*, **2002**, *2* (6), 325-332.

[65] Byrd, J.C.; Marcucci, G.; Parthun, M.R.; Xiao, J.J.; Klisovic, R.B.; Moran, M.; Lin, T.S.; Liu, S.; Sklenar, A.R.; Davis, M.E.; Lucas, D.M.; Fischer, B.; Shank, R.; Tejaswi, S.L.; Binkley, P.; Wright, J.; Chan, K.K.; Grever, M.R. A phase 1 and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia. *Blood*, **2005**, *105* (3), 959-967.

[66] Heinemann, V.; Quietzsch, D.; Gieseler, F.; Gonnermann, M.; Schonekas, H.; Rost, A.; Neuhaus, H.; Haag, C.; Clemens, M.; Heinrich, B.; Vehling-Kaiser, U.; Fuchs, M.; Fleckenstein, D.; Gesierich, W.; Uthgenannt, D.; Einsele, H.; Holstege, A.; Hinke, A.; Schalhorn, A.; Wilkowski, R. Randomized phase III trial of gemcitabine plus cisplatin compared with gemcitabine alone in advanced pancreatic cancer. *J. Clin. Oncol.*, **2006**, *24* (24), 3946-3952.

[67] Paz-Ares, L.; Douillard, J.Y.; Koralewski, P.; Manegold, C.; Smit, E.F.; Reyes, J.M.; Chang, G.C.; John, W.J.; Peterson, P.M.; Obasaju, C.K.; Lahn, M.; Gandara, D.R. Phase III study of gemcitabine and cisplatin with or without aprinocarsen, a protein kinase C-alpha antisense oligonucleotide, in patients with advanced-stage non-small-cell lung cancer. *J. Clin. Oncol.*, **2006**, *24* (9), 1428-1434.

[68] Halloran, C.M.; Ghaneh, P.; Shore, S.; Greenhalf, W.; Zumstein, L.; Wilson, D.; Neoptolemos, J.P.; Costello, E. 5-Fluorouracil or gemcitabine combined with adenoviral-mediated reintroduction of p16INK4A greatly enhanced cytotoxicity in Panc-1 pancreatic adenocarcinoma cells. *J. Gene Med.*, **2004**, *6* (5), 514-525.

[69] Lee, W.P.; Tai, D.I.; Tsai, S.L.; Yeh, C.T.; Chao, Y.; Lee, S.D.; Hung, M.C. Adenovirus type 5 E1A sensitizes hepatocellular carcinoma cells to gemcitabine. *Cancer Res.*, **2003**, *63* (19), 6229-6236.

[70] Liu, D.; Kojima, T.; Ouchi, M.; Kuroda, S.; Watanabe, Y.; Hashimoto, Y.; Onimatsu, H.; Urata, Y.; Fujiwara, T. Preclinical evaluation of synergistic effect of telomerase-specific oncolytic virotherapy and gemcitabine for human lung cancer. *Mol. Cancer Ther.*, **2009**, *8* (4): 980-987.

[71] Huang, P.; Chubb, S.; Hertel, L.W.; Grindey, G.B.; Plunkett, W. Action of 2',2'-difluorodeoxycytidine on DNA synthesis. *Cancer Res.*, **1991**, *51* (22), 6110-6117.

[72] Wang, H.G.; Draetta, G.; Moran, E. E1A induces phosphorylation of the retinoblastoma protein independently of direct physical association between the E1A and retinoblastoma products. *Mol. Cell Biol.*, **1991**, *11* (8), 4253-4265.

[73] Hollyoake, M.; Stuhler, A.; Farrell, P.; Gordon, J.; Sinclair, A. The normal cell cycle activation program is exploited during the infection of quiescent B lymphocytes by Epstein-Barr virus. *Cancer Res.*, **1995**, *55* (21), 4784-4787.

[74] Morozov, A.; Shiyanov, P.; Barr, E.; Leiden, J.M.; Raychaudhuri, P. Accumulation of human papillomavirus type 16 E7 protein bypasses G1 arrest induced by serum deprivation and by the cell cycle inhibitor p21. *J. Virol.*, **1997**, *71* (5), 3451-3457.

[75] Nahle, Z.; Polakoff, J.; Davuluri, R.V.; McCurrach, M.E.; Jacobson, M.D.; Narita, M.; Zhang, M.Q.; Lazebnik, Y.; Bar-Sagi, D.; Lowe, S.W. Direct coupling of the cell cycle and cell death machinery by E2F. *Nat. Cell Biol.*, **2002**, *4* (11), 859-864.

[76] Kuroda, S.; Fujiwara, T.; Shirakawa, Y.; Yamasaki, Y.; Yano, S.; Uno, F.; Tazawa, H.; Hashimoto, Y.; Watanabe, Y.; Noma, K.; Urata, Y.; Kagawa, S.; Fujiwara, T. Telomerase-dependent oncolytic adenovirus sensitizes human cancer cells to ionizing radiation via inhibition of DNA repair machinery. *Cancer Res.*, **2010**, *70* (22): 9339-9348.

[77] Kojima, T.; Watanabe, Y.; Hashimoto, Y.; Kuroda, S.; Yamasaki, Y.; Yano, S.; Ouchi, M.; Tazawa, H.; Uno, F.; Kagawa, S.; Kyo, S.; Mizuguchi, H.; Urata, Y.; Tanaka, N.; Fujiwara, T. *In vivo* biological purging for lymph node metastasis of human colorectal cancer by telomerase-specific oncolytic virotherapy. *Ann. Surg.*, **2010**, *251* (6),1079-1086.

Received: May 03, 2010 **Accepted: September 21, 2010**

Acta Med. Okayama, 2012
Vol. 66, No. 2, pp. 83-92

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Review

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

Shinji Kuroda[a,b*§], Yasuo Urata[c], and Toshiyoshi Fujiwara[a]

[a]*Department of Gastroenterological Surgery, Okayama University Graduate School for Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan,* [b]*Department of Thoracic and Cardiovascular Surgery, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA, and* [c]*Oncolys BioPharma Inc., Minato-ku, Tokyo 105-0001, Japan*

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

Received June 21, 2011; accepted July 25, 2011.
*Corresponding author. Phone:+1-713-792-8905; Fax:+1-713-794-4669
E-mail:skuroda@mdanderson.org (S. Kuroda)
§The winner of the 2010 Hayashibara Prize of the Okayama Medical Association

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.

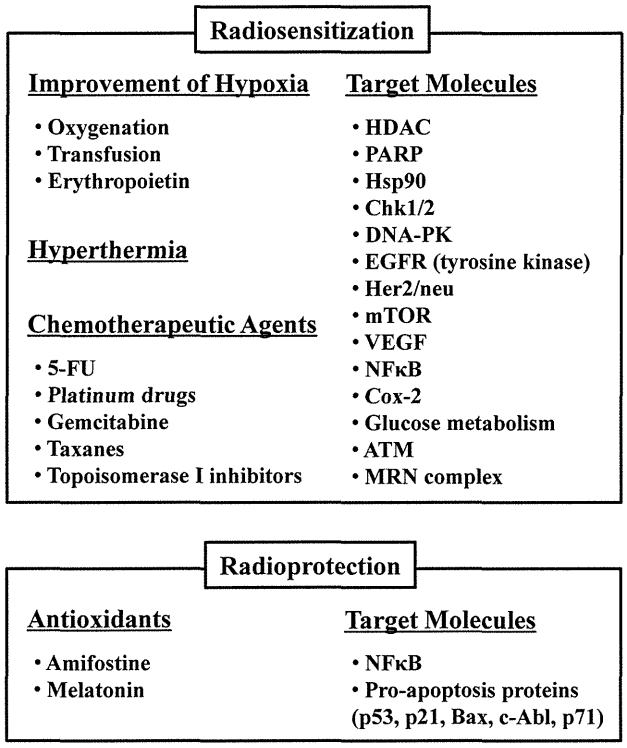

Fig. 1 Approaches to improvement of radiotherapy. Radiosensitizing agents are designed to increase the cytotoxic effects of radiation on cancer cells, and radioprotective agents are designed to decrease the adverse effects of radiation on normal cells. Hsp90, heat shock protein 90; NF-κB, nuclear factor-κB;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

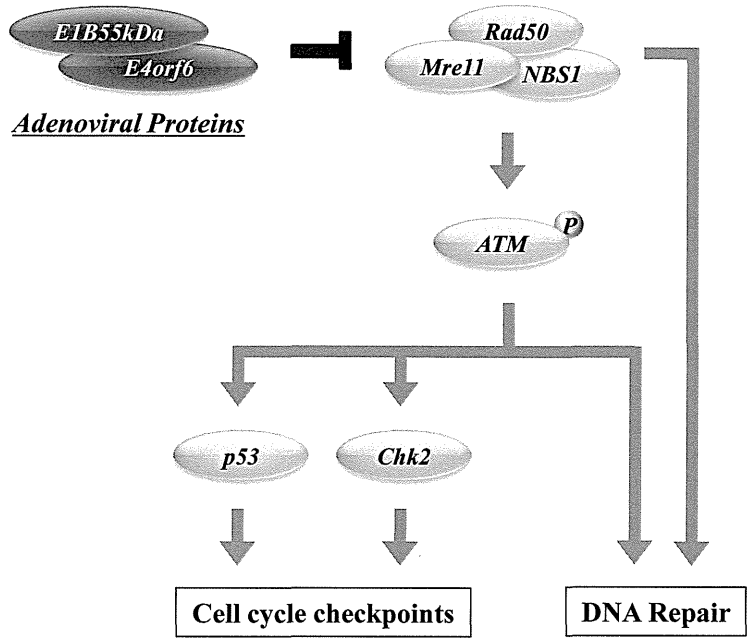

Fig. 2 The molecular mechanism of radiosensitization via the adenoviral E1B55kDa protein. E1B55kDa inhibits the function of the MRN complex in cooperation with the adenoviral E4orf6 protein, which inhibits the ATM signaling pathway and leads to cell-cycle-checkpoint abrogation and DNA-repair dysfunction.

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.

