Research Article

p53-armed oncolytic adenovirus induces autophagy and apoptosis in KRAS and BRAF-mutant colorectal cancer cells

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Abstract

Colorectal cancer (CRC) cells harboring KRAS or BRAF mutations show a more-malignant phenotype than cells with wild-type KRAS and BRAF. KRAS/BRAF–wild-type CRCs are sensitive to epidermal growth factor receptor (EGFR)-targeting agents, whereas KRAS/BRAF–mutant CRCs are resistant due to constitutive activation of the EGFR-downstream KRAS/BRAF signaling pathway. Novel therapeutic strategies to treat KRAS/BRAF mutant CRC cells are thus needed. We recently demonstrated that the telomerase-specific replication-competent oncolytic adenoviruses OBP-301 and p53-armed OBP-702 exhibit therapeutic potential against KRAS-mutant human pancreatic cancer cells. In this study, we evaluated the therapeutic potential of OBP-301 and OBP-702 against human CRC cells with differing KRAS/BRAF status. Human CRC cells with wild-type KRAS/BRAF (SW48, Colo320DM, CACO-2), mutant KRAS (DLD-1, SW620, HCT116), and mutant BRAF (RKO, HT29, COLO205) were used in this study. The antitumor effect of OBP-301 and OBP-702 against CRC cells was analyzed using the XTT assay. Virus-mediated modulation of apoptosis, autophagy, and the EGFR-MEK-ERK and AKT-mTOR signaling pathways was analyzed by Western blotting. Wild-type and KRAS-mutant CRC cells were sensitive to OBP-301 and OBP-702, whereas BRAF-mutant CRC cells were sensitive to OBP-702 but resistant to OBP-301. Western blot analysis demonstrated that OBP-301 induced autophagy and that OBP-702 induced autophagy and apoptosis in human CRC cells. In BRAF-mutant CRC cells, OBP-301 and OBP-702 suppressed the expression of EGFR, MEK, ERK, and AKT proteins, whereas mTOR expression was suppressed only by OBP-702. Our results suggest that p53-armed oncolytic virotherapy is a viable therapeutic option for treating KRAS/BRAF-mutant CRC cells via induction of autophagy and apoptosis.

Introduction

Colorectal cancer (CRC) is the third leading cause of death worldwide [1]. CRCs harboring KRAS and BRAF mutations are often refractory to chemotherapy, resulting in a poorer prognosis than cases involving wild-type KRAS and BRAF due to tumor recurrence and metastasis [2]. KRAS/BRAF wild-type CRCs are sensitive to the epidermal growth factor receptor (EGFR)-targeting agents cetuximab and panitumumab [3]. However, KRAS/BRAF–mutant CRCs are highly resistant to EGFR-targeting therapy due to constitutive activation of the EGFR-downstream RAS-RAF-MEK-ERK signaling pathway [3]. Moreover, microsatellite stable (MSS) CRC reportedly exhibits greater resistance to immunotherapy compared with microsatellite instable (MSI) CRC [4]. Therefore, novel therapeutic strategies that will improve the clinical outcome in patients with KRAS/BRAF–mutant MSS CRC are needed.

KRAS–mutant CRCs harboring mutations at codon 12 or 13 are associated with worse prognosis [5]. BRAF–mutant CRCs frequently exhibit V600E mutation, leading to poor prognosis [6]. Constitutive activation of the RAS-RAF-MEK-ERK pathway plays a crucial role in the resistance to EGFR-targeting agents in KRAS/BRAF–mutant CRC cells [7]. Targeting the RAS-RAF-MEK-ERK pathway using BRAF, MEK, and ERK inhibitors improves the resistance to EGFR-targeting therapy in KRAS/BRAF-mutant CRC cells [8]. Dual inhibition of the RAS-RAF-MEK-ERK and EGFR-downstream PI3K-AKT-mTOR pathways using BRAF, MEK, ERK, and EGFR inhibitors has been suggested to be effective for treating KRAS/BRAF–mutant CRC cells [9]. Therefore, novel therapeutic strategies that suppress the RAS-RAF-MEK-ERK and EGFR-PI3K-AKT-mTOR pathways is needed for treating KRAS/BRAF–mutant CRCs.

