Thesis

Enhancement of the activity of oncolytic adenovirus by augment HuR export of host cells

(RNA 結合タンパク HuR の核外輸送活性化による腫瘍溶解アデノウイルスの効果増強)

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1. **Ishraque Ahmed**, Mohammad Towfik Alam, Aya Yanagawa-Matsuda, Elora Hossain, Tetsuya Kitamura, Kazuyuki Minowa and Fumihiro Higashino

   Enhanced oncolytic activity of E4orf6-deficient adenovirus by facilitating nuclear export of HuR. *Biochemical and Biophysical Research Communications (BBRC), accepted, April 30, 2020.*

2. Yohei Mikawa, Mohammad Towfik Alam, Elora Hossain, Aya Yanagawa-Matsuda, Tetsuya Kitamura, Motoaki Yasuda, Umme Habiba, **Ishraque Ahmed**, Yoshimasa Kitagawa, Masanobu Shindoh and Fumihiro Higashino

   Conditionally Replicative Adenovirus Controlled by the Stabilization System of AU-Rich Elements Containing mRNA. *Cancers, published May 11, 2020.*

3. Elora Hossain, Umme Habiba, Aya Yanagawa-Matsuda, Arefin Alam, **Ishraque Ahmed**, Mohammad Towfik Alam, Motoaki Yasuda and Fumihiro Higashino

   Advantages of Using Paclitaxel in Combination with Oncolytic Adenovirus Utilizing RNA Destabilization Mechanism, *Cancers, Published May 12, 2020.*

List of presentations

Part of this study was presented in the following academic conferences:

1. **Ishraque Ahmed**, Mohammad Towfik Alam, Aya Yanagawa-Matsuda, Tetsuya Kitamura, Kazuyuki Minowa, and Fumihiro Higashino

   Enhancement of the activity of oncolytic adenovirus by augment HuR export of host cells-*Poster presentation*

   (The 5th Hokkaido University Interdisciplinary Symposium), November 6, 2019, Sapporo, Japan.

2. **Ishraque Ahmed**, Mohammad Towfik Alam, Aya Yanagawa-Matsuda, Tetsuya Kitamura, Kazuyuki Minowa and Fumihiro Higashino

   Combination effect of oncolytic adenovirus with ethanol for human cancer cells-*Oral presentation*

   (The 4th Hokkaido University interdisciplinary Symposium), January 25, 2019, Sapporo, Japan.

3. **Ishraque Ahmed**, Mohammad Towfik Alam, Tetsuya Kitamura, Aya Yanagawa-Matsuda, Kazuyuki Minowa and Fumihiro Higashino

   Combination effect of oncolytic adenovirus with ethanol for human cancer cells-*Poster presentation*

   (The 66th Annual Meeting of Japanese Association for Dental Research), November 17-18, 2018, Sapporo, Japan.

4. **Ishraque Ahmed**, Mohammad Towfik Alam, Tetsuya Kitamura, Aya Yanagawa-Matsuda and Fumihiro Higashino

   Combination effect of oncolytic adenovirus with ethanol for human cancer cells-*Poster presentation*

   (The 107th Annual Meeting of the Japanese Society of Pathology), June 21-23, 2018, Sapporo, Japan.

5. **Ishraque Ahmed**, Mohammad Towfik Alam, Tetsuya Kitamura, Aya Yanagawa Matsuda and Fumihiro Higashino

   Combination effect of oncolytic adenovirus with ethanol for human cancer cells-*Oral presentation*

   (Hokkaido University Graduate school of Medical Sciences 1st Student Research presentation Meeting), November 22, 2017, Sapporo, Japan.

Chapter 1

General Introduction

Cancer

Cancer is defined as an abnormal growth of cells. More than 100 types of cancers, which are very egregious type. Symptoms vary depending on the type of cancers and all are potentially life-threatening. Chemotherapy, radiation, and/or surgery are the most common treatment plan of cancer. According to GLOBOCAN 2018 database (Press release from WHO, 2018), globally cancer burden is estimated to have risen to 18.1 million new cases and 9.6 million death in 2018.

For human death, cancer is now 2nd leading cause. Cancer incidence rates are growing rapidly globally. Mainly the increase is expected to be concentrated in developing countries due to heavy smoking, poor diet, physical inactivity and environmental pollutions and others. According to the global cancer data in 2018, lung cancer is responsible for the largest number of deaths (1.8 million deaths, 18.4% of the total), colorectal cancer (0.8 million deaths, 9.2%), stomach cancer (0.7 million deaths, 8.2%), liver cancer (0.78 million deaths, 8.2%), and female breast cancer is now fifth leading cancer death (0.62 million death, 6.6%).

After human ailment, more than 1000 years ago cancer has been recognized but only in the past century, medical science has been understood what cancer really is and how it progresses. Nowadays, with modern technology cancer diagnosis is easier than before. And also using the modern technique for cancer therapy people with cancer are now living longer. However, some form of the cancers remain frustratingly difficult to treat.

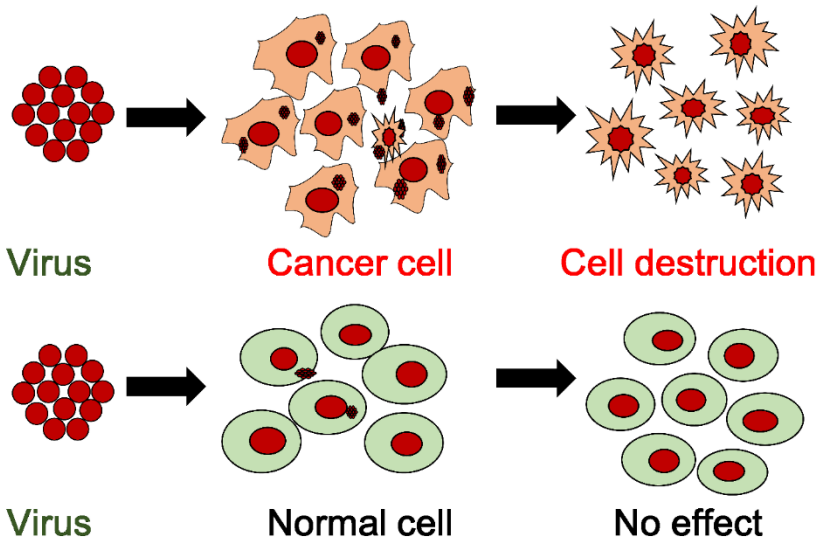
The conventional method of cancer treatment

The selection of cancer treatment and its progress depends on the type of cancer, grading, and staging. The spectrum of the traditional treatment of cancer includes three basic methods: surgical removal of tumor, chemotherapy, and radiotherapy. Depends on cancer type, combined with surgery and chemotherapy or a combination of chemotherapy and radiotherapy is the treatment of choice.

Some of the modern modalities are hormone-based therapy, anti-angiogenic modalities, stem cell therapies, immunotherapy and dendritic cell-based immunotherapy (Zaigham et al., 2018) are seldom to be appeared. Variant unique therapy exploited for the treatment of malignancy like treatment against the angiogenic ability of cancers, oncolytic virotherapy, hereditary control of apoptotic and tumor-attacking path-ways, antisense and RNAi techniques (Zaigham et al., 2018).

Oncolytic Virotherapy

Oncolytic Virotherapy is now one of the fast-developing and an exciting tool in cancer research. In the early 20th century, it was recognized that virus can destroy cancer cells (Kelly et al., 2007). The advanced genetic engineering techniques has been made possible to alter the viral gene, and using this virus for cancer regression. Oncolytic viruses are those viruses that eliminate the cancer cells by targeting the malignancy site without harming the noncancerous cells (Schematic diagram. 1).



Schematic diagram. 1. Oncolytic adenovirus selectively replicates in cancer cell

Among the oncolytic viruses being studied in preclinical and clinical trials are Adenovirus, Herpes Simplex Virus, Vesticular Stomatic Virus, Reovirus, Coxsackie virus and Vaccinia Virus (https://en.wikipedia.org/wiki/Oncolytic_virus).

