厚生労働科学研究費補助金

医療機器開発推進研究事業

テロメラーゼ依存性蛍光発現ナノバイオ・ウイルス製剤を標識薬剤とする高感度リアルタイム微小癌転移イメージングシステムの開発

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平成21～23年度　総合研究報告書

研究代表者　**藤原　俊義**

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テロメラーゼ依存性蛍光発現ナノバイオ・ウイルス製剤を標識薬剤とする高感度リアルタイム微小癌転移イメージングシステムの開発

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【研究要旨】

天然に存在する生物由来の蛍光タンパク質は、至適な波長の励起光を吸収することにより強い蛍光を発し、導入した細胞を生きたままの状態で可視化することができる。Green Fluorescent Protein（GFP）をはじめとする蛍光タンパク質を用いた分子イメージングは、最先端の生命科学の研究や技術開発には広範囲に利用されているが、医療への応用は未だ研究段階であり、実際にヒトに臨床応用された事例はない。平成17-19年度の厚生労働科学研究費にて、標識薬剤としてテロメラーゼ活性依存性に癌細胞で選択的に増殖して*GFP*遺伝子を発現するウイルス製剤TelomeScan（開発コード：OBP-401）を作成し、携帯用接触プローブ型蛍光検出装置の有用性を明らかにしてきた。本研究では、新たに鏡視下手術用の高感度蛍光感知機能を付与したビデオスコープを試作し、大動物でその操作性と有用性を評価することで、最近の腹腔鏡・胸腔鏡手術の普及に対応した、より実践的な低侵襲治療の確立を目指す。

まず、高感度GFP蛍光検出ビデオスコープを試作し、大動物の胃粘膜下へ注入した蛍光ビーズでリンパ流がリアルタイムに可視化できることを確認した。次に、*GFP*遺伝子を導入したヒト癌細胞をペレット状にして大動物に投与し、緑色蛍光が明瞭に観察可能であることを明らかにした。さらに、近赤外蛍光を感知できる高感度蛍光感知ビデオスコープを試作し、近赤外蛍光*Katushka*遺伝子を挿入した非増殖型アデノウイルスベクター（Ad-Katushka）を感染させたヒト癌細胞を大動物の消化管や近傍のリンパ節に投与して赤色蛍光を観察した。

A．研究目的

天然に存在する生物由来の蛍光タンパク質は、至適な波長の励起光を吸収することにより強い蛍光を発し、導入した細胞を生きたままの状態で可視化することができる。GFP（Green Fluorescent Protein）をはじめとする蛍光タンパク質を用いた分子イメージングは、最先端の生命科学の研究や技術開発には広範囲に利用されているが、医療への応用は未だ研究段階であり、実際にヒトに臨床応用された事例はない。われわれは、テロメラーゼ活性（hTERT遺伝子発現）に依存して癌細胞で選択的に増殖し細胞死を誘導する改変アデノウイルス製剤Telomelysin（開発コード：OBP-301）を開発し、米国にて臨床試験を行い、その安全性と臨床効果を確認した。このウイルスのゲノム配列は完全に明らかになっており、癌選択的ベクターとして外来遺伝子を搭載することができる。すなわち、蛍光タンパク質をコードする遺伝子を組み込み、癌細胞で選択的に蛍光発現を誘導することができる。

本研究では、テロメラーゼ活性依存性に癌細胞で選択的に増殖してGFP遺伝子を発現するナノバイオ・ウイルス製剤TelomeScan（OBP-401）を標識薬剤とし、鏡視下手術用の高感度蛍光感知ビデオスコープを用いたリアルタイム微小癌転移診断用の外科ナビゲーション・システムを開発する。また、GFPの蛍光波長より長く、組織透過性の高い近赤外蛍光を発する新しい蛍光タンパク質遺伝子を搭載した新規ウイルス標識薬剤を開発し、鏡視下手術時における有用性や汎用性をTelomeScanと比較検討する。術前に原発病巣や体腔に投与されたナノバイオ・ウイルス製剤は、微小転移巣で癌細胞に感染・増殖して選択的に蛍光を発するので、高感度蛍光検出ビデオスコープを用いてリアルタイムにハイビジョン・モニター上で可視化することができる。

平成21年度から23年度までの3年間で、GFP蛍光および近赤外蛍光を感知できる高感度蛍光検出ビデオスコープをそれぞれ試作し、大動物において蛍光ビーズ、あるいはそれぞれの蛍光遺伝子導入ヒト癌細胞をリアルタイムに検出でき、リンパ流や臓器近傍のリンパ節、組織を可視化できることを検証してきた。

B．研究方法

1）TelomeScan（OBP-401）の構造と機能

TelomeScanは幼児の「かぜ」症状の原因となるアデノウイルス5型を基本骨格とし、テロメラーゼ構成成分であるhTERT（human telomerase reverse transcriptase）遺伝子のプロモーターの下流にウイルス増殖に必須のE1AおよびE1B遺伝子がIRES配列で連結して組み込まれている。また、ウイルスゲノムのE3領域に、オワンクラゲ由来のGFP（Green Fluorescent Protein）蛍光発現遺伝子が挿入されている。TelomeScanは癌細胞で選択的に増殖してGFP蛍光を発するとともに、最終的には細胞死を誘導する。一方、テロメラーゼ活性を持たない正常細胞では、その増殖は抑制され、GFPもみられず、細胞死も生じることはない。

