厚生労働科学研究費補助金

第3次対がん総合戦略研究事業

光感受性ROS産生蛍光タンパク質を発現する遺伝子改変アデノウイルス製剤を用いた新たな癌の光線力学療法システムの開発

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研究代表者　**藤原　俊義**

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【研究要旨】
テロメライシンは岡山大学で開発された国産の抗癌アデノウイルス製剤であり、テロメラーゼ構成成分である*hTERT*遺伝子のプロモーターにより、癌細胞のみで選択的に増殖して腫瘍融解を引き起す。本研究では、テロメライシンに光感受性蛍光タンパク質をコードする遺伝子を搭載した改変アデノウイルス製剤を開発する。腫瘍選択的に光感受性蛍光タンパク質を発現させ、治療用デバイスにて励起光照射することで癌細胞のみで活性酸素（reactive oxygen species：ROS）を産生して細胞死を誘導する新たな癌の光線力学療法を確立する。本研究の目的は、テロメラーゼ依存性増殖機能を持つアデノウイルス製剤テロメライシンをベクターとして癌細胞選択的に光感受性蛍光タンパク質*KillerRed*遺伝子を発現する次世代型武装化アデノウイルス製剤を作成し、新たな癌の光線力学療法としての臨床応用を行うことである。
平成24年度は、テロメライシンのE3領域に*KillerRed*遺伝子を搭載したTelomeKillerウイルスを作成し、TelomeKillerを感染させ48時間経過したヒト癌細胞に励起光照射することで有意に強い抗腫瘍効果がみられることを確認した。また、ミニブタの開腹下に*KillerRed*遺伝子発現ヒト癌細胞をリンパ節などに注入し、励起光照射硬性ビデオスコープにて赤色蛍光を検出可能であることを検証した。

A．研究目的

テロメライシン（Telomelysin、開発コード：OBP-301）は岡山大学で開発された国産の抗癌アデノウイルス製剤であり、癌細胞で選択的に増殖・複製する機能を有しており、自立的に癌細胞を破壊することで増殖したウイルスが拡散していく。したがって、テロメライシン自体が新しい作用機構に基づく新規の画期的な医薬品となるが、ゲノム構造が明らかなアデノウイルスを基本骨格としているため、テロメライシンはベクターとして応用可能であり、導入遺伝子の癌選択的かつ長期的発現が期待できる。

最近、GFPをはじめとする蛍光タンパク質は分子イメージングのマーカーとして注目されているが、マーカー機能では細胞毒性が低いものが選択される。研究代表者らも、*GFP*遺伝子を搭載したテロメスキャン（TelomeScan、OBP-401）を診断用医薬品として開発してきたが、GFP自体の細胞毒性は極めて低い（Kishimoto *et al.*, Nature Med., 12, 1213-1219, 2006）。その発想を転換し、本研究では診断と治療を融合した新しい研究分野「Theranostics」の医薬品として、光誘導細胞死を生じる新規蛍光赤色タンパク質*KillerRed*遺伝子を用いた光感受性武装化アデノウイルス製剤を開発する。標的癌細胞でウイルス増殖に伴う強い蛍光タンパク質発現により癌細胞の可視化を可能とし、また同時に励起光照射で産生されるROSによる強力な細胞傷害活性が発揮される。すなわち、遺伝子改変ウイルス製剤を基盤とする本技術は、分子イメージングと分子手術による全く新しい独創的な治療戦略と言える。

本研究では、テロメラーゼ依存性増殖機能を持つアデノウイルス製剤テロメライシンをベクターとして癌細胞選択的に光感受性蛍光タンパク質遺伝子を発現する次世代型武装化アデノウイルス製剤を作成し、新たな癌の光線力学療法としてその機能解析、小動物・大動物を用いた有効性･安全性の検討を終了することを目指す。

B．研究方法

1）*KillerRed*遺伝子発現テロメライシンの作成

KillerRedは*Anthomedusae*クラゲの発色タンパク質であるanm2CPを改変して開発された新しい赤色蛍光タンパク質であり、540～580nmの緑色光照射によって活性酸素（reactive oxygen species：ROS）を産生して細胞死を誘導することができる。テロメライシンはhTERTプロモーターでアデノウイルスの増殖に必要な*E1A*、*E1B*遺伝子を駆動する増殖カセットを搭載しており、今回、サイトメガロウイルス・プロモーターの下流に*KillerRed*遺伝子を組み込み、テロメライシン・ゲノムのE3領域に搭載することで、*KillerRed*遺伝子発現テロメライシンであるTelomeKillerを作成した。癌細胞で選択的に増殖

し、強力な*KillerRed*遺伝子発現を来す。

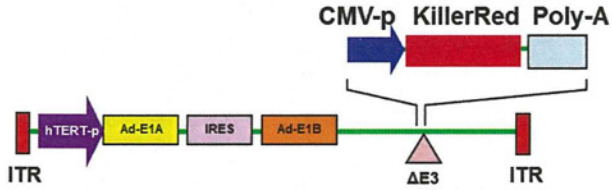

図1　TelomeKillerウイルスの構造

2）KillerRed用励起光照射デバイスの試作

オリンパスと共同で、KillerRedタンパク質に蛍光発現させるための励起光照射用ビデオスコープを試作した。励起波長570～590nmの励起光を発し、*KillerRed*遺伝子発現細胞にて蛍光波長600～655nmの近赤外蛍光を誘導することができる。

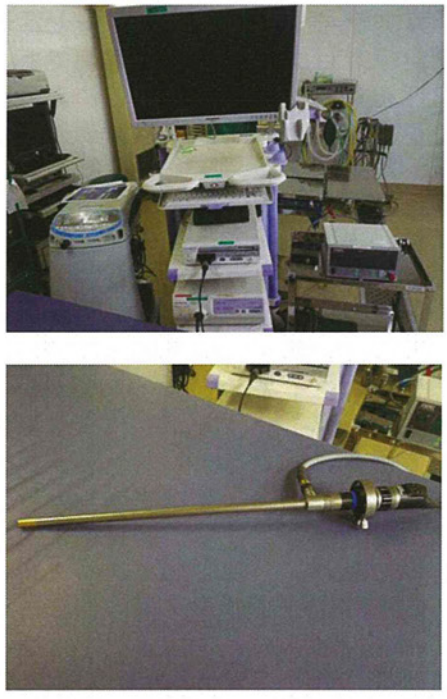

図2　KillerRedタンパク質励起用ビデオスコープ

3）大動物における*KillerRed*遺伝子発現ヒト癌細胞の可視化

*KillerRed*遺伝子発現H1299ヒト肺癌細胞を濃縮懸濁液にして、全身麻酔下のミニブタに開腹にて胃壁や近傍リンパ節に注入した。気腹下に励起光照射用ビデオスコープを用いて静止画、動画撮影を行った。

（倫理面への配慮）

制限増殖型ウイルス製剤を用いる本研究は「大臣確認実験」となるため、「第二種使用等拡散防止措置確認申請書」を作成、学内の担当部署での検討の後に文部科学省に申請し、研究計画実施の承認を得ている。

C．研究結果

1）TelomeKiller感染による*KillerRed*遺伝子発現

H1299ヒト肺癌細胞に各種濃度のTelomeKillerウイルスを感染させ、24時間後に蛍光顕微鏡下に赤色蛍光発現を確認した。10 MOI（multiplicity of infection）のTelomeKillerウイルスの感染で、ほぼ100%の*KillerRed*遺伝子発現を確認できた。

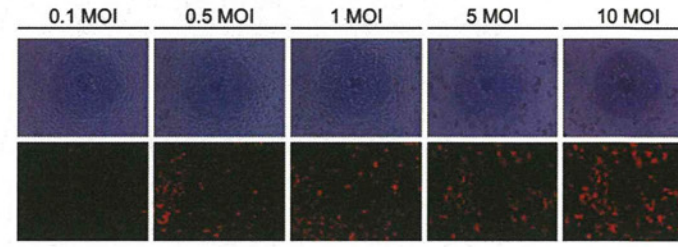

図3 TelomeKillerによる*KillerRed*遺伝子発現

2）TelomeKiller感染による抗腫瘍効果の検討

H1299ヒト肺癌細胞において、励起光非照射状態でのTelomeKillerの抗腫瘍効果をTelomelysinや*GFP*遺伝子を発現するTelomeScanと比較したところ、同等かより強い細胞障害活性が観察された。

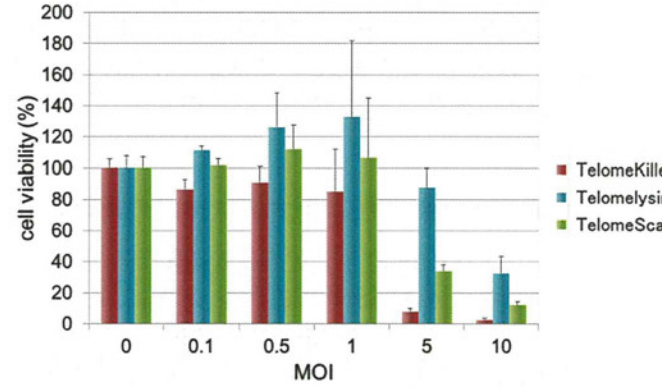

図4 TelomeKiller単独の抗腫瘍効果

また、TelomeKillerを感染させ48時間経過したヒト大腸癌細胞HCT-116に励起光照射することで、有意に強い抗腫瘍効果を確認できた。

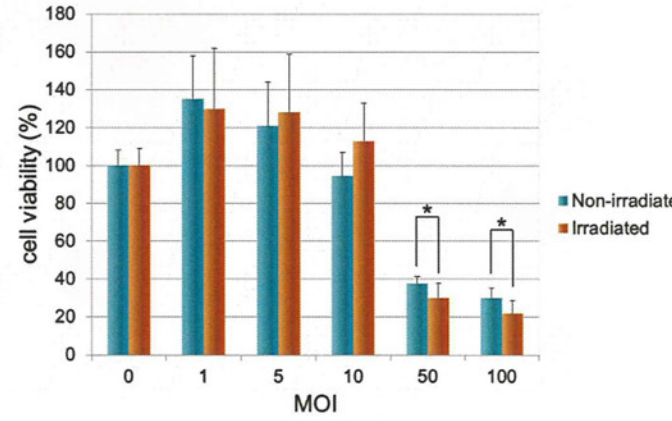

図5 励起光照射による抗腫瘍効果の増強

ヒト肺癌細胞H1299にTelomeKillerを感染させ励起光照射を行うと、5分以内に急速なPhotobleachingが生じ、1時間以内でGFP蛍光が減弱する完全細胞死を誘導することが可能であった。

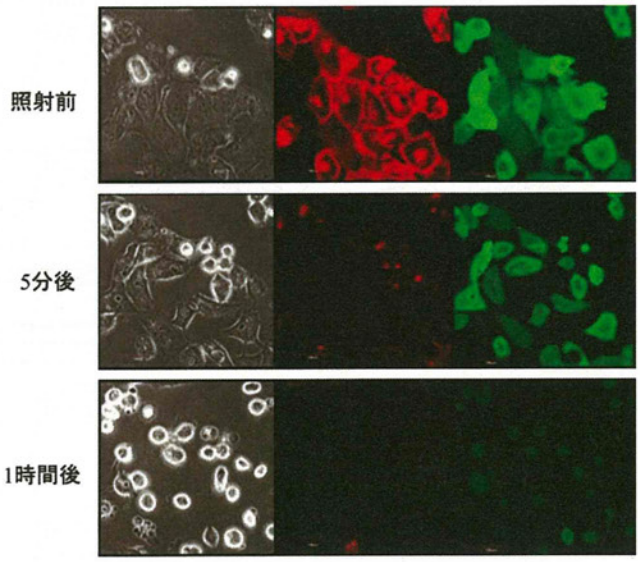

図6 励起光照射による蛍光発現と細胞死誘導

しかし、励起光照射による増強効果はそれぞれの癌細胞のTelomelysinの感受性に依存しており、Telomelysinのみで殺細胞効果が十分得られる癌細胞では有意な上乗せ効果を証明するのは困難であった。