Viruses are nanoparticle with ranging from 20 to 500 nm in diameter. During tumor development, oncolytic viruses attain their specificity by exploiting cell surface receptors or intracellular abnormality in gene expression that arise in malignancies. It is one of the benefits that the oncolytic virus compromises over chemotherapeutic agent due to its genetic manipulation. In 1950s, the vaccine strain of Rabies viruses was first time used as an oncolytic virus in clinical studies for

melanomatosis treatment, and 8 of 30 patients showed tumor regression. (Pack et al., 1950). After that, several tested with oncolytic adenovirus has been performed in various animal and human model to find out the anti-cancer potency, tumor specificity and safety (Ring. et al., 2002). Some of them are West Nile virus strain Egypt 101, Mumps, Newcastle disease, measles, autonomous parvo, adeno, reo, vesicular stomatitis, herpes simplex and enteroviruses (Vaha-Koskela et al., 2007). Conversely, many viruses showed side effects which ultimately terminated the trials and interest in viruses as anti-cancer agents declined during the 1970s. After the apparition of modern biotechnology, gene therapy and better understanding of cancer biology, resurrection of interest in viral therapy in cancer treatment again. (Ravindra et al., 2009 and Guo et al., 2008). The original pioneer research of virus therapy was conducted with Herpes simplex virus type-1 and multiple clinical trials were performed with adenovirus ONYX-015 (Kelly et al., 2007). In 2005, China's State Food & Drug Administration approved first time genetically modified adenovirus-H101 type 552, in which E1B-55 kD and partial E3 genes have been deleted as oncolytic virus. For oncolytic virotherapy, both DNA and RNA viruses have been used but DNA viruses most reliable because these are more amenable to genetic manipulation (Guo et al., 2008).

Human adenovirus

The adenoviruses (Ad) are non-enveloped viruses composed of linear, double-stranded DNA of approximately 36 kb, and associated proteins surrounded by an icosahedral nucleocapsid of about 80-100 nm with 12 protruding fiber knobs. The viral genome consists of early (E) and late (L) genes (Berk et al., 2006, Seth et al., 2000). The L genes encode structural proteins (L1, L2, L3, L4, and L5), which package the viral DNA into the ad virion during the final stages of replication. The E genes include E1, E2, E3, E4, and E7 which regulate viral replication (Russel et al., 2000). The early gene E1A, a subunit of the E1 gene, produced immediately after infection of modulating the cycle, recruit cellular proteins, and regulate the expression of cellular and viral genes (Ben-Israel et al., 2002). The ad E1B gene encodes two major polypeptides, termed 55 k, which is 55 kilodaltons (KDa) and 19k, 19 kDa, respectively. Both E1B55K and E1B19K expression are essential to transform rodent cells following viral transduction and DNA transfection (Barker et al., 1987, Stillman et al., 1986). These two proteins also defend infected cells from E1A-induced stabilization of TP53 (Tumor suppressor protein 53) and apoptosis. (Lowe et al., 1993). E1B55K also augments viral E1A expression (Zheng et al., 2006) and is responsible for the induction of cyclin E gene expression, which is required for efficient Ad replication (Rao et al., 2006, Zheng et

al., 2008). The Ad E1B19K protein is a putative B-cell lymphoma 2 Protein (BCL-2) functional homolog and a strong inhibitor of apoptosis (Debbas et al., 1993). E1B19K also protect E1A-induced apoptosis by interfering with the pro-apoptotic proteins Bak and Bax action (Cuconati et al., 2002). During Ad infection, E1B19K can also transactivate E1A protein (Herrmann et al., 1987). Consequently, it is thought that the function of E1B-encoded proteins maximizes Ad replication. Among those early genes, E4orf6, which is necessary for virus replication, participates in ARE-mRNA export and stabilization.

Oncolytic Adenovirus

Oncolytic viruses (OVs) are engineered and/or produced to replicate selectively in cancerous tissues. There are twin machinery of action; the direct killing of infected cancer cells cross-primes anticancer immunity to boost the killing of uninfected cancer cells (Maroun et al., 2017).

Due to adenoviruses are not capable to specifically aim to kill tumor cells, various modifications, like genetic manipulation, are needed. Two main changes in Ads genome have been applied to attain tumor selectivity. The first modification was a small deletion of crucial viral genes that are prerequisite for viral replication in normal cells. These minor deletions terminate the phenotype alterations in cancer cells, thus Oncolytic adenovirus replication limited to tumor cells (Baker et al., 2018). Bischoff et al. (1996) first introduced ONYX15 (dl1520), which lacks a functional E1B55K gene and replicates only in cells with mutations in the p53 gene. Besides, supplementary deletion in the E3B region sensitives ONYX-15 against antiviral immune responses (Ries et al., 2002). And the other section of E1B gene, E1B19K deletion, consequences down-regulate the multiplication of selective oncolytic adenovirus (OAd). The other main alteration is the addition of tissue-or tumor-specific promoters to control viral reproduction. Rodriguez et al., 1997 applied this approach 1st time by addition of prostate-specific antigen (PSA) promoter for E1A expression. Other tissue-specific promoters including α-fetoprotein for liver cancer (Kim et al., 2002), tyrosine for melanoma (Zhang et al., 2002), and carcinoembryonic antigen (CEA) for colorectal cancer (Li et al., 2003) has been implemented.

Combination treatment with oncolytic adenovirus for cancer therapy

Though oncolytic adenovirus has antitumor properties but oncolytic virotherapy by itself, has not been effective to complete tumor abolition in both preclinical animal models and clinical studies. There are also many limitations of chemotherapy and radiotherapy for their incompetence to treat metastatic disease. For the best chance of complete tumor eradication, combination with current treatment strategies of chemo and radiation therapies and the developing field of clinical gene therapy. Oncolytic virotherapy, in parallel with other conventional cancer therapy, might be one of the best treatment options. Several clinical trials showed the effectiveness of adding oncolytic adenoviruses with radiation therapy, chemotherapy and gene therapy (Chu et al., 2004).

With these combination treatments, various reports have been published and showed the effectiveness of the oncolytic virus. Different mechanisms are involved to increase virus activity. For example, CV787 and Docetaxel combination showed increased p53 expression in LNCaP cells (Yu et al., 2001). And also for the clinical trial with combination treatment with oncolytic adenovirus expressed good result like with intratumoral dl1520 injection and combination of cisplatin and 5-fluorouracil therapy in recurrent squamous cell carcinoma of the head and neck, sixty three percent of patients (19 of 30) had an effectively decreased in tumor size (50%). Eight of 30 (27%) showed complete response (no measurable disease), although 11 of 30 (36%) patients had a partial response (decrease of 50-100% in tumor area). (Khuri et al., 2000 and Lamont et al., 2000).

Besides chemotherapy, oncolytic adenovirus expressed the effectiveness against cancer with radiation combination. Although the mechanism to kill cancer cells is different between these two options. Combination of dl1520 Intra-arterial injections and total body irradiation (5 Gy) were evaluated in subcutaneous human malignant glioma xenograft models those were both p53 mutant and p53 wild-type (Geoerger et al., 2003). Tumor growth was significantly delayed in combination-treated p53 mutant tumors compared to monotherapies (30 days versus 10-15 days). Partial and complete responses in combination-treated group were also higher in number compared with animals treated with only radiation or dl1520. Improved antitumor efficacy was also observed in combination studies involving Ad5-24RGD, CV706, and AdFGR (Aghi et al., 2000, Wildner et al., 1999, Freytag et al., 2003). Of note is that 106 plaque-forming units of Ad5-24RGD in combination with radiation therapy acquired similar antitumor effects as 107 plaque-forming units of Ad5-24RGD alone, revealing that reduced viral treatment doses can

be effective in combination therapy. From these findings, it is ensured that radiation did not hamper viral replication (Rogulski et al., 2000, Geoerger et al., 2003, Lamfers et al., 2002, Chen et al., 2001) and also no additive toxicities showed in the virus and radiation combination-treated groups compared with the monotherapy groups (Rogulski et al., 2000 and Chen et al., 2001).

All these studies ensured that during the combination therapy with oncolytic adenovirus, the chemotherapeutic agent or the radiation act as a regulator that influences the viruses to perform enhance oncolytic activity. Although most of the mechanisms are yet to elucidate.