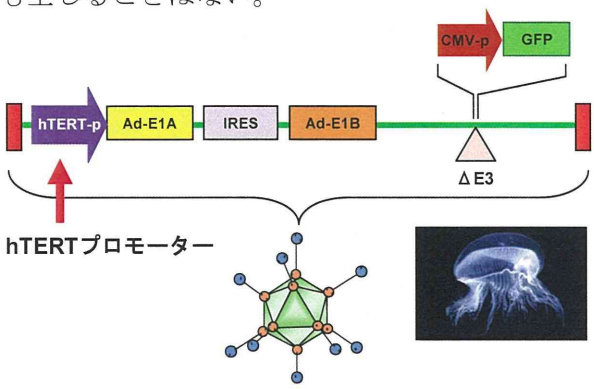


図１　TelomeScanウイルスの構造

2）近赤外蛍光*Katushka*遺伝子発現アデノウイルスベクター（Ad-Katushka）の構造と機能

GFPより深部の微小癌組織を高感度に検出するために、組織透過性の高い近赤外蛍光を発する蛍光タンパク質を搭載した新たなウイルス標識薬剤を作成した。GFPの蛍光波長505 nmより長く組織透過性の高い635 nmの近赤外蛍光を発するイソギンチャク*Entacmaea quadricolor*由来の新しい蛍光タンパク質Katushkaをコードする遺伝子を、幼児の「かぜ」症状の原因となるアデノウイルス5型のE1欠失領域にサイトメガロウイルス・プロモーターとともに挿入した。

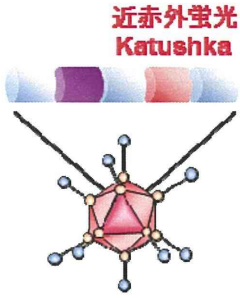


図2　Ad-Katushkaアデノウイルスベクター

3）高感度GFP蛍光検出ビデオスコープの試作

GFP蛍光を可視化することができる高感度蛍光検出ビデオスコープの第1号機を試作した。明視野モード時は白色光（400~700 nm）、蛍光モード時は励起光（460~480 nm）を照明光とし、反射光と蛍光をスコープに装着したカメラアダプタで分離して、反射光画像と蛍光画像を同時に表示するシステムとした。明視野モードと蛍光モードは光源で切り替える方式を採用した。

第1号試作機の使用経験に基づき、第2号試作機を作成した。カメラヘッドを100~200g程度に小型化し、カメラヘッドのスイッチにて明視野モードと蛍光モードのフィルタ切り替えがワンタッチでできるようにした。また、カメラヘッド部での切り替えに連動して光源フィルタとモニタ表示が切り替わり、明視野と蛍光視野を切り替えて表示するシステムとなっている。

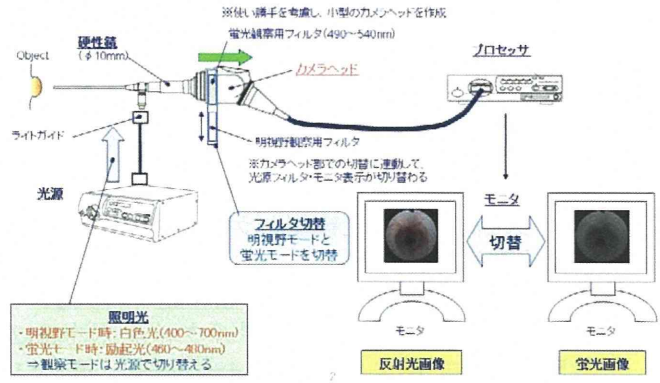


図3　ビデオスコープ第2号試作機のシステム図

4）近赤外蛍光を観察可能な高感度蛍光検出ビデオスコープの試作

近赤外蛍光を可視化することができる高感度蛍光検出ビデオスコープを試作した。光源、スコープ、カメラアダプタ、カメラ、プロセッサ、モニターから成り、励起波長570～590nm、蛍光波長600～655nmで観察する。

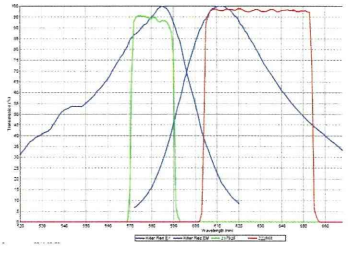


図4　励起波長と蛍光波長プロファイル

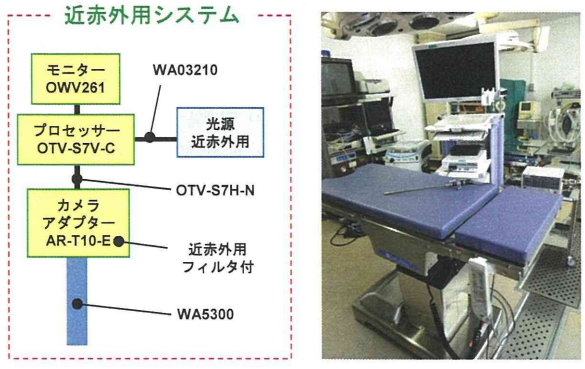


図5　近赤外蛍光感知ビデオスコープシステム

5）ミニブタを用いた大動物実験

A）蛍光ビーズでの実験

ビデオスコープ試作機を用いて、GFP蛍光が大動物にて検出可能かどうかを検討した。TelomeScanは担癌動物モデルでのみGFP蛍光を発するが、大動物では適した担癌モデルが存在しないため、本実験ではTelomeScanと同一の蛍光特性を持つ蛍光ビーズ（Fluoresbrite蛍光ミクロスフェア、フナコシ株式会社）（50、200、500 nm）を使用した。

全身麻酔下にミニブタの下腹部にトロッカーを挿入し、炭酸ガスにて気腹した後、試作したビデオスコープで腹腔内を観察した。

B）*GFP*遺伝子導入ヒト癌細胞での実験

非増殖型*GFP*遺伝子発現アデノウイルスベクター（Ad-GFP）をH1299ヒト肺癌細胞に感染させ、24-48時間後に遠心にて回収、ペレット状にして、全身麻酔下のミニブタに経口的内視鏡にて粘膜下に、あるいは開腹にて胃壁漿膜下に注入し、初年度に作成した高感度蛍光検出ビデオスコープ第2号機にて蛍光観察を行った。

C）*Katushka*遺伝子導入ヒト癌細胞での実験

非増殖型Ad-KatushkaアデノウイルスをH1299ヒト肺癌細胞に感染させ、24-48時間後に遠心にて回収、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する。気腹下に近赤外蛍光観察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）高感度GFP蛍光検出ビデオスコープ第1号試作機による大動物実験

