INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy in the world and the third leading cause of cancer death in both men and women in 2020. Although the mortality rate of CRC is decreasing due to improved treatment strategies, the prognosis for patients with distant metastases in advanced stages remains poor, with a constant low 5-year survival rate of 12.5%.

The main cause of death in patients with CRC is distant metastasis, of which the liver is the most common distant metastatic organ. Currently, the only treatment for unresectable liver metastasis of CRC is systemic chemotherapy. Hence, there is an urgent need to develop alternative treatment options.

Dual-specificity tyrosine-regulated kinase 2 (DYRK2) is a protein kinase of the DYRK family that localizes primarily in the cytoplasm and shows intracellular serine/threonine kinase activity. It functions...
as a pro-apoptotic kinase that induces apoptosis in response to DNA damage through phosphorylation of p53 at Ser46. In addition, DYRK2 has been reported to regulate cell cycle progression by degrading c-Myc, c-Jun, and telomeric reverse transcriptase. Previous studies have reported that DYRK2 expression is downregulated in breast cancer, lung cancer, hepatocellular carcinoma, CRC, and liver metastases of CRC.

To focus on the tumor-suppressive function of DYRK2 in this study, we attempted to develop a novel gene therapy for CRC by overexpressing DYRK2 using Adv. Adenovirus vectors are widely used for gene therapy in humans because they can express a target gene in infected cells with high efficiency. Gene transfer using Adv is safe because a foreign gene is not integrated into the genome of the host cell. In fact, we have previously reported that overexpression of DYRK2 by local injection of Adv into subcutaneously implanted tumors in mice with hepatocellular carcinoma exerted an antitumor effect. In this study, we aimed to examine whether DYRK2 overexpression using Adv could be a novel therapeutic option for unresectable liver metastases of CRC.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human CRC cell lines HCT116, RKO, LS174T, LoVo, DLD-1, SW480, and SW620 were obtained from the JCRB Cell Bank or the ATCC. Lenti-X 293T cells were obtained from Takara for lentivirus production. Cells were cultured in DMEM with 10% FBS, 1% penicillin-streptomycin (Nacalai Tesque), and 1% l-glutamine (Gibco). All cell lines were maintained at 37°C in a 5% CO₂ incubator.

2.2 | Immunoblotting

Cells were harvested, washed with PBS, and resuspended in lysis buffer (50 mmol/L Tris-HCl [pH 7.6], 150 mmol/L NaCl, 1 mmol/L PMSF, 1 mmol/L DTT, 10 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 1% NP-40) with phosphatase inhibitor (10 mmol/L NaF and 1 mmol/L Na₃VO₄) for 20 minutes on ice. After centrifugation, the supernatants were isolated and used as cell lysates. The cell lysates were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with blocking buffer (0.1% casein, 0.1% gelatin, and 0.1% Tween-20 in TBS) and incubated with the following primary Abs: rabbit anti-DYRK2, mouse anti-phospho-p53, c-Myc, Cyclin D1 and GAPDH and rabbit anti-Cyclin D2 (Santa Cruz Biotechnology), mouse anti-phospho-p53-Ser46 (Bio Academia), mouse anti-cleaved PARP, and rabbit anti-cleaved caspase 3 (Cell Signaling Technology). Membranes were then washed three times in TBS with 0.05% Tween-20, and incubated with peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) or peroxidase-conjugated anti-mouse IgG κ-BP (Santa Cruz Biotechnology). Signals were detected using a chemiluminescent regent, ImmunoStar LD (Wako). Signals were observed and band intensity was measured using a Fusion-Solo system (M and S Instruments).

2.3 | Cell growth assay

Cells were seeded into 96-well plates (2 × 10³ cells/well) and MTS assay was carried out in triplicate using a CellTiter 96 AQ Solution Cell Proliferation Assay Kit (Promega) according to the manufacturer’s instructions. The absorbance was measured at 490 nm with a multiple counter (Infinite 200PRO; TECAN). A colony formation assay was carried out according to a previous report. Briefly, cells were plated into 6-well culture plates (2 × 10⁵ cells/well) and cultured for 8 days to allow colony formation.

2.4 | Apoptosis assay

Cells were resuspended in 100 μL binding buffer and incubated with annexin V-FITC and PI (Nacalai Tesque) for 15 minutes at 4°C in the dark, according to the manufacturer’s instructions. The cells were analyzed using a MACS Quant flow cytometer (Miltenyi Biotec).