HuR is one of the key mediators for cancer

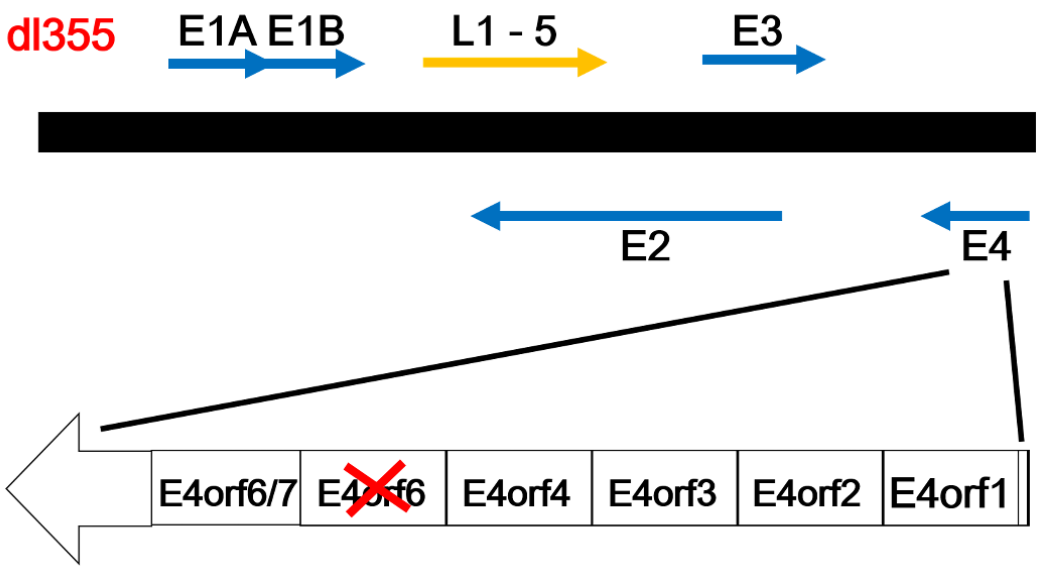
Human Antigen R or HuR is a member of the embryonic lethal abnormal vision (ELAV) family of RNA binding protein. It is well documented that HuR is a nuclear protein and under various stimulations to AU-rich (ARE), which is RNA element included in growth-related mRNA such as oncogene. It interacts with ARE-mRNAs in nucleus and makes HuR-ARE-mRNAs complex to transport them to the cytoplasm. After HuR binding with the targeted transcript, the message is stabilized and revive it from rapid degradation by exonucleases (Wang et al., 2013). From the mRNA, HuR releases itself and after stabilization, the mRNA it returns rapidly to the nucleus (Wang et al., 2013). It has been reported that many stimulators as an example, Ultraviolet radiation (UVR), lipopolysaccharide (LPS), viral infection, Ethanol, etc induce HuR export. With the stimulation of these stimuli, the outcome is significantly upregulated the HuR accumulation in the cytoplasm. Though the mechanism of transportation is incompletely understood but some associated proteins of HuR such as transportin 2 (Trn 2) (Rebane et al., 2004) and chromosome maintenance region 1 (CRM1) which implicates nuclear export signal recognition by the export receptor (Gallouzi et al., 2001 and Brennan et al., 2000). It was previously documented that HuR protein expression is enhanced in most of the cancer cells. (Dalamu et al., 1992, King et al., 1996, King et al., 1997). HuR is the 1st RNA binding protein that plays a critical role in cancer progression and overexpression of HuR is significantly related to cancer progression, advanced stage and also poor survival (Wang et al., 2013).

HuR and virus replication

The adenovirus E4 region contains seven open reading frames (ORFs); those play a critical role for virus growth, DNA replication and particle assembly (Leppard et al., 1997). Specifically, E4orf3 and E4orf6 are requisite for mRNA splicing and accumulation (Bridge et al., 1989), And E4orf6 can stabilize ARE-mRNA, resulting exert the oncogenic activity and persuade malignant transformation by HuR mediated pathway. (Kuroshima et al., 2011). Different virus gene products like HSV UL41 (Esclatine et al., 2004), KSHV Kaposin B (McCormic et al., 2005) have been exhibited the control of the destiny of ARE-mRNA. Our other report proved that cells infected with adenovirus type 5, ARE-mRNAs are exported to the cytoplasm and are stabilized by E4orf6, which is required for the virus replication (Higashino et al., 2005).

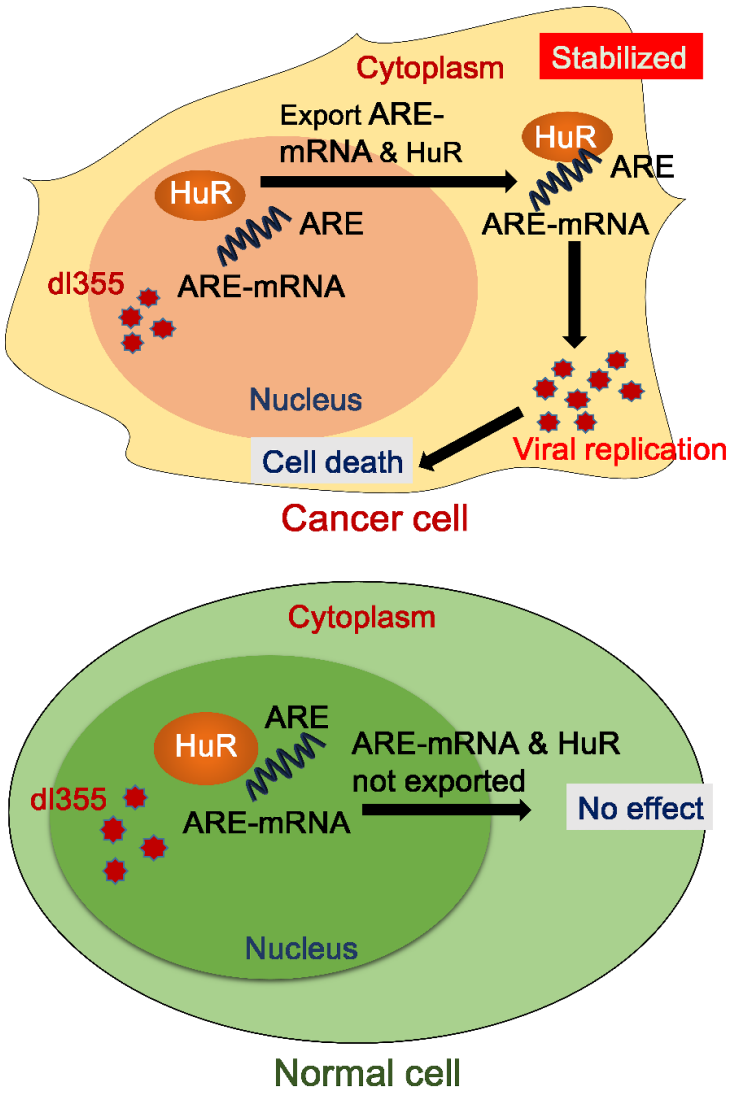
E4orf6 deleted mutant dl355

The dl355 oncolytic adenovirus from which the 14 base-pair deletion in the E4orf6 of the wild type adenovirus type 5 (Ad5) (Halbert et al., 1985). (Schematic diagram. 2).



Schematic diagram. 2. Oncolytic adenovirus dl355 from which orf6 gene has been deleted from E4 region.

The deletion of E4orf6 has been reduced the capability of replication activity of dl355 in the normal cells due to less efficiency of ARE-mRNA stabilization activity. On the other side, E4orf6 is not obligate to presence for viral replication where ARE-mRNA is constitutively stabilized by HuR like cancer cells (Schematic diagram. 3). Our previous study with dl355 has been shown the enhanced oncolytic activity in cancer cells. (Matsuda et al., 2019).



Schematic diagram. 3. Both HuR and ARE-mRNA export to the cytoplasm constitutively in cancer cells but not in normal cell. And dl355 replicates specifically in cancer cells in which ARE-mRNA has been stabilized by HuR.

Enhanced HuR expression using exogenous regulator may increase the oncolytic activity

In cancer cell, a wide-ranging exploration of the biological activity of HuR revealed that the HuR is an ultimate controller of post-transcriptional gene expression and has a principal role in cancer. Establishing HuR molecular machinery containment could be effective in detecting new targets for drug design. Many exogenous regulators like Ethanol, X-Ray, UV Radiation, LPS, Tamoxifen, and Gemcitabine and others showed enhanced cytoplasmic HuR expression (Wang et al., 2013) in cancer cells. For virus replication, ARE-mRNA induced by HuR plays a crucial role. So we hypothesized that the treatment with an exogenous regulator may upregulate the HuR activity which can regulate the enhancement of oncolytic viral replication. In this study, Ethanol, X-ray and UVR have been used as a HuR up-regulator. Though these agents have cancerous property but very limited usages as therapy may encourage oncolytic virus replication in cancer cell. HuR mediated oncolytic adenovirus dl355 may one of the pioneering therapy with these HuR regulators due to its ineffectiveness in the normal cells (Matsuda et al., 2019).

Aim of the study

To evaluate the oncolytic activity of E4orf6 deleted mutant dl355 by augmented HuR using exogenous regulators in cancer cells.