全身麻酔下にミニブタにトロッカー5本を挿入し、臍下部のポートから第1号試作機のビデオスコープを挿入して腹腔内を観察した。次に、経口的に上部消化管用内視鏡を挿入し、胃粘膜下に注射ニードルにて蛍光ビーズ1 mlを注入したところ、腹腔内からの蛍光視野にてリアルタイムに所属リンパ節領域へのリンパ流が可視化できた。しかし、この試作機はカメラヘッドが約1Kgと重く、視野切り替えを光源サイドで行うため、操作性に難点がみられた。

2）高感度GFP蛍光検出ビデオスコープ第2号試作機による大動物実験

第1号機での問題点を解決するために、カメラヘッドを小型化し、カメラヘッドのスイッチにて視野モードのフィルタ切り替えができるようにした。また、明視野と蛍光視野を同一モニターで表示するシステムとすることで、視野を動かさずに手術操作が可能となった。小型化によってCCDの感度はやや低下したが、上部消化管内視鏡による胃粘膜下への蛍光ビーズ注入によって、極めて良好にリアルタイムにリンパ流を確認することが可能であった。

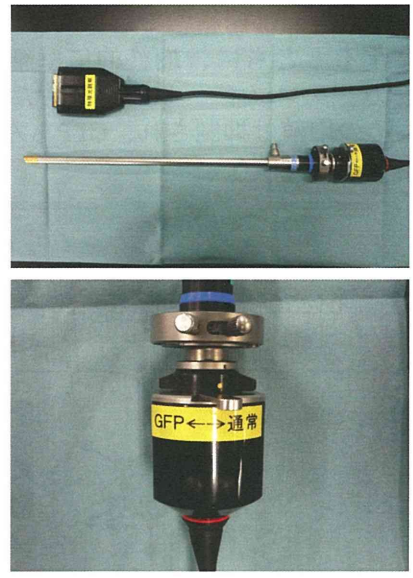


図6　第2号試作機のカメラヘッド

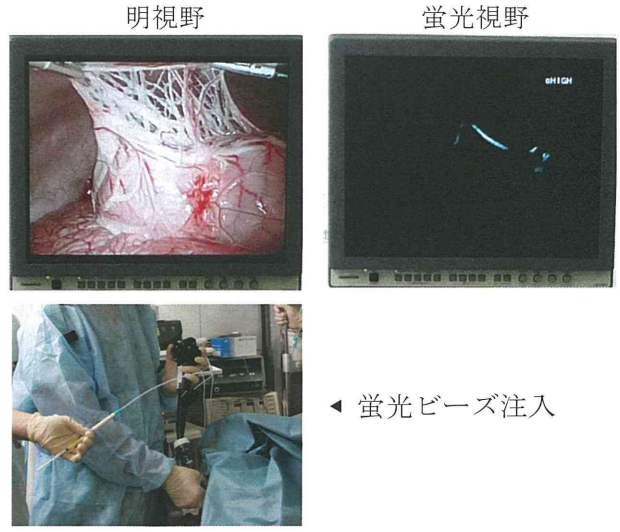


図7　第2号試作機の蛍光視野

3）大動物における*GFP*遺伝子発現ヒト癌細胞の可視化の試み

全身麻酔下のミニブタに*GFP*遺伝子発現H1299ヒト肺癌細胞をペレット状にして経口的内視鏡にて粘膜下に、あるいは開腹にて胃壁漿膜下に注入し、高感度蛍光検出ビデオスコープ第2号試作機にて腹腔内を観察したところ、GFP蛍光が明瞭に検出可能であった。

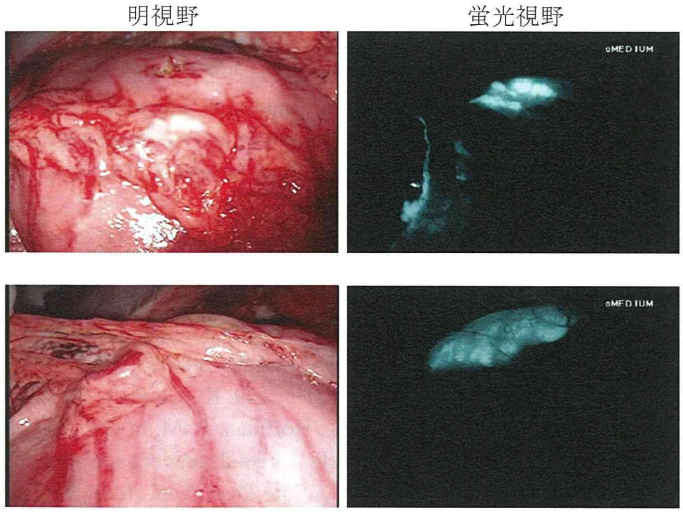
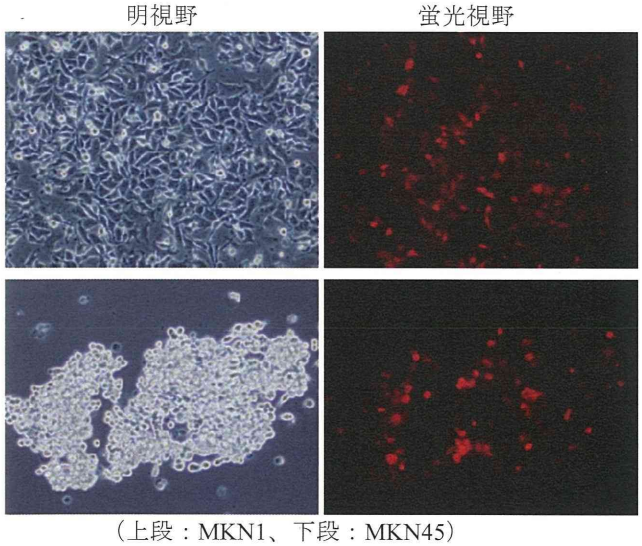