2.5 | Adenovirus vector and cell infection

Adenovirus construction and infections were undertaken according to a previous report. Briefly, this adenovirus is a nonproliferative vector that lacks the E1 and E3 regions required for proliferation, and hence gene expression is transient. As it was impossible to produce adenovirus directly expressing DYRK2 from the EF1α promoter for uncertain reasons, Adv expressing Flag-DYRK2 were designed to depend upon Cre expression. Flag-DYRK2 and Flag-DYRK2-KR, as described previously, were inserted into the SwiI site of pAxE-FLNLwi2, which was the cosmid cassette for pAXEFCRE-dependent expression Adv construction. pAxEFLNLDYRK2it2 and pAXE-FLNLfDYRK2KRit2 had neomycin-resistant genes flanked by two loxPs in front of these expression units as stuffers. Ad-DYRK2-WT and Ad-DYRK2-KR were generated as described previously. Ax1wi1, which bears no insert, was used as a control (hereafter “Adv. empty”). The Ad-Cre-carrying Cre expression unit under control of the EF1α promoter was previously described. Adenovirus vectors were titrated with the previously reported method. The MOIs of 20 and 100 for HCT116 and RKO, respectively, were determined using a GFP-expressing Adv.

2.6 | Plasmid transfection and virus transduction

The iRFP720 sequence was amplified from pIRFP720-N1 (Addgene) using the primers 5′-CACCGATCCGGCGGCATGGCGGAGGA TC-3′ and 5′-CACACCGTTTACTTCCATCACGC-3′. The
amplified product was inserted into a pLenti6 lentiviral vector and the modified plasmid was transfected into Lenti-X 293T cells using poly-ethyleneimine (PEI)-MAX (Polysciences). HCT116 cells were infected with the virus-containing supernatants with 10 μg/mL polybrene (Sigma-Aldrich). Stable HCT116 cells expressing iRFP720 were established by sorting positive cells with a MoFlo XDP cell sorter (Beckman Coulter) and named HCT116-iRFP720. To generate a cell line stably expressing Cre, the NLS+Cre (NCre) sequence from pxsAvNCre-dp was amplified using the primers 5′-GGGAGA CCCAAGCTTATGAGCGGCCCTCCAAAAAGAAGA-3′ and 5′-A TATCTGCAAGATTCATGCCATCTTCCAGCGGCCG-3′ and inserted into pcDNA3 (Addgene) to construct a plasmid for NCre expression. A stable cell line was established by transfection of this plasmid to HCT116-iRFP720 using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer’s protocol, and named HCT116-RFP-NCre.

2.7 | Mouse xenografts and in vivo treatment with adenovirus

Our animal experiment protocol was approved by the Institutional Animal Care and Use Committee of Jikei University (No. 2019-053), and the studies were carried out in accordance with the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan. For subcutaneous space injections, 7-week-old male nude mice (BALB/cAJcl-nu/nu; CLEA) were injected with HCT116-iRFP720 cells (1 × 10⁶) suspended in Matrigel Basement Membrane Matrix (Corning). Tumors were monitored using the IVIS Lumina in vivo imaging system (Perkin Elmer). Tumor volumes were calculated according to the following formula: V (mm³) = 0.5 × (larger diameter × smaller diameter³). Mice were subcutaneously injected on one side with HCT116-iRFP720 cells (1 × 10⁶ cells per mouse). Two weeks after injection, the mice were randomly divided into three equal groups: Ad-DYRK2-WT, Ad-DYRK2-KR, and Adv.empty as control. In each group, the mice received multisite intratumoral injections of the corresponding recombinant virus at 1 × 10⁹ pfu/mouse through single injections. Tumor growth was evaluated based on the tumor size. Before the maximum diameter of the tumor exceeded 200 mm, mice were killed and tumors were harvested. Tumor growth was assessed based on the size and weight of the removed tumor.

2.8 | Immunohistochemistry

For immunostaining, resected xenografts were fixed, followed by immersion in 30% trehalose in 20 mmol/L HEPES to cryoprotect the tissue. Depending on the Ab, the cryosections (7 μm thickness) were antigen retrieved by an ImmunoSaver (Nisshin EM) for 60 minutes at 80°C. The sections were incubated with 0.75% BSA and 0.4% Triton X-100 in HEPES buffer (blocking buffer). After washing, the sections were stained using primary Abs: rabbit anti-DYRK2 (Sigma-Aldrich), mouse anti-Ki-67 (BD Biosciences), rabbit anti-cleaved caspase 3 (Cell Signaling Technology), and mouse anti-DYKDDDDK (Transgenic) in blocking buffer at 4°C overnight. After the immunoreaction, the sections were incubated with secondary Abs using Cy3- or Cy5-conjugated AffiniPure donkey anti-rabbit and mouse IgG (Jackson ImmunoResearch). The sections were washed and incubated in VECTASHIELD Mounting Medium (Vector Laboratories) containing DAPI. The sections were observed under a BZ-X800 fluorescence microscope (Keyence). The number and proportion of immunopositive cells were measured by counting each 10 field of view (0.0156 mm² per view).