3）励起光照射硬性ビデオスコープの効果の検証

ミニブタの開腹下に*KillerRed*遺伝子発現H1299細胞の懸濁液を胃壁や周辺リンパ節に注入し、硬性ビデオスコープから励起光を照射した。しかし、赤色蛍光は極めて微弱であり、細胞障害活性は認められなかった。現行のシステムでは光強度が不十分であり、レーザー照射のような工夫が必要であると考えられた。

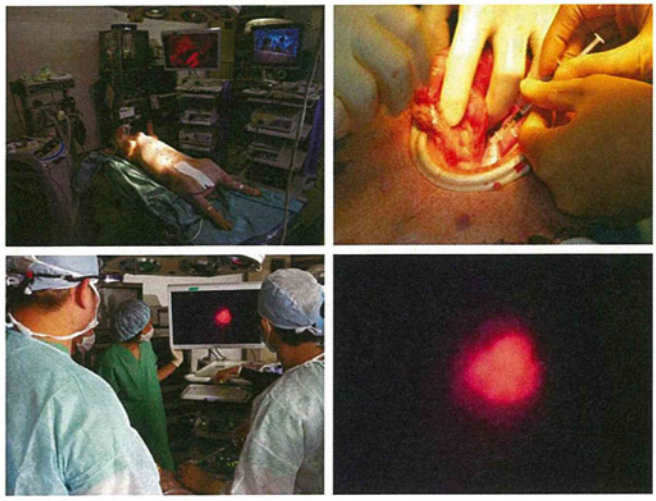

図7　ミニブタによる励起光照射実験

D．考察

光感受性物質と励起光照射を組み合わせて行う光線力学療法は、侵襲が少ない癌治療として一部の早期癌で臨床応用が試みられている。しかし、光感受性物質の標的とする生体内分子に対する選択性が小さいことや、光感受性物質の投与のために光線過敏症を引き起こすなどの問題点を残していた。本研究では、癌への選択性が強いテロメライシンをベクターとして*KillerRed*遺伝子を発現するため、癌集積性は極めて高い。生物製剤、特にウイルス製剤と光照射を組み合わせた光線力学療法は、未だ世界で報告がなく、臨床応用に向けた試みももちろん存在しない。一方、蛍光タンパク質自体の発見や開発は最近のトピックスであり、これらを結びつけることで現実的な治療開発につながる可能性が強い。

テロメライシンはテロメラーゼ活性を標的とする癌治療を目的とした生物製剤であり、生体内で自律性を持って増殖することによる従来の抗癌剤や分子標的薬剤にない抗腫瘍効果の増強が期待できる。本研究では、米国の臨床試験で安全性が確認されたこのテロメライシンをベクターとして全く新たな光線力学療法への応用を目指しており、その安全性と効果に関する理論的根拠が得られれば、創薬シーズとしては極めて魅力的なものになると思われる。中国では、すでに2種類の局所療法剤としてのウイルス製剤（Gendicine、H101）が中国FDAの承認を受けており、一般市場にて日常臨床に使用されている。しかし、遺伝子治療やペプチド医薬などに関しては中国以外の海外でも未だ実績がない分野だけに、新技術が製品の形で現実のものになれば、社会的インパクトは極めて大きく、抗癌剤市場はさらに高い伸びを示すと予測されている。

本研究では、TelomeKillerウイルス製剤によりKillerRedタンパク質を発現することで、癌細胞に選択的に光感受性を誘導することができ、Telomelysin本来の抗腫瘍効果の増強が可能であることが検証された。しかし、大動物用に試作した励起光照射硬性ビデオスコープからの照射では光強度が十分ではなく、レーザー照射等を検討する必要が示唆された。また、in vivoにおける予備実験では、三次元における励起光照射深度は比較的浅く、対象となる癌組織も表在型のものに限られる可能性が出てきた。今後は、早期病変に標的を絞り、塗布用のDrug Delivery System（DDS）などを応用した技術を開発していきたいと考えている。

光感受性武装化アデノウイルス製剤は低侵襲な局所制御療法であり、光線力学療法への応用で選択的な抗腫瘍活性を増強することで、集学的治療として癌患者の生活の質（QOL）の向上に貢献する。また、有効な癌治療戦略の一つとなることで、

国民の健康増進や医療経済の節減にも役立つと期待される。

E．結論

緑色励起光により*KillerRed*遺伝子発現ヒト癌細胞を選択的に殺傷することができ、光感受性武装化アデノウイルス製剤を用いたKillerRedの光線力学療法への応用の可能性が示唆された。

F．研究発表

1. 論文発表

【英文】

1. Hashimoto, Y., Tazawa, H., Teraishi, F., Kojima, T., Watanabe, Y., Uno, F., Yano, S., Urata, Y., Kagawa, S., Fujiwara, T. The hTERT promoter enhances the antitumor activity of an oncolytic adenovirus under a hypoxic microenvironment. ***PLoS One***, (in press), 2013.
2. Nobuoka, D., Yoshikawa, T., Takahashi, M., Iwama, T., Horie, K., Shimomura, M., Suzuki, S., Sakemura, N., Nakatsugawa, M., Sadamori, H., Yagi, T., Fujiwara, T., Nakatsura, T. Intratumoral peptide injection enhances tumor cell antigenicity recognized by cytotoxic T lymphocytes: a potential option for improvement in antigen-specific cancer immunotherapy. ***Cancer Immunol. Immunother.***, (in press), 2013.
3. Hasei, J., Sasaki, T., Tazawa, H., Osaki, S., Yamakawa, Y., Kunisada, T., Yoshida, A., Hashimoto, Y., Onishi, T., Uno, F., Kagawa, S., Urata, Y., Ozaki, T., Fujiwara, T. Dual programmed cell death pathways induced by p53 transactivation overcome resistance to oncolytic adenovirus in human osteosarcoma cells. ***Mol. Cancer Ther.***, 12: 314-325, 2013.
4. Ohara, T., Noma, K., Urano, S., Watanabe, S., Nishitani, S., Tomono, Y., Kimura, F., Kagawa, S., Shirakawa, Y., Fujiwara, T. A novel synergistic effect of iron depletion on anti-angiogenic cancer therapy. ***Int. J. Cancer***, 132: 2705-2713, 2013.
5. Sasaki, T., Tazawa, H., Hasei, J., Osaki, S., Kunisada, T., Yoshida, A., Hashimoto, Y., Yano, S., Yoshida, R., Kagawa, S., Uno, F., Urata, Y., Ozaki, T., Fujiwara, T. A simple detection system for adenovirus receptor expression using a telomerase-specific replication-competent adenovirus. ***Gene Ther.***, 20: 112-118,, 2013.
6. Shigeyasu, K., Kagawa, S., Uno, F., Nishizaki, M., Kishimoto, H., Gochi, A., Kimura, T., Takahata, T., Nonaka, Y., Ninomiya, M., Fujiwara, T. Multicenter phase II study of S-1 and docetaxel combination chemotherapy for advanced or recurrent gastric cancer patients with peritoneal dissemination. ***Cancer Chemother. Pharmacol.***, 71: 937-943, 2013.
7. Sadamori, H., Yagi, T., Shinoura, S., Umeda, Y., Yoshida, R., Satoh, D., Nobuoka, D., Utsumi, M., Fujiwara, T. Risk factor for major morbidity after liver resection for hepatocellular carcinoma. ***Brit. J. Surg.***, 100: 122-129, 2013.
8. Sadamori, H., Yagi, T., Shinoura, S., Umeda, Y., Yoshida, R., Satoh, D., Nobuoka, D., Utsum, M., Fujiwara, T. New surgical approach to large splenorenal shunt in living donor liver transplantation: diversion of SMV and SPV blood flow. ***J. Gastrointest. Surg.***, 17: 403-407, 2013.
9. Sadamori, H., Yagi, T., Shinoura, S., Umeda, Y., Yoshida, R., Satoh, D., Nobuoka, D., Utsumi, M., Yoshida, K., Fujiwara, T. Risk factors for organ/space surgical site infection after hepatectomy for hepatocellular carcinoma in 359 recent cases. ***J. Hepato-Bil- Panc. Sci.***, 20:186-196, 2013.
10. Umeda, Y., Nagasaka, T., Mori, Y., Sadamori, H., Sun, D. S , Shinoura, S., Yoshida, R., Satoh, D., Nobuoka, D., Utsumi, M., Yoshida, K., Yagi, T., Fujiwara T. Poor prognosis of KRAS or BRAF mutant colorectal liver metastasis without microsatellite instability. ***J. Hepato-Biliary Pancreat. Sci.***, 20: 223-233, 2013.
11. Nobuoka, D., Motomura, Y., Shirakawa, H., Yoshikawa, T., Kuronuma, T., Takahashi, M., Nakachi, K., Ishii, H., Furuse, J., Gotohda, N., Takahashi, S., Nakagohri, T., Konishi, M., Kinoshita, T., Komori, H., Baba, H., Fujiwara, T., Nakatsura, T. Radiofrequency ablation for hepatocellular carcinoma induces glypican-3 peptide-specific cytotoxic T lymphocytes. ***Int. J. Oncol.***, 40: 63-70, 2012.
12. Yamasaki, Y., Tazawa, H., Hashimoto, Y., Kojima, T., Kuroda, S., Yano, S., Yoshida, R., Uno, F., Mizuguchi, H., Ohtsuru, A., Urata, Y., Kagawa, S., Fujiwara, T. A novel apoptotic mechanism of genetically engineered adenovirus-mediated tumour-specific p53 overexpression through E1A-dependent p21 and MDM2 suppression. ***Eur. J. Cancer***, 48: 2282-2291, 2012.
13. Tazawa, H., Yano, S., Yoshida, R., Yamasaki, Y., Sasaki, T., Hashimoto, Y., Kuroda, S., Ouchi, M., Onishi, T., Uno, F., Kagawa, S., Urata, Y., Fujiwara, T. Genetically engineered oncolytic adenovirus induces autophagic cell death through an E2F1-microRNA-7-epidermal growth factor receptor axis. ***Int. J. Cancer***, 131, 2939–2950, 2012.
14. Yoshida, R., Tazawa, H., Hashimoto, Y., Yano, S., Onishi, T., Sasaki, T., Shirakawa, Y., Kishimoto, H., Uno, F., Nishizaki, M., Kagawa, S., Fujiwara, T. Mechanism of resistance to trastuzumab and molecular sensitization via ADCC activation by exogenous expression of HER2-extracellular domain in human cancer cells. ***Cancer Immuno. Immunother.***, 61: 1905–1916, 2012.
15. Watanabe, Y., Hashimoto, Y., Kagawa, S., Kawamura, H., Nagai, K., Tanaka, N., Urata, Y., Fujiwara, T. Enhanced antitumor efficacy of telomerase-specific oncolytic adenovirus with valproic acid against human cancer cells. ***Cancer Gene Ther.***, 19: 767-772, 2012.
16. Sadamori, H., Yagi, T., Matsuda, H., Shinoura, S., Umeda, Y., Fujiwara, T. Intractable bile leakage after hepatectomy for hepatocellular carcinoma in 359 recent cases. ***Dig. Surg.***, 29: 149-156, 2012.
17. Fujiwara, T., Kagawa, S., Tazawa, H. Synergistic interaction of telomerase-specific oncolytic virotherapy and chemotherapeutic agents for human cancer. ***Curr. Pharm. Biotechno.***, 13: 1809-1816, 2012.
18. Kuroda, S., Urata, Y., Fujiwara, T. Ataxia-Telangiectasia Mutated and the Mre11-Rad50-NBS1 Complex:Promising Targets for Radiosensitization. ***Acta Med. Okayama***, 66: 83-92, 2012.

【邦文】

1. 永坂岳司、藤原俊義：糞便を用いた大腸がんの遺伝子診断（2）～便中メチル化 CpG 検出による大腸がんスクリーニング～. ***大腸癌 Frontier*** 5: 50-57、2012.
2. 永坂岳司、母里淑子、楳田祐三、藤原俊義：疾患バイオ

マーカーの臨床研究：大腸がん. ***日本臨床*** 70: 802-808、2012.