図8　GFP蛍光観察ビデオスコープによる蛍光検出

4）近赤外蛍光遺伝子発現ウイルス製剤の作成

GFPより深部の微小癌組織を高感度に検出するために近赤外蛍光遺伝子Katushkaを搭載する非増殖型アデノウイルス（Ad-Katushka）の遺伝子改変を行った。Ad-Katushkaを50 multiplicity of infection (MOI)でヒト胃癌細胞（MKN1、MKN45）、ヒト大腸癌細胞（SW620、SW480）に感染させたところ、48時間後に明瞭な近赤外蛍光発現が観察された。



（上段：MKN1、下段：MKN45）

図9　ヒト癌細胞における近赤外蛍光遺伝子発現

5）大動物における*Katushka*遺伝子発現ヒト癌細胞の可視化の試み

全身麻酔下のミニブタに*Katushka*遺伝子発現H1299ヒト肺癌細胞を濃縮懸濁液にして開腹にて胃壁漿膜下や近傍リンパ節に注入し、高感度近赤外蛍光検出ビデオスコープにて腹腔内を観察したところ、近赤外蛍光が明瞭に検出可能であった。

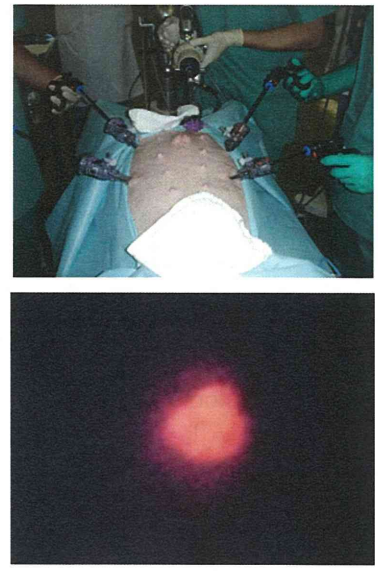


図10　近赤外観察ビデオスコープによる蛍光検出

また、陽性コントロールとして赤色ビーズを同様に胃漿膜下やリンパ節に投与して同様に観察可能であることを確認した。さらに、胃漿膜下にインジゴカルミン色素を注入し、リンパ流が近傍リンパ節に流入することを検証し、病変近辺に投与したウイルス製剤がリンパ節まで到達できることを確認した。

D．考察

蛍光タンパク質を用いた*in vivo*蛍光イメージングは、最先端の生命科学の研究に重要な技術であるが、医療の現場で実用化された事例はまだない。テロメラーゼ活性に反応して増殖する遺伝子改変アデノウイルスは80-100 nmの天然のバイオ・ナノマシーンであり、癌細胞で選択的に蛍光遺伝子を発現するために適したベクターと成り得る。投与されたウイルス標識薬剤はリンパ流や血流に乗って拡散し、微小転移病巣で増殖するとともに蛍光発現を生じる。高感度蛍光感知ビデオスコープを組み合わせることで鏡視下手術用外科ナビゲーション・システムの臨床応用が実現すれば、手術中にリアルタイムにリンパ節転移などの微小癌組織を同定することができ、必要最小限の領域を切除する超縮小手術の施行が可能となる。

たとえば早期消化器癌の場合、本技術による診査腹腔鏡や経管腔的内視鏡手術（NOTES、Natural Orifice Translumenal Endoscopic Surgery）で確実にリンパ節転移や播種がないことを確認できれば、原発巣は内視鏡的粘膜下切開術（ESD、Endoscopic Submucosal Dissection）のみで切除可能なケースも増えてくる。すなわち、本研究成果によって、外科切除範囲を最小限に留めることで大部分の臓器を温存したり、あるいはリンパ節郭清そのものを省略したりすることができれば、画期的な機能温

存が可能となり、治療後の患者の生活の質の著しい向上に貢献することができる。

初年度である平成21年度は、GFPを可視化することができる高感度蛍光感知ビデオスコープの第1号試作機、および小型・軽量化した第2号試作機を作成した。カメラヘッドのスイッチにて明視野モードと蛍光モードのフィルター切り替えがワンタッチでできる、その切り替えに連動して光源フィルターとモニター表示が切り替わり、明視野と蛍光視野を切り替えて表示するシステムとした。全身麻酔下のミニブタで、経口的に挿入した消化管内視鏡で胃粘膜下にTelomeScanと同一の蛍光特性を持つ蛍光ビーズを注入したところ、腹腔内から第2号試作機にて極めて良好に胃から所属リンパ節へのリンパ流をリアルタイムに確認することができた。

研究2年目には、高感度蛍光感知ビデオスコープ第2号試作機によるミニブタの組織における*GFP*遺伝子発現の検出を試みた。Ad-GFPの投与では蛍光発現はみられなかったが、細胞レベルでGFP発現していると高感度に検出可能であることが明らかとなった。このGFP蛍光は、平成19年度までに本研究課題にて試作した携帯型蛍光検出プローブにおいても同様に検出することができた。

また、近赤外蛍光*Katushka*遺伝子を挿入した非増殖型アデノウイルスベクター（Ad-Katushka）を作成し、各種ヒト癌細胞において近赤外蛍光の発現を確認した。当初、近赤外蛍光遺伝子を発現する癌特異的制限増殖型ウイルスの作成を試みる予定であったが、非担癌動物での発現は期待できないため、まず基盤研究としてAd-Katushkaを構築した。

最終年度には、近赤外蛍光感知ビデオスコープを試作し、Ad-Katushkaを*ex vivo*で感染させたヒト癌細胞を投与して空間的検出能を含めたビデオスコープの機能解析を行った。

今後は、診断用イメージング医薬品としてのウイルスの安全性と有効性、高感度蛍光感知ビデオスコープの医療機器としての薬事申請を計画し、外科ナビゲーション・システムとしての臨床試験の立案を行い、胃癌をはじめとする消化器癌治療現場への臨床展開を目指す。

E．結論

蛍光遺伝子発現で組織を特異的にラベリングすることができ、大動物において高感度蛍光検出ビデオスコープを用いてリンパ流や臓器近傍のリンパ節、組織を可視化することが可能であった。

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研究成果の刊行に関する一覧表（1）

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研究成果の刊行に関する一覧表（2）

雑誌

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Telomerase-Specific Virotheranostics for Human Head and Neck Cancer

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Abstract

Purpose: Long-term outcomes of patients with squamous cell carcinoma of the head and neck (SCCHN) remain unsatisfactory despite advances in combination of treatment modalities. SCCHN is characterized by locoregional spread and it is clinically accessible, making it an attractive target for intratumoral biological therapies.