Oncolytic virotherapy has recently emerged as a novel antitumor therapy against CRCs [10]. Oncolytic viruses induce tumor-specific death of malignant tumor cells via modulated viral replication [11]. Telomerase activity is higher in malignant tumor cells than normal cells [12]. To target malignant tumor cells with telomerase activity, we developed two types of telomerase-specific replication-competent oncolytic adenoviruses, OBP-301 [13] and OBP-702 armed with the wild-type *p53* tumor suppressor gene [14]. We previously demonstrated that OBP-301 induces autophagy-related death in human lung cancer cells by suppressing EGFR expression [15]. Moreover, we recently demonstrated that OBP-301 and p53-armed OBP-702 exhibit high antitumor efficacy against KRAS-mutant human pancreatic cancer cells via the KRAS-MEK-ERK signaling pathway [16]. Therefore, we hypothesized that OBP-301 and OBP-702 would be effective for use in eliminating KRAS/BRAF–mutant CRC cells.

In the present study, we investigated the therapeutic potential of the telomerase-specific replication-competent oncolytic adenoviruses OBP-301 and p53-armed OBP-702 for eliminating human CRC cells with differing KRAS/BRAF mutation status and microsatellite stability. The ability of OBP-301 and OBP-702 to induce apoptosis and autophagy and modulate the EGFR-MEK-ERK and AKT-mTOR signaling pathways was analyzed by Western blotting.

Materials and Methods

Cell lines

The human CRC cell line Colo320DM was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The human CRC cell lines SW48,

SW620, DLD-1, HCT116, HT29, and RKO were obtained from the American Type Culture Collection (Manassas, VA, USA). The human CRC cell lines CACO-2 and COLO205 were obtained from the Riken BioResource Research Center (Tsukuba, Ibaraki, Japan). Colo320DM cells were maintained in Dulbecco's modified Eagle's medium. SW48, SW620, DLD-1, and COLO205 cells were maintained in RPMI-1640 medium. CACO-2 and RKO cells were maintained in Eagle's Minimal Essential Medium. HCT116 and HT-29 cells were maintained in McCoy's 5A medium. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Culture medium for CACO-2 cells were supplemented with 20% FBS and 1% non-essential amino acids solution. The cells were routinely maintained at 37°C in a humidified atmosphere with 5% CO_2.

Recombinant adenoviruses

The telomerase-specific replication-competent adenovirus OBP-301 (suratadenoturev), in which the promoter element of the human telomerase reverse transcriptase gene drives expression of the *E1A* and *E1B* genes, was previously constructed and characterized [13, 17] (**Fig 1A**). OBP-702 was generated by modifying OBP-301 via insertion of a human wild-type *p53* gene expression cassette into the *E3* region of OBP-301 [14, 18] (**Fig 1B**).

Cell viability assay

Cells were seeded in 96-well plates at a density of 10^3 cells/well. After 24 h, cells were infected with OBP-301 or OBP-702 at a multiplicity of infection (MOI) of 0, 1, 5, 10, 50, or 100 plaque-forming units (PFU)/cell. Uninfected (mock-treated) cells were used

as virus-infected cells at an MOI of 0. Cell viability was determined on day 3 after virus infection using a Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's protocol.

Western blot analysis

Cells were seeded in a 100-mm dish at a density of 10^5 cells/dish 24 h before virus infection. The cells were then infected for 72 h with OBP-301 or OBP-702 at the indicated MOI. Uninfected (mock-treated) cells were used as virus-infected cells at an MOI of 0. Whole-cell lysates were prepared in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Complete Mini; Roche Applied Science, Mannheim, Germany). Proteins (20 μg per lane) were electrophoresed on 6-10% SDS polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (Hybond-P; GE Health Care, Buckinghamshire, UK). Blots were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) at room temperature for 30 min. The primary antibodies used were as follows: rabbit anti–poly (ADP-ribose) polymerase (PARP) polyclonal antibody (pAb) (1:1000, 9542; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-p62 pAb (1:1000, 5114; Cell Signaling Technology), mouse anti-E1A monoclonal antibody (mAb) (1:500, 554155; BD Bioscience, Franklin Lakes, NJ, USA), mouse anti-p53 mAb (1:1000, 18032; Cell Signaling Technology), rabbit anti-EGFR pAb (1:1000, 2232; Cell Signaling Technology), mouse anti-MEK1/2 mAb (1:1000, 4694; Cell Signaling Technology), rabbit anti-ERK1/2 mAb (1:1000, 4695; Cell Signaling Technology), rabbit anti-AKT mAb (1:1000, 4691; Cell Signaling Technology), rabbit anti-mTOR mAb (1:1000, 2983; Cell Signaling Technology), and mouse anti–β-Actin mAb (1:5000, A5441;