3. 藤原俊義：アカデミアにおける探索的医薬品開発の方向性～国産ウイルス製剤の米国での臨床試験への道程～. ***Jpn. Pharmacol. Ther.（薬理と治療）*** 40: 303-304、2012.
4. 佐々木剛、田澤大、長谷井嬢、国定俊之、吉田晶、橋本悠里、矢野修也、吉田亮介、宇野太、香川俊輔、森本裕樹、浦田泰生、藤原俊義、尾崎敏文：テロメラーゼ依存的腫瘍融解ウイルス療法の骨・軟部肉腫に対する前臨床的検討（平成23年度岡山医学会賞（山田賞）受賞論文）. ***岡山医学会雑誌*** 124: 105-110、2012.
5. 藤原俊義：癌遺伝子と癌抑制遺伝子の多様性と治療への応用. ***臨床と研究*** 90: 38-42、2013.

2. 学会発表

【国際学会】

1. Shigeyasu, K., Hashimoto, Y., Morikawa, T., Mori, Y., Sun, D-S., Kagawa, S., Uno, F., Tazawa, H., Nagasaka, T., Kyo, S., Mizuguchi, H., Urata, Y., Fujiwara, T. A highly sensitive detection system of genetic alterations in circulating tumor cells using a telomerase-specific replication-competent adenovirus. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.
2. Kikuchi, S., Kishimoto, H., Tazawa, H., Hashimoto, Y., Uno, F., Nishizaki, M., Kagawa, S., Urata, Y., Hoffman, R. B., Fujiwara, T. A precise orthotopic rectal tumor model for evaluating therapeutic response of cancer treatment. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.
3. Osaki, S., Sasaki, T., Tazawa, H., Hasei, J., Hashimoto, Y., Nouso, K., Urata, Y., Ozaki, T., Fujiwara, T. A simple biological imaging system for detecting adenovirus receptor expression in tumor cells using a telomerase-specific replication-competent adenovirus. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.
4. Hasei, J., Sasaki, T., Tazawa, H., Hashimoto, Y., Kunisada, T., Urata, Y., Ozaki, T., Fujiwara, T. p53-mediated apoptotic signaling overcomes the resistance to oncolytic adenovirus in human osteosarcoma cells. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.
5. Ohara, T., Noma, K., Nishitani, S., Watanabe, S., Tomono, Y., Hasimoto, Y., Tazawa, H., Fujiwara, T. Iron chelator contributes to anti-angiogenic therapy via selective induction of VEGF-A. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.
6. Watanabe, S., Nishitani, S., Noma, K., Ohara, T., Tomono, Y., Hasimoto, Y., Tazawa, H., Fujiwara, T. Iron deficiency suppresses EMT through down-regulation of N-cadherin in esophageal cancer. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.
7. Tazawa, H., Sasaki, T., Hashimoto, Y., Kikuchi, S., Kishimoto, H., Mizuguchi, H., Fujiwara, T. Preclinical evaluation of cytotoxic effect of photosensitive fluorescent protein in human cancer cells. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.
8. Hashimoto, Y., Tazawa, H., Urata, Y., Kagawa, S., Fujiwara, T. Inhibitory effect of oncolytic adenovirus on transforming growth factor- β-induced epithelial-mesenchymal transition in human cancer cells. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.
9. Kurihara, Y., Sato, D., Shirota, T., Kondo, S., Kataoka, M., Urata, Y., Fujiwara, T., Shintani, S. Telomerase-specific replication-selective virotherapy combined with radiation therapy for oral squamous cell carcinoma cells. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.
10. Yano, S., Li, S., Han, Q., Tan, Y., Fujiwara, T., Hoffman, R. B. Color-coded selective chemotherapy of methioninase-synchronized S-phase cancer cells expressing cell-cycle-specific fluorescent reporters: A general approach to the treatment of cancer. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.
11. Yano, S., Li, S., Han, Q., Tan, Y., Fujiwara, T., Hoffman, R. B. Methioninase S-phase block of cancer cells imaged in real time by cell-cycle-specific fluorescence reporters. ***2012 Annual Meeting of the American Association for Cancer Research***, Chicago, April, 2012.

【国内学会】

1. 香川俊輔、岸本浩行、宇野太、黒田新士、石田道拡、橋本悠里、重安邦俊、西崎正彦、白川靖博、永坂岳司、藤原俊義：胃癌における蛍光発現ウイルスによる腹腔洗浄液診断：生物学的悪性度指標としての可能性. ***第113回日本外科学会定期学術集会（シンポジウム）***、福岡、2013年4月.
2. 重安邦俊、橋本悠里、宇野太、永坂岳司、田澤大、香川俊輔、水口裕之、浦田泰生、藤原俊義：テロメラーゼ活性を指標とする血中遊離癌細胞の高感度検出法の開発と遺伝子解析技術への応用. ***第113回日本外科学会定期学術集会（ワークショップ）***、福岡、2013年4月.
3. 楳田祐三、八木孝仁、貞森裕、内海方嗣、篠浦先、吉田龍一、佐藤太祐、信岡大輔、藤智和、保田裕子、藤原俊義：生体肝移植の短期・晩期予後向上に向けた治療戦略個別化の可能性. ***第113回日本外科学会定期学術集会（パネルディスカッション）***、福岡、2013年4月.
4. 稲田涼、永坂岳司、母里淑子、楳田祐三、森川達也、久保田暢人、近藤喜太、宇野太、貞森裕、八木孝仁、三嶋秀行、藤原俊義：遺伝子情報を用いた多臓器転移大腸癌の検討および治療戦略の構築. ***第113回日本外科学会定期学術集会（パネルディスカッション）***、福岡、2013年4月.
5. 藤原俊義：消化器がん治療における遺伝子工学技術に基づく先端医療開発. ***第20回日本消化器関連学会週間（第10回日本消化器外科学会大会）（特別企画「消化器癌治療を目指したトランスレーショナルリサーチ<若手医師へのメッセージ>」***、神戸、2012年10月.
6. 永坂岳司、森川達也、藤原俊義：便中メチル化CpG検出による消化器癌スクリーニング. ***第20回日本消化器関連学会週間（シンポジウム）***、神戸、2012年10月.
7. 永坂岳司、母里淑子、森川達也、久保田暢人、横道直祐、重安邦俊、竹原清人、吉田一博、楳田祐三、貞森裕、藤原俊義：大腸癌の再発を予測する新規バイオマーカーの検討 –Stage II/III 根治切除症例の検討から. ***第45回制癌剤適応研究会（シンポジウム）***、東京、2012年3月.

研究成果の刊行に関する一覧表

雑誌

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Hashimoto, Y., Tazawa, H., Teraishi, F., Kojima, T., Watanabe, Y., Uno, F., Yano, S., Urata, Y., Kagawa, S., Fujiwara, T.	The hTERT promoter enhances the antitumor activity of an oncolytic adenovirus under a hypoxic microenvironment.	**PLoS One**		In press	2013
Hasei, J., Sasaki, T., Tazawa, H., Osaki, S., Yamakawa, Y., Kunisada, T., Yoshida, A., Hashimoto, Y., Onishi, T., Uno, F., Kagawa, S., Urata, Y., Ozaki, T., Fujiwara, T.	Dual programmed cell death pathways induced by p53 transactivation overcome resistance to oncolytic adenovirus in human osteosarcoma cells.	**Molecular Cancer Therapeutics**	12	314-325	2013
Ohara, T., Noma, K., Urano, S., Watanabe, S., Nishitani, S., Tomono, Y., Kimura, F., Kagawa, S., Shirakawa, Y., Fujiwara, T.	A novel synergistic effect of iron depletion on antiangiogenic cancer therapy.	**International Journal of Cancer**	132	2705-2713	2013
Fujiwara, T., Kagawa, S., Tazawa, H.	Synergistic interaction of telomerase-specific oncolytic virotherapy and chemotherapeutic agents for human cancer.	**Current Pharmaceutical Biotechnology**	13	1809-1816	2012
Kuroda, S., Urata, Y., Fujiwara, T.	Ataxia-Telangiectasia Mutated and the Mre11-Rad50-NBS1 Complex:Promising Targets for Radiosensitization.	**Acta Medica Okayama**	66	83-92	2012

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The hTERT Promoter Enhances the Antitumor Activity of an Oncolytic Adenovirus under a Hypoxic Microenvironment

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Abstract

Hypoxia is a microenvironmental factor that contributes to the invasion, progression and metastasis of tumor cells. Hypoxic tumor cells often show more resistance to conventional chemoradiotherapy than normoxic tumor cells, suggesting the requirement of novel antitumor therapies to efficiently eliminate the hypoxic tumor cells. We previously generated a tumor-specific replication-competent oncolytic adenovirus (OBP-301: Telomelysin), in which the human telomerase reverse transcriptase (*hTERT*) promoter drives viral E1 expression. Since the promoter activity of the *hTERT* gene has been shown to be upregulated by hypoxia, we hypothesized that, under hypoxic conditions, the antitumor effect of OBP-301 with the *hTERT* promoter would be more efficient than that of the wild-type adenovirus 5 (Ad5). In this study, we investigated the antitumor effects of OBP-301 and Ad5 against human cancer cells under a normoxic (20% oxygen) or a hypoxic (1% oxygen) condition. Hypoxic condition induced nuclear accumulation of the hypoxia-inducible factor-1α and upregulation of *hTERT* promoter activity in human cancer cells. The cytopathic activity of OBP-301 was significantly higher than that of Ad5 under hypoxic condition. Consistent with their cytopathic activity, the replication of OBP-301 was significantly higher than that of Ad5 under the hypoxic condition. OBP-301-mediated E1A was expressed within hypoxic areas of human xenograft tumors in mice. These results suggest that the cytopathic activity of OBP-301 against hypoxic tumor cells is mediated through hypoxia-mediated activation of the *hTERT* promoter. Regulation of oncolytic adenoviruses by the *hTERT* promoter is a promising antitumor strategy, not only for induction of tumor-specific oncolysis, but also for efficient elimination of hypoxic tumor cells.

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Introduction

Solid tumor tissues often contain hypoxic regions, in which the supply of oxygen and nutrition is reduced because of an immature vascular network, and in which there is rapid tumor progression [1]. Hypoxia is a critical microenvironmental factor that contributes to tumor angiogenesis, invasion, progression and metastasis [1,2]. Indeed, hypoxic conditions have been shown to be associated with cancer progression and poor prognosis [3–5]. Furthermore, recent accumulated evidence suggests that hypoxia induces cancer progression-related characteristics such as epithelial-mesenchymal transition (EMT) [6,7] and stemness properties [8–11] of tumor cells. Acquisition of such properties by tumor cells within hypoxic areas of tumor tissues would greatly contribute to tumor progression and recurrence.

Hypoxic tumor cells are known to be highly resistant to conventional chemoradiotherapy, leading to poor prognosis [5,12]. To improve clinical outcome, novel antitumor agents that efficiently eradicate tumor cells under hypoxic conditions as well as under normoxic conditions are required. Oncolytic virotherapy has emerged as a promising novel antitumor therapy [13]. We previously generated a telomerase-specific replication-competent oncolytic adenovirus (OBP-301: Telomelysin), in which the human telomerase reverse transcriptase (*hTERT*) promoter element drives E1 gene expression. OBP-301 efficiently kills human cancer cells but not normal human somatic cells [14]. hTERT is a catalytic subunit of human telomerase and is highly expressed in tumor cells, but not in normal cells. hTERT expression closely correlates with telomerase activity [15–17]. Tumor-specific antitumor activity of OBP-301 against various types of human cancer cells with high telomerase activity has been demonstrated in both *in vitro* and *in vivo* settings [14,18,19]. Furthermore, the feasibility of OBP-301 for clinical use has been demonstrated in a recently completed phase I clinical trial in the USA of OBP-301 in patients with advanced solid tumors [20]. However, whether OBP-301 has an antitumor effect against hypoxic tumor cells remains unclear.