Following objectives were set to acquire our aim

1. To investigate the HuR translocation with exogenous sources like ethanol treatment in cancer cells.

2. To analyze the oncolytic activity of dl355 after enhancing cytoplasmic HuR with ethanol treatment.

3. To find out the enhanced viral replication with ethanol combination and also to find out the death mechanism.

4. To find out the cytolytic activity of dl355 in combination with two other different radiation therapies like X-Ray and UVR.

List of Abbreviations

Ad	Adenovirus
ARE	Adenylate-uridylate-rich element
CEA	Carcinoembryonic antigen
CRAd	Conditionally replicative adenovirus
CRM1	Chromosome maintenance region 1
DNA	Deoxyribonucleic acid
HuR	Human Antigen R
ifu	Infectious unit
MOI	Multiplicity of infection
mRNA	Messenger RNA
nm	Nanometer
ORFS	Open reading frames
OVS	Oncolytic virus
PEI	Percutaneous ethanol injection
PMLE	Polymorphic light eruption
PSA	Prostate specific antigen
PUVA	Ultraviolet A with a skin sensitizing psoralen
q-RT PCR	quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RNAi	Double-stranded RNA-mediated interference
Trn 2	Transportin 2
TP53	Tumor suppressor protein 53
UV	Ultraviolet

UVR	Ultraviolet radiation
VP	Virus particle

Chapter 2

Enhancement of the activity of oncolytic adenovirus by augment HuR export of host cells by ethanol

Introduction

Conditionally replicative adenovirus (CRAd) is an attractive anticancer tool due to the ability of cell-specific propagation and also releases a large number of progeny viruses to infect other surrounding cancer cells (Kruyt et al., 2002). Furthermore, combination with gene therapy, radiotherapy or chemotherapeutic drugs, oncolytic adenovirus plays a more potent antitumor effect (Bressy et al., 2014). The early gene products of adenovirus like E1B55k and also E4orf6, retain the modulatory functions of viral and host cells mRNA (Berk et al.,2013, Dobner et al., 2001). E1B55k and E4orf6 proteins are responsible for creating specific complex within infected cells (Sarnow et al., 1984). After binding of E1B55k and E4orf6, the protein complex is assimilated with the E3 ubiquitin-ligase to smoothed the ubiquitination of various host proteins like p53 (Querido et al., 2001). This is requisite for the virus's late mRNAs exportation during the late phase of virus infection (Woo et al., 2007, Blanchette et al., 2008).

Recently we found that E4orf6 protein is capable to stabilize AU-rich element (ARE) containing mRNA (Higashino et al., 2005, Kuroshima et al., 2011) and this stabilization plays an important role for adenovirus replication (Jehung et al., 2018). ARE is one of the targets of rapid RNA degradation and is present in mRNAs transcripted from genes such as oncogene and cytokine, which are related to cell growth. The decay of ARE-mRNA is regulated by several RNA-binding proteins. HuR is an RNA binding protein of ELAV (embryonic lethal abnormal vision) family that binds to ARE to protect ARE-mRNA from rapid degradation (Hinman et al., 2008). HuR regulates the ARE-mRNA export from the nucleus to the cytoplasm and its stabilization. Some associated proteins of HuR such as transportin 2 (Trn 2) (Rebane et al., 2004) and chromosome maintenance region 1 (CRM1) which implicates nuclear export signal recognition by the export receptor (Gallouzi et al., 2001 and Brennan et al., 2000). In normal condition, HuR localized in the nucleus but transiently relocalized to the cytoplasm, however, in most of the cancer cells, HuR constitutively localized in the cytoplasm (Lopez de Silances et al., 2005).

As ARE-mRNA is always stabilized in cancer cells, that’s the reason why without the presence of E4orf6, dl355 oncolytic adenovirus is able to replicate in cancer cells. (Matsuda et al., 2019). In the previous study, dl355 had higher virus growth efficiency in cancer cells, than dl1520, which had previously shown oncolytic activity, and dl355 also had higher cytolytic activity for cancer cells (Matsuda et al., 2019).

In this study, to examine whether the enhancement of ARE-mRNA stabilization up-regulates the oncolytic adenovirus dl355, we have checked the cytolytic effect of dl355 with ethanol treatment. Ethanol is using as an alcoholic drink worldwide although it has cancerous property. Nevertheless, nowadays ethanol is using as an anti-cancerous drug-like direct percutaneous ethanol injection (PEI), results in dehydration and necrosis of the tumor cells in small hepatocellular carcinoma (Lencioni et al., 2005). In addition, different authors have reported that percutaneous ethanol injection (PEI) showed good result in the treatment of benign thyroid cyst (Del Prete et al., 2004, Sung et al., 2008) as well as hyperfunctioning thyroid adenomas (Livraghi et al., 1994) and benign parathyroid adenomas (Veldman et al., 2008). It was also reported, ethanol exposure increases the cytoplasmic HuR accumulation which facilitates the ARE-mRNA stabilization (Wang et al., 2013).

In this study, we examined the oncolytic activity of dl355 with ethanol. Cytoplasmic HuR relocation was comparatively higher in ethanol-treated cells. Virus production was higher in ethanol condition and the cytolytic effect was significantly higher in ethanol-treated cancer cells compared to those of dl355 alone. In addition, a cleaved PARP expression was higher in the combination-treated cancer cells indicated the apoptotic cell death exceedingly in the cancer cells. These findings indicate that the oncolytic effect of dl355 was easily augmented by the reagent which is able to export HuR to the cytoplasm of the cancer cells. Since a lot of reagents can be involved in the export of HuR (Wang et al., 2013) so these methods are available to activate dl355 oncolytic activity, and that character is the benefit of dl355.

Material and Method

Cells and adenoviruses

HeLa (human cervical carcinoma cell), A549 (human lung carcinoma cell), and BJ (human normal fibroblast cell) were used. These cells were collected from the American Type Culture Collection (ATCC; USA). To generate viruses, W162 cells (a generous gift from T. Dobner) were used due to the presence of an integrated copy of the Ad5 E4 region. Both cancer and normal cells were cultured at 37°C with 5% CO2 in Dulbecco's modified Eagle minimal essential medium (DMEM) (SIGMA-ALDRICH, D 5796, UK) containing 10% fetal bovine serum (BIOWEST, S-1650, Mexico) without antibiotics.

In this study, we used E4orf6-deleted mutant (dl355) (Halbert et al., 1985) and wild type adenovirus type 5 (WT300) (generous gifts from Dr. T. Shenk).

Western blotting

We focused on to find out the best treatment options of Ethanol in terms of its doses and effective time courses. To check cytoplasmic HuR exportation with ethanol treatment, cancer cells (A549, HeLa) were seeded in 6 cm dishes and treated with 0.1%, 0.2%, 0.3%, 0.5%, 1%, 2% and 5% ethanol and incubated for 12 hours in 37°C. Again to find out the best timing of cytoplasmic HuR export, cancer and normal cells treated with 0.3% ethanol and incubated for 12, 18, 24, 48 and 72 hours. Cytoplasmic and nuclear protein was separated using fractionation buffer (10 mM Tris HCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40 and protease inhibitor cocktail) followed by 5 minutes vigorous shaking and centrifugation at 12, 000 rpm for 30 sec. The supernatant was collected as a cytoplasmic protein. Western blot experiment was performed using 10% SDS PAGE gel.

Total protein was collected after the dl355 infection (MOI 100 vp/cell) with or without ethanol treatment (0.3%). To collect total protein, cells were lysed using RIPA Buffer (CST-9806, Cell Signaling Technology) containing PMSF. After three rounds of sonication, lysates were centrifuged at 14000 G for 10 minutes at 4° C. E1A and Hexon expression was detected by using 10% SDS PAGE gel, and Cleaved PARP expression was evaluated by using 8% SDS page gel. On the other hand, the upstream molecules of PARP, Caspase 3, 7, and 9 were checked by using 12% SDS PAGE gel.

To detect protein expression, anti-HuR 3A2 (sc-5261; Santa Cruz Biotechnology), anti-Adenovirus-5 E1A M58 (sc-58658; Santa Cruz Biotechnology), anti-adenovirus late proteins (L133; generous gifts from Dr. T. Dobner), anti-PARP (46D11) (CST- 9532, Cell Signaling Technology), anti-Caspase-3 (CST-9662, Cell Signaling Technology), anti-Caspase 7 (CST-9492, Cell Signaling Technology), anti-Caspase 9 (CST-9502, Cell Signaling Technology), anti-β-tubulin (cat. no. 05-661; EMD Millipore Corp., Darmstadt, Germany) and anti-β-Actin (sc-47778; Santa Cruz Biotechnology) antibodies were used. For secondary antibodies, HRP-anti-mouse IgG (cat. no. 115-035-062; Jackson Immuno Research Laboratories) and HRP anti-rabbit IgG (sc-2054, Santa Cruz Biotechnology) were used.