Sigma-Aldrich, St. Louis, MO, USA). The secondary antibodies used were horseradish peroxidase–conjugated antibodies against mouse IgG (1:2500, NA931; GE Healthcare) or rabbit IgG (1:5000, NA934; GE Healthcare). Immunoreactive bands on the blots were visualized using enhanced chemiluminescence substrate (ECL Prime; GE Healthcare).

Statistical analysis

Data are expressed as means ± SD. The significance of differences was assessed using the Student's *t*-test. Statistical significance was defined as $P<0.05$.

Results

In vitro cytopathic effect of OBP-301 against human CRC cells with different KRAS/BRAF mutation status

To investigate the therapeutic potential of oncolytic adenoviruses against human CRC cells, we used three KRAS/BRAS wild-type human CRC cell lines, Colo320DM (MSS type), SW48 (MSI type), and CACO-2 (MSS type); three KRAS-mutant human CRC cell lines, SW620 (MSS type), DLD-1 (MSI type), and HCT116 (MSI type); and three BRAF-mutant human CRC cell lines, HT29 (MSS type), RKO (MSI type), and COLO205 (MSS type). The viability of CRC cells after infection with OBP-301 for 72 h was assessed using an XTT assay. OBP-301 treatment significantly suppressed the viability of KRAS/BRAF wild-type and KRAS-mutant CRC cells, independent of microsatellite status (**Fig 2A and B**). BRAF-mutant CRC cells were relatively less sensitive to OBP-301 compared with KRAS/BRAF wild-type or KRAS-mutant CRC cells (**Fig 2C**). These results suggest that OBP-301 has therapeutic potential against

human CRC cells with wild-type KRAS/BRAF or mutant KRAS, but not mutant BRAF.

OBP-301 induces autophagy in human CRC cells with different KRAS/BRAF mutation status

Oncolytic adenoviruses have been shown to induce autophagy-related cell death in tumor cells [19-21]. We previously demonstrated that OBP-301 and OBP-702 exhibit cytopathic activity in association with autophagy and apoptosis in a variety of human cancer cells [15, 18]. To investigate whether OBP-301 induces autophagy and apoptosis in human CRC cells, human CRC cells with different KRAS/BRAF mutation status were infected with OBP-301 for 72 h, and cell lysates were then prepared and subjected to Western blotting. OBP-301 treatment induced apoptosis with upregulation of C-PARP (but not autophagy due to a lack of p62 expression) in KRAS/BRAF wild-type Colo320DM cells (**Fig 3A and Fig S1**). Following OBP-301 treatment, KRAS/BRAF wild-type SW48 cells exhibited autophagy with downregulation of p62 (**Fig 3A and Fig S1**). OBP-301 treatment further induced autophagy (but not apoptosis) in KRAS-mutant and BRAF-mutant CRC cells (**Fig 3B and C and Figs S2 and S3**). The expression of p53 protein was decreased by OBP-301 treatment in both p53-intact (SW48, RKO) and p53-mutant (Colo320DM, SW620, HT29, DLD-1) CRC cells (**Fig 3**). These results suggest that OBP-301 has therapeutic potential to induce autophagy in human CRC cells with wild-type KRAS/BRAF or mutant KRAS. However, OBP-301-induced autophagy was not effective to treat BRAF-mutant CRC cells.

***In vitro* cytopathic effect of OBP-702 against human CRC cells with different KRAS/BRAF mutation status**

To investigate the therapeutic potential of p53-armed OBP-702 against human CRC cells, human CRC cells with different KRAS/BRAF mutation status were treated with OBP-702 for 72 h, and the viability of the cells was then assessed by XTT assay. OBP-702 treatment significantly decreased the viability of KRAS/BRAF wild-type, KRAS-mutant, and BRAF-mutant CRC cells, independent of microsatellite status (**Fig 4**). These results suggest that OBP-702 has therapeutic potential against human CRC cells independent of KRAS/BRAF mutation and microsatellite status.