Hypoxia-inducible factor 1 (HIF-1) is a master transcription factor that is activated by hypoxia [1]. HIF-1 consists of α and β subunits and HIF-1α expression is tightly regulated by oxygen concentration. The HIF-1α protein is stabilized under hypoxic conditions, whereas it is immediately degraded under normoxic conditions. HIF-1α induces the expression of many down-stream target genes that are associated with cellular metabolism, proliferation, survival, apoptosis, neovascularization and migration [4]. The expression of many target genes is activated by HIF-1 through binding to a *cis*-acting hypoxia response element (HRE) located at their enhancer or promoter regions [4,21,22]. The *hTERT* gene is also a HIF-1-target gene. Two HREs that are present in the *hTERT* gene promoter are involved in hypoxia-mediated *hTERT* gene upregulation [23–25]. In contrast, it has also been shown that hypoxic conditions impair the replication of wild-type adenovirus in tumor cells [26,27]. Based on these findings, we hypothesized that the cytopathic activity of OBP-301 that is regulated by the *hTERT* gene promoter would be much stronger against hypoxic tumor cells than that of wild-type adenovirus due to hypoxia-induced enhancement of OBP-301 virus replication.

In the present study, we evaluated whether hypoxic conditions affect the expression levels of hTERT and the coxsackie and adenovirus receptor (CAR) in human cancer cells. We next assessed the antitumor effects of OBP-301 and Ad5 against human cancer cells under normoxic or hypoxic conditions. We further evaluated the replication of OBP-301 within hypoxic areas of human xenograft tumors.

Results

Maintenance of human cancer cells under hypoxic conditions

A hypoxia chamber filled with a gas mixture of 1% O_2, 5% CO_2 and 94% N_2 was used to maintain human cancer cells under hypoxic conditions. Human cancer cells were also maintained under normoxic conditions, consisting of 20% O_2 and 5% CO_2. To first confirm that the tumor cells were efficiently exposed to hypoxia in the chamber, the expression of HIF-1α, which is the main transcription factor induced by hypoxia [1], was evaluated using Western blot analysis. Consistent with HIF-1α induction by exposure to cobalt chloride ($CoCl_2$), HIF-1α expression was strongly induced in human cancer cells (HT29, DLD-1, H1299) maintained in the hypoxia chamber (Fig. 1A). However, no, or slight, HIF-1α expression was detected under normoxic conditions. Moreover, using immunocytochemistry, we further confirmed that HIF-1α was expressed and accumulated in the nuclei of human cancer cells under hypoxic conditions, but not under normoxic conditions (Fig. 1B). These results indicate that human cancer cells are maintained under hypoxic conditions in the hypoxia chamber.

Expression of hTERT and the adenovirus receptor in human cancer cells under hypoxic conditions

OBP-301 contains the *hTERT* gene promoter, which allows tumor-specific regulation of the gene expression of *E1A* and *E1B* that are required for viral replication [14]. The activity of the *hTERT* gene promoter in human cancer cells has been shown to be upregulated under hypoxic conditions [23–25], suggesting that hypoxia would enhance OBP-301 replication through upregulation of *hTERT* gene promoter activity. To evaluate the effect of hypoxic conditions on the activity of the *hTERT* gene promoter in tumor cells, we first investigated the expression level of *hTERT* mRNA in human tumor cells under normoxic or hypoxic conditions by quantitative real-time RT-PCR analysis. The expression of *hTERT* mRNA was increased in all tumor cells under the hypoxic condition by 1.3 to 4.3-fold compared to the normoxic condition (Fig. 2A). Despite evident inductions of HIF-1α by hypoxia, the increases were not statistically significant in HT29 and DLD-1 cells. Because it is known that hTERT expression is regulated not only transcriptionally but also post-transcriptionally by alternative splicing [25], we further examined the effects of hypoxia on activity of exogenous *hTERT* gene promoter using luciferase reporter assay. Hypoxia activated the *hTERT* gene promoter by at least 3-fold compared to the *hTERT* gene promoter activity under normoxia (Fig. 2B). To further confirm hypoxia-induced *hTERT* promoter activation, we used chemical inhibitor of HIF-1α. The protein expression of HIF-1α and the activity of *hTERT* gene promoter were significantly decreased in HT29 and H1299 cells treated with 30 μM HIF-1α inhibitor LW6 and cultured in hypoxic condition (Fig. S1 and Fig. 2C). Moreover, we confirmed that hTERT protein was expressed and accumulated in nuclei of human cancer cells under hypoxic conditions by immunofluorescence staining (Fig. 2D). These results suggest that the *hTERT* gene promoter in OBP-301 is more strongly activated under the hypoxic condition than under the normoxic condition.

The infection efficiency of Ad5-based viral vectors depends mainly on the expression of the adenoviral receptor CAR in target cells [28]. Therefore, to evaluate whether hypoxic conditions affect the expression of CAR in tumor cells, we examined the expression level of CAR in all tumor cells under normoxic or hypoxic conditions by flow cytometry. CAR expression was clearly detected in all tumor cells tested: the percentage of CAR-positive cells was 99.5%, 99.3% and 98.8% for HT29, DLD-1 and H1299 cells, respectively (Fig. 2E). All tumor cell lines showed similar expression levels of CAR under normoxic and hypoxic conditions. These results indicate that tumor cells show high CAR expression under hypoxic conditions as well as under normoxic conditions.

Antitumor activities of OBP-301 and Ad5 against hypoxic tumor cells

To explore the potential antitumor activities of the telomerase-dependent oncolytic adenovirus OBP-301 against normoxic and hypoxic tumor cells, we investigated the cytopathic activities of OBP-301 and Ad5 against tumor cells under normoxic or hypoxic conditions. Under normoxic conditions, the cytopathic activities of OBP-301 and Ad5 against HT29 and DLD-1 cells were very similar. The cytopathic activity of OBP-301 against H1299 cells was significantly higher than that of Ad5 at a low dose of infection, whereas it was similar to that of Ad5 at a high dose of infection (Fig. 3A). In contrast, under hypoxic conditions, the cytopathic activity of OBP-301 against all tumor cells was significantly higher than that of Ad5, especially at a high dose of infection (Fig. 3B). To further evaluate the antitumor activities of OBP-301 and Ad5 against hypoxic tumor cells, the 50% inhibiting dose (ID_{50}) values of OBP-301 and Ad5 under a hypoxic or a normoxic condition were calculated. The calculated ID_{50} values indicated that the cytopathic activity of OBP-301 against all tumor cells under hypoxic conditions was higher than that of Ad5, although the cytopathic activities of OBP-301 and Ad5 were very similar under normoxic conditions (Table 1). These results suggest that the cytopathic activity of OBP-301 against hypoxic tumor cells is more efficient than that of Ad5.

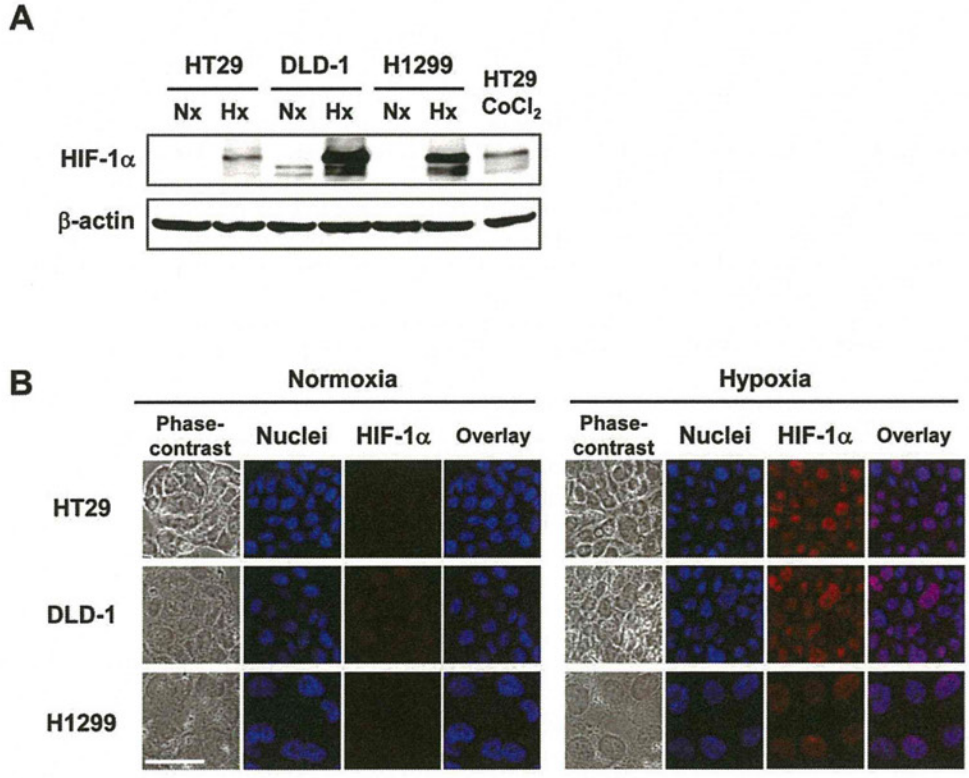

Figure 1. Increased HIF-1α expression in human cancer cells under hypoxic conditions. A, Western blot analysis of HIF-1α protein expression in human cancer cells (HT29, DLD-1 and H1299) under normoxic (Nx) or hypoxic (Hx) conditions. Cells were maintained under a normoxic (20% O_2) or a hypoxic (1% O_2) condition for 18 h. HT29 cells were also exposed to $CoCl_2$ as a positive control. Cell lysates were subjected to Western blot analysis using an anti- HIF-1α antibody. β-actin was assayed as a loading control. **B,** Subcellular localization of HIF-1α expression in human cancer cells under normoxia or hypoxia was assessed using immunofluorescent staining. Cells cultured under a normoxic or a hypoxic condition for 18 h were stained with anti-HIF-1α antibody (red). Nuclei were counterstained with DAPI (blue). Scale bars = 50 μm.
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Increased replication of OBP-301 compared to that of Ad5 under hypoxic conditions

We next examined the ability of OBP-301 and Ad5 to replicate in HT29, DLD-1 and H1299 cells, which showed almost similar sensitivity to OBP-301 and Ad5 under normoxic conditions but different sensitivity under hypoxic conditions (Fig. 3). The replication ability of OBP-301 and Ad5 was quantified by measuring viral E1A DNA in tumor cells infected with OBP-301 or Ad5 using quantitative real-time PCR analysis. Under normoxic conditions, the amount of virus production was very similar in tumor cells after infection with OBP-301 or Ad5 (Fig. 4). In contrast, under hypoxic conditions, viral production was significantly increased in OBP-301-infected tumor cells compared to Ad5-infected cells (Fig. 4). These results suggest that the replication of OBP-301 within hypoxic tumor cells is more efficient than that of Ad5.

OBP-301-mediated E1A expression in the hypoxic regions of xenograft tumor tissues

To investigate whether OBP-301 actually replicates in the hypoxic regions of tumor tissues, we examined HT29 and DLD-1 xenograft tumors after intratumoral injection of OBP-301. OBP-301-mediated E1A protein expression was assessed in HT29 and DLD-1 xenograft tumors by immunohistochemistry. Hypoxic areas in tumor tissues were detected by immunohistochemical analysis of the exogenous hypoxic marker, pimonidazole hydrochloride. OBP-301-mediated E1A was expressed in the normoxic regions (Fig. 5Aa and 5Ba) and the regions that were confirmed to be hypoxic by detection of pimonidazole expression (Fig. 5Ab and 5Bb). Moreover, the quantitative image analysis of immunohistochemical stains showed that the E1A-positive areas were almost equal in pimonidazole-negative and pimonidazole-positive regions (Fig. 5C and 5D). These results suggest that OBP-301 replicates in hypoxic tumor cells within the hypoxic areas of tumor tissues.