Experimental Design: OBP-301 is a type 5 adenovirus that contains the replication cassette in which the human telomerase reverse transcriptase promoter drives expression of the *E1* genes. OBP-401 contained the replication cassette and the green fluorescent protein (*GFP*) gene. The antitumor effects of OBP-301 were evaluated *in vitro* by the sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate assay and *in vivo* in an orthotopic xenograft model. Virus spread into the lymphatics was also orthotopically assessed by using OBP-401.

Results: Intratumoral injection of OBP-301 resulted in the shrinkage of human SCCHN tumors orthotopically implanted into the tongues of BALB/c *nu/nu* mice and significantly recovered weight loss by enabling oral ingestion. The levels of GFP expression following *ex vivo* infection of OBP-401 may be of value as a positive predictive marker for the outcome of telomerase-specific virotherapy. Moreover, whole-body fluorescent imaging revealed that intratumorally injected OBP-401 could visualize the metastatic lymph nodes, indicating the ability of the virus to traffic to the regional lymphatic area and to selectively replicate in neoplastic lesions, resulting in GFP expression and cell death in metastatic lymph nodes.

Conclusions: These results illustrate the potential of telomerase-specific oncolytic viruses for a novel therapeutic and diagnostic approach, termed theranostics, for human SCCHN.

Cancer remains a leading cause of death worldwide despite improvements in diagnostic techniques and clinical management (1, 2). An estimated 500,000 patients worldwide are diagnosed with squamous cell carcinoma of the head and neck (SCCHN) annually. This aggressive epithelial malignancy is associated with a high mortality rate and severe morbidity among the long-term survivors (3). Current treatment strategies for advanced SCCHN include surgical resection, radiation, and cytotoxic chemotherapy. Although a combination of these modalities can improve survival, most patients eventually experience disease progression that leads to death; disease progression is often the result of intrinsic or acquired resistance to treatment (4, 5). A lack of specificity for tumor cells is the primary limitation of radiotherapy and chemotherapy. To improve the therapeutic index, there is a need for anticancer agents that selectively target only tumor cells and spare normal cells.

Replication-selective tumor-specific viruses present a novel approach for cancer treatment (6, 7). We reported previously that telomerase-specific replication-competent adenovirus (OBP-301, Telomelysin), in which the human telomerase reverse transcriptase (hTERT) promoter element drives the expression of *E1A* and *E1B* genes linked with an IRES, induced selective E1 expression, and efficiently killed human cancer cells but not normal cells (8–10). We also found that intratumoral injection of telomerase-specific replication-selective adenovirus expressing the green fluorescent protein (*GFP*) gene (OBP-401, TelomeScan) causes viral spread into the regional lymphatic area with subsequent selective replication in

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Translational Relevance

Despite new therapeutic modalities, long-term outcomes of patients with squamous cell carcinoma of the head and neck (SCCHN) remain unsatisfactory. Thus, the development of efficient treatment methods to enable the reduction of tumors in these patients is clearly imperative. Tumor-targeted oncolytic viruses have the potential to selectively infect target tumor cells, multiply, and cause cell death and release of viral particles, leading to the spread of viral-mediated antitumor effects. We developed a telomerase-specific oncolytic adenovirus OBP-301 (Telomelysin) as well as OBP-401 – expressing *GFP* gene (TelomeScan). Our data showed that telomerase-specific oncolytic viruses can be effective to kill human SCCHN cells *in vitro* and *in vivo* as well as to identify the patients who will likely benefit from virotherapy, suggesting that an oncolytic virus-based approach exhibited desirable features of a novel "virotheranostics," the combination of a diagnostic assay with a therapeutic entity for human SCCHN. This is a preclinical study for the future clinical trials.

metastatic lymph nodes in *nu/nu* mice (11). Although up to 25% of patients with SCCHN develop distant metastasis to the lung, liver, or bone, lymph node metastases are more common in SCCHN patients (12); therefore, locoregional disease control with telomerase-specific oncolytic viruses may be a novel therapeutic strategy that is clinically applicable for the treatment of human SCCHN.

In the present study, we explore the therapeutic as well as diagnostic ability of telomerase-specific oncolytic viruses *in vitro* and *in vivo*. To this end, we adopted an orthotopic head and neck cancer xenograft model by inoculating human SCCHN cells into the tongues of *nu/nu* mice; this model resembles human SCCHN in a number of biological properties (13).

Materials and Methods

Cell lines and cell culture. The human oral squamous carcinoma cell lines SAS-L, SCC-4, SCC-9, HSC-2, HSC-3, and HSC-4 were maintained *in vitro* as monolayers in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin (complete medium). The human non–small-cell lung cancer cell line H460 and the human esophageal cancer cell line TE8 were routinely propagated in monolayer culture in RPMI 1640 supplemented with 10% fetal bovine serum. The normal human lung diploid fibroblast cell line WI38 (JCRB0518) was obtained from the Health Science Research Resources Bank (Osaka, Japan) and grown in Eagle's MEM with 10% fetal bovine serum. The normal human lung fibroblast NHLF (TaKaRa Biomedicals) and the normal human embryonic lung fibroblast MRC-5 (RIKEN BioResource Center) were cultured according to the vendors' specifications.