OBP-702 induces apoptosis and autophagy in human CRC cells with different KRAS/BRAF mutation status

To investigate whether OBP-702 induces autophagy and apoptosis in human CRC cells, human CRC cells with different KRAS/BRAF mutation status were infected with OBP-702 for 72 h, after which cell lysates were prepared and subjected to Western blotting. OBP-702 treatment induced apoptosis in all CRC cells with different KRAS/BRAF mutation status (**Fig 5 and Figs S4-6**). Moreover, OBP-702 treatment induced autophagy in all CRC cells except KRAS/BRAF wild-type Colo320DM cells, which lack p62 expression (**Fig 5 and Figs S4-6**). The expression of p53 protein was increased by OBP-702 treatment in both p53-intact (SW48, RKO) and p53-mutant (Colo320DM, SW620, HT29, DLD-1) CRC cells (**Fig 5 and Figs S4-6**). These results suggest that OBP-702 has therapeutic potential to induce apoptosis and autophagy in human CRC cells independent of KRAS/BRAF mutation status.

OBP-702 suppresses the EGFR-MEK-ERK and AKT-mTOR signaling pathways in BRAF-mutant human CRC cells more strongly than OBP-301

BRAF-mutant CRC cells have been shown to exhibit activation of the EGFR-MEK-ERK and AKT-mTOR signaling pathways, resulting in malignant progression [22]. To explore the underlying mechanism of differing sensitivity to OBP-301 and OBP-702 in BRAF-mutant CRC cells, we investigated whether OBP-301 and OBP-702 suppress the EGFR-MEK-ERK and AKT-mTOR signaling pathways in BRAF-mutant CRC cells. BRAF-mutant HT29 cells were treated with OBP-301 or OBP-702 for 72 h, after which cell lysates were prepared and subjected to Western blotting. OBP-301 and OBP-702 efficiently suppressed the expression of EGFR, MEK, ERK, and AKT proteins (**Fig 6A and Figs S7 and S8**). However, mTOR expression was suppressed by OBP-702 but not OBP-301 (**Fig 6A and Figs S7 and S8**). These results suggest that OBP-702 is superior to OBP-301 in suppressing the AKT-mTOR signaling pathway, although the EGFR-MEK-ERK signaling pathway was similarly suppressed by OBP-301 and OBP-702.

To evaluate whether oncolytic virus–mediated p53 activation suppresses the expression of mTOR in BRAF-mutant CRC cells, BRAF-mutant HT29 cells were treated with OBP-301 and p53-expressing Ad-p53 or non-expressing control DL312 for 72 h. OBP-301 monotherapy induced autophagy with downregulation of p62, but it did not induce apoptosis, whereas treatment with Ad-p53 or DL312 did not induce either apoptosis or autophagy (**Fig 6B and Fig S9**). Combination therapy with OBP-301 and Ad-p53 induced apoptosis with upregulation of C-PARP and p53, in addition to autophagy, more strongly than combination treatment with OBP-301 and DL312 (**Fig 6B and Fig S9**). The expression of mTOR protein was suppressed by combination treatment with OBP-301 and Ad-p53 but not combination treatment with OBP-301 and DL312, although the expression of EGFR, MEK, ERK, and AKT proteins was similarly suppressed by combination treatment with OBP-301 and Ad-p53 or DL312 (**Fig 6B and**

Fig S9). These results suggest that oncolytic virus–mediated p53 activation plays a crucial role in suppression of the AKT-mTOR signaling pathway and apoptosis induction in BRAF-mutant CRC cells.

Discussion

KRAS/BRAF-mutant CRC cells are thought to exhibit greater malignant potential in association with a greater likelihood of progression, metastasis, and resistance to EGFR-targeted therapies compared with KRAS/BRAF wild-type CRC cells [2, 3]. Therefore, novel therapeutic strategies to treat KRAS/BRAF-mutant CRC are needed. In this study, we investigated the therapeutic potential of OBP-301 and OBP-702 against human CRC cells with different KRAS/BRAF mutation and microsatellite status. KRAS wild-type and KRAS-mutant CRC cells were sensitive to both OBP-301 and OBP-702 independent of microsatellite status. BRAF-mutant CRC cells were less sensitive to OBP-301 compared with OBP-702. OBP-301 induced autophagy in association with downregulation of p62 and p53 protein expression in CRC cells, whereas OBP-702 induced apoptosis and autophagy in association with upregulation of C-PARP and p53 protein expression in CRC cells. OBP-301 and OBP-702 efficiently suppressed the expression of EGFR, MEK, ERK, and AKT proteins in BRAF-mutant CRC cells (**Fig 6C**). However, mTOR expression was suppressed by OBP-702 but not OBP-301 (**Fig 6C**). Thus, p53-armed oncolytic virotherapy appears to be a promising antitumor strategy that strongly induces apoptosis and autophagy in KRAS/BRAF-mutant CRC cells by suppressing the EGFR-MEK-ERK and AKT-mTOR signaling pathways.