2.9 | Mouse liver metastasis model and treatment with adenovirus

The liver metastasis model of CRC in mice was generated by transferring HCT116-RFP-NCre cells into the spleen at 3 × 10⁶ cells/mouse (7-week-old nude mice) as previously reported. Two weeks after the cells were transferred, the mice were observed by IVIS imaging, and mice were divided equally into two groups: Ad-DYRK2 and Adv.empty as control. Each group was given a single dose of Adv at 1 × 10⁷ pfu/mouse through the tail vein of the mice. Ten days after injection of the adenovirus, the regions of interest of the liver metastatic tumors were set uniformly in each individual mouse, and the fluorescence intensity was measured using IVIS. At the appropriate time, the mice were killed, and the liver was removed to evaluate the number of metastatic tumors and tumor weight.

2.10 | Statistical analyses

Data are presented as mean ± SEM. Statistical significance of differences was assessed by two-tailed Student’s t test or two-way ANOVA; P < .05 difference was considered significant. All statistical analyses were undertaken using GraphPad Prism (version 8).

3 | RESULTS

3.1 | Forced expression of DYRK2 by adenovirus in CRC cells inhibits growth and induces apoptosis in vitro

It has been reported that the expression of DYRK2 is decreased in CRC tissues. In addition, low expression levels of DYRK2 are associated with poor prognosis. Based on these findings, we hypothesized that overexpression of DYRK2 by adenovirus (Ad-DYRK2) could be applied as a novel therapeutic strategy for CRC. First, we compared the protein levels of DYRK2 in human colon cancer cell lines (Figure 1A). As the expression level of DYRK2 was lower in HCT116 and RKO cell lines than in others, we decided to
FIGURE 1 Legend on next page
mainly investigate the effect of DYRK2 overexpression in HCT116 and RKO cells.

To evaluate the effect of DYRK2 on cell proliferation and apoptosis of CRC, we transiently expressed DYRK2 (Ad-DYRK2-WT), kinase-dead (K251R) DYRK2 mutant (Ad-DYRK2-KR), or Adv.empty as control (Figure 1B). Overexpression of DYRK2 suppressed the expression levels of c-Myc, Cyclin D1, and Cyclin D2, which are cell cycle progression markers (Figures 1B and S1). The results of the cell proliferation assay (MTS assay) indicated that tumor growth was significantly attenuated by overexpressing Ad-DYRK2-WT (Figure 1C). Similar effects were obtained in the colony formation assay (Figure 1D). Importantly, the effects of Ad-DYRK2-WT were partly cancelled by the overexpression of the kinase-dead construct (Ad-DYRK2-KR) (Figure 1B-D). In RKO cells, the difference between Ad-DYRK2-WT and Ad-DYRK2-KR was observed to be less significant in the MTS assay; however, in the colony formation assay, the difference was statistically significant in both HCT116 and RKO cells (Figure 1D).

We have previously shown that DYRK2 induces p53AIP1 expression and apoptosis in a p53-Ser46 phosphorylation-dependent manner. Overexpression of DYRK2 in CRC cell lines using Ad-DYRK2-WT induced phosphorylation of p53-Ser46 (Figures 2A and S2). Accordingly, cleaved PARP and cleaved caspase 3 were detected in Ad-DYRK2-WT (Figures 2A and S2). To confirm whether

![Diagram](image_url)
overexpression of DYRK2 induces apoptosis, we assessed the rate of apoptosis using flow cytometry analysis. The number of both annexin V+/PI− (early apoptosis) and annexin V+/PI+ (late apoptosis) HCT116 and RKO cells was significantly higher in Ad-DYRK2-WT cells compared to that in Adv.empty or Ad-DYRK2-KR cells (Figure 2B,C). Although apoptosis was also induced in Ad-DYRK2-KR cells, the amount of change was markedly less compared to Ad-DYRK2-WT cells (Figure 2B,C). These data suggest that DYRK2 overexpression induces apoptosis in a kinase-dependent manner.