References

1. Mendelsohn FA, Divino CM, Reis ED and Kerstein MD: Wound care after radiation therapy. Adv Skin Wound Care (2002) 15: 216-224.

2. Timmerman RD, Kavanagh BD, Cho LC, Papiez L and Xing L: Stereotactic body radiation therapy in multiple organ sites. J Clin Oncol (2007) 25: 947-952.
3. Bentzen SM, Harari PM and Bernier J: Exploitable mechanisms for combining drugs with radiation: concepts, achievements and future directions. Nat Clin Pract Oncol (2007) 4: 172-180.
4. Okunieff P, de Bie J, Dunphy EP, Terris DJ and Hockel M: Oxygen distributions partly explain the radiation response of human squamous cell carcinomas. Br J Cancer Suppl (1996) 27: S185-190.
5. Harrison LB, Chadha M, Hill RJ, Hu K and Shasha D: Impact of tumor hypoxia and anemia on radiation therapy outcomes. Oncologist (2002) 7: 492-508.
6. Varlotto J and Stevenson MA: Anemia, tumor hypoxemia, and the cancer patient. Int J Radiat Oncol Biol Phys (2005) 63: 25-36.
7. Overgaard J and Horsman MR: Modification of Hypoxia-Induced Radioresistance in Tumors by the Use of Oxygen and Sensitizers. Semin Radiat Oncol (1996) 6: 10-21.
8. Krempler A, Deckbar D, Jeggo PA and Lobrich M: An imperfect G2M checkpoint contributes to chromosome instability following irradiation of S and G2 phase cells. Cell Cycle (2007) 6: 1682-1686.
9. Mimitou EP and Symington LS: DNA end resection: many nucleases make light work. DNA Repair (Amst) (2009) 8: 983-995.
10. Lamarche BJ, Orazio NI and Weitzman MD: The MRN complex in double-strand break repair and telomere maintenance. FEBS Lett (2010) 584: 3682-3695.
11. Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB, Kish JA, Kim HE, Cmelak AJ, Rotman M, Machtay M, Ensley JF, Chao KS, Schultz CJ, Lee N and Fu KK: Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. N Engl J Med (2004) 350: 1937-1944.
12. Bernier J, Domenge C, Ozsahin M, Matuszewska K, Lefebvre JL, Greiner RH, Giralt J, Maingon P, Rolland F, Bolla M, Cognetti F, Bourhis J, Kirkpatrick A and van Glabbeke M: Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. N Engl J Med (2004) 350: 1945-1952.
13. Longley DB, Harkin DP and Johnston PG: 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer (2003) 3: 330-338.
14. Spalding AC and Lawrence TS: New and emerging radiosensitizers and radioprotectors. Cancer Invest (2006) 24: 444-456.
15. Hermann RM, Rave-Frank M and Pradier O: Combining radiation with oxaliplatin: a review of experimental results. Cancer Radiother (2008) 12: 61-67.
16. Morgan MA, Parsels LA, Maybaum J and Lawrence TS: Improving gemcitabine-mediated radiosensitization using molecularly targeted therapy: a review. Clin Cancer Res (2008) 14: 6744-6750.
17. Wachters FM, van Putten JW, Maring JG, Zdzienicka MZ, Groen HJ and Kampinga HH: Selective targeting of homologous DNA recombination repair by gemcitabine. Int J Radiat Oncol Biol Phys (2003) 57: 553-562.
18. Morgan MA, Parsels LA, Parsels JD, Mesiwala AK, Maybaum J and Lawrence TS: Role of checkpoint kinase 1 in preventing premature mitosis in response to gemcitabine. Cancer Res (2005) 65: 6835-6842.
19. Momm F, Kaden M, Tannock I, Schumacher M, Hasse J and Henke M: Dose escalation of gemcitabine concomitant with radiation and cisplatin for nonsmall cell lung cancer: a phase 1-2 study. Cancer (2010) 116: 4833-4839.
20. Cooke EW and Hazard L: Curative radiation therapy for pancreatic malignancies. Surg Clin North Am (2010) 90: 341-354.
21. Benasso M, Vigo V, Bacigalupo A, Ponzanelli A, Marcenaro M, Corvo R and Margarino G: A phase II trial of low-dose gemcitabine and radiation alternated to cisplatin and 5-fluorouracil: an active and manageable regimen for stage IV squamous cell carcinoma of the head and neck. Radiother Oncol (2008) 89: 44-50.
22. Milas L, Milas MM and Mason KA: Combination of taxanes with radiation: preclinical studies. Semin Radiat Oncol (1999) 9: 12-26.
23. Chen Y, Pandya K, Keng PP, Feins R, Raubertas R, Smudzin T, Rosenblatt J and Okunieff P: Schedule-dependent pulsed paclitaxel radiosensitization for thoracic malignancy. Am J Clin Oncol (2001) 24: 432-437.
24. Zhao J, Kim JE, Reed E and Li QQ: Molecular mechanism of antitumor activity of taxanes in lung cancer (Review). Int J Oncol (2005) 27: 247-256.
25. Lau D, Leigh B, Gandara D, Edelman M, Morgan R, Israel V, Lara P, Wilder R, Ryu J and Doroshow J: Twice-weekly paclitaxel and weekly carboplatin with concurrent thoracic radiation followed by carboplatin/paclitaxel consolidation for stage III non-small-cell lung cancer: a California Cancer Consortium phase II trial. J Clin Oncol (2001) 19: 442-447.
26. Tishler RB, Norris CM Jr, Colevas AD, Lamb CC, Karp D, Busse PM, Nixon A, Frankenthaler R, Lake-Willcutt B, Costello R, Case M and Posner MR: A Phase I/II trial of concurrent docetaxel and radiation after induction chemotherapy in patients with poor prognosis squamous cell carcinoma of the head and neck. Cancer (2002) 95: 1472-1481.
27. Kleinberg L and Forastiere AA: Chemoradiation in the management of esophageal cancer. J Clin Oncol (2007) 25: 4110-4117.
28. Chen AY, Chou R, Shih SJ, Lau D and Gandara D: Enhancement of radiotherapy with DNA topoisomerase I-targeted drugs. Crit Rev Oncol Hematol (2004) 50: 111-119.
29. Murphy BA: Topoisomerases in the treatment of metastatic or recurrent squamous carcinoma of the head and neck. Expert Opin Pharmacother (2005) 6: 85-92.
30. Ilson DH, Bains M, Kelsen DP, O'Reilly E, Karpeh M, Coit D, Rusch V, Gonen M, Wilson K and Minsky BD: Phase I trial of escalating-dose irinotecan given weekly with cisplatin and concurrent radiotherapy in locally advanced esophageal cancer. J Clin Oncol (2003) 21: 2926-2932.
31. Takeda K, Negoro S, Tanaka M, Fukuda H, Nakagawa K, Kawahara M, Semba H, Kudoh S, Sawa T, Saijo N and Fukuoka M: A phase II study of cisplatin and irinotecan as induction chemotherapy followed by concomitant thoracic radiotherapy with weekly low-dose irinotecan in unresectable, stage III, non-small cell lung cancer: JCOG 9706. Jpn J Clin Oncol (2011) 41: 25-31.
32. Feun L and Savaraj N: Topoisomerase I inhibitors for the treatment of brain tumors. Expert Rev Anticancer Ther (2008) 8: 707-716.
33. Zhukov NV and Tjulandin SA: Targeted therapy in the treatment of solid tumors: practice contradicts theory. Biochemistry (Mosc) (2008) 73: 605-618.
34. Press MF and Lenz HJ: EGFR, HER2 and VEGF pathways: validated targets for cancer treatment. Drugs (2007) 67: 2045-2075.
35. Janku F, Stewart DJ and Kurzrock R: Targeted therapy in non-small-cell lung cancer--is it becoming a reality? Nat Rev Clin Oncol (2010) 7: 401-414.
36. Camphausen K and Tofilon PJ: Inhibition of histone deacetylation:

a strategy for tumor radiosensitization. J Clin Oncol (2007) 25: 4051-4056.
37. Almenara J, Rosato R and Grant S: Synergistic induction of mitochondrial damage and apoptosis in human leukemia cells by flavopiridol and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA). Leukemia (2002) 16: 1331-1343.
38. Marks PA: The clinical development of histone deacetylase inhibitors as targeted anticancer drugs. Expert Opin Investig Drugs (2010) 19: 1049-1066.
39. Camphausen K, Burgan W, Cerra M, Oswald KA, Trepel JB, Lee MJ and Tofilon PJ: Enhanced radiation-induced cell killing and prolongation of gammaH2AX foci expression by the histone deacetylase inhibitor MS-275. Cancer Res (2004) 64: 316-321.
40. Munshi A, Kurland JF, Nishikawa T, Tanaka T, Hobbs ML, Tucker SL, Ismail S, Stevens C and Meyn RE: Histone deacetylase inhibitors radiosensitize human melanoma cells by suppressing DNA repair activity. Clin Cancer Res (2005) 11: 4912-4922.
41. Ree AH, Dueland S, Folkvord S, Hole KH, Seierstad T, Johansen M, Abrahamsen TW and Flatmark K: Vorinostat, a histone deacetylase inhibitor, combined with pelvic palliative radiotherapy for gastrointestinal carcinoma: the Pelvic Radiation and Vorinostat (PRAVO) phase 1 study. Lancet Oncol (2010) 11: 459-464.
42. Shabason JE, Tofilon PJ and Camphausen K: Grand Rounds at the National Institutes of Health: HDAC Inhibitors as Radiation Modifiers, from Bench to Clinic. J Cell Mol Med (2011).
43. Noel G, Giocanti N, Fernet M, Megnin-Chanet F and Favaudon V: Poly (ADP-ribose) polymerase (PARP-1) is not involved in DNA double-strand break recovery. BMC Cell Biol (2003) 4: 7.
44. Schultz N, Lopez E, Saleh-Gohari N and Helleday T: Poly (ADP-ribose) polymerase (PARP-1) has a controlling role in homologous recombination. Nucleic Acids Res (2003) 31: 4959-4964.
45. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC and Ashworth A: Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature (2005) 434: 917-921.
46. Loser DA, Shibata A, Shibata AK, Woodbine LJ, Jeggo PA and Chalmers AJ: Sensitization to radiation and alkylating agents by inhibitors of poly (ADP-ribose) polymerase is enhanced in cells deficient in DNA double-strand break repair. Mol Cancer Ther (2010) 9: 1775-1787.
47. Chalmers AJ, Lakshman M, Chan N and Bristow RG: Poly (ADP-ribose) polymerase inhibition as a model for synthetic lethality in developing radiation oncology targets. Semin Radiat Oncol (2010) 20: 274-281.
48. Camphausen K and Tofilon PJ: Inhibition of Hsp90: a multitarget approach to radiosensitization. Clin Cancer Res (2007) 13: 4326-4330.
49. Kabakov AE, Kudryavtsev VA and Gabai VL: Hsp90 inhibitors as promising agents for radiotherapy. J Mol Med (Berl) (2010) 88: 241-247.
50. Lapenna S and Giordano A: Cell cycle kinases as therapeutic targets for cancer. Nat Rev Drug Discov (2009) 8: 547-566.
51. Tofilon PJ and Camphausen K: Molecular targets for tumor radiosensitization. Chem Rev (2009) 109: 2974-2988.
52. Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazi M, Pecker I, Frydman M, Harnik R, Patanjali SR, Simmons A, Clines GA, Sartiel A, Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NG, Taylor AM, Arlett CF, Miki T, Weissman SM, Lovett M, Collins FS and Shiloh Y: A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science (1995) 268: 1749-1753.
53. Shiloh Y: Ataxia-telangiectasia and the Nijmegen breakage syndrome: related disorders but genes apart. Annu Rev Genet (1997) 31: 635-662.
54. Eriksson D and Stigbrand T: Radiation-induced cell death mechanisms. Tumour Biol (2010) 31: 363-372.
55. Postiglione I, Chiaviello A and Palumbo G: Twilight effects of low doses of ionizing radiation on cellular systems: a bird's eye view on current concepts and research. Med Oncol (2010) 27: 495-509.
56. Riesterer O, Matsumoto F, Wang L, Pickett J, Molkentine D, Giri U, Milas L and Raju U: A novel Chk inhibitor, XL-844, increases human cancer cell radiosensitivity through promotion of mitotic catastrophe. Invest New Drugs (2011) 29: 514-522.
57. Morgan MA, Parsels LA, Zhao L, Parsels JD, Davis MA, Hassan MC, Arumugarajah S, Hylander-Gans L, Morosini D, Simeone DM, Canman CE, Normolle DP, Zabludoff SD, Maybaum J and Lawrence TS: Mechanism of radiosensitization by the Chk1/2 inhibitor AZD7762 involves abrogation of the G2 checkpoint and inhibition of homologous recombinational DNA repair. Cancer Res (2010) 70: 4972-4981.
58. Shrivastav M, De Haro LP and Nickoloff JA: Regulation of DNA double-strand break repair pathway choice. Cell Res (2008) 18: 134-147.
59. Alao JP and Sunnerhagen P: The ATM and ATR inhibitors CGK733 and caffeine suppress cyclin D1 levels and inhibit cell proliferation. Radiat Oncol (2009) 4: 51.
60. Sarkaria JN, Tibbetts RS, Busby EC, Kennedy AP, Hill DE and Abraham RT: Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. Cancer Res (1998) 58: 4375-4382.
61. Zhou BB, Chaturvedi P, Spring K, Scott SP, Johanson RA, Mishra R, Mattern MR, Winkler JD and Khanna KK: Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. J Biol Chem (2000) 275: 10342-10348.
62. Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NM, Orr AI, Reaper PM, Jackson SP, Curtin NJ and Smith GC: Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. Cancer Res (2004) 64: 9152-9159.
63. Won J, Kim M, Kim N, Ahn JH, Lee WG, Kim SS, Chang KY, Yi YW and Kim TK: Small molecule-based reversible reprogramming of cellular lifespan. Nat Chem Biol (2006) 2: 369-374.
64. Rainey MD, Charlton ME, Stanton RV and Kastan MB: Transient inhibition of ATM kinase is sufficient to enhance cellular sensitivity to ionizing radiation. Cancer Res (2008) 68: 7466-7474.
65. Collis SJ, Swartz MJ, Nelson WG and DeWeese TL: Enhanced radiation and chemotherapy-mediated cell killing of human cancer cells by small inhibitory RNA silencing of DNA repair factors. Cancer Res (2003) 63: 1550-1554.
66. Guha C, Guha U, Tribius S, Alfieri A, Casper D, Chakravarty P, Mellado W, Pandita TK and Vikram B: Antisense ATM gene therapy: a strategy to increase the radiosensitivity of human tumors. Gene Ther (2000) 7: 852-858.
67. Ajimura M, Leem SH and Ogawa H: Identification of new genes required for meiotic recombination in Saccharomyces cerevisiae. Genetics (1993) 133: 51-66.
68. Parry JM, Davies PJ and Evans WE: The effects of "cell age" upon the lethal effects of physical and chemical mutagens in the

yeast, Saccharomyces cerevisiae. Mol Gen Genet (1976) 146: 27–35.
69. Carney JP, Maser RS, Olivares H, Davis EM, Le Beau M, Yates JR, 3rd, Hays L, Morgan WF and Petrini JH: The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. Cell (1998) 93: 477–486.
70. Stewart GS, Maser RS, Stankovic T, Bressan DA, Kaplan MI, Jaspers NG, Raams A, Byrd PJ, Petrini JH and Taylor AM: The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. Cell (1999) 99: 577–587.
71. Waltes R, Kalb R, Gatei M, Kijas AW, Stumm M, Sobeck A, Wieland B, Varon R, Lerenthal Y, Lavin MF, Schindler D and Dork T:Human RAD50 deficiency in a Nijmegen breakage syndrome-like disorder. Am J Hum Genet (2009) 84: 605–616.
72. Williams RS, Williams JS and Tainer JA: Mre11-Rad50-Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. Biochem Cell Biol (2007) 85: 509–520.
73. Nelms BE, Maser RS, MacKay JF, Lagally MG and Petrini JH: In situ visualization of DNA double-strand break repair in human fibroblasts. Science (1998) 280: 590–592.
74. Paull TT: Making the best of the loose ends: Mre11/Rad50 complexes and Sae2 promote DNA double-strand break resection. DNA Repair (Amst) (2010) 9: 1283–1291.
75. Di Virgilio M and Gautier J: Repair of double-strand breaks by nonhomologous end joining in the absence of Mre11. J Cell Biol (2005) 171: 765–771.
76. Dupre A, Boyer-Chatenet L, Sattler RM, Modi AP, Lee JH, Nicolette ML, Kopelovich L, Jasin M, Baer R, Paull TT and Gautier J: A forward chemical genetic screen reveals an inhibitor of the Mre11-Rad50-Nbs1 complex. Nat Chem Biol (2008) 4: 119–125.
77. Garner KM, Pletnev AA and Eastman A: Corrected structure of mirin, a small-molecule inhibitor of the Mre11-Rad50-Nbs1 complex. Nat Chem Biol (2009) 5: 129–130; author reply 130.
78. Kuroda S, Fujiwara T, Shirakawa Y, Yamasaki Y, Yano S, Uno F, Tazawa H, Hashimoto Y, Watanabe Y, Noma K, Urata Y and Kagawa S: Telomerase-dependent oncolytic adenovirus sensitizes human cancer cells to ionizing radiation via inhibition of DNA repair machinery. Cancer Res (2010) 70: 9339–9348.
79. Kawashima T, Kagawa S, Kobayashi N, Shirakiya Y, Umeoka T, Teraishi F, Taki M, Kyo S, Tanaka N and Fujiwara T: Telomerase-specific replication-selective virotherapy for human cancer. Clin Cancer Res (2004) 10: 285–292.
80. Umeoka T, Kawashima T, Kagawa S, Teraishi F, Taki M, Nishizaki M, Kyo S, Nagai K, Urata Y, Tanaka N and Fujiwara T: Visualization of intrathoracically disseminated solid tumors in mice with optical imaging by telomerase-specific amplification of a transferred green fluorescent protein gene. Cancer Res (2004) 64: 6259–6265.
81. Hashimoto Y, Watanabe Y, Shirakiya Y, Uno F, Kagawa S, Kawamura H, Nagai K, Tanaka N, Kumon H, Urata Y and Fujiwara T: Establishment of biological and pharmacokinetic assays of telomerase-specific replication-selective adenovirus. Cancer Sci (2008) 99: 385–390.
82. Nemunaitis J, Tong AW, Nemunaitis M, Senzer N, Phadke AP, Bedell C, Adams N, Zhang YA, Maples PB, Chen S, Pappen B, Burke J, Ichimaru D, Urata Y and Fujiwara T: A phase I study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. Mol Ther (2010) 18: 429–434.
83. Blackford AN and Grand RJ: Adenovirus E1B 55-kilodalton protein: multiple roles in viral infection and cell transformation. J Virol (2009) 83: 4000–4012.
84. Stracker TH, Carson CT and Weitzman MD: Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature (2002) 418: 348–352.
85. Carson CT, Schwartz RA, Stracker TH, Lilley CE, Lee DV and Weitzman MD: The Mre11 complex is required for ATM activation and the G2/M checkpoint. EMBO J (2003) 22: 6610–6620.
86. Schwartz RA, Lakdawala SS, Eshleman HD, Russell MR, Carson CT and Weitzman MD: Distinct requirements of adenovirus E1b55K protein for degradation of cellular substrates. J Virol (2008) 82: 9043–9055.
87. Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M, Ng L, Nye JA, Sampson-Johannes A, Fattaey A and McCormick F: An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science (1996) 274: 373–376.
88. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R and Langer R: Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol (2007) 2: 751–760.
89. Majumdar D, Peng XH and Shin DM: The medicinal chemistry of theragnostics, multimodality imaging and applications of nanotechnology in cancer. Curr Top Med Chem (2010) 10: 1211–1226.
90. Eto Y, Yoshioka Y, Mukai Y, Okada N and Nakagawa S: Development of PEGylated adenovirus vector with targeting ligand. Int J Pharm (2008) 354: 3–8.