Oncolytic virotherapy with various viruses has been shown to induce the antitumor effect against KRAS/BRAF-mutant cancers, including CRC [23-26]. Jiffry et al.

265 demonstrated that oncolytic reovirus-induced autophagy is effective to treat KRAS-
266 mutant CRC cells [24]. Oncolytic adenovirus-induced autophagy has also been
267 suggested to contribute to antitumor effect via mediating oncolysis and autophagy-
268 related cell death [27]. We previously reported that OBP-301 induces autophagy-related
269 cell death in human lung cancer cells by suppressing the EGFR expression [15]. OBP-
270 301 and OBP-702 efficiently induced autophagy in KRAS/BRAF wild-type and KRAS-
271 mutant CRC cells, although p53 expression was differentially modulated by OBP-301
272 and OBP-702. These findings suggest that autophagy induction may be involved in the
273 therapeutic effect of OBP-301 and OBP-702 independent of p53 modulation.
274 Autophagy plays both pro-survival and antitumoral roles in CRC cells [28]. The EGFR
275 signaling pathway modulates autophagy in association with cell survival and cell death
276 [29]. Li et al. demonstrated that the anti-EGFR antibody cetuximab induces autophagy
277 in association with cell death in human CRC cells by activating the Beclin 1/hVps34
278 complex [30]. Giannopoulou et al. showed that the anti-EGFR antibody pantitumumab
279 induces autophagy-related death in KRAS-mutant CRC cells by increasing the Beclin 1
280 protein level [31]. We also demonstrated that OBP-301 and OBP-702 induce autophagy-
281 related death in KRAS-mutant human pancreatic cancer cells by suppressing the KRAS
282 signaling pathway [16]. Thus, oncolytic virotherapy appears to be a promising
283 antitumor strategy for inducing autophagy-related death in KRAS/BRAF wild-type and
284 KRAS-mutant CRC cells by suppressing the EGFR-KRAS signaling pathway.
285 p53-armed OBP-702 induced apoptosis in BRAF-mutant CRC cells, whereas non-
286 armed OBP-301 or p53-expressing Ad-p53 did not induce apoptosis, suggesting that
287 p53-activating oncolytic virotherapy would be effective for treating BRAF-mutant CRC
288 via apoptosis induction. With regard to the molecular mechanism of the OBP-702–

289 mediated cytopathic effect against BRAF-mutant CRC cells, our data showed that OBP-
290 702 suppresses the expression of mTOR protein in BRAF-mutant CRC cells.
291 Accumulating evidence indicates that p53 activation inhibits the mTOR pathway at
292 transcriptional and non-transcriptional levels [32]. With regard to the role of mTOR
293 inhibition in BRAF-mutant CRC cells, Mao et al. demonstrated that suppression of the
294 AKT-mTOR signaling pathway enhances the sensitivity to BRAF inhibition in BRAF-
295 mutant CRC cells [33]. Garcia-Garcia et al. showed that dual blockade of the MEK-
296 ERK and AKT-mTOR signaling pathways results in the induction of apoptosis in
297 BRAF-mutant RKO cells (p53 wild-type) but not BRAF-mutant HT-29 cells (p53
298 mutant) [34], suggesting that p53 activation plays an important role in suppression of
299 the MEK-ERK and AKT-mTOR signaling pathways, followed by apoptosis induction.
300 Although whether suppression of mTOR is associated with OBP-702-induced apoptosis
301 remains unclear, He et al. demonstrated that mTOR inhibitors induce apoptosis in
302 KRAS/BRAF-mutant CRC cells via the extrinsic apoptotic pathway [35]. Thus, OBP-
303 702 treatment may contribute to the induction of apoptosis in BRAF-mutant CRC cells
304 by suppressing the AKT-mTOR and EGFR-MEK-ERK signaling pathways and
305 activating the p53 signaling pathway.
306 Combination of oncolytic virotherapy with small molecules targeting the MEK-
307 ERK pathway has also been shown to induce more profound antitumor efficacy than
308 monotherapy in *in vivo* tumor models with KRAS/BRAF mutations [36]. Lee et al.
309 showed that combination of MEK inhibitor promotes the antitumor efficacy of oncolytic
310 vaccinia virus against chemo-resistant human ovarian cancer cells via enhancement of
311 virus replication [37]. Bommareddy et al. demonstrated that combination of MEK
312 inhibitor enhances antitumor effect of oncolytic herpes simplex virus against KRAS-