Discussion

Hypoxic microenvironments contribute to tumor invasion, progression, metastasis and resistance to conventional antitumor therapy, such as chemotherapy and radiotherapy, leading to poor prognosis [1–5]. The development of novel antitumor therapies that efficiently eliminate hypoxic tumor cells is an urgent issue for improvement of the clinical outcome of cancer patients. Although adenovirus-based oncolytic virotherapy has recently emerged as a promising antitumor therapy, a hypoxic microenvironment has been shown to reduce the replication of wild-type adenovirus in target tumor cells [26,27]. Therefore, efficient replication of an oncolytic adenovirus under hypoxic conditions is a critical factor for the eradication of hypoxic tumor cells. In this study, our goal was to assess whether the telomerase-specific oncolytic adenovirus OBP-301 that is regulated by the *hTERT* gene promoter shows cytopathic activity against human tumor cells under hypoxic conditions. We demonstrated that the cytopathic activity of OBP-301 against hypoxic tumor cells was much stronger than that of wild-type adenovirus (Fig. 3 and Table 1). Hypoxia-mediated

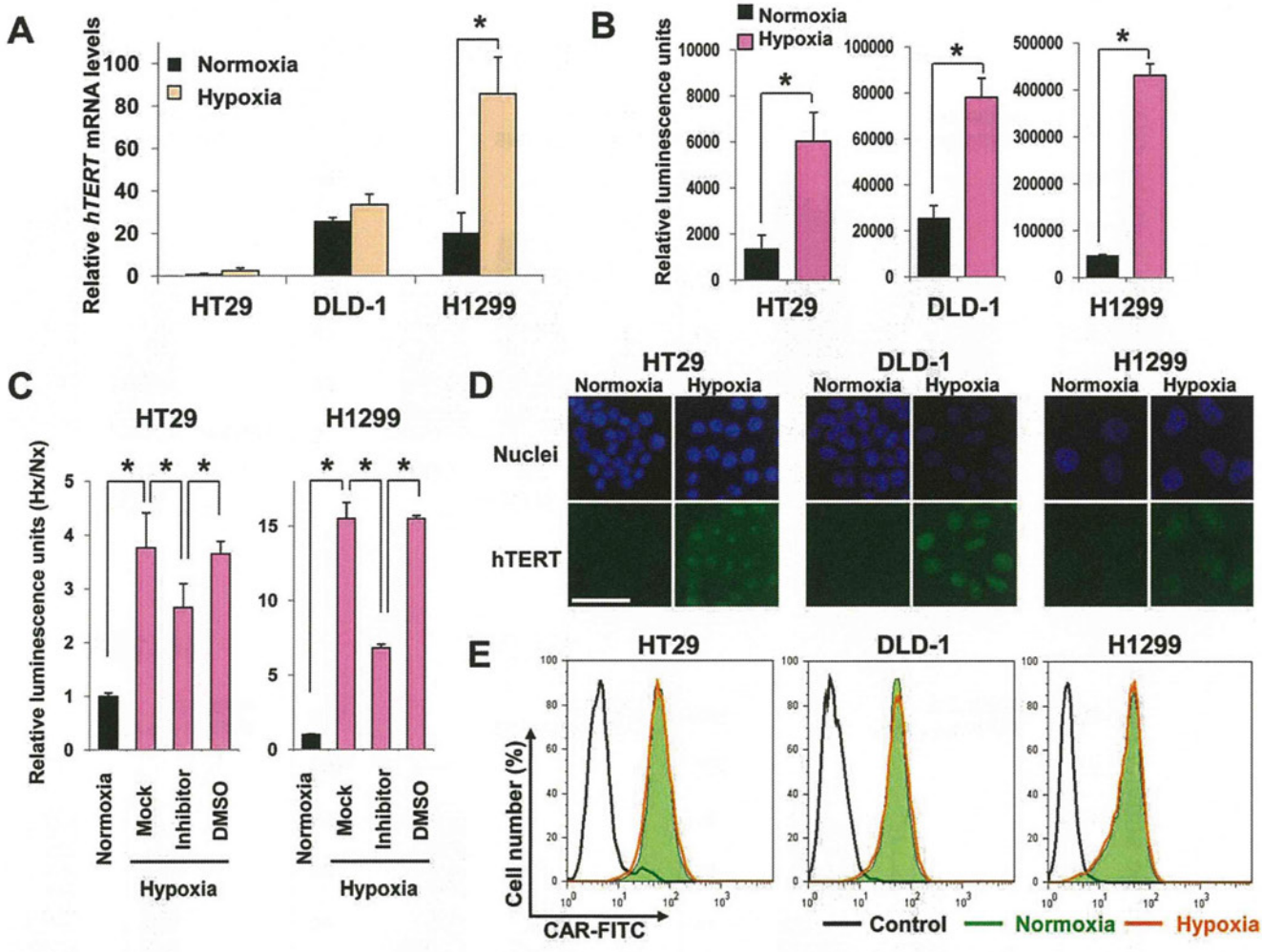

Figure 2. Effect of hypoxia on hTERT and CAR expression in human cancer cells. A, *hTERT* mRNA expression was assessed in human cancer cells that were maintained under normoxia (Nx) or hypoxia (Hx) for 18 h, using quantitative real-time RT- PCR analysis. The levels of *hTERT* mRNA were plotted as fold induction relative to the values of *hTERT* mRNA in HT29 cells incubated under normoxia, which was set at 1.0. Data are shown as mean values ± SD of triplicate experiments. Statistical significance (*) was determined as $P<0.05$ (Student's *t* test). **B** and **C**, *hTERT* gene promoter activity was assessed in human cancer cells that were transfected with the hTERT reporter vector (pGL3-hTERT) and then cultured under normoxia or hypoxia for 24 h, using luciferase reporter assay. The GFP expression vector (pCMV-EGFP) was used as a reporter for transfection efficiency, and the activities of hTERT promoter were determined as ratio of luciferase activity to GFP expression. Data are shown as mean values ± SD of triplicate experiments. Statistical significance (*) was determined as $P<0.05$ (Student's *t* test). **C**, HT29 and H1299 cells were treated with 30 μM HIF-1α inhibitor or DMSO solvent control in hypoxic condition. The levels of luminescence were plotted as fold induction relative to the values of luminescence in cancer cells incubated under normoxia, which were set at 1.0. **D**, Subcellular localization of hTERT protein expression in human cancer cells under normoxia or hypoxia was assessed using immunofluorescent staining. Cells cultured under a normoxic or hypoxic condition for 48 h were stained with anti-hTERT antibody (green). Nuclei were counterstained with DAPI (blue). Scale bars = 50 μm. **E,** Flow cytometric analysis of CAR expression in human cancer cells maintained under normoxia (green) or hypoxia (red) for 18 h. Cells were incubated with a mouse anti-CAR antibody followed by FITC-labeled rabbit anti-mouse IgG. An isotype-matched normal mouse IgG was used as a control (black).
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activation of the *hTERT* gene promoter was involved in the enhancement of virus replication in hypoxic tumor cells (Fig. 2 and 4). These results suggest that the *hTERT* gene promoter is useful for regulation of the replication of oncolytic adenoviruses in tumor cells in a hypoxic microenvironment.

The replication of OBP-301 depends on the activity of the *hTERT* gene promoter, which contains two HREs and is activated by HIF-1α under hypoxic conditions [23–25]. Hypoxic conditions that induced nuclear accumulation of HIF-1α (Fig. 1) upregulated *hTERT* gene promoter activity in human cancer cells (Fig. 2B and 2C). Consistent with this *hTERT* gene promoter activation, OBP-301 replication was significantly higher than that of Ad5 with the endogenous *E1* promoter (Fig. 4). These findings suggest that hypoxia enhances OBP-301 virus replication through HIF-1α-mediated activation of the *hTERT* gene promoter.

Recently, oncolytic virotherapy has garnered interest as potential therapeutic strategy for hypoxic tumors [29]. A hypoxia-responsive promoter that is upregulated by HIF-1 has been used for the tumor-specific replication of an oncolytic adenovirus [30–32]. Although an oncolytic adenovirus that is regulated by a hypoxia-responsive promoter will also be effective against hypoxic tumor cells following HIF-1 activation, non-hypoxic tumor cells in which HIF-1 is not activated may be less sensitive to these viruses. In contrast, the *hTERT* gene promoter-regulated oncolytic adenovirus OBP-301 would be effective

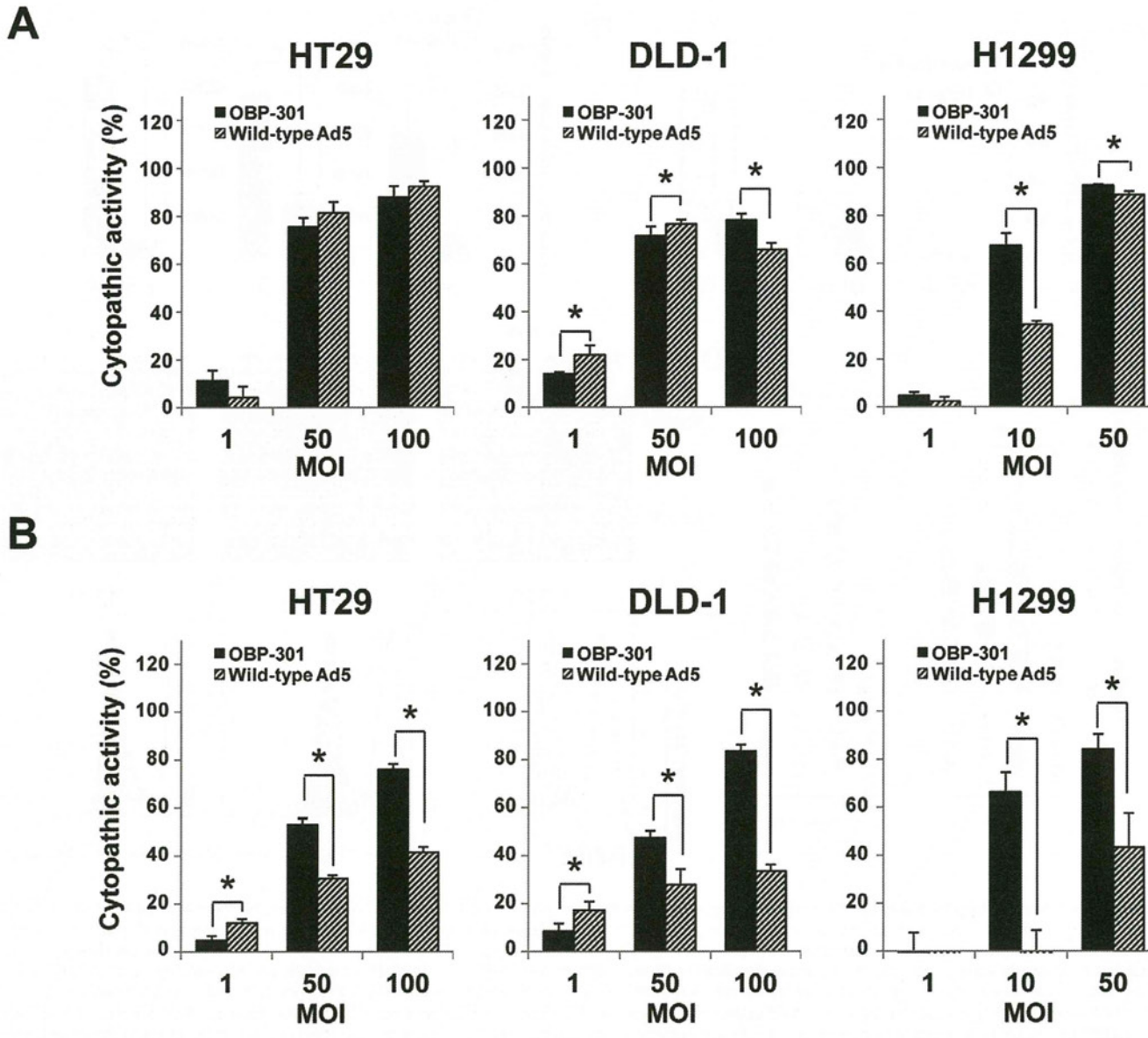

Figure 3. Cytopathic effect of OBP-301 and wild-type adenovirus serotype 5 (Ad5) under normoxic or hypoxic conditions. Cells were infected with OBP-301 (solid bars) or wild-type Ad5 (diagonal bars) at the indicated MOIs under normoxic (**A**) or hypoxic (**B**) conditions for 3 days. Cell viability was determined using an XTT assay. Cell viability was calculated relative to that of mock-treated cells, whose viability was set at 100%. Cytopathic activity was further calculated using the following formula; Cytopathic activity (%) = 100 (%) – cell viability (%). The results shown are the mean values ± SD of quadruplicate experiments. Statistical significance (*) was determined as $P<0.05$ (Student's *t* test).
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against both hypoxic and normoxic tumor cells through hTERT activation.