Adenoviruses. The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 (Telomelysin), in which the hTERT promoter element drives the expression of *E1A* and *E1B* genes linked with an IRES, was previously constructed and characterized (8–10). OBP-401 is a telomerase-specific replication-competent adenovirus variant with the replication cassette, and *GFP* gene under the control of the cytomegalovirus promoter was inserted into the E3 region for monitoring viral replication (11, 14). The viruses were purified by ultracentrifugation in 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. An sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) assay was done to assess the viability of tumor cells. Human SCCHN cells (1,000 per well) were seeded onto 96-well plates 18 to 20 h before viral infection. Cells were then infected with OBP-301 at a multiplicity of infection (MOI) of 1, 10, 50, and 100 plaque-forming units (pfu) per cell. Cell viability was determined at the indicated time points by using a Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the protocol provided by the manufacturer.

Fluorescence microplate reader. Cells were infected with OBP-401 at the indicated MOI values in a 96-well black-bottomed culture plate and further incubated for the indicated time periods. GFP fluorescence was measured by using a fluorescence microplate reader (DS Pharma Biomedical) with excitation/emission at 485 nm/528 nm.

Animal experiments. SAS-L and HSC-3 human oral squamous cell carcinoma cells were harvested and suspended at a concentration of 5×10^6/mL in the medium. To generate an orthotopic head and neck cancer model, 6-wk-old female BALB/c *nu/nu* mice were anesthetized and injected directly with 20 μL of cell suspension at a density of 10^5 cells. The cells were injected into the right lateral border of the tongue with a 27-gauge needle. When the tumor grew to 2 to 3 mm in diameter ~5 to 7 days later, 20 μL of solution containing 1×10^8 pfu of OBP-301, OBP-401, or PBS were injected into the tumor. The perpendicular diameter of each tumor was measured every 3 d, and tumor volume was calculated by using the following formula: tumor volume (mm^3) = $a \times b^2 \times 0.5$, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. The body weights of mice were monitored and recorded. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University.

In vivo *fluorescence imaging.* *In vivo* GFP fluorescence imaging was acquired by illuminating the animal with a Xenon 150-W lamp. The reemitted fluorescence was collected through a long-pass filter on a Hamamatsu C5810 3-chip color charge-coupled device camera (Hamamatsu Photonics Systems). High-resolution image acquisition was accomplished by using an EPSON PC. Images were processed for contrast and brightness with the use of Adobe Photoshop 4.0.1J software (Adobe). A fluorescence stereomicroscope (SZX7; Olympus) was also used to visualize GFP-positive tissues.

Statistical analysis. The statistical significance of the differences in the *in vitro* and *in vivo* antitumor effects of viruses was determined by using the Student's t test (two-tailed). The antitumor effect viruses on orthotopically implanted tumors in nude mice were assessed by plotting survival curves according to the Kaplan-Meier method. P values <0.05 were considered statistically significant.

Results

In vitro *cytopathic efficacy of OBP-301 on human SCCHN cell lines.* We examined the cytopathic effect of OBP-301, which is an attenuated adenovirus in which the hTERT promoter element drives expression of *E1A* and *E1B* genes linked with an internal ribosome entry site (IRES; Fig. 1A), on various human SCCHN cell lines by the XTT cell viability assay. OBP-301 infection induced cell death in human SCCHN cells in a dose-dependent manner; the sensitivity, however, varied among different cell lines (Fig. 1B). The ID_{50} values calculated from the dose-response curves confirmed that SAS-L cells could be efficiently killed by OBP-301 at an multiplicity of infection (MOI) of <150 (ID_{50} = 148), whereas HSC-3 cells were less sensitive to OBP-301 (ID_{50} = 500; Fig. 1C).