Gene Therapy (2012) 1 - 7

www.nature.com/gt

SHORT COMMUNICATION

A simple detection system for adenovirus receptor expression using a telomerase-specific replication-competent adenovirus

T Sasaki[1], H Tazawa[2,3], J Hasei[1], S Osaki [1], T Kunisada[1,4], A Yoshida[1], Y Hashimoto[3], S Yano[3], R Yoshida[3], S Kagawa[3], F Uno[3], Y Urata[5], T Ozaki[1] and T Fujiwara[3]

Adenovirus serotype 5 (Ad5) is frequently used as an effective vector for induction of therapeutic transgenes in cancer gene therapy or of tumor cell lysis in oncolytic virotherapy. Ad5 can infect target cells through binding with the coxsackie and adenovirus receptor (CAR). Thus, the infectious ability of Ad5-based vectors depends on the CAR expression level in target cells. There are conventional methods to evaluate the CAR expression level in human target cells, including flow cytometry, western blotting and immunohistochemistry. Here, we show a simple system for detection and assessment of functional CAR expression in human tumor cells, using the green fluorescent protein (GFP)-expressing telomerase-specific replication-competent adenovirus OBP-401. OBP-401 infection induced detectable GFP expression in CAR-expressing tumor cells, but not in CAR-negative tumor cells, nor in CAR-positive normal fibroblasts, 24 h after infection. OBP-401-mediated GFP expression was significantly associated with CAR expression in tumor cells. OBP-401 infection detected tumor cells with low CAR expression more efficiently than conventional methods. OBP-401 also distinguished CAR-positive tumor tissues from CAR-negative tumor and normal tissues in biopsy samples. These results suggest that GFP-expressing telomerase-specific replication-competent adenovirus is a very potent diagnostic tool for assessment of functional CAR expression in tumor cells for Ad5-based antitumor therapy.

Gene Therapy advance online publication, 12 January 2012; doi:10.1038/gt.2011.213

Keywords: oncolytic virus; adenovirus; telomerase; sarcoma; GFP

INTRODUCTION

Adenovirus serotype 5 (Ad5) is widely and frequently used as an effective vector in cancer gene therapy and oncolytic virotherapy.[1-3] Adenovirus-mediated transgene transduction is a highly efficient method for induction of ectopic transgene expression in tumor cells.[1,2] The p53 tumor suppressor gene, which is a potential therapeutic transgene that may induce a very strong antitumor effect, has been transduced into tumor cells using a replication-deficient adenovirus vector (Ad-p53, Advexin, Intorgen Therapeutics, Inc., Austin, TX, USA), and Ad-p53 has been reported to induce an antitumor effect in clinical studies.[4-7] Recently, an Ad5-based replication-competent oncolytic adenovirus has been developed as a promising anticancer reagent for induction of tumor-specific cell lysis.[8,9] Ad5-based vectors infect human target cells through binding with the coxsackie and adenovirus receptor (CAR).[10] Thus, the infection efficiency of Ad5-based vectors mainly depends on the CAR expression level in tumor tissues.[11-17] Increased CAR expression has been frequently shown in tumor cells in various organs such as the brain,[18] thyroid,[19] esophagus,[20] gastrointestinal tract,[21] prostate,[14] bone and soft tissues.[22-24] However, tumor cells often show reduced CAR expression following tumor progression.[18,21,25,26] Decreased CAR expression has also been shown in tumor tissues after repeated injection of Ad-p53.[27,28] It is therefore necessary to assess the CAR expression level of target tumor tissues before and after Ad5-based cancer gene therapy and oncolytic virotherapy.

There are some conventional methods for evaluation of the CAR expression level in tumor tissues, such as flow cytometry, immunohistochemistry, western blotting and reverse transcription (RT)-PCR . Flow cytometry is mainly used to detect CAR-positive human tumor cell lines.[13,24,28,29] Immunohistochemistry is frequently used to assess CAR expression in various human tumor tissues.[11,14,20,23,25] Western blotting is usually performed to confirm the expression of many types of proteins including CAR in molecular biological experiments. Quantitative RT-PCR is also a useful method for evaluation of the mRNA expression of CAR.[18,22] Although these conventional methods can detect CAR expression in tumor tissues, it still remains unclear whether Ad5-based vectors really infect target tumor cells through binding with the CAR that is detected using conventional methods. Therefore, the development of a novel method for assessment of the level of expression of functional CAR in tumor tissues, which is what the Ad5-based vectors really bind, is required for Ad5-based anticancer therapy.

We previously developed a telomerase-specific replication-competent adenovirus OBP-301 (Telomelysin, Oncolys BioPharma, Inc., Tokyo, Japan) that drives the *E1A* and *E1B* genes under the human telomerase reverse transcriptase (*hTERT*) promoter.[8,29-31] OBP-301 infects both normal and tumor cells that express CAR, but replicates only in CAR-positive tumor cells in a telomerase-dependent manner. Furthermore, we recently generated a green fluorescent protein (GFP)-expressing telomerase-specific replication-

[1]Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [2]Center for Gene and Cell Therapy, Okayama University Hospital, Okayama, Japan; [3]Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [4]Department of Medical Materials for Musculoskeletal Reconstruction, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan and [5]Oncolys BioPharma, Inc., Tokyo, Japan. Correspondence: Professor T Fujiwara, Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan.
E-mail: toshi_f@md.okayama-u.ac.jp
Received 15 July 2011; revised 7 November 2011; accepted 5 December 2011

competent adenovirus OBP-401, which induces ectopic GFP expression in tumor cells, but not in normal cells.[32] OBP-401 infection efficiently induces GFP expression in metastatic tumor cells at regional lymph nodes[32] and liver,[33] circulating tumor cells in blood flow[34] and disseminated tumor cells in the abdominal cavity.[35] These results suggest that OBP-401 is a highly sensitive tool for the detection of tumor cells. Furthermore, Ad5-based OBP-401 would also be useful for induction of GFP expression in CAR-positive tumor cells, but not in CAR-negative tumor cells.

In the present study, we evaluated whether induction of GFP expression by OBP-401 infection is associated with CAR expression in tumor cells. OBP-401-mediated GFP induction was further examined in xenograft tumor tissues that have different levels of CAR expression and in surrounding normal tissues.

RESULTS AND DISCUSSION

Assessment of an OBP-401 infection protocol for the detection of CAR-positive tumor cells

We recently demonstrated that the level of CAR expression that was detected using flow cytometry was significantly associated with OBP-301-mediated cytopathic activity in human bone and soft tissue sarcoma cells.[29] Furthermore, OBP-401 infection has been shown to induce GFP expression 24 h after infection of human sarcoma cells.[34] To evaluate whether GFP expression that is induced by OBP-401 infection is associated with CAR expression in tumor cells, we used three human sarcoma cell lines (OST, NMFH-1 and OUMS-27) that have different levels of CAR expression, as previously reported.[29] Flow cytometric analysis confirmed that OST cells showed detectable CAR expression, whereas cells of the NMFH-1 and OUMS-27 sarcoma cell lines had no detectable CAR expression (Figure 1a).

To determine suitable conditions for OBP-401 infection in order to detect CAR-positive tumor cells, OST sarcoma cells were infected with OBP-401 at multiplicity of infections (MOIs) of 1, 10 and 100 plaque-forming units (PFU) per cell over 24 h (Figure 1b and c). Twelve hours after infection, only OBP-401 infection at an MOI of 100 had induced GFP expression in all of the OST cells. Twenty-four hours after infection, OBP-401 infection at MOIs of 10 and 100 had induced ectopic GFP expression in all of the OST cells, whereas OBP-401 infection at an MOI of 1 had induced GFP expression in about 80% of the OST cells. These results indicate that OBP-401 infection at an MOI of greater than 10 is necessary to efficiently detect CAR-positive tumor cells 24 h after infection.

To subsequently determine a suitable condition for OBP-401 infection that would exclude CAR-negative tumor cells, the NMFH-1 and OUMS-27 sarcoma cells that do not express CAR were infected with OBP-401 at MOIs of 10 and 100 for 60 h (Figures 1d and e). NMFH-1 cells expressed GFP at 24 and 48 h after OBP-401 infection at MOIs of 100 and 10, respectively. In contrast, OUMS-27 cells exhibited no GFP expression after OBP-401 infection. To investigate the different GFP expression between these CAR-negative tumor cells, expression of integrins, αvβ3 and αvβ5, was further examined by flow cytometry. NMFH-1 cells showed twofold higher expression of integrin αvβ3 compared with OUMS-27 cells, whereas αvβ5 expression was similar in these cells (Supplementary Figure S1a). These results indicate that OBP-401 infection at an MOI of 10 for 24 h is a suitable protocol for distinguishing CAR-negative tumor cells from CAR-positive tumor cells, when CAR-negative tumor cells express integrin molecules.

Relationship between OBP-401-induced GFP expression and CAR expression

To evaluate whether OBP-401-induced GFP expression correlates with CAR expression in tumor cells, six human sarcoma cell lines (OST, U2OS, NOS-10, MNNG/HOS, NMFH-1 and OUMS-27) and normal human lung fibroblasts (NHLF) cells that have different levels of CAR expression (Figure 1a and Supplementary Figure S1b) were infected with OBP-401 at an MOI of 10 for 24 h, and the GFP-positive cells in each cell type were analyzed under fluorescence microscopy (Figures 2a and b). OBP-401 infection-induced GFP expression from 12 h after infection and, after 24 h, more than 40% of all CAR-positive tumor cells (OST, U2OS, NOS-10 and MNNG/HOS) were detected as GFP-positive cells. However, no GFP-positive cells were detected in the CAR-negative tumor cells (NMFH-1, OUMS-27), or in the normal NHLF cells, 24 h after infection. Furthermore, OBP-401-mediated GFP induction in CAR-positive tumor cells was suppressed by blocking CAR proteins with anti-CAR antibody (Supplementary Figure S2). To assess the GFP expression level in all tumor and normal cells in a more quantitative manner, we quantified the level of GFP fluorescence in each cell type 24 h after infection using a fluorescence microplate reader (Figure 2c). We also quantified the level of CAR expression in these cells by calculating the mean fluorescence intensity in flow cytometric analysis (Figure 2d). GFP fluorescence was detected in CAR-positive tumor cells, but not in either CAR-negative tumor cells or in CAR-positive normal cells. There was a significant relationship between the CAR expression level and the GFP fluorescence level ($r = 0.885$; $P = 0.019$) (Figure 2e). These results indicate that OBP-401-mediated GFP expression is highly associated with CAR expression in tumor cells.