RNA isolation and q-RT PCR analysis

Total RNA was isolated by using ReliaPrep™ RNA Cell Miniprep System (Promega Corporation, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed in the DNA Engine Opticon2 (MJ Research, Watertown, MA, USA), and cDNA was amplified using the following primers: *Iva2*, FW: 5′-TGACTCGCATGTTTTCCCTGAC-3′, RV: 5′- GCTTGGTCAAACGCTCAAAAGC-3′ and *Actb*: FW: 5′- TTTGCACATGCCGGAGCCGTTG-3′, RV: 5′-TTTGCAGCTCCTTCGTTGCCGG-3′. For this experiment, virus infection was at an MOI 100 vp/cell, and ethanol treatment was 0.3%.

Virus proliferation assay

Cells were harvested into 12-well plates at a density of 1×10^5 cells/well for BJ cells and 5×10^4 cells/well for HeLa and A549 cells. After 12 hours of treatment of 0.3% ethanol, cells were infected with dl355 at an MOI of 10 vp/cell. Cells were collected 48 hours after infection and lysed with three cycles of freezing and thawing in liquid nitrogen. W162 cells were seeded in 24-well plates at a density of 2.5×10^5 cells/well and then infected with 3-fold serially diluted viruses. Viral titers (ifu/ml) were measured by using the Adeno-X™ Rapid Titer kit (Cat no-632250, Clontech Laboratories, USA) according to the manufacturer's protocol.

Cytopathic effect assay (CPE assay) and cell viability assay (XTT assay)

To evaluate the most potent dose of dl355 infection, Cells were cultured in 24 well plates (2.4×10^4 cells/well). After 24 hours, cells were infected with dl355 in different MOIs (MOI 0.1/1/10/50/100 vp/cell) and incubated for 7 days. Cells were stained with Coomasie brilliant blue on day 7.

To check the cytolytic effect with ethanol and dl355 combination, the cells were harvested in 24-well plates (2.4×10^4 cells/well). After 24 hours of incubation, both cancer and normal cells were treated with 0.3% ethanol and incubated at 37°C for 12 hours. Cells were then infected with dl355 at an MOI of 100 (vp/cell), with and without 0.3% ethanol. After infection, cells were maintained for an additional 5 and 7 days and were stained with Coomassie brilliant blue.

To measure cell viability, cancer and normal cells seeded on 96-well plates at a density of 3.0×10^3 cells/well. XTT assays were performed using the Cell Proliferation KIT II (XTT) (Cat. No.11465015001, Roche, Germany). Cell viability was determined on days 0, 1, 3, 5, and 7.

Results

Ethanol increases HuR relocation to the cytoplasm

In order to identify the optimal ethanol concentration required to induce HuR translocation, we examined the levels of cytoplasmic HuR protein in cancer cells at various concentrations.

We found that 0.3% Ethanol resulted in observable HuR translocation with minimal negative effects on cell viability (Fig. 1A). We then treated both cancer (A549 and HeLa) and normal (BJ) cells with 0.3% ethanol for various amounts of time (12, 18, 24, 48, and 72 hours) (Fig. 1B).

Cytoplasmic fractions were isolated, and HuR was detected by Western blotting. 12 hours after ethanol treatment, levels of cytoplasmic HuR were enhanced by 2 to 3 times in both cancer cell lines (Fig. 1B). In contrast, cytoplasmic HuR did not increase in normal cells with this treatment. These results indicate that ethanol exposure specifically induces cytoplasmic HuR accumulation in cancer cells while not affect normal, healthy cells.

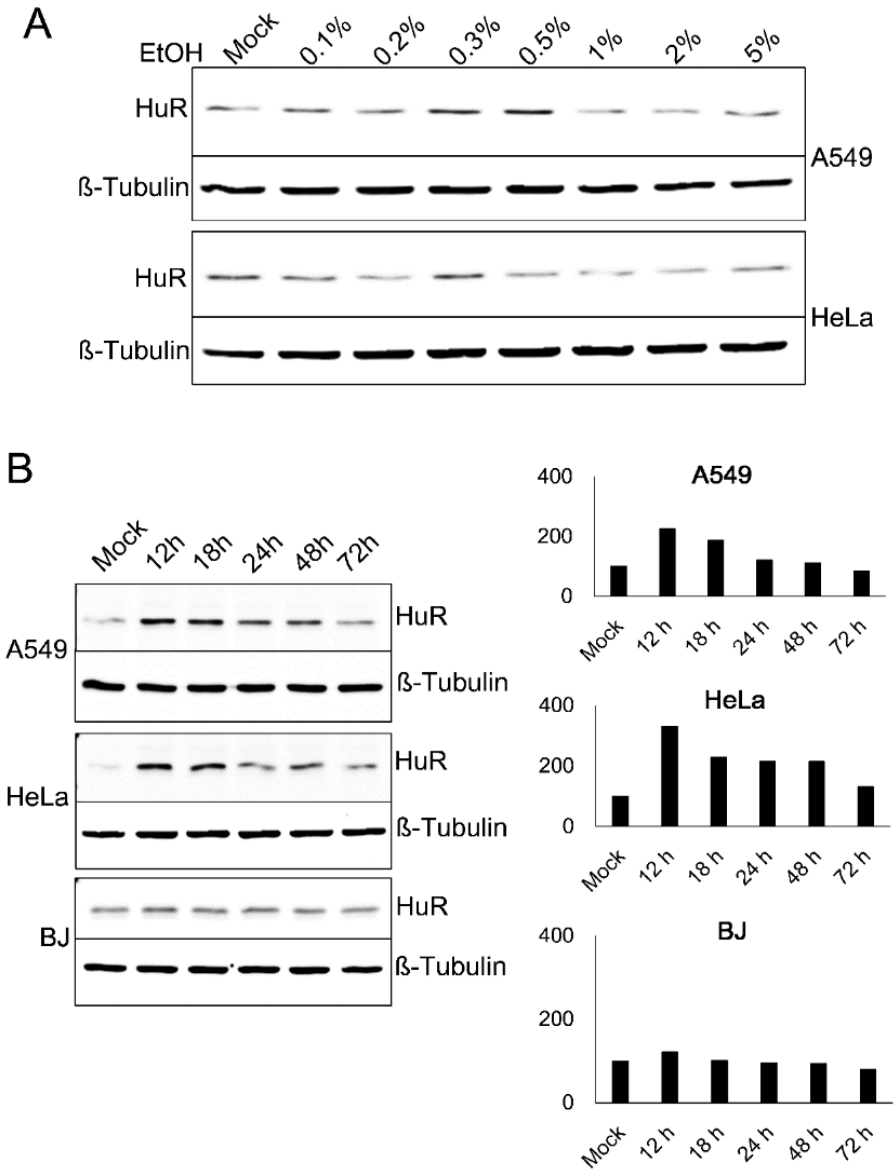


Fig. 1. Relocation of cytoplasmic HuR with ethanol treatment.

(A) Cytoplasmic HuR levels in cancer cells treated with various concentrations of ethanol for 12 hours. Protein abundance was determined by western blotting with β-tubulin as an internal control. (B) Cancer (A549, HeLa) and normal cells (BJ) cells were treated with 0.3% ethanol. Cytoplasmic HuR levels were examined by western blotting 12 hours after treatment. β-tubulin expression was also measured as an internal control (left). The amount of HuR in each cell type and condition is shown (right)

MOI 100 vp/cell is the most potent dose for the cytolytic effect of cancer cells

We tried to detect the most effective dose of dl355 for cancer cell lysis without the harmful effect of normal cell. For the confirmation, we infected both cancer (A549, HeLa) and normal (BJ) cells with different doses of MOI (0.1/1/10/50/100 vp/cell) and incubated at 37°C for 7 days. The survived cells were observed by staining Coomassie brilliant blue (Fig. 2).

Infection with MOI 100 showed an enhanced cytolytic effect in both cancer cells. Interestingly, we could not detect any toxicity with MOI 100 in normal cells. This result ensured that MOI 100 infection is a safer dose for normal cells but has the potency to kill cancer cells.