The infection efficacy of Ad5-based oncolytic adenoviruses has been suggested to depend mainly on the expression level of CAR on the target cell surface [28]. Hypoxia has been shown to downregulate CAR expression in tumor cells in a HIF-1α dependent manner [33]. However, in the present study, high CAR expression was maintained in all of the human cancer cells tested, even under hypoxic conditions (Fig. 2E). These results are consistent with a previous report [27], which demonstrated that hypoxia has no influence on adenoviral infectivity of target cancer cells. The expression levels of integrin αvβ3 and αvβ5 are also involved in the infection efficacy of adenoviruses [34]. Previous reports have shown that hypoxia upregulates the expression levels of integrin αvβ3 and αvβ5 in tumor cells [35,36]. These results suggest that hypoxic conditions would mainly suppress the replication of an adenovirus rather than the infection efficiency of the adenovirus.

Tumor tissues frequently contain hypoxic areas due to an immature vascular network. Various exogenous and endogenous hypoxia-related proteins have recently been developed as markers for identification of hypoxic regions of tumor tissues. Increased HIF-1 expression is a useful endogenous marker of hypoxic areas close to blood vessels. Expression of the exogenous hypoxia marker, Pimonidazole, is as effective a marker as HIF-1 for the detection of severely hypoxic regions [37]. In this study, OBP-301-mediated E1A expression was detected in pimonidazole-positive regions as well as normoxic regions (Fig. 5). These results indicate that the telomerase-specific oncolytic adenovirus OBP-301 could

Table 1. Comparison of the ID_{50} values of OBP-301 and Ad5 against human cancer cells under normoxia and hypoxia.

Cell lines	Viruses	ID_{50} value[a] (MOI)		Ratio[b] (Hx/Nx)
		Normoxia	Hypoxia	
HT29	OBP-301	20.2±1.5	51.7±7.3	2.5
	Ad5	25.6±4.3	212.8±52.0	9.4
DLD-1	OBP-301	8.2±2.7	26.3±10.3	3.0
	Ad5	6.6±1.9	2359.6±440.7	597.1
H1299	OBP-301	8.1±1.2	5.3±0.9	0.8
	Ad5	15.1±0.9	18.8±7.8	1.3

[a]The ID_{50} values of OBP-301 and Ad5 were calculated from the data of the XTT assay at day 3 after infection. Data are shown as the mean values ± SE of triplicate experiments.
[b]The ratio was calculated by division of the ID_{50} value under hypoxia (Hx) by the ID_{50} value under normoxia (Nx).
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infect and replicate in tumor cells under a hypoxic microenvironment including in tumor cells in which HIF-1 was active.

Recent advances in our knowledge of tumor microenvironments have provided evidence that hypoxic tumor cells contribute to cancer progression. For example, hypoxia activates the metastatic potential of tumor cells by inducing EMT [6,7] and facilitates the maintenance of cancer stem cells [8–11]. Therefore, the complete elimination of hypoxic tumor cells with metastatic and stemness properties is important for improvement of the clinical outcome of cancer patients. Recent reports have suggested that tumor cells undergoing EMT show reduced CAR expression [38,39], suggesting that tumor cells undergoing EMT are less sensitive to oncolytic adenovirus infection. Further study to investigate the cytopathic effect of OBP-301 in tumor cells undergoing EMT is warranted. In contrast, recent reports have shown that an oncolytic adenovirus induces oncolytic cell death in cancer stem cells [40–43]. Cancer stem cells have recently been shown to have increased hTERT expression compared to non-cancer stem cells [44,45]. Consistent with this high hTERT expression in cancer stem cells, Hemminki *et al.* has suggested that an oncolytic adenovirus that is regulated by specific promoters for hTERT, cyclooxygenase-2 or multidrug resistance, shows efficient cytopathic activity against human breast cancer stem cells [46]. Thus, the hTERT promoter-regulated oncolytic adenovirus OBP-301 may have the potential to eliminate highly progressive tumor cells in a hypoxic microenvironment, thereby contributing to the improvement of its therapeutic benefit against malignant tumors.

In conclusion, we have clearly demonstrated that the antitumor effect of the telomerase-specific oncolytic adenovirus OBP-301 against tumor cells in a hypoxic microenvironment is much stronger than that of a wild-type adenovirus. Regulation of virus replication by the *hTERT* gene promoter would be an effective antitumor strategy that would enhance the cytopathic activity of an oncolytic adenovirus against hypoxic tumor cells.

Materials and Methods

Cell lines

The human colorectal cancer (DLD-1 and HT29) and non-small cell lung cancer (H1299) cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Although cell lines were not authenticated by the authors, cells were immediately expanded after receipt and stored in liquid N_2. Cells were not cultured for more than 5 months following resuscitation. DLD-1 and H1299 cells were propagated as monolayer cultures in RPMI-1640 medium. HT29 was grown in McCoy's 5A medium. The transformed embryonic kidney cell line 293 obtained from the ATCC was maintained in Dulbecco's

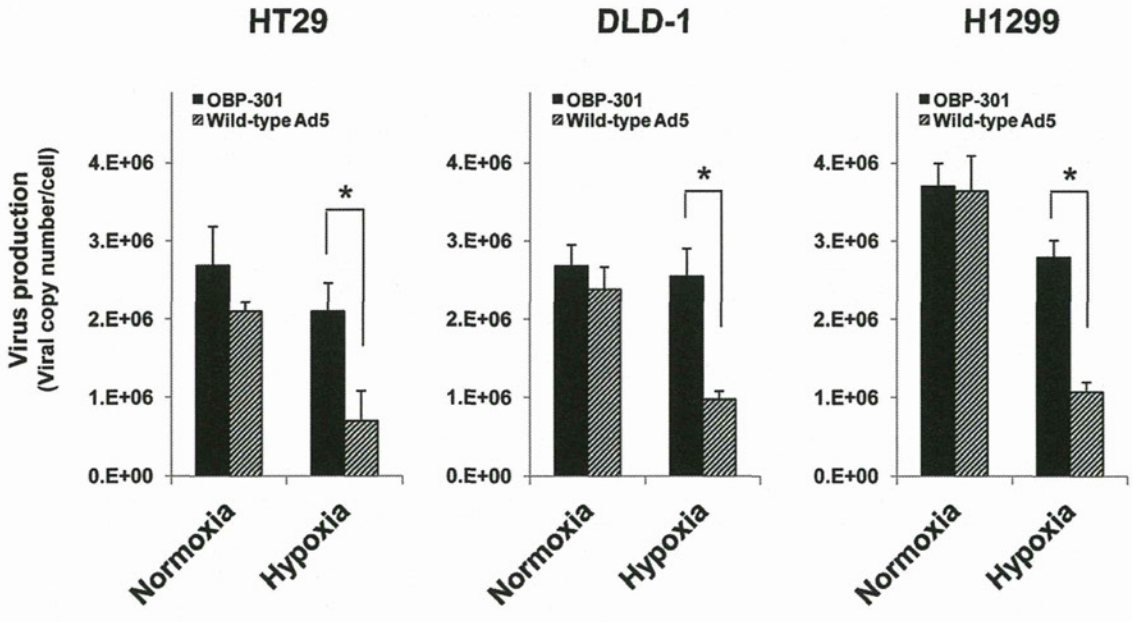

Figure 4. Quantification of viral DNA replication in human cancer cells under normoxia or hypoxia. The indicated human cancer cells were infected with OBP-301 or Ad5 at an MOI of 50 PFU/cell for 1 h, and were further incubated under normoxic (Nx) or hypoxic (Hx) conditions for 48 h. After incubation, cells were harvested and counted. *E1A* copy number in the cells at 48 h after incubation under normoxia or hypoxia was analyzed by quantitative PCR analysis. The amount of virus production was defined as the value of the *E1A* copy number relative to the number of cancer cells. Data are shown as the mean values ± SE of triplicate experiments. Statistical significance (*) was determined as $P<0.05$ (Student's *t* test).
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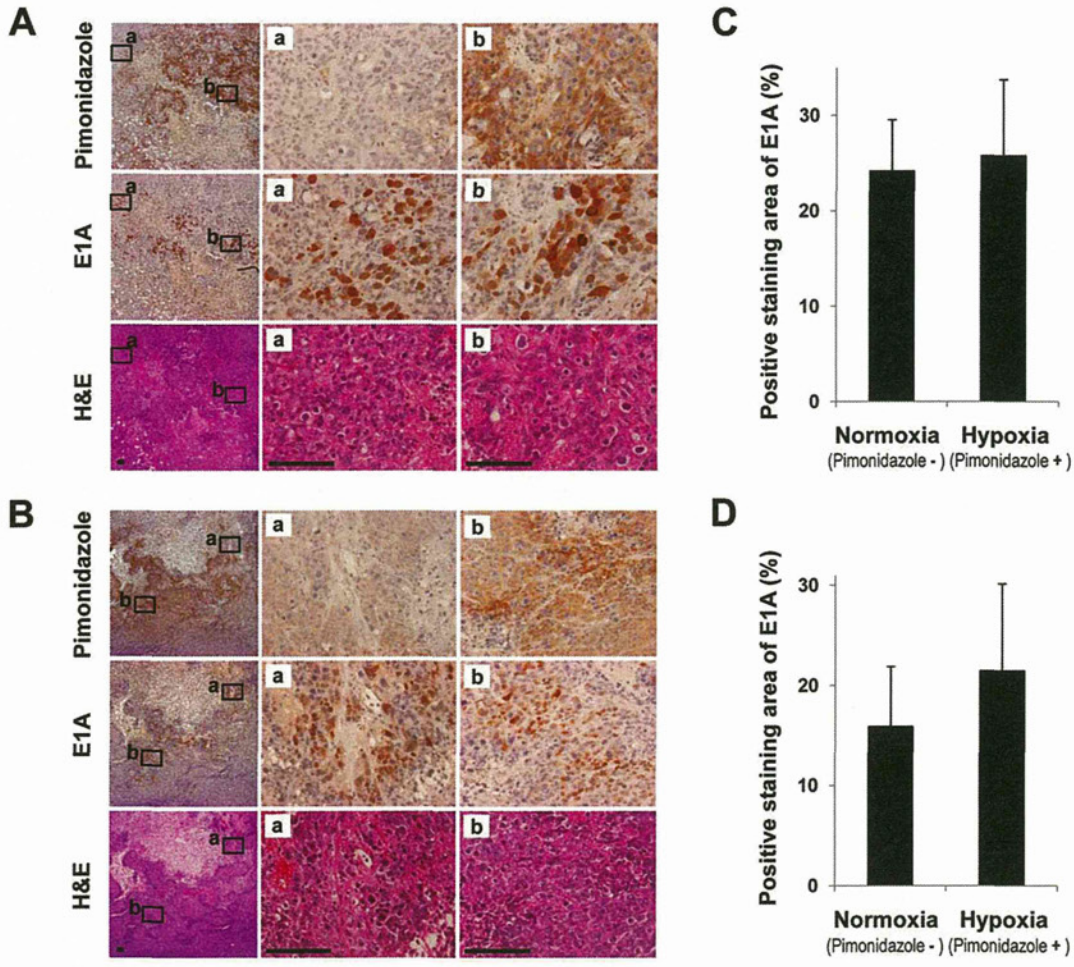

Figure 5. E1A expression in hypoxic areas of human xenograft tumors intratumorally injected with OBP-301. HT29 (**A** and **C**) and DLD-1 (**B** and **D**) tumor cells (5×10^6cells/mouse) were injected subcutaneously into the flank of athymic nude mice. Two weeks after inoculation, OBP-301 (1×10^8 PFU/tumor) was injected into the tumor for three cycles every 2 days. One day after final administration of OBP-301, the mice were intraperitoneally injected with the hypoxia marker pimonidazole hydrochloride (120 mg/kg). Thirty minutes after injection of pimonidazole hydrochloride, the mice were sacrificed and the tumors were harvested. Paraffin-embedded sections of HT29 and DLD-1 tumors were stained with hematoxylin and eosin (H&E). Tumor sections were also immunostained with an anti-pimonidazole antibody and an anti-adenovirus E1A antibody. **A** and **B**, Middle (**a**) and right (**b**) panels are higher magnifications of the boxed regions in the left panels. Original magnification: ×4 (left panels), ×40 (middle and right panels). Scale bars = 100 μm. **C** and **D**, Quantitative analysis of the E1A-positive areas in the normoxic and hypoxic regions of human xenografts tumor tissues. Data are shown as mean values ± SD of quadruplicate experiments.
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modified Eagle's medium containing high glucose (4.5 g/L). All media were supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin G and 100 μg/ml streptomycin. To maintain human cancer cells under hypoxic conditions, the cells were incubated in a hypoxic chamber (Modular Incubator Chamber; Billups-Rothenberg, Del Mar, CA, USA) filled with a gas mixture of 1% O_2, 5% CO_2 and N_2. The cells were also incubated under normoxic conditions at 37°C in a humidified atmosphere with 5% CO_2 and 20% O_2. HIF-1α inhibitor LW6 was purchased from Calbiochem (San Diego, CA, USA) and used at the concentration of 30 μM.