Comparison of the potential of OBP-401-mediated GFP induction and of conventional methods for CAR detection

To estimate the potential of OBP-401-mediated GFP induction for the detection of CAR-positive tumor cells, we compared the above protocol using OBP-401 with western blot analysis and immunocytochemistry. CAR expression was detected in OST, U2OS and NOS-10 sarcoma cells, but not in CAR-positive MNNG/HOS sarcoma cells, using western blot analysis (Supplementary Figure S3a). In contrast, only OST cells displayed a positive CAR signal using immunocytochemistry, whereas the CAR signal of the other three CAR-positive tumor cells was almost as weak as that from CAR-negative tumor cells (Supplementary Figure S3b). CAR expression was also not detected in CAR-positive NHLF cells by either western blot analysis or by immunocytochemistry. These results suggest that the GFP induction protocol using OBP-401 is more sensitive for the detection of CAR-positive tumor cells than conventional methods.

OBP-401-mediated GFP induction was detected in MNNG/HOS sarcoma cells that expressed a low level of CAR (Figure 2c), although neither western blot analysis nor immunocytochemistry detected CAR in these cells (Supplementary Figure S3). Furthermore, although conventional methods may be able to detect high CAR expression in tumor cells, whether the CAR expression that is detected by conventional methods is really functional for binding with Ad5-based vectors still remains unclear. In contrast, as OBP-401 is an Ad5-based vector that expresses a fluorescent *GFP* gene, OBP-401-induced GFP expression directly proves that the CAR that is expressed is functional for Ad5-based vector binding. Thus, the OBP-401-mediated GFP induction strategy is a potential diagnostic method that can efficiently and directly assess functional CAR expression in tumor cells.

OBP-401-mediated GFP induction in xenograft tumor and normal tissues with different CAR expression

Finally, to investigate the potential of the OBP-401-mediated method for the detection of CAR expression in tumor and normal tissues, we used this method to analyze CAR expression of human xenograft tumor tissues, that do or do not express CAR, as well as of surrounding normal muscle tissues, which have been previously shown to lose CAR expression.[36] CAR-positive OST sarcoma cells or CAR-negative OUMS-27 sarcoma cells were inoculated into nude

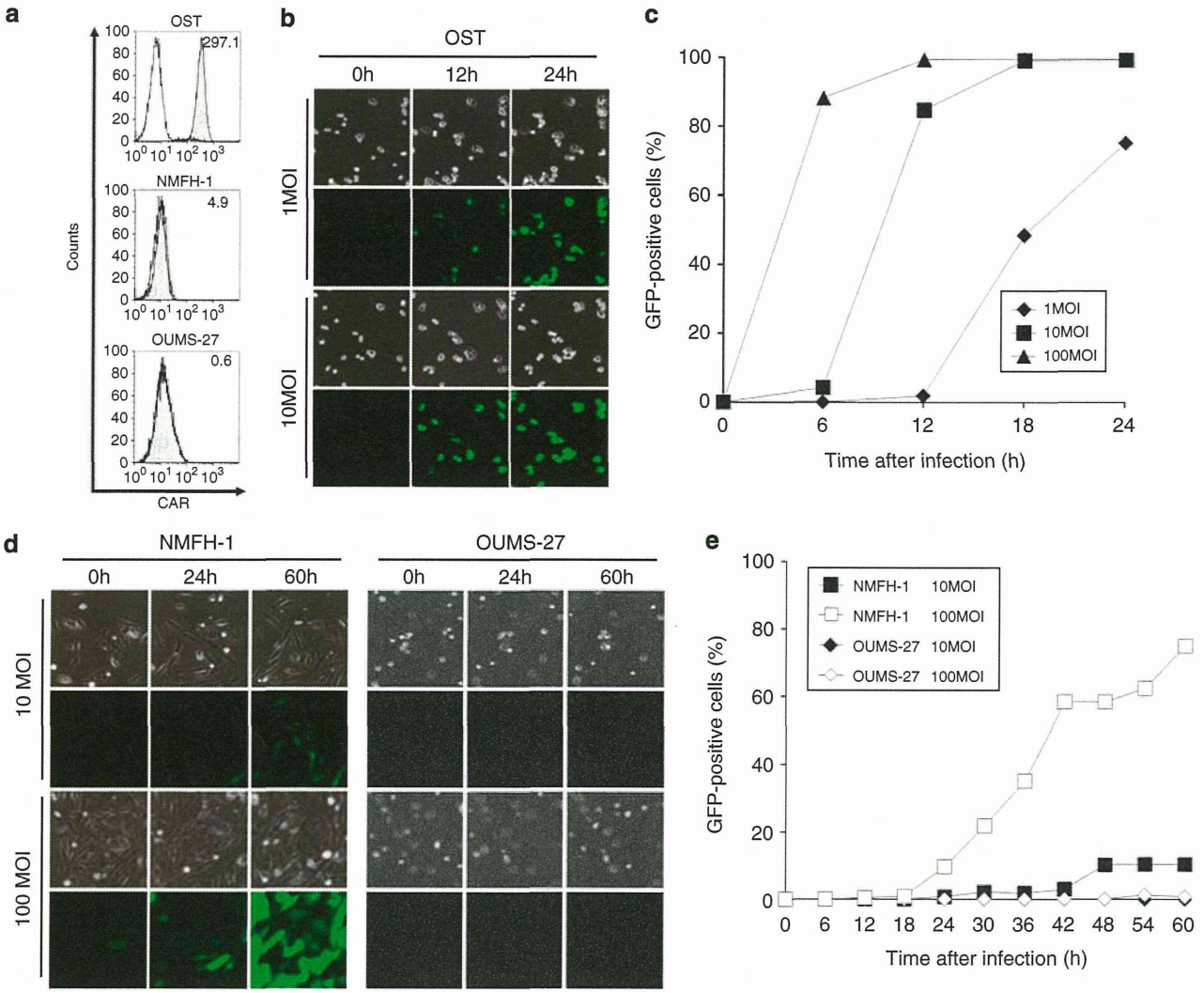

Figure 1. Establishment of a suitable protocol for the detection of CAR expression using OBP-401. (**a**) The level of CAR expression on three human sarcoma cell lines (OST, NMFH-1 and OUMS-27) was analyzed using flow cytometry. The cells were incubated with a monoclonal anti-CAR (RmcB) antibody and the signal was detected using a fluorescent isothiocyanate (FITC)-labeled secondary antibody. The mean fluorescence intensity (MFI), which is a measure of CAR and integrin expression, was calculated for each cell and is shown at the top right of each graph. (**b**) Time-lapse images of OST cells, which displayed the highest CAR expression, were recorded for 24 h after OBP-401 infection at MOIs of 1 and 10 PFU per cell. Representative images taken at the indicated time points and MOIs show cell morphology that was analyzed using phase-contrast microscopy (top panels) and GFP expression that was analyzed using fluorescence microscopy (bottom panels). Original magnification: × 80. (**c**) The percentage of GFP-positive cells was counted in OST cells at the indicated time points after OBP-301 infection at MOIs of 1, 10 and 100 PFU per cell. (**d**) Time-lapse images of non-CAR-expressing OUMS-27 and NMFH-1 cells were recorded for 60 h after OBP-401 infection at MOIs of 10 and 100 PFU per cell. Representative images taken at the indicated time points and MOIs show cell morphology that was analyzed using phase-contrast microscopy (top panels) and GFP expression that was analyzed using fluorescence microscopy (bottom panels). Original magnification: × 80. (**e**) The percentage of OUMS-27 and NMFH-1 GFP-positive cells was counted at the indicated time points after OBP-301 infection at MOIs of 10 and 100 PFU per cell.

mice to develop xenograft tumors. After resection of the OST tumors, the OUMS-27 tumors and normal muscle tissue, the tissues were subjected to the protocol for OBP-401-mediated GFP induction using a three-step procedure (Figure 3a) as follows; step 1: OBP-401 infection for 24 h, step 2: washing with PBS and step 3: observation under a fluorescence microscope. As shown in Figure 3b, OBP-401 infection-induced GFP expression in CAR-positive OST tumor tissues, but not in CAR-negative OUMS-27 tumor tissues or in normal muscle tissue. These results suggest that OBP-401-mediated GFP induction is a simple and useful method for the detection of CAR expression by tumor tissues.

Flow cytometry is a highly sensitive conventional method for the detection of cell surface CAR expression, which is associated with the therapeutic efficacy of Ad5-based vectors in tumor cells.[13,24,28,29] However, as many tumor cells tightly bind to each other or to normal stromal cells within tumor tissues, the preparation of single tumor cells is not easy, and therefore flow cytometry is an inadequate method for the detection of CAR expression in tumor tissues. In contrast, the preparation of single tumor cells is not necessary for the OBP-401-mediated GFP induction protocol. Furthermore, assay of OBP-401-induced GFP expression was more sensitive than flow cytometry (Figure 2d) in distinguishing CAR-positive normal cells from CAR-positive tumor cells (Figure 2c). Thus, the OBP-401-mediated GFP induction method is a simple and tumor-specific system for the detection of CAR expression in tumor tissues.