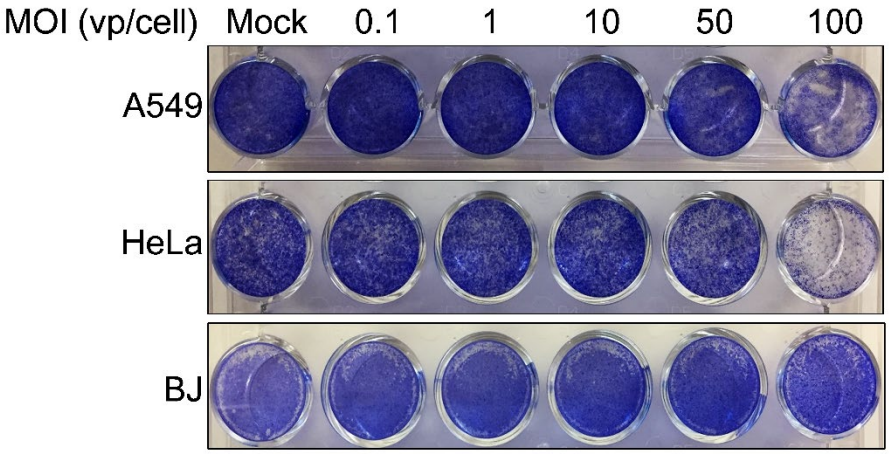


Fig. 2. Cytotoxic effect was enhanced in both cancer cells with dl355 MOI 100 vp/cell infection.

Ethanol up-regulates dl355 replication

As shown in previous studies, the efficient production of dl355 oncolytic adenovirus progeny is much higher in cancer cells than in normal cells (Matsuda et al., 2019). However, depletion of cytoplasmic HuR protein impairs dl355 replication. These data suggest that viral replication is dependent upon the presence of cytoplasmic HuR protein. To further explore this, we examined whether ethanol treatment, which leads to increased cytoplasmic HuR levels, affects the replication efficiency of dl355.

dl355 replication was assessed using two cancer cell lines, A549 and HeLa and also normal human fibroblast cell line BJ. In both cell types, the virus titer in ethanol treated-cells was higher than that of cells infected dl355 in the absence of ethanol (Fig. 3).

Viral propagation in A549 cells was higher than HeLa cells in both the combination and virus-only treatments. Furthermore, in A549 cells, virus production was 4.26×10^8 ifu/ml in the combination treatment condition, and 1.90×10^8 in dl355 infection only. On the other hand, virus production in HeLa cells infected with dl355 in the presence of ethanol was 3.3×10^8 ifu/ml, and 1.3×10^8 ifu/ml with dl355 infection alone. And also virus production in BJ cells with ethanol and dl355 combination was 1.8×10^7 and dl355 alone was 4×10^6. Together, these results suggest that ethanol influences dl355 replication in cancer cells.

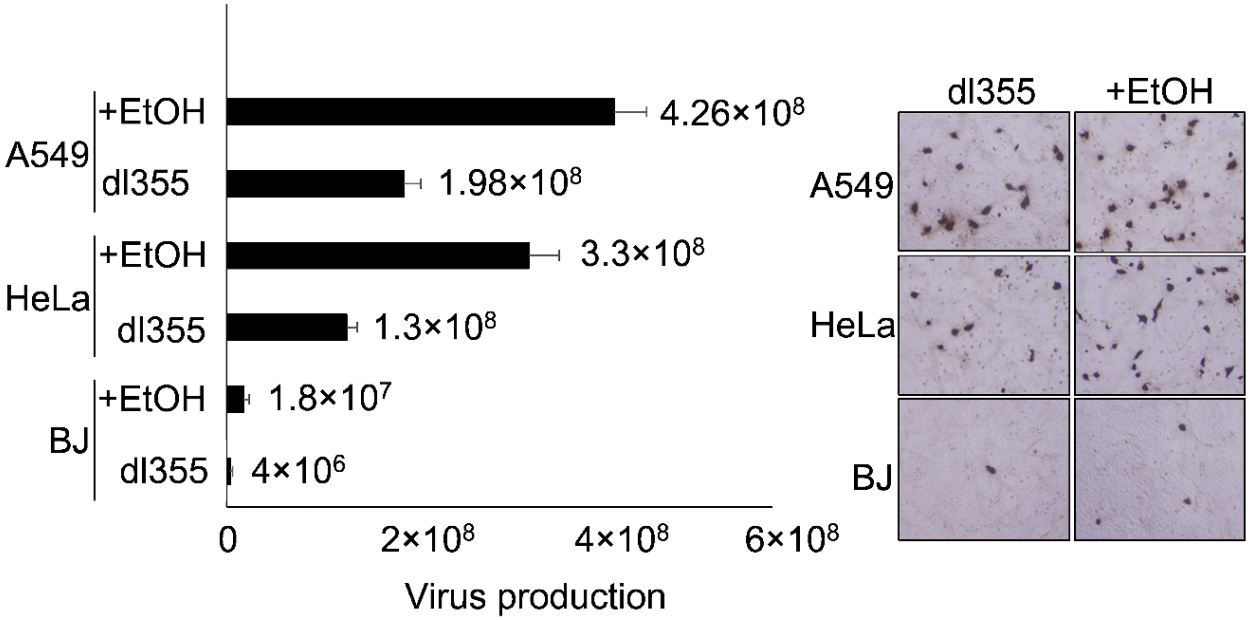


Fig. 3. Ethanol treatment that upregulate viral replication.

Virus production was evaluated in 48 hours after infection in both cancer and normal cells treated with ethanol alone, infected with dl355 alone, or infected with dl355 in the presence of ethanol. Each bar on the graph indicates the virus production (ifu/ml) (left). Hexon staining of W162 cells was used to determine viral load (right).

Ethanol increases the cytolytic potential of dl355

We examined whether the cytolytic potential of dl355 is augmented by ethanol treatment. Cytolytic activity was analyzed using a cytopathic effects (CPE) assay. Cancer (A549 and HeLa) and normal (BJ) cells were either treated with ethanol alone, infected with dl355 (MOI 100 vp/cell), or infected with dl355 in the presence of ethanol, and living cells were stained by Coomassie brilliant blue 5 and 7 days after infection (Fig. 4A).

Interestingly, cytotoxicity was enhanced in cells infected with dl355 in combination with ethanol treatment compared to cells infected with dl355 alone. This was true for both cancer cell types. Furthermore, most of the normal cells persisted in both treatments (dl355 infection alone or dl355 infection in the presence of ethanol), and ethanol-treatment alone showed no cytotoxic effects in either cancer or normal cell lines.

Next, cell viability after each treatment was estimated by XTT assay. A549, HeLa, and BJ cells were treated with the same conditions as in Fig. 4A and an XTT assay was executed. 7 days after infection, the viability of both cancer cell lines was decreased with dl355 infection in the presence of ethanol when compared to the cell viability with dl355 infection alone (Fig. 4B). Cell viabilities were unchanged in uninfected cells, both cancer and normal, treated with ethanol only. Moreover, 7 days after dl355 infection alone, or in combination with ethanol, most normal cells survived. Together, these results suggest that ethanol can augment the cytolytic activity and oncolytic activities of dl355 and that these effects are much more potent in cancer cells than in non-cancerous cell types.