3.2 | Antitumor effects of Ad-DYRK2 in colorectal xenograft models

To extend our in vitro findings, we examined the antitumor effect by infection of Ad-DYRK2 on HCT116 cells using xenograft tumors. The design of the experiment is shown in the schema in Figure 3A. Two weeks after injection, tumors treated with the Ad-DYRK2-WT were significantly smaller than those treated with Adv. empty or Ad-DYRK2-KR (Figure 3B,C). Similarly, tumor weight in the Ad-DYRK2-WT group was significantly less than in other groups (Figure 3D). These results indicate that the infection of the Ad-DYRK2-WT vector induces an antitumor effect in vivo.

3.3 | Immunohistochemical staining of subcutaneous tumors in a xenograft model

No study has examined in vivo the effects of DYRK2 overexpression at a single cell level. To clarify whether the kinase activity of overexpressed DYRK2 suppresses cell proliferation and induces apoptosis in subcutaneous tumors, similar to the in vitro results in the liver, we analyzed DYRK2-overexpressed xenograft models by immunohistochemistry. HCT116-iRFP720 cells were transplanted subcutaneously into nude mice, and the Adv.s were injected into each tumor. Overexpressed DYRK2 could be detected in the tumors at day 4 after injection of adenovirus, but not at day 14 (Figure S3). Therefore, we analyzed the xenografts at day 4 after injection. In this analysis, Ki-67 and caspase 3 were used as markers for cell proliferation and apoptosis induction, respectively. First, the immunohistochemistry showed that the proportion of Ki-67-positive cells (Ki-67-positive cells in DAPI) in the Ad-DYRK2-WT group was significantly reduced compared to that in the Ad-DYRK2-KR group or in the control group (Figure 4A,C). Notably, in DYRK2-WT-overexpressed cells (Ad-DYRK2-WT), the proportion of Ki-67-positive cells was significantly reduced compared to the Ad-DYRK2-KR group (Figure 4A,D). Conversely, the proportion of cleaved caspase 3-positive cells in DYRK2-WT-overexpressed cells (Ad-DYRK2-WT) was significantly increased compared to the Ad-DYRK2-KR group (Figure 4B,E). These

FIGURE 3 Adv.s expressing dual-specificity tyrosine-regulated kinase 2 (Ad-DYRK2) inhibits growth in HCT116 xenograft tumors. A, Timeline of the procedure for animal studies. Cells were injected into the subcutaneous space of male nude mice. After 2 wk, mice received multisite intratumor injections with Adv. empty, Ad-DYRK2-WT, or Ad-DYRK2-KR. Tumors were imaged by near-infrared fluorescent protein (iRFP)-positive cells were examined by an in vivo imaging system. B, Images of tumors in nude mice at necropsy are shown (left) and tumor volumes were measured at indicated time points (right). n = 7 per group. Data are presented as mean ± SEM (n = 7/group). C, iRFP-positive cells on days 0 and 14 after injection of adenovirus were imaged with an in vivo imaging system. D, Weight of tumors at 14 days is shown. Data are presented as mean ± SEM (n = 7 per group) ***P < .001, ****P < .0001. ns, not significant.
FIGURE 4  Cell proliferation is inhibited and apoptosis is induced in dual-specificity tyrosine-regulated kinase 2 (DYRK2)-WT overexpressing cells by immunohistochemical analysis. A, B, Tumors infected with Adv.empty, Ad-DYRK2-WT, or Ad-DYRK2-KR were stained by immunocytochemistry for DYRK2 (green) and Ki-67 (red) (A), and caspase 3 (green) and FLAG-DYRK2 (red) (B). Nuclei were stained with DAPI (blue). Boxed areas are enlarged in lower panels. Longer and shorter scale bars are 100 and 10 μm, respectively. White arrowheads indicate double-positive cells for DYRK2 and Ki-67 (A) or caspase 3 and FLAG-DYRK2 (B). C-E, Proportion of Ki-67-positive cells in DAPI (C), Ki-67-positive cells in DYRK2-positive cells (D), and caspase 3-positive cells in FLAG-DYRK2-positive cells (E). Data are presented as mean ± SEM (n = 3 biological replicates in each condition, more than 500 cells were scored in each experiment). **P < .01, ***P < .001. ns, not significant.
FIGURE 5  Legend on next page
results collectively indicated that DYRK2 overexpression inhibits cell proliferation and induces apoptosis in a kinase-dependent manner in colorectal xenograft tumors.