313 mutant murine CRC tumors via enhancement of cytopathic activity and antitumor
314 immunity [38]. Thus, *in vivo* experiments using immune-deficient and immune-
315 competent mice are warranted to evaluate the therapeutic potential of OBP-301 and
316 OBP-702 against KRAS/BRAF-mutant CRC tumors in monotherapy and combination
317 therapy with small molecules targeting the MEK-ERK pathway.

318 Clinical application of OBP-301 and OBP-702 is expected as treatment modalities
319 for CRC. Administration route of OBP-301 and OBP-702 is limited to intratumoral
320 injection to avoid the antiviral immune response mediated by circulating neutralizing
321 antibody. Therefore, rectal cancer may be suitable in treating accessible CRC tumors.
322 We previously reported that intratumoral injection of OBP-301 suppresses lymph node
323 metastasis in an orthotopic rectal tumor model with KRAS-mutant HCT116 cells [39]. It
324 has also been shown that intratumoral injection of OBP-702 exhibits antitumor effect in
325 subcutaneous and orthotopic tumor models with human pancreatic cancer cells
326 harboring in-frame *BRAF* deletion [16]. More recently, we demonstrated that
327 endoscopic intratumoral injection of OBP-301 with radiotherapy was feasible and well
328 tolerated in esophageal cancer patients [40]. Although whether the feasibility and safety
329 of intratumoral injection of OBP-702 in CRC patients remains to be elucidated, rectal
330 cancers with KRAS/BRAF mutations may be potent candidate for treating with OBP-
331 301 and OBP-702.

332 In conclusion, we demonstrated that the telomerase-specific oncolytic adenoviruses
333 OBP-301 and OBP-702 have therapeutic potential for inducing autophagy-related death
334 in KRAS wild-type and KRAS-mutant CRC cells. Moreover, OBP-702–mediated p53
335 activation may provide a novel therapeutic option for inducing apoptosis in BRAF-
336 mutant CRC cells. *In vivo* experiments are needed to evaluate the therapeutic potential

of OBP-301 and OBP-702 against KRAS/BRAF-mutant CRC tumors.

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Authors' contributions

Conception and design: H.T., S.Ka., T.F.

Development of methodology: S.T., N.H., Y.L., M.Y.

Acquisition of data: S.T., N.H., Y.L., M.Y.

Analysis and interpretation of data: S.T., N.H., Y.L., M.Y.

Writing, review, and/or revision of the manuscript: S.T., H.T., T.F.

Administrative, technical, or material support: Y.U.

Study supervision: H.T., S.Ki., S.Ku, S.Ka., T.F.

Conflict of interest

Y.U. is the President & CEO of Oncolys BioPharma, Inc. H.T. and T.F. are consultants for Oncolys BioPharma, Inc. The other authors have no potential conflicts of interest to disclose. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Abbreviations

CRC, colorectal cancer; EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; MOI, multiplicity of infection; MSI, microsatellite instable; MSS, microsatellite stable; pAb, polyclonal antibody; PARP, poly (ADP-ribose) polymerase;

361 PFU, plaque-forming units.

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546 **Figure legends**

547 **Fig 1. Structures of telomerase-specific replication-competent oncolytic**
548 **adenoviruses. A** OBP-301 is a telomerase-specific replication-competent oncolytic
549 adenovirus in which the *hTERT* promoter drives expression of the *E1A* and *E1B* genes.
550 **B** OBP-702 is a p53-armed OBP-301 variant in which the *Egr1* promoter drives
551 expression of the human wild-type *p53* gene. Ad5, adenovirus serotype 5; hTERT,
552 human telomerase reverse transcriptase; IRES, internal ribosome entry site; ITR,
553 inverted terminal repeat.