Fluorescent proteins including GFP have great potentials to visualize tumor cells in real time on the *in vivo* setting.[37,38]

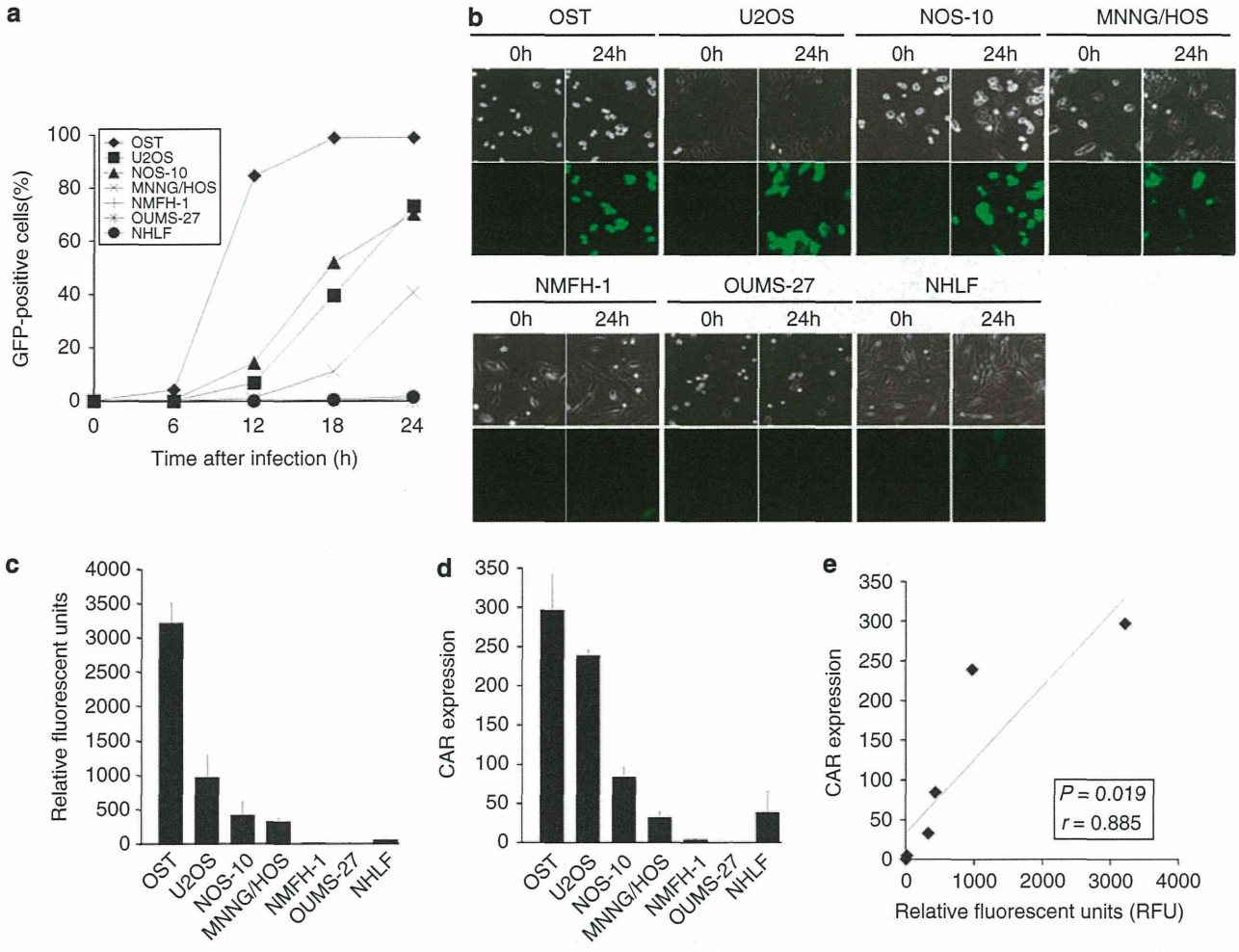

Figure 2. *In vitro* CAR-dependent GFP expression induced by OBP-401 infection. (**a**) The percentage of GFP-positive cells in all tumor and normal cells was counted at the indicated time points after OBP-301 infection at an MOI of 10 PFU per cell. (**b**) Time-lapse images of all tumor and normal cells were recorded for 24 h after infection with OBP-401 at an MOI of 10 PFU per cell. Representative images taken at the indicated time points show cell morphology that was analyzed using phase-contrast microscopy (top panels) and GFP expression that was analyzed using fluorescence microscopy (bottom panels). Original magnification: ×80. (**c**) Quantitative assessment of the level of GFP fluorescence in all tumor and normal cells 24 after OBP-401 infection at an MOI of 10 PFU per cell, using a fluorescent microplate reader with excitation/emission at 485 nm/528 nm. The intensity of GFP fluorescence was evaluated based on the brightness determinations used as relative fluorescence units (RFU). (**d**) The mean fluorescent intensity (MFI) of (CAR) expression on human sarcoma cells and normal fibroblasts. The cells were incubated with a monoclonal anti-CAR (RmcB) antibody, followed by a FITC-labeled secondary antibody, and were analyzed using flow cytometry. (**e**) Relationship between the level of GFP fluorescence and CAR expression in all tumor and normal cells after OBP-401 infection. The slope represents the inverse correlation between these two factors. Statistical significance was determined as $P<0.05$, after analysis of Pearson's correlation coefficient.

We previously reported that OBP-401 can efficiently induce GFP expression in small populations of metastatic tumor cells at various regions *in vivo*.[32-35] In this study, we further demonstrated that OBP-401-mediated GFP expression provides us the important information for detection of CAR-positive tumor cells. OBP-401 with *hTERT* gene promoter-induced GFP expression in CAR-positive tumor cells with telomerase activity, but not CAR-positive normal cells without telomerase activity (Figure 2c). There was significant relationship between the CAR expression and the GFP expression in tumor cells (Figure 2d). Among the four CAR-positive tumor cells, U2OS cells showed low GFP expression compared with high CAR expression (Figure 1a and 2c). As we recently reported that U2OS cells showed low *hTERT* mRNA expression, the low activity of *hTERT* gene promoter in tumor cells would affect OBP-401-mediated GFP expression. However, as various types of human cancer cells frequently show high telomerase activities,[39] OBP-401-mediated GFP induction system would be widely useful method to evaluate CAR expression in tumor cells.

Previous reports have suggested that *ex vivo* infection of human cancer specimens with a GFP-expressing replication-deficient adenovirus[40] or a replication-selective oncolytic adenovirus[41] is a useful method for assessment of the transduction efficacy or cytopathic activity, respectively, of Ad5-based vectors in individual tumor tissues. In this study, we confirmed that the GFP-expressing telomerase-specific oncolytic adenovirus OBP-401 is useful for detection of CAR-positive tumor tissues through induction of GFP expression (Figure 3b). Interestingly, OBP-401-infected OST tumor tissues showed heterogenous GFP expression (Figure 3b), although GFP expression was induced in all OBP-401-infected OST cells *in vitro* (Figure 2b). Our finding of heterogenous GFP expression in tumor tissues, which indicates heterogenous CAR expression, is consistent with a previously reported heterogeneity in CAR expression.[42] As several factors such as hypoxia[43] and cell cycle status[44] have been suggested to affect CAR expression in tumor cells, factors in the tumor microenvironment may be involved in the heterogenous CAR expression in tumor cells.

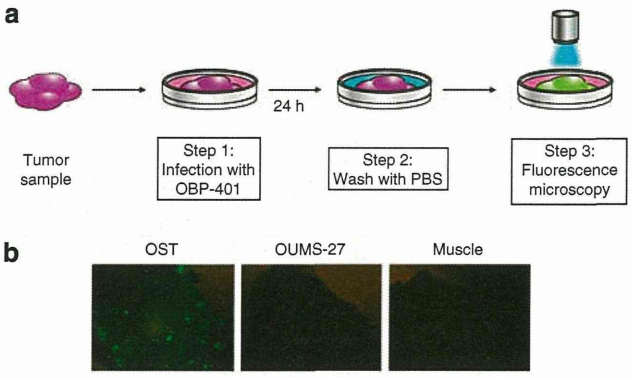

Figure 3. A simple method for detection of CAR expression in tumor tissues using OBP-401 infection. (**a**) Outline of the 3-step procedure; step 1: infection with OBP-401, step 2: washing with PBS and step 3: observation under a fluorescence microscope. Tumor tissues ($2 \times 2 \times 2\,mm^3$) were infected with OBP-401 at a concentration of 2.4×10^6 PFU for 24 h, were washed with PBS and were observed using fluorescence microscopy. (**b**) Assessment of GFP expression in the CAR-positive OST tumor (left panel), the CAR-negative OUMS-27 tumor (middle panel) and normal muscle tissues (right panel) under a fluorescence microscope. Original magnification: $\times$ 30.

Furthermore, as OBP-401 induces tumor-specific GFP expression, normal stromal or epithelial cells may be involved in heterogenous GFP expression in tumor tissues.

In conclusion, we have demonstrated that the GFP-expressing telomerase-specific replication-competent adenovirus OBP-401 is a promising fluorescence imaging tool for the detection of functional and tumor-specific CAR expression in tumor tissues. OBP-401-mediated GFP induction is a simple and highly sensitive method for analysis of tumor cells compared with conventional methods. This novel CAR detection system using OBP-401 has the potential of being widely applicable to assessment of predictive biomarkers for Ad5-based vector-mediated anticancer therapy.

MATERIALS AND METHODS

Cell lines

The human osteosarcoma cell line OST was kindly provided by Dr Satoru Kyo (Kanazawa University, Ishikawa, Japan). The human osteosarcoma cell line U2OS and the transformed embryonic kidney cell line 293 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The human osteosarcoma cell line NOS-10[45] and the human malignant fibrous histiocytoma cell line NMFH-1[46] were kindly provided by Dr Hiroyuki Kawashima (Niigata University, Niigata, Japan). The human osteosarcoma cell line MNNG/HOS was purchased from DS Pharma Biomedical (Osaka, Japan). The chondrosarcoma cell line OUMS-27 was previously established in our laboratory.[47] The normal human lung fibroblast cell line NHLF was obtained from TaKaRa Biomedicals (Kyoto, Japan). These cells were propagated as monolayer cultures in the medium recommended by the manufacturer. All media were supplemented with 10% heat-inactivated fetal bovine serum, 100 units ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Recombinant adenoviruses

We previously generated and characterized OBP-401, which is a telomerase-specific replication-competent adenovirus variant, in which the *hTERT* promoter element drives the expression of *E1A* and *E1B* genes that are linked to an internal ribosome entry site, and in which the *GFP* gene is inserted into the E3 region under a cytomegalovirus promoter.[32,34] The virus was purified by ultracentrifugation using cesium chloride step gradients. Viral titers were determined by a plaque-forming assay using 293 cells and viruses were stored at −80 °C.