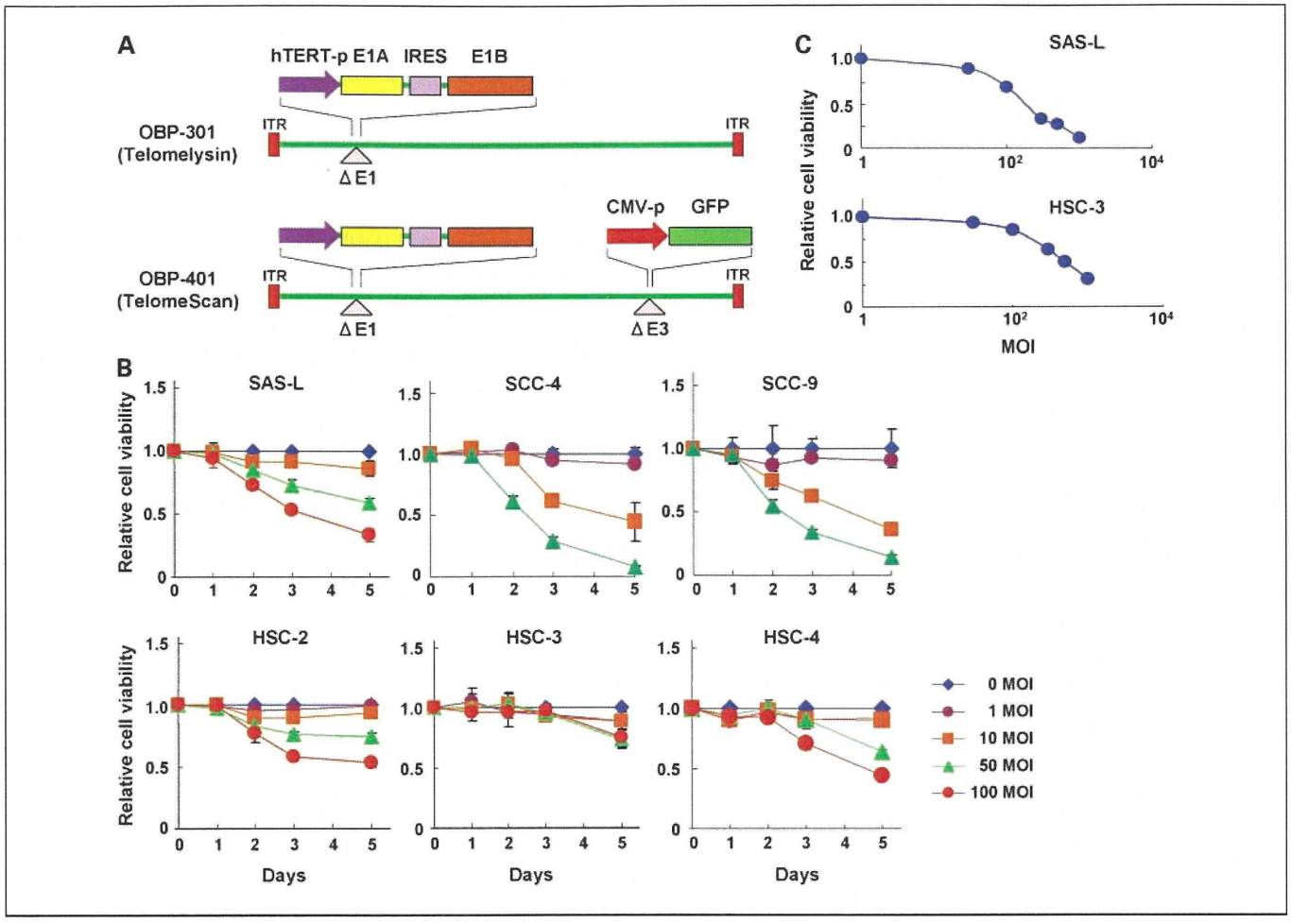

Fig. 1. Schematic DNA structures of telomerase-specific viruses and selective cytopathic effect in human SCCHN cell lines *in vitro*. *A,* OBP-301 is a telomerase-specific replication-competent adenovirus containing the hTERT promoter sequence inserted into the adenovirus genome to drive transcription of the E1A and E1B bicistronic cassette linked by the IRES. OBP-401 is a variant of OBP-301, in which the *GFP* gene is inserted under the cytomegalovirus (*CMV*) promoter into the E3 region for monitoring viral replication. *B,* human SCCHN cell lines were infected with OBP-301 at the indicated MOI values, and surviving cells were quantitated over 5 d by the XTT assay. The cell viability of mock-treated cells on day 0 was considered 1.0, and the relative cell viability was calculated. Points, mean of triplicate experiments; bars, SD. *C,* effects of various concentrations of OBP-301 on SAS-L and HSC-3 cells assessed 5 d after the XTT assay. Results are expressed as the relative cell viability of untreated control cells.

To confirm the specificity of telomerase activity in human SCCHN cells, we next measured the expression of *hTERT* mRNA in a panel of human SCCHN cell lines and normal cell lines by using a real-time reverse transcription-PCR method. Although the levels of expression varied widely, all SCCHN cell lines expressed detectable levels of *hTERT* mRNA, whereas human fibroblast cells such as NHLF and WI38 were negative for *hTERT* expression (Supplementary Fig. S1A). We also examined the expression levels of coxsackievirus and adenovirus receptor on the cell surface of each type of cell by flow cytometric analysis. Apparent amounts of coxsackievirus and adenovirus receptor expression were detected on SAS-L and HSC-3 human SCCHN cells (Supplementary Fig. S1B).

To assess whether viral replication was restricted to tumor cells, we next examined the replication ability of OBP-301 by measuring the relative amounts of E1A DNA. SAS-L human SCCHN cells and MRC-5 human fibroblasts were harvested at indicated time points over 72 h after infection with OBP-301 and subjected to quantitative real-time PCR analysis. The ratios were normalized by dividing the value of cells obtained 2 h after viral infection. OBP-301 replicated 3 to 4 logs within 48 h after infection; the viral replication, however, was attenuated up to 2 logs in normal MRC-5 cells (Supplementary Fig. S2).

The response of tumor cells to DNA-damaging stimuli such as chemotherapeutic drugs and ionizing radiation is predetermined by the functional status of their *p53* gene (15); however, the p53 status of human SCCHN cell lines (wild-type p53 [SAS-L], mutant p53 [SCC-4, HSC-2, HSC-3, HSC-4], and deleted p53 [SCC-9]) is not related to their sensitivity to OBP-301. Indeed, OBP-301 similarly killed parental SAS-L cells and cells stably transfected with the mutant *p53* gene (Supplementary Fig. S3), suggesting that OBP-301 induces cell death in a p53-independent manner.

***Selective replication of OBP-401 in human SCCHN cell lines* in vitro.** OBP-401 is a genetically engineered adenovirus that expresses GFP by inserting the *GFP* gene under the control of the cytomegalovirus promoter at the deleted E3 region of OBP-301 (Fig. 1A). To determine whether OBP-401 replication is associated with selective GFP expression in human SCCHN cells, cells were analyzed and recorded by using a time-lapse fluorescent microscope after OBP-401 infection. Representative images at the indicated time points are shown (Fig. 2A). SAS-L