Recombinant adenoviruses

The recombinant replication-selective, tumor-specific adenovirus OBP-301 (Telomelysin), in which elements within the *hTERT* gene promoter drive the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site, was previously constructed and characterized [14]. The wild-type Ad5 was used as a control vector. OBP-301 and Ad5 were generated in 293 cells and purified by cesium chloride step-gradient ultracentrifugation. Their infectious titers were determined by a plaque-forming assay using 293 cells. The ratios of viral particle/plaque-forming unit of OBP-301 and Ad5 are 26 and 27, respectively. Viruses were stored at −80°C.

Western blot analysis

Cells were maintained under a hypoxic or a normoxic condition for 18 h or 24 h. Whole cell lysates were then prepared in a lysis buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40) containing a protease inhibitor mixture (Complete Mini; Roche, Indianapolis, IN, USA). Lysates were electrophoresed on 4%–7% SDS polyacrylamide gels and proteins were transferred to polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK). The primary antibodies used for Western blotting were: mouse anti-HIF-1α monoclonal antibody (mAb) (BD Biosciences, San Diego, CA, USA) and mouse anti-β-actin mAb (Sigma, St. Louis, MO, USA). Horseradish peroxidase-conjugated antibody against mouse IgG

(GE Healthcare) was used as the secondary antibody. Immunoreactive bands on the blots were visualized using enhanced chemiluminescence substrates (ECL Plus; GE Healthcare).

Immunofluorescence staining

Cells grown in chamber slides were washed twice with ice-cold PBS, and then fixed with cold 4% paraformaldehyde in PBS for 15 min on ice. The cells were permeabilized by incubation with 0.2% Triton X-100 in PBS for 5 min on ice and then blocked with 3% bovine serum albumin in PBS for 30 min at room temperature. The slides were subsequently incubated with mouse anti-HIF-1α mAb (BD Biosciences) or mouse anti-hTERT mAb (KYOWA Medex, Tokyo, JP) for 1 h at room temperature. After two washes with PBS, the slides were incubated with Alexa Fluor 488- or Alexa Fluor 568-labeled goat anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA) for 1 h. The slides were further stained with 10 mg/ml 4′,6-diamidino-2-phenylindole (DAPI), mounted using Fluorescence Mounting Medium (Dako, Glostrup, Denmark), and then photographed using a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Quantitative real-time RT-PCR analysis

Total RNA was extracted from cancer cells maintained under hypoxic or normoxic conditions for 18 h using the RNA-Bee regent (Tel-test; Friendswood, TX, USA). The *hTERT* mRNA copy number was determined by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) using a LightCycler instrument and a LightCycler Telo*TAGGG* hTERT Quantification Kit (Roche Diagnostics, Bagel, Switzerland). Data analysis was performed using LightCycler Software. The expression of *hTERT* mRNA was defined from the threshold cycle (Ct), and relative expression levels were calculated after normalization with reference to the expression of porphobilinogen deaminase (PBGD).

Transfection and luciferase reporter assay

Cells were seeded on 6-well plates at a density of 4×10^5 cells/well and incubated overnight. Each cell line was transfected with 3 μg of hTERT reporter plasmid (pGL3-hTERT) and 3 μg of GFP expression vector (pCMV-EGFP) as a reporter for transfection efficiency, using Lipofectamin LTX (Invitrogen) following the manufacturer's recommendations. Cells were then incubated under normoxic or hypoxic conditions. After 24 h incubation, luciferase activity was determined using a Bright-Glo reagent (Promega Corporation, Madison, WI, USA). Results presented are the ratios of luciferase activity to GFP fluorescent intensity and the means of three independent experiments.

Flow cytometric analysis

The cells that were maintained under hypoxic or normoxic conditions for 18 h were labeled with a mouse anti-CAR mAb (Upstate Biotechnology, Lake Placid, NY, USA) for 30 min at 4°C. An isotype-matched normal mouse IgG1 (Serotec, Oxfordshire, UK) was used as a negative control. The cells were then incubated with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG second antibody (Zymed Laboratories, San Francisco, CA, USA) and were analyzed using flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, CA, USA).

Cell viability assay

Cells were seeded on 96-well plates at a density of 1×10^4 cells/well 20 h before viral infection. All cell lines were infected with OBP-301 or wild-type Ad5 at multiplicity of infections (MOI) of 0, 1, 5, 10, 50 or using 100 plaque-forming units (PFU)/cell. The cells were then incubated under normoxic or hypoxic conditions for 3 days. Cell viability was determined using a Cell Proliferation Kit II (Roche Diagnostics) that was based on a sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay according to the manufacturer's protocol. The cytotoxic activity and the ID_{50} value of each virus was calculated using cell viability data. Each experiment was performed in quadruplicate during the same day and repeated at least three times.

In vitro virus replication assay

Cells were seeded on 6-well plates at a density of 3×10^5 cells/well 20 h before viral infection and were infected with OBP-301 or wild-type Ad5 at an MOI of 50 for 1 h. Following removal of the viral inocula, the cells were further maintained under hypoxic or normoxic conditions and were then harvested at 48 h after virus infection. After cell counting, DNA was purified using the QIAmp DNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. E1A copy numbers were determined by quantitative real-time PCR using the StepOnePlus Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and TaqMan Gene Expression Assays (Applied Biosystems). The sequences of the specific primers and probe used in this experiment were: E1A primers, 5′-CCT GAG ACG CCC GAC ATC-3′ and 5′-GGA CCG GAG TCA CAG CTA TCC-3′; E1A probe, 5′-FAM-CTG TGT CTA GAG AAT GC-MGB-3′. Data analysis was carried out using StepOne Software (Applied Biosystems).

In vivo human xenograft tumor models

Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine (Approval ID: OKU-2009051). The HT29 and DLD-1 cells (5×10^6 cells per site) were inoculated subcutaneously into the flank of 5- to 6-week-old female BALB/c *nu/nu* mice (Japan SLC, Shizuoka, Japan). When the tumor size reached approximately 10 mm in diameter, OBP-301 was injected into the tumors at a dose of 1×10^8 PFU/tumor every 2 days for three cycles. To detect hypoxic areas within tumor tissues, pimonidazole hydrochloride (Hypoxyprobe -1; Hypoxyprobe Inc., Burlington, MA, USA) was injected intraperitoneally at a dose of 120 mg/kg body weight 24 h after the final treatment. The mice were then sacrificed and the tumors were harvested 30 min after pimonidazole injection. Four mice were used for each group.

Immunohistochemistry

Tumors were fixed in 10% neutralized formalin and embedded in paraffin blocks. Sections (4 μm) were prepared for hematoxylin/eosin staining and also for immunohistochemical examination. After deparaffinization and rehydration, antigen retrieval was performed by microwave irradiation in 10 mM citrate buffer (pH 6.0). After quenching of endogenous tissue peroxidase, tissue sections were incubated with mouse anti-adenovirus type 5 E1A mAb (BD Biosciences) and mouse anti-Hypoxyprobe-1 mAb (Hypoxyprobe Inc.). The sections were then incubated using the Histofine Mouse Stain Kit (Nichirei Biosciences, Tokyo, Japan). Immunoreactive signals were visualized by using 3,3′-diaminobenzidine tetrahydrochloride solution, and the nuclei were counterstained with hematoxylin. Signals were viewed under a microscope (BX50; Olympus). The percentage of the positive area in each field was analyzed using Image J software (version 1.45).

Statistical analysis

Determination of significant differences among groups was assessed by using the Student's t test. $P<0.05$ was considered significant.

Supporting Information

Figure S1 Suppression of HIF-1α expression in human cancer cells under hypoxic conditions by HIF-1 inhibitor. A, Western blot analysis of HIF-1α protein expression in human cancer cells (HT29 and H1299) under normoxic or hypoxic conditions. Cells were treated with 30 mM HIF-1α inhibitor or DMSO solvent control under hypoxic condition for 24 h. Cell lysates were subjected to Western blot analysis using an anti- HIF-1α antibody. β-actin was assayed as a loading control. **B,** Subcellular localization of HIF-1α expression in human cancer cells treated with 30 mM HIF-1α inhibitor or DMSO solvent control under hypoxia was assessed using immunofluorescent staining. Cells cultured under a hypoxic condition for 24 h were stained with anti-HIF-1α antibody (red). Nuclei were counter-stained with DAPI (blue). Scale bars = 50 μm.
(TIF)

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Author Contributions

Conceived and designed the experiments: YH HT FT TF. Performed the experiments: YH FT TK YW SY. Analyzed the data: YH HT FT FU SK TF. Contributed reagents/materials/analysis tools: YU. Wrote the paper: YH HT TF.