554

555 **Fig 2. OBP-301 reduces the viability of human CRC cells with wild-type and**
556 **mutant KRAS. A, B, C** Human CRC cells with different KRAS/BRAF mutation status,
557 KRAS/BRAF wild type (**A**), mutant KRAS (**B**), and mutant BRAF (**C**), were treated
558 with OBP-301 at the indicated multiplicity of infection (MOI) for 72 h. Cell viability
559 was quantified using the XTT assay. Uninfected (mock-treated) cells were shown as
560 virus-infected cells at an MOI of 0. Cell viability was calculated relative to that of the
561 mock-treated group, which was set at 100%. Cell viability data are expressed as mean ±
562 SD (n = 5). The Student's *t*-test was used to evaluate the significance of differences.
563 **P*<0.05; ***P*<0.01 (versus an MOI of 0).

564

565 **Fig 3. OBP-301 induces autophagy in human CRC cells. A, B, C** Human CRC cells
566 with different KRAS/BRAF mutation status, KRAS/BRAF wild type (**A**), mutant
567 KRAS (**B**), and mutant BRAF (**C**), were treated with OBP-301 at the indicated MOI for
568 72 h. Cell lysates were prepared and subjected to Western blot analysis of PARP,
569 cleaved PARP (C-PARP), p62, E1A, and p53 expression. β-Actin was assayed as a

loading control. Uninfected (mock-infected) cells were shown as virus-infected cells at an MOI of 0.

Fig 4. OBP-702 reduces the viability of human CRC cells independent of KRAS/BRAF mutation status. A, B, C Human CRC cells with different KRAS/BRAF mutation status, KRAS/BRAF wild type (**A**), mutant KRAS (**B**), and mutant BRAF (**C**), were treated with OBP-702 at the indicated MOI for 72 h. Cell viability was quantified using the XTT assay. Uninfected (mock-treated) cells were shown as virus-infected cells at an MOI of 0. Cell viability was calculated relative to that of the mock-treated group, which was set at 100%. Cell viability data are expressed as mean ± SD (n = 5). The Student's *t*-test was used to evaluate the significance of differences. **P*<0.05; ***P*<0.01 (versus an MOI of 0).

Fig 5. OBP-702 induces apoptosis and autophagy in human CRC cells. A, B, C Human CRC cells with different KRAS/BRAF mutation status, KRAS/BRAF wild type (**A**), mutant KRAS (**B**), and mutant BRAF (**C**), were treated with OBP-702 at the indicated MOI for 72 h. Cell lysates were prepared and subjected to Western blot analysis of PARP, cleaved PARP (C-PARP), p62, E1A, and p53 expression. β-Actin was assayed as a loading control. Uninfected (mock-infected) cells were shown as virus-infected cells at an MOI of 0.

Fig 6. OBP-702 suppresses the EGFR-MEK-ERK and AKT-mTOR signaling pathways in BRAF-mutant CRC cells. A BRAF-mutant HT29 cells were treated with OBP-301 or OBP-702 at the indicated MOI for 72 h. Cell lysates were prepared and

subjected to Western blot analysis of EGFR, MEK, ERK, AKT, and mTOR expression. **B** BRAF-mutant HT29 cells were treated with OBP-301, DL312, or Ad-p53 at the indicated MOI for 72 h. Cell lysates were prepared and subjected to Western blot analysis of PARP, C-PARP, p62, E1A, p53, EGFR, MEK, ERK, AKT, and mTOR expression. β-Actin was assayed as a loading control. Uninfected (mock-infected) cells were shown as virus-infected cells at an MOI of 0. **C** Outline of the EGFR-MEK-ERK and AKT-mTOR signaling pathways in BRAF-mutant CRC cells infected with OBP-301 or OBP-702.

Supporting information

S1 Fig. Full image of Figure 3A

S2 Fig. Full image of Figure 3B

S3 Fig. Full image of Figure 3C

S4 Fig. Full image of Figure 5A

S5 Fig. Full image of Figure 5B

S6 Fig. Full image of Figure 5C

S7 Fig. Full image of Figure 6A (OBP-301)

S8 Fig. Full image of Figure 6A (OBP-702)

S9 Fig. Full image of Figure 6B