Flow cytometry

The cells (5×10^5 cells) were labeled with the mouse monoclonal anti-CAR (RmcB; Upstate Biotechnology, Lake Placid, NY, USA) antibody for 30 min at 4 °C. The cells were then incubated with fluorescent isothiocyanate-conjugated rabbit anti-mouse IgG second antibody (Zymed Laboratories, San Francisco, CA, USA) and were analyzed using flow cytometry (FACS Array; Becton Dickinson, Mountain View, CA, USA). The mean fluorescence intensity of CAR for each cell line was determined by calculating the differences between the mean fluorescence intensity in antibody-treated and non-treated cells in triplicate experiments.

Time-lapse confocal laser microscopy

The cells (1×10^5 cells per dish) were seeded in 35 mm glass-based dishes 20 h before virus infection. OST cells were infected with OBP-401 at an MOI of 1, 10 or 100 PFU per cell for 24 h. NMFH-1 and OUMS-27 cells were infected with OBP-401 at an MOI of 10 or 100 PFU per cell for 60 h. Other cells were infected with OBP-401 at an MOI of 10 PFU per cell for 24 h. Phase-contrast and fluorescence time-lapse recordings were obtained to concomitantly analyze cell morphology and GFP expression using an inverted FV10i confocal laser scanning microscopy (OLYMPUS; Tokyo, Japan). Photographic images were taken every 5 min. The percentage of GFP-positive cells in each field was calculated using the formula: the number of CAR-positive cells / the total number of CAR-positive and CAR-negative cells $\times$ 100.

Fluorescence microplate assay

The cells (5×10^3 cells per well) were seeded on 96-well black bottomed culture plates and were incubated for 20 h before virus infection. The cells were infected with OBP-401 at an MOI of 10 for 24 h. The level of expression of GFP fluorescence was measured using a fluorescent microplate reader (DS Pharma Biomedical; Osaka, Japan) with excitation/emission at 485 nm/528 nm. The mean expression of GFP fluorescence in each cell was calculated in triplicate experiments, as previously reported.[34]

Animal experiments

Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine. OST and OUMS-27 cells (5×10^6 cells per site) were inoculated into the flank of female athymic nude mice aged 6 to 7 weeks (Charles River Laboratories, Wilmington, MA, USA). Palpable tumors developed within 14 to 21 days and were permitted to grow to ~5 to 6 mm in diameter. At that stage, tumor and normal muscle tissues were resected. The tumor and normal tissues ($2 \times 2 \times 2\,mm^3$) were placed in 96-well plates with culture medium. As single tumor cell is about 10 μm in diameter, we considered that there are 2.4×10^5 cells on the surface area of each sample tissue. Then, we infected each sample tissue with 2.4×10^6 PFU (10 MOI per sample) of OBP-401 for 24 h. After washing with PBS, tumor and normal tissues were again placed in 96-well plates with culture medium and analyzed using an inverted fluorescence microscope (OLYMPUS).

Statistical analysis

Data are expressed as means ± s.d. Student's *t*-test was used to compare differences between groups. Pearson's product-moment correlation coefficients were calculated using PASW statistics software version 18 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as when the *P* value was less than 0.05.

ABBREVIATIONS

Ad5, Adenovirus serotype 5; CAR, coxsackie and adenovirus receptor; GFP, green fluorescent protein; RT-PCR, reverse transcription-polymerase chain reaction; hTERT, human telomerase reverse transcriptase; MOI, multiplicity of infection;

PFU, plaque-forming unit; IRES, internal ribosome entry site; FITC, fluorescent isothiocyanate; MFI, mean fluorescence intensity.

CONFLICT OF INTEREST

Y Urata is an employee of Oncolys BioPharma, Inc., the manufacturer of OBP-401 (Telomescan). The remaining authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Satoru Kyo (Kanazawa University) for providing the OST cells; Dr Hiroyuki Kawashima (Niigata University) for providing the NOS-10 and NMFH-1 cells; and Tomoko Sueishi for her excellent technical support. This study was supported by grants-in-Aid from the Ministry of Education, Science and Culture, Japan and grants from the Ministry of Health and Welfare, Japan.

REFERENCES

1 Kanerva A, Hemminki A. Adenoviruses for treatment of cancer. *Ann Med* 2005; **37**: 33-43.
2 Rein DT, Breidenbach M, Curiel DT. Current developments in adenovirus-based cancer gene therapy. *Future Oncol* 2006; **2**: 137-143.
3 Yamamoto M, Curiel DT. Current issues and future directions of oncolytic adenoviruses. *Mol Ther* 2010; **18**: 243-250.
4 Clayman GL, el-Naggar AK, Lippman SM, Henderson YC, Frederick M, Merritt JA *et al.* Adenovirus-mediated p53 gene transfer in patients with advanced recurrent head and neck squamous cell carcinoma. *J Clin Oncol* 1998; **16**: 2221-2232.
5 Swisher SG, Roth JA, Nemunaitis J, Lawrence DD, Kemp BL, Carrasco CH *et al.* Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer. *J Natl Cancer Inst* 1999; **91**: 763-771.
6 Shimada H, Matsubara H, Shiratori T, Shimizu T, Miyazaki S, Okazumi S *et al.* Phase I/II adenoviral p53 gene therapy for chemoradiation resistant advanced esophageal squamous cell carcinoma. *Cancer Sci* 2006; **97**: 554-561.
7 Fujiwara T, Tanaka N, Kanazawa S, Ohtani S, Saijo Y, Nukiwa T *et al.* Multicenter phase I study of repeated intratumoral delivery of adenoviral p53 in patients with advanced non-small-cell lung cancer. *J Clin Oncol* 2006; **24**: 1689-1699.
8 Fujiwara T, Urata Y, Tanaka N. Telomerase-specific oncolytic virotherapy for human cancer with the hTERT promoter. *Curr Cancer Drug Targets* 2007; **7**: 191-201.
9 Pesonen S, Kangasniemi L, Hemminki A. Oncolytic adenoviruses for the treatment of human cancer: focus on translational and clinical data. *Mol Pharm* 2011; **8**: 12-28.
10 Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS *et al.* Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997; **275**: 1320-1323.
11 Hemmi S, Geertsen R, Mezzacasa A, Peter I, Dummer R. The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures. *Hum Gene Ther* 1998; **9**: 2363-2373.
12 Hutchin ME, Pickles RJ, Yarbrough WG. Efficiency of adenovirus-mediated gene transfer to oropharyngeal epithelial cells correlates with cellular differentiation and human coxsackie and adenovirus receptor expression. *Hum Gene Ther* 2000; **11**: 2365-2375.
13 You Z, Fischer DC, Tong X, Hasenburg A, Aguilar-Cordova E, Kieback DG. Coxsackievirus-adenovirus receptor expression in ovarian cancer cell lines is associated with increased adenovirus transduction efficiency and transgene expression. *Cancer Gene Ther* 2001; **8**: 168-175.
14 Rauen KA, Sudilovsky D, Le JL, Chew KL, Hann B, Weinberg V *et al.* Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: potential relevance to gene therapy. *Cancer Res* 2002; **62**: 3812-3818.
15 Kim M, Zinn KR, Barnett BG, Sumerel LA, Krasnykh V, Curiel DT *et al.* The therapeutic efficacy of adenoviral vectors for cancer gene therapy is limited by a low level of primary adenovirus receptors on tumour cells. *Eur J Cancer* 2002; **38**: 1917-1926.
16 Qin M, Chen S, Yu T, Escuadro B, Sharma S, Batra RK. Coxsackievirus adenovirus receptor expression predicts the efficiency of adenoviral gene transfer into non-small cell lung cancer xenografts. *Clin Cancer Res* 2003; **9**: 4992-4999.
17 Douglas JT, Kim M, Sumerel LA, Carey DE, Curiel DT. Efficient oncolysis by a replicating adenovirus (ad) *in vivo* is critically dependent on tumor expression of primary ad receptors. *Cancer Res* 2001; **61**: 813-817.
18 Fuxe J, Liu L, Malin S, Philipson L, Collins VP, Pettersson RF. Expression of the coxsackie and adenovirus receptor in human astrocytic tumors and xenografts. *Int J Cancer* 2003; **103**: 723-729.
19 Marsee DK, Vadysirisack DD, Morrison CD, Prasad ML, Eng C, Duh QY *et al.* Variable expression of coxsackie-adenovirus receptor in thyroid tumors: implications for adenoviral gene therapy. *Thyroid* 2005; **15**: 977-987.
20 Anders M, Rosch T, Kuster K, Becker I, Hofler H, Stein HJ *et al.* Expression and function of the coxsackie and adenovirus receptor in Barrett's esophagus and associated neoplasia. *Cancer Gene Ther* 2009; **16**: 508-515.
21 Korn WM, Macal M, Christian C, Lacher MD, McMillan A, Rauen KA *et al.* Expression of the coxsackievirus- and adenovirus receptor in gastrointestinal cancer correlates with tumor differentiation. *Cancer Gene Ther* 2006; **13**: 792-797.
22 Gu W, Ogose A, Kawashima H, Ito M, Ito T, Matsuba A *et al.* High-level expression of the coxsackievirus and adenovirus receptor messenger RNA in osteosarcoma, Ewing's sarcoma, and benign neurogenic tumors among musculoskeletal tumors. *Clin Cancer Res* 2004; **10**: 3831-3838.
23 Kawashima H, Ogose A, Yoshizawa T, Kuwano R, Hotta Y, Hotta T *et al.* Expression of the coxsackievirus and adenovirus receptor in musculoskeletal tumors and mesenchymal tissues: efficacy of adenoviral gene therapy for osteosarcoma. *Cancer Sci* 2003; **94**: 70-75.
24 Rice AM, Currier MA, Adams LC, Bharatan NS, Collins MH, Snyder JD *et al.* Ewing sarcoma family of tumors express adenovirus receptors and are susceptible to adenovirus-mediated oncolysis. *J Pediatr Hematol Oncol* 2002; **24**: 527-533.
25 Matsumoto K, Shariat SF, Ayala GE, Rauen KA, Lerner SP. Loss of coxsackie and adenovirus receptor expression is associated with features of aggressive bladder cancer. *Urology* 2005; **66**: 441-446.
26 Anders M, Vieth M, Rocken C, Ebert M, Pross M, Gretschel S *et al.* Loss of the coxsackie and adenovirus receptor contributes to gastric cancer progression. *Br J Cancer* 2009; **100**: 352-359.
27 Yamamoto S, Yoshida Y, Aoyagi M, Ohno K, Hirakawa K, Hamada H. Reduced transduction efficiency of adenoviral vectors expressing human p53 gene by repeated transduction into glioma cells *in vitro*. *Clin Cancer Res* 2002; **8**: 913-921.
28 Tango Y, Taki M, Shirakiya Y, Ohtani S, Tokunaga N, Tsunemitsu Y *et al.* Late resistance to adenoviral p53-mediated apoptosis caused by decreased expression of Coxsackie-adenovirus receptors in human lung cancer cells. *Cancer Sci* 2004; **95**: 459-463.
29 Sasaki T, Tazawa H, Hasei J, Kunisada T, Yoshida A, Hashimoto Y *et al.* Preclinical evaluation of telomerase-specific oncolytic virotherapy for human bone and soft tissue sarcomas. *Clin Cancer Res* 2011; **17**: 1828-1838.
30 Kawashima T, Kagawa S, Kobayashi N, Shirakiya Y, Umeoka T, Teraishi F *et al.* Telomerase-specific replication-selective virotherapy for human cancer. *Clin Cancer Res* 2004; **10** (1 Pt 1): 285-292.
31 Hashimoto Y, Watanabe Y, Shirakiya Y, Uno F, Kagawa S, Kawamura H *et al.* Establishment of biological and pharmacokinetic assays of telomerase-specific replication-selective adenovirus. *Cancer Sci* 2008; **99**: 385-390.
32 Kishimoto H, Kojima T, Watanabe Y, Kagawa S, Fujiwara T, Uno F *et al. In vivo* imaging of lymph node metastasis with telomerase-specific replication-selective adenovirus. *Nat Med* 2006; **12**: 1213-1219.
33 Kishimoto H, Urata Y, Tanaka N, Fujiwara T, Hoffman RM. Selective metastatic tumor labeling with green fluorescent protein and killing by systemic administration of telomerase-dependent adenoviruses. *Mol Cancer Ther* 2009; **8**: 3001-3008.
34 Kojima T, Hashimoto Y, Watanabe Y, Kagawa S, Uno F, Kuroda S *et al.* A simple biological imaging system for detecting viable human circulating tumor cells. *J Clin Invest* 2009; **119**: 3172-3181.
35 Kishimoto H, Zhao M, Hayashi K, Urata Y, Tanaka N, Fujiwara T *et al. In vivo* internal tumor illumination by telomerase-dependent adenoviral GFP for precise surgical navigation. *Proc Natl Acad Sci USA* 2009; **106**: 14514-14517.
36 Feero WG, Rosenblatt JD, Huard J, Watkins SC, Epperly M, Clemens PR *et al.* Viral gene delivery to skeletal muscle: insights on maturation-dependent loss of fiber infectivity for adenovirus and herpes simplex type 1 viral vectors. *Hum Gene Ther* 1997; **8**: 371-380.
37 Hoffman RM. The multiple uses of fluorescent proteins to visualize cancer *in vivo*. *Nat Rev Cancer* 2005; **5**: 796-806.
38 Hoffman RM, Yang M. Subcellular imaging in the live mouse. *Nat Protoc* 2006; **1**: 775-782.
39 Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer* 1997; **33**: 787-791.
40 Marsman WA, Buskens CJ, Wesseling JG, Offerhaus GJ, Bergman JJ, Tytgat GN *et al.* Gene therapy for esophageal carcinoma: the use of an explant model to test adenoviral vectors *ex vivo*. *Cancer Gene Ther* 2004; **11**: 289-296.
41 Wang Y, Thorne S, Hannock J, Francis J, Au T, Reid T *et al.* A novel assay to assess primary human cancer infectibility by replication-selective oncolytic adenoviruses. *Clin Cancer Res* 2005; **11**: 351-360.
42 Zeimet AG, Muller-Holzner E, Schuler A, Hartung G, Berger J, Hermann M *et al.* Determination of molecules regulating gene delivery using adenoviral vectors in ovarian carcinomas. *Gene Therapy* 2002; **9**: 1093-1100.
43 Kuster K, Koschel A, Rohwer N, Fischer A, Wiedenmann B, Anders M. Downregulation of the coxsackie and adenovirus receptor in cancer cells by hypoxia depends on HIF-1alpha. *Cancer Gene Ther* 2010; **17**: 141-146.