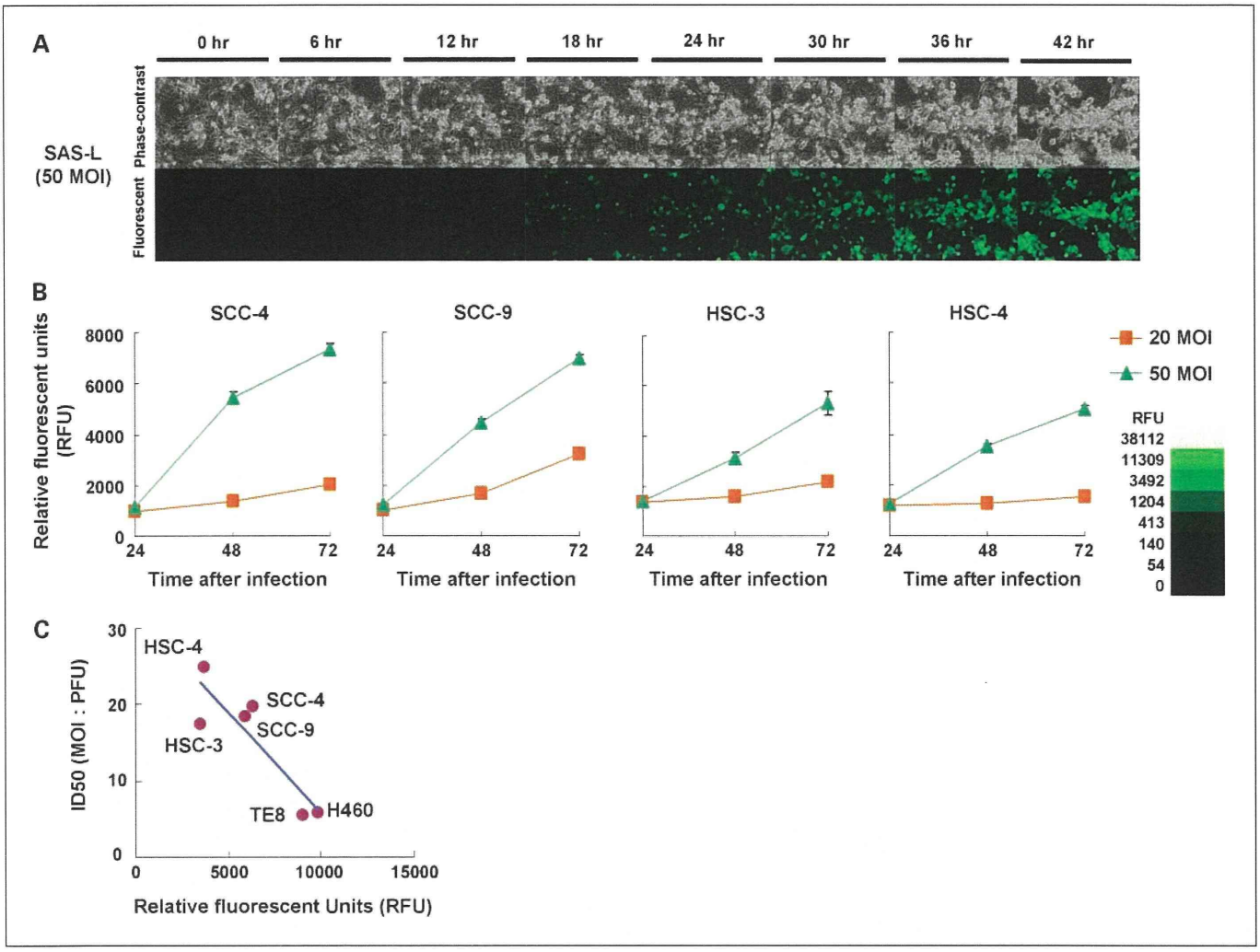

Fig. 2. Selective visualization of human SCCHN cells *in vitro* by OBP-401. *A,* time-lapse images of SAS-L cells were recorded for 42 h after OBP-401 infection at a MOI of 50. Representative images taken at the indicated time points show cell morphology by phase-contrast microscopy (*top*) and GFP expression under fluorescence microscopy (*bottom*). Magnification, ×200. *B,* quantitative assessment of GFP labeling by OBP-401 in human SCCHN cell lines. Cells were infected with OBP-401 at the indicated MOI values, and GFP fluorescence was measured over 72 h by the fluorescence microplate reader. The intensity of green fluorescence was evaluated based on the brightness determinations used as relative fluorescence units (*RFU*). The relative fluorescence unit and time after infection were plotted on the ordinate and abscissa, respectively. A green color calibration bar for the indicated relative fluorescence unit is shown on the right. *C,* relationship between GFP fluorescence after OBP-401 infection and ID_{50} values after OBP-301 infection in human cancer cell lines, including SCCHN cells. Relative GFP fluorescence was measured by the fluorescence microplate reader 72 h after OBP-401 infection at a MOI of 50. The ID_{50} values of OBP-301 on cell viability at 5 d after infection were calculated and expressed as ID_{50} values. The slope represents the inverse correlation between these two factors ($R^2 = 0.7839$).

human SCCHN cells expressed bright GFP fluorescence as early as 12 h after OBP-401 infection at a MOI of 50. The fluorescence intensity gradually increased in a dose-dependent manner, followed by rapid cell death due to the cytopathic effect of OBP-401, as evidenced by floating, highly light-refractile cells under phase-contrast photomicrographs.

We also quantified GFP expression in human SCCHN cells following OBP-401 infection by using a fluorescence plate reader. Relative expression levels of GFP gradually increased in a dose-dependent manner (Fig. 2B). Moreover, we found an apparent inverse correlation between relative GFP expression at 72 h after OBP-401 infection and the ID_{50} values of OBP-301 in various human cancer cell lines including SCCHN cell lines (Fig. 2C), indicating that the outcome of OBP-301 treatment could be predicted by measuring GFP expression following OBP-401 infection.

In vivo *antitumor effect of intratumoral injection of OBP-301 in an orthotopic nude mouse model of human SCCHN.* To assess the effect of OBP-301 on SCCHN *in vivo*, we used an orthotopic animal model for SCCHN in which SAS-L cells were implanted into the tongues of BALB/c *nu/nu* mice. Histopathologic examination of the excised primary tumors showed a tumor formation composed of implanted SAS-L cells with a solid architecture (Fig. 3A). Mice bearing palpable SAS-L tumors with a diameter of 3 to 5 mm received three courses of intratumoral injections of 10^8 pfu of OBP-301 or PBS (mock treatment) every 3 days beginning on the 7th day (regimen 1) or 10th day (regimen 2) after the initial tumor inoculation (Fig. 3B). Representative images from each group showed that tumors treated with OBP-301 starting on day 7 after tumor inoculation were consistently smaller than those of mock-treated mice 28 days after the first viral injection (Fig. 3C).