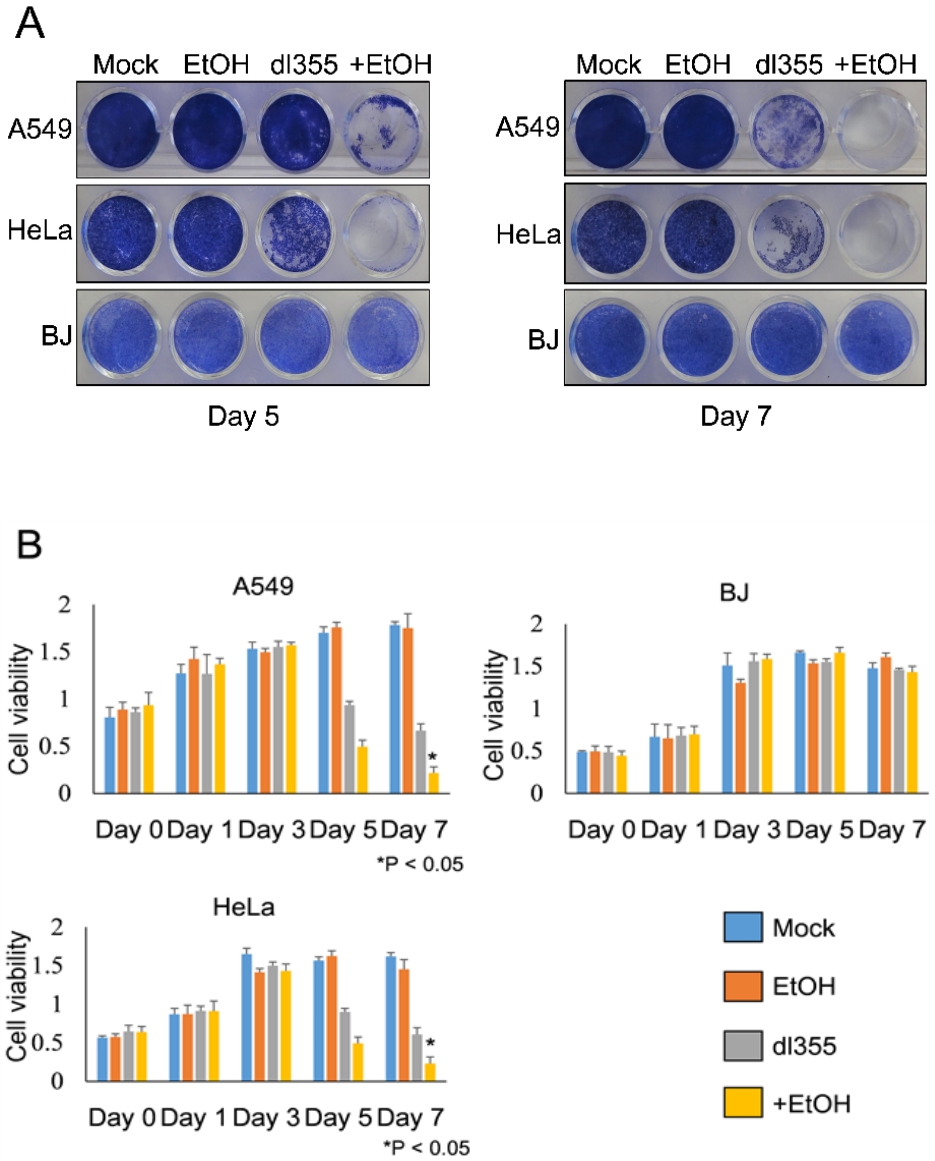


Fig. 4. The effect of ethanol for the cytolytic activity of dl355.

(A) Using a cytopathic effect assay (CPE), both cancer and normal cells were treated with ethanol, infected with dl355, or infected with dl355 in combination with ethanol treatment. 7 days after infection, cells were stained with Coomassie brilliant blue. Living cells appear blue in color. (B) Cells treated as described in A were subjected to an XTT assay to measure each cell viability 7 days after treatment (* = p value <0.05). Data are representation of three individual experiments.

Ethanol can enhance cancer cell apoptosis mediated by dl355

To identify the mechanisms of cell death mediated by dl355, we examined the apoptotic activity of dl355 infected cells. PARP cleavage, an indicator of apoptosis, was assessed in A549 (cancer) and BJ (normal control) cells 96 hours after each treatment by western blotting. As shown in Fig. 5A cleaved-PARP was present in A549 cells infected with dl355 (MOI 100 vp/cell), both with and without ethanol, with higher levels in infected cells exposed to ethanol, whereas no band for cleaved PARP appeared in the ethanol only condition. On the other hand, cleaved PARP was not detected in the normal cells (Fig. 5A). These results indicate that, in combination with ethanol, dl355 increases apoptosis in cancer cells, but not in normal cells.

To evaluate the upstream molecules for PARP in terms of apoptosis, Caspase 3, 7, and 9 were also investigated in dl355 infected in A549 (cancer) cells with or without the presence of ethanol. Total protein was collected 24, 48, 72 and 96 hours after infection. Unfortunately, no cleaved caspase was detected up to 96 hours of dl355 infection and also combination therapy (Fig. 5B). From these results, it is concluded that dl355 induced apoptosis is not related to caspase 3/7/9 mechanism but there might be another mechanism is involved. More studies are recommended to find out the mechanism of cell death by dl355.

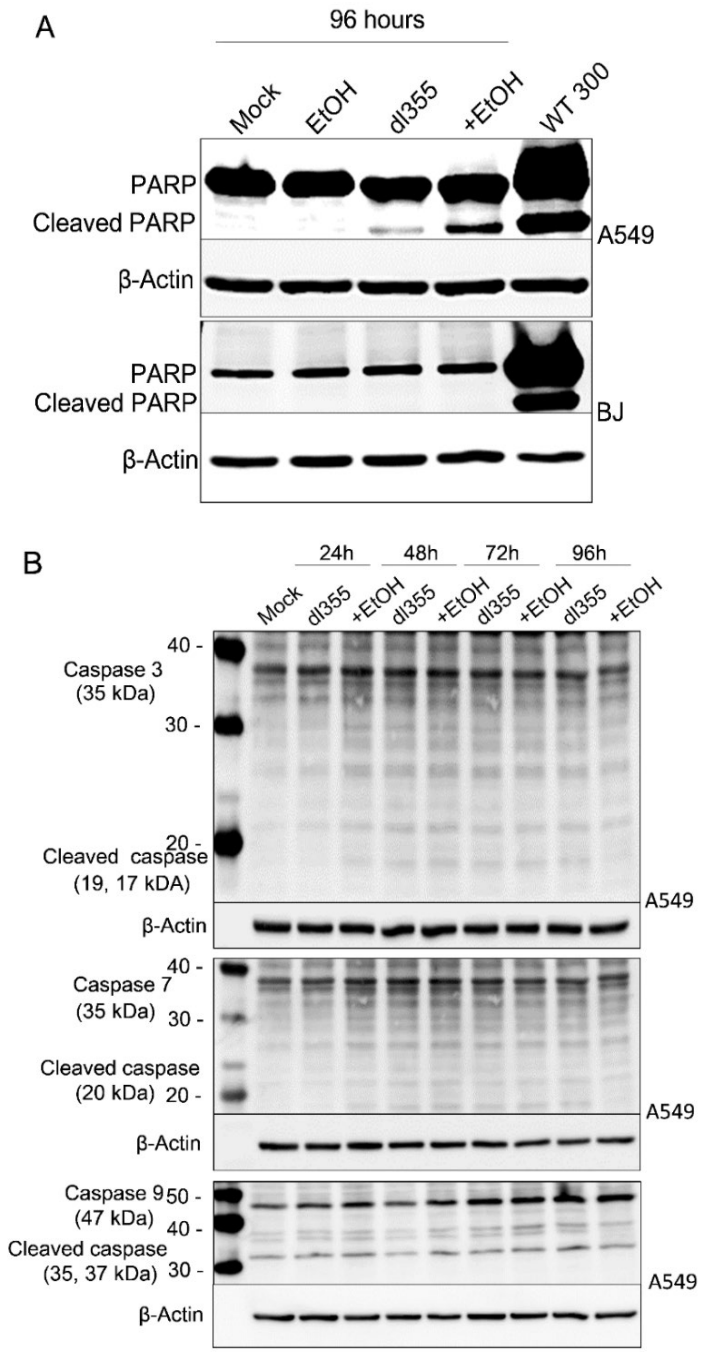


Fig. 5. Ethanol treatment enhances apoptotic cell death in cancer cells.

(A) Cancer (A549) and normal (BJ) cells were treated with ethanol, infected with dl355 alone, or infected with dl355 in combination with ethanol treatment. Apoptosis-associated protein cleaved PARP was detected by western blotting 96 hours after treatment. WT300 infection was performed as a positive control, and β-Actin was probed as an internal control. The data are representative of three independent experiments. (B) Cleaved of Caspase 3, 7 and 9 with dl355 and combination treatment up to 96 hours were examined by western blotting.

E1A and Hexon protein expressions in ethanol treated-cells

The E1A is the first viral protein expressed during productive infection in host cells and is necessary for virus replication. In order to understand the mechanisms of virus replication in ethanol-treated cells, we examined E1A protein expression. Although E1A expression was enhanced in ethanol-treated cancer cells after 24 hours of treatment, no change was observed beyond 24 hours (Fig. 6A). In normal cells, the onset of expression was very slow, no expression was observed after 24 hours of treatment, and slight E1A expression appeared after 48 hours. Furthermore, the amount of E1A expressed in normal cells was very low compared to the levels observed in cancer cells even in the long exposure figure (Fig. 6A). Thus, E1A expression was found to be less related to virus growth in ethanol-treated cells.