References

1. Majmundar AJ, Wong WJ, Simon MC (2010) Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell 40: 294–309.
2. Wilson WR, Hay MP (2011) Targeting hypoxia in cancer therapy. Nat Rev Cancer 11: 393–410.
3. Höckel M, Vaupel P (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93: 266–276.
4. Bertout JA, Patel SA, Simon MC (2008) HYPOXIA AND METABOLISM SERIES – TIMELINE The impact of O-2 availability on human cancer. Nature Reviews Cancer 8: 967–975.
5. Harrison L, Blackwell K (2004) Hypoxia and anemia: Factors in decreased sensitivity to radiation therapy and chemotherapy? Oncologist 9: 31–40.
6. Yang MH, Wu MZ, Chiou SH, Chen PM, Chang SY, et al. (2008) Direct regulation of TWIST by HIF-1alpha promotes metastasis. Nat Cell Biol 10: 295–305.
7. Yoo YG, Christensen J, Gu J, Huang LE (2011) HIF-1{alpha} Mediates Tumor Hypoxia to Confer a Perpetual Mesenchymal Phenotype for Malignant Progression. Sci Signal 4: pt4.
8. Keith B, Simon MC (2007) Hypoxia-inducible factors, stem cells, and cancer. Cell 129: 465–472.
9. Heddleston JM, Li Z, Lathia JD, Bao S, Hjelmeland AB, et al. (2010) Hypoxia inducible factors in cancer stem cells. Br J Cancer 102: 789–795.
10. Li Z, Bao S, Wu Q, Wang H, Eyler C, et al. (2009) Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. Cancer Cell 15: 501–513.
11. Chang CJ, Yang JY, Xia W, Chen CT, Xie X, et al. (2011) EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-beta-catenin signaling. Cancer Cell 19: 86–100.
12. Tredan O, Galmarini CM, Patel K, Tannock IF (2007) Drug resistance and the solid tumor microenvironment. Journal of the National Cancer Institute 99: 1441–1454.
13. Kirn D, Martuza RL, Zwiebel J (2001) Replication-selective virotherapy for cancer: Biological principles, risk management and future directions. Nat Med 7: 781–787.
14. Kawashima T, Kagawa S, Kobayashi N, Shirakiya Y, Umeoka T, et al. (2004) Telomerase-specific replication-selective virotherapy for human cancer. Clinical Cancer Research 10: 285–292.
15. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, et al. (1994) Specific association of human telomerase activity with immortal cells and cancer. Science 266: 2011–2015.
16. Shay JW, Wright WE (1996) Telomerase activity in human cancer. Curr Opin Oncol 8: 66–71.
17. Nakayama J, Tahara H, Tahara E, Saito M, Ito K, et al. (1998) Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. Nat Genet 18: 65–68.
18. Umeoka T, Kawashima T, Kagawa S, Teraishi F, Taki M, et al. (2004) Visualization of intrathoracically disseminated solid tumors in mice with optical imaging by telomerase-specific amplification of a transferred green fluorescent protein gene. Cancer Research 64: 6259–6265.
19. Hashimoto Y, Watanabe Y, Shirakiya Y, Uno F, Kagawa S, et al. (2008) Establishment of biological and pharmacokinetic assays of telomerase-specific replication-selective adenovirus. Cancer Science 99: 385–390.
20. Nemunaitis J, Tong AW, Nemunaitis M, Senzer N, Phadke AP, et al. (2010) A phase I study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. Mol Ther 18: 429–434.
21. Pouysségur J, Dayan F, Mazure NM (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 441: 437–443.
22. Ke Q, Costa M (2006) Hypoxia-inducible factor-1 (HIF-1). Mol Pharmacol 70: 1469–1480.
23. Nishi H, Nakada T, Kyo S, Inoue M, Shay JW, et al. (2004) Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT). Molecular and Cellular Biology 24: 6076–6083.
24. Yatabe N, Kyo S, Maida Y, Nishi H, Nakamura M, et al. (2004) HIF-1-mediated activation of telomerase in cervical cancer cells. Oncogene 23: 3708–3715.
25. Anderson CJ, Hoare SF, Ashcroft M, Bilsland AE, Keith WN (2006) Hypoxic regulation of telomerase gene expression by transcriptional and post-transcriptional mechanisms. Oncogene 25: 61–69.
26. Pipiya T, Sauthoff H, Huang YQ, Chang B, Cheng J, et al. (2005) Hypoxia reduces adenoviral replication in cancer cells by downregulation of viral protein expression. Gene Ther 12: 911–917.
27. Shen BH, Hermiston TW (2005) Effect of hypoxia on Ad5 infection, transgene expression and replication. Gene Ther 12: 902–910.
28. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, et al. (1997) Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275: 1320–1323.
29. Guo ZS (2011) The impact of hypoxia on oncolytic virotherapy. Virus Adaptation and Treatment 3: 71–82.
30. Hernandez-Alcoceba R, Pihalja M, Qian D, Clarke MF (2002) New oncolytic adenoviruses with hypoxia- and estrogen receptor-regulated replication. Hum Gene Ther 13: 1737–1750.
31. Post DE, Van Meir EG (2003) A novel hypoxia-inducible factor (HIF) activated oncolytic adenovirus for cancer therapy. Oncogene 22: 2065–2072.
32. Kwon OJ, Kim PH, Huyn S, Wu L, Kim M, et al. (2010) A hypoxia- and {alpha}-fetoprotein-dependent oncolytic adenovirus exhibits specific killing of hepatocellular carcinomas. Clin Cancer Res 16: 6071–6082.
33. Küster K, Koschel A, Rohwer N, Fischer A, Wiedenmann B, et al. (2010) Downregulation of the coxsackie and adenovirus receptor in cancer cells by hypoxia depends on HIF-1alpha. Cancer Gene Ther 17: 141–146.
34. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR (1993) Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 73: 309–319.
35. Cowden Dahl KD, Robertson SE, Weaver VM, Simon MC (2005) Hypoxia-inducible factor regulates alphavbeta3 integrin cell surface expression. Mol Biol Cell 16: 1901–1912.
36. Niu JX, Zhang WJ, Ye LY, Wu LQ, Zhu GJ, et al. (2007) The role of adhesion molecules, alpha v beta 3, alpha v beta 5 and their ligands in the tumor cell and endothelial cell adhesion. Eur J Cancer Prev 16: 517–527.
37. Kizaka-Kondoh S, Konse-Nagasawa H (2009) Significance of nitroimidazole compounds and hypoxia-inducible factor-1 for imaging tumor hypoxia. Cancer Sci 100: 1366–1373.
38. Lacher MD, Tiirikainen MI, Saunier EF, Christian C, Anders M, et al. (2006) Transforming growth factor-beta receptor inhibition enhances adenoviral infectability of carcinoma cells via up-regulation of Coxsackie and Adenovirus Receptor in conjunction with reversal of epithelial-mesenchymal transition. Cancer Res 66: 1648–1657.
39. Lacher MD, Shiina M, Chang P, Keller D, Tiirikainen MI, et al. (2011) ZEB1 limits adenoviral infectability by transcriptionally repressing the coxsackie virus and adenovirus receptor. Mol Cancer 10: 91.
40. Jiang H, Gomez-Manzano C, Aoki H, Alonso MM, Kondo S, et al. (2007) Examination of the therapeutic potential of Delta-24-RGD in brain tumor stem cells: role of autophagic cell death. J Natl Cancer Inst 99: 1410–1414.
41. Eriksson M, Guse K, Bauerschmitz G, Virkkunen P, Tarkkanen M, et al. (2007) Oncolytic adenoviruses kill breast cancer initiating CD44+CD24−/low cells. Mol Ther 15: 2088–2093.

42. Zhang X, Komaki R, Wang L, Fang B, Chang JY (2008) Treatment of radioresistant stem-like esophageal cancer cells by an apoptotic gene-armed, telomerase-specific oncolytic adenovirus. Clin Cancer Res 14: 2813–2823.
43. Short JJ, Curiel DT (2009) Oncolytic adenoviruses targeted to cancer stem cells. Mol Cancer Ther 8: 2096–2102.
44. Hiyama E, Hiyama K (2007) Telomere and telomerase in stem cells. Br J Cancer 96: 1020–1024.
45. Castelo-Branco P, Zhang C, Lipman T, Fujitani M, Hansford L, et al. (2011) Neural tumor-initiating cells have distinct telomere maintenance and can be safely targeted for telomerase inhibition. Clin Cancer Res 17: 111–121.
46. Bauerschmitz GJ, Ranki T, Kangasniemi L, Ribacka C, Eriksson M, et al. (2008) Tissue-specific promoters active in CD44+CD24−/low breast cancer cells. Cancer Res 68: 5533–5539.

Molecular Cancer Therapeutics

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

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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

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soft-tissue sarcomas (9, 10). Telomerase activation is closely correlated with the expression of the human telomerase reverse transcriptase (*hTERT*) gene (11). On the basis of these data, we previously developed a telomerase-specific replication-competent oncolytic adenovirus OBP-301 (Telomelysin) in which the *hTERT* gene promoter drives the expression of the *E1A* and *E1B* genes (12). A phase I clinical trial of OBP-301, which was conducted in the United States on patients with advanced solid tumors, indicated that OBP-301 was well tolerated by patients (13). Recently, we reported that OBP-301 efficiently killed human bone and soft-tissue sarcoma cells (14, 15). However, some osteosarcoma cell lines were not sensitive to the antitumor effect of OBP-301. Therefore, to efficiently eliminate tumor cells with OBP-301, its antitumor effects need to be enhanced.

Cancer gene therapy is defined as the treatment of malignant tumors via the introduction of a therapeutic tumor suppressor gene or the abrogation of an oncogene. The tumor suppressor *p53* gene has an attractive tumor suppressor profile as a potent therapeutic transgene for induction of cell-cycle arrest, senescence, apoptosis, and autophagy (16). Dual cell death pathways, such as apoptosis and autophagy, induced by p53 transactivation are mainly involved in the suppression of tumor initiation and progression. However, among the p53 downstream target genes, p21, which is most rapidly and strongly induced during the DNA damage response, mainly induces cell-cycle arrest through suppression of apoptotic and autophagic cell death pathways (17, 18). Thus, p21 suppression may be a more effective strategy for the induction of apoptotic and autophagic cell death pathways in tumor cells, particularly when the tumor suppressor *p53* gene is overexpressed in tumor cells in response to cancer gene therapy.

A p53-expressing replication-deficient adenovirus (Ad-p53, Advexin) has previously been reported to induce an antitumor effect in the *in vitro* and *in vivo* settings (19, 20) as well as in some clinical studies (21–24). We recently reported that combination therapy with OBP-301 and Ad-p53 resulted in a more profound antitumor effect than monotherapy with either OBP-301 or Ad-p53 (25). Moreover, we generated armed OBP-301 expressing the wild-type *p53* tumor suppressor gene (OBP-702) and showed that OBP-702 suppressed the viability of various types of epithelial malignant cells more efficiently than did OBP-301 (26). OBP-702 induced a more profound apoptotic cell death effect than Ad-p53, likely via adenoviral E1A-mediated suppression of anti-apoptotic p21 in human epithelial malignant cells. However, it remained unclear whether OBP-702 efficiently induces an antitumor effect in human nonepithelial malignant cells, including osteosarcomas.

In the present study, we investigated the *in vitro* cytopathic efficacy of the p53-expressing telomerase-specific replication-competent oncolytic adenovirus, OBP-702, in human osteosarcoma cells, and we compared the induction level of apoptotic and autophagic cell deaths in OBP-301–resistant human osteosarcoma cells infected with OBP-301, OBP-702, and Ad-p53. The molecular mechanism by which OBP-702 mediates induction of apoptosis and autophagy was also investigated. Finally, the *in vivo* antitumor effect of OBP-702 was evaluated using an orthotopic OBP-301–resistant human osteosarcoma xenograft tumor model.

Materials and Methods

Cell lines

The human osteosarcoma cell lines, HOS and SaOS-2, were kindly provided by Dr. Satoru Kyo (Kanazawa University, Ishikawa, Japan). These cells were propagated as monolayer cultures in Dulbecco's Modified Eagle's Medium. The human osteosarcoma cell line, U2OS, was obtained from the American Type Culture Collection and was grown in McCoy's 5a Medium. The human osteosarcoma cell line, MNNG/HOS, was purchased from DS Pharma Biomedical and was maintained in Eagle's Minimum Essential Medium containing 1% nonessential amino acids. All media were supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. The normal human lung fibroblast (NHLF) cell line, NHLF, was obtained from TaKaRa Biomedicals. NHLF cells were propagated as monolayer culture in the medium recommended by the manufacturer. Although cell lines were not authenticated by the authors, cells were immediately expanded after receipt and stored in liquid N_2. Cells were not cultured for more than 5 months following resuscitation. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Recombinant adenoviruses

The recombinant telomerase-specific replication-competent adenovirus OBP-301 (Telomelysin), in which the promoter element of the *hTERT* gene drives the expression of *E1A* and *E1B* genes, was previously constructed and characterized (12, 27). For OBP-301–mediated induction of exogenous *p53* gene expression, we recently generated OBP-702, in which a human wild-type *p53* gene expression cassette was inserted into the *E3* region (Supplementary Fig. S1; ref. 26). Ad-p53 is a replication-defective adenovirus serotype 5 vector with a *p53* gene expression cassette at the *E1* region (19, 20). Recombinant viruses were purified by ultracentrifugation using cesium chloride step gradients, their titers were determined by a plaque-forming assay using 293 cells and they were stored at −80°C.

Cell viability assay

Cells were seeded on 96-well plates at a density of 1×10^3 cells/well 24 hours before viral infection. All cell lines were infected with OBP-702 at multiplicity of infections (MOI) of 0, 0.1, 1, 10, 50, or 100 plaque-forming units (PFU)/cell. Cell viability was determined on days 1, 2, 3, and 5 after virus infection using Cell Proliferation Kit II (Roche Molecular Biochemicals). The 50% inhibiting dose (ID_{50}) value of OBP-702 for each cell line was calculated