44 Seidman MA, Hogan SM, Wendland RL, Worgall S, Crystal RG, Leopold PL. Variation in adenovirus receptor expression and adenovirus vector-mediated transgene expression at defined stages of the cell cycle. *Mol Ther* 2001; **4**: 13-21.
45 Hotta T, Motoyama T, Watanabe H. Three human osteosarcoma cell lines exhibiting different phenotypic expressions. *Acta Pathol Jpn* 1992; **42**: 595-603.
46 Kawashima H, Ogose A, Gu W, Nishio J, Kudo N, Kondo N *et al.* Establishment and characterization of a novel myxofibrosarcoma cell line. *Cancer Genet Cytogenet* 2005; **161**: 28-35.
47 Kunisada T, Miyazaki M, Mihara K, Gao C, Kawai A, Inoue H *et al.* A new human chondrosarcoma cell line (OUMS-27) that maintains chondrocytic differentiation. *Int J Cancer* 1998; **77**: 854-859.

Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)

Molecular Cancer Therapeutics

Dual Programmed Cell Death Pathways Induced by p53 Transactivation Overcome Resistance to Oncolytic Adenovirus in Human Osteosarcoma Cells

Joe Hasei[1], Tsuyoshi Sasaki[1], Hiroshi Tazawa[2,4], Shuhei Osaki[1], Yasuaki Yamakawa[1], Toshiyuki Kunisada[1,3], Aki Yoshida[1], Yuuri Hashimoto[2], Teppei Onishi[2], Futoshi Uno[2], Shunsuke Kagawa[2], Yasuo Urata[5], Toshifumi Ozaki[1], and Toshiyoshi Fujiwara[2]

Abstract

Tumor suppressor p53 is a multifunctional transcription factor that regulates diverse cell fates, including apoptosis and autophagy in tumor biology. p53 overexpression enhances the antitumor activity of oncolytic adenoviruses; however, the molecular mechanism of this occurrence remains unclear. We previously developed a tumor-specific replication-competent oncolytic adenovirus, OBP-301, that kills human osteosarcoma cells, but some human osteosarcoma cells were OBP-301–resistant. In this study, we investigated the antitumor activity of a p53-expressing oncolytic adenovirus, OBP-702, and the molecular mechanism of the p53-mediated cell death pathway in OBP-301–resistant human osteosarcoma cells. The cytopathic activity of OBP-702 was examined in OBP-301–sensitive (U2OS and HOS) and OBP-301–resistant (SaOS-2 and MNNG/HOS) human osteosarcoma cells. The molecular mechanism in the OBP-702–mediated induction of two cell death pathways, apoptosis and autophagy, was investigated in OBP-301–resistant osteosarcoma cells. The antitumor effect of OBP-702 was further assessed using an orthotopic OBP-301–resistant MNNG/HOS osteosarcoma xenograft tumor model. OBP-702 suppressed the viability of OBP-301–sensitive and -resistant osteosarcoma cells more efficiently than OBP-301 or a replication-deficient p53-expressing adenovirus (Ad-p53). OBP-702 induced more profound apoptosis and autophagy when compared with OBP-301 or Ad-p53. E1A-mediated *miR-93/106b* upregulation induced p21 suppression, leading to p53-mediated apoptosis and autophagy in OBP-702–infected cells. p53 overexpression enhanced adenovirus-mediated autophagy through activation of damage-regulated autophagy modulator (DRAM). Moreover, OBP-702 suppressed tumor growth in an orthotopic OBP-301–resistant MNNG/HOS xenograft tumor model. These results suggest that OBP-702–mediated p53 transactivation is a promising antitumor strategy to induce dual apoptotic and autophagic cell death pathways via regulation of miRNA and DRAM in human osteosarcoma cells. *Mol Cancer Ther; 12(3); 314–25. ©2012 AACR.*

Introduction

Osteosarcoma is one of the most common malignant tumors in young children (1, 2). Current treatment strategies, which consist of multi-agent chemotherapy and aggressive surgery, have significantly improved the cure rate and prognosis of patients with osteosarcoma. In fact, over the past 30 years, the 5-year survival rate has increased from 10% to 70% (3–5). Even in patients with osteosarcoma with metastases at diagnosis, the 5-year survival rate has reached 20% to 30% in response to chemotherapy and surgical removal of primary and metastatic tumors (6). However, treatment outcomes for patients with osteosarcomas have further improved over the last few years. Therefore, the development of novel therapeutic strategies is required to improve the clinical outcomes in patients with osteosarcomas.

Tumor-specific replication-competent oncolytic viruses are being developed as novel anticancer therapy, in which the promoters of cancer-related genes are used to regulate virus replication in a tumor-dependent manner. More than 85% of all human cancers express high telomerase activity to maintain the length of the telomeres during cell division, whereas normal somatic cells seldom show this enhanced telomerase activity (7, 8). Telomerase activity has also been detected in 44% to 81% of bone and

Authors' Affiliations: Departments of [1]Orthopaedic Surgery, [2]Gastroenterological Surgery, and [3]Medical Materials for Musculoskeletal Reconstruction, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences; [4]Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama; and [5]Oncolys BioPharma, Inc., Tokyo, Japan

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Toshiyoshi Fujiwara, Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan. Phone: 81-86-235-7257; Fax: 81-86-221-8775; E-mail: toshi_f@md.okayama-u.ac.jp

doi: 10.1158/1535-7163.MCT-12-0869