Hexon is one of the virus late gene products and is a very important component of viral particles. Hexon expression in cancer cells began to appear 48 hours later, slightly after the onset of E1A expression, an early gene product. Low levels of hexon were expressed in normal cells after 72 hours, even later than what was observed in cancer cells (Fig. 6B). Therefore, the expression of the late protein is synchronized with the expression of E1A examined.

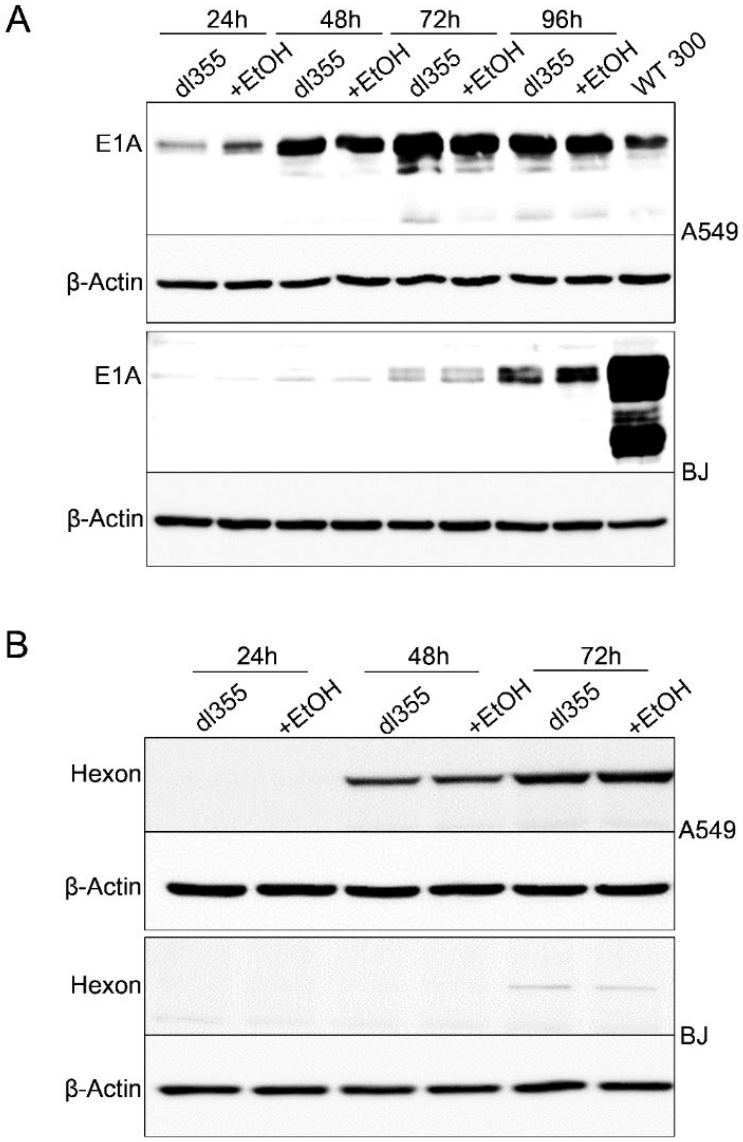


Fig. 6. The expression of E1A protein of dl355 by ethanol-treated cells.

(A) Both cancer and normal cells were infected with dl355 with and without ethanol, and total protein was purified 24, 48, 72, and 96 hours after infection. E1A expression was detected by western blotting. The data of BJ cells show the results of prolonged exposure. (B) The virus late protein hexon was examined by western blotting in cells infected with dl355, with and without ethanol treatment 24, 48 and 72 hours after infection. β-Actin was used as an internal control for both experiments (A and B).

***IVa2* mRNA expression was up-regulated by ethanol treatment**

Given that the *IVa2* gene transcript contains an ARE, we expected that increased *IVa2* mRNA stability may be involved in the enhanced dl355 replication in the presence of ethanol. We performed q-RT PCR to measure the amounts of *IVa2* mRNA in both normal and cancer cells treated with ethanol. As expected, the quantities of *IVa2* mRNA were markedly higher in ethanol-treated cells compared to levels in dl355 infected cells in the absence of ethanol (Fig. 7 A). In addition, mRNA up-regulation was maintained up to 48 hours after infection (Fig. 7 B). These results suggest that enhanced dl355 oncolytic activity was due to, at least in part, the stabilization of *IVa2* mRNA mediated by ethanol.

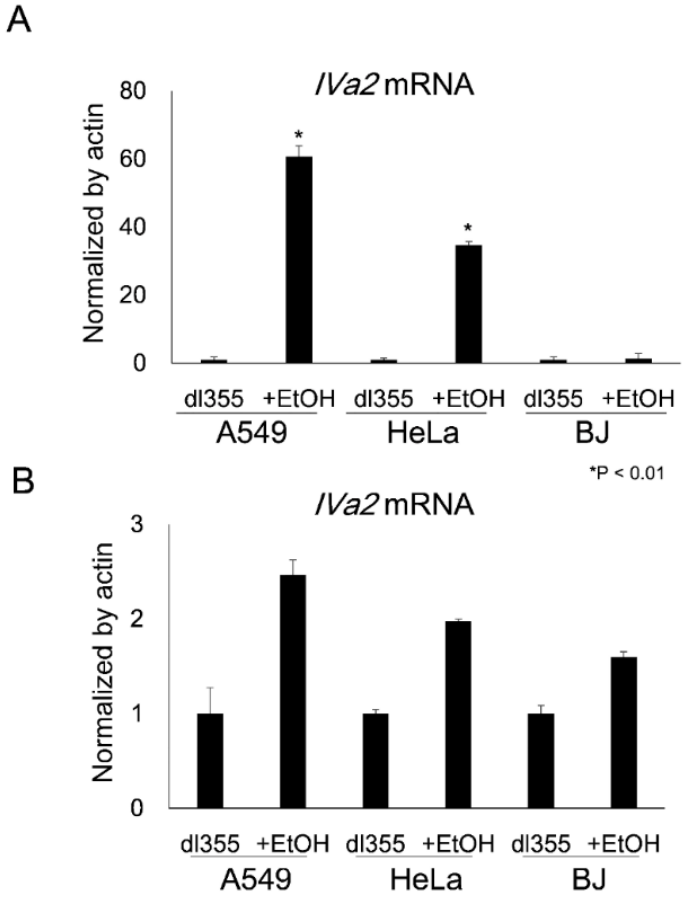


Fig. 7. The expression of IVa2 mRNA of dl355 by ethanol-treated cells.

(A) *IVa2* mRNA expression in cancer and normal cells was evaluated by q-RT PCR 24 hours after treatment (*= p value <0.01). (B) *IVa2* mRNA expression with infection by dl355, with and without ethanol treatment, was evaluated in cancer and normal cells by q- RT PCR analysis. Total RNA was extracted 48 hours after infection.

Discussion

In this study, we determined that the oncolytic effect of E4orf6-deleted adenovirus dl355 is enhanced by increased HuR export to the cytoplasm in the presence of ethanol. Ethanol was able to activate the replication and apoptosis-mediated cytolytic activity of dl355 in cancer cells though we do not know the mechanism, and more studies need to carry out. *IVa2* mRNA, rather than E1A, was up-regulated in response to ethanol in dl355-infected cells. These findings indicate that ethanol is capable of augmenting the oncolytic activity of viruses regulated by the ARE-mRNA stabilization system.

As shown in Fig. 1A and Fig. 1B, ethanol increases the amount of HuR in the cytoplasm. However, the cytolytic activity of dl355 was not increased by ethanol treatment (Fig. 4A). That is, nuclear HuR export to the cytoplasm in response to ethanol treatment increased cell death in cancer cells, but not in normal cells. These findings indicate that a certain minimum amount of HuR is required in the cytoplasm for dl355 to sufficiently proliferate and induce cell lysis.

As shown in our previous report, ARE-mRNA export to the cytoplasm, followed by ARE-mRNA stabilization, is essential for adenovirus replication (Jehung et al., 2018). In particular, *IVa2* mRNA stability was found to be critical given that *IVa2* mRNA is the only adenovirus transcript that contains an ARE (Vakalopoulou et al., 1991). In this study, the expression of the E1A protein, which is most important for replication of dl355, was increased by ethanol treatment within the first 24 hours after infection but did not change thereafter. Instead, as shown in Fig. 7A, the amount of *IVa2* mRNA increased, suggesting increased transcript stability. Therefore, we conclude that *IVa2* mRNA is important for the enhanced oncolytic effect of dl355 that occurs with ethanol. Future studies will examine whether other viral mRNAs are also stabilized by this mechanism.