3.4 | Antitumor effects of DYRK2 expression using Advs in a mouse model of CRC liver metastasis

To examine the effects of DYRK2 expression using Advs in metastatic CRC, we constructed a therapeutic model in which Advs were administered intravenously to a mouse liver metastasis model. The design of the experiment is shown in the schema shown in Figure 5A. The Adv that we used in this study requires dual infection with a Cre expression vector in order to express DYRK2 in infected cells. Therefore, in order to create a system that expresses DYRK2 with a single vector, we generated a cell line that stably expresses Cre. We constructed HCT116-RFP-NCre, which stably expresses iRFP720 and Cre, using the aforementioned HCT116-iRFP720 as a parental line (Figure S4A). HCT116-RFP-NCre showed similar responses to the parental strain in terms of growth inhibition and apoptosis induction upon DYRK2 overexpression by the Adv (Figure S4B-D).

The liver metastasis mouse model of CRC was generated by the method previously published by our group. After 10 days of vector administration, the total radiant efficiency was measured by IVIS, and the mice were killed to observe the liver and to measure the number of metastatic nodules and tumor weight. The results with IVIS indicated that the total radiant efficiency (photon/s) was significantly lower in the Ad-DYRK2 group than that in the Adv.empty group (Figure 5B). Similarly, the number of metastatic nodules and the tumor weight in the Ad-DYRK2 group were significantly lower than that in the Adv.empty group (Figure 5C,D). In addition, the Adv.empty group showed a decrease in the body weight of mice as the metastatic tumor increased, while the Ad-DYRK2 group significantly suppressed the weight loss (Figure 5E). These results show that intravenous injection of Ad-DYRK2 induces antitumor effects for liver metastasis of colon cancer in a mouse model.

We have previously reported that silencing DYRK2 suppresses cell proliferation in human breast, ovarian, and colon cancer cells by promoting the degradation of c-Myc and c-Jun, and that DYRK2 induces the expression of p53AIP1 and induces apoptosis in a Ser46 phosphorylation-dependent manner.7,9 We also reported that the expression of DYRK2 is decreased in CRC tissues compared to that in normal mucosal tissues, and the expression levels might correlate with prognosis.16,17 These findings led us to consider the possibility that overexpression of DYRK2 in CRC could become a novel gene therapy. In this regard, we showed that overexpression of DYRK2, despite its transient expression, produces antitumor effects both in vitro and in vivo.

Adenovirus vectors have been widely used for human gene therapy because of their ability to efficiently transfer genes into infected cells.18-20 Gene transfer by Advs is considered to be suitable for gene therapy as the genes are rarely incorporated into the genome of the host cells.20 We have constructed Advs using the EF1α promoter, which has been shown to be less inflammatory and toxic in the liver.27 Indeed, in a mouse model, there were no systemic effects on mice associated with adenovirus injection or death after administration (data not shown), and no obvious effect on liver parenchyma cells was observed in mouse liver tissue early after intravenous administration of adenovirus (Figure S5).

Currently, systemic chemotherapy is the only treatment option for unresectable CRC liver metastasis.3,5 As shown in the results of this study, DYRK2 gene transfer by intravenous administration of Advs could be a novel treatment option for liver metastasis of CRC.

One of the problems in applying the adenovirus used in this study to cancer treatment is that it is a nonproliferative vector. As it does not have the E1A genes necessary for viral replication,24 the virus does not proliferate in the infected cells. Therefore, the overexpression of DYRK2 in adenovirus-infected tumors is transient, and the effect of overexpression is expected to fade as the tumor grows. In fact, DYRK2 expression in tumors can be observed at 4 days postinfection, but was undetectable in tumors after 2 weeks (Figure S1). Considering the actual therapeutic application, we believe that loading the DYRK2 gene into a proliferative vector carrying the E1A/E1B genes could provide an ideal antitumor effect. Another issue in the future therapeutic application of the adenovirus used in this study is the selectivity of the adenovirus for infection. In the liver metastasis model in this study, Cre was expressed in the metastasized tumor to allow it to express DYRK2 specifically in the tumor using an adenovirus. However, the adenovirus we used expresses the gene
through the EF1α promoter, which might affect normal cells in actual therapeutic situations. Although the effects of DYRK2 overexpression may be different between normal cells and cancer cells, it is desirable to minimize the effects on normal cells as much as possible. The construction of Ads carrying tumor-specific promoters (eg, telomerase reverse transcriptase or survivin promoter) to express target genes in a tumor-specific manner should be considered.28-30

In conclusion, this is the first report that DYRK2 overexpression by adenovirus inhibits tumor growth through inhibition of proliferation and induction of apoptosis in colorectal xenograft and liver metastasis models. Overexpression of DYRK2 by adenovirus could be a new gene therapy option for unresectable metastatic CRC.

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DISCLOSURE
The authors have no conflict of interest.

ORCID
Kiyotsugu Yoshida https://orcid.org/0000-0003-3108-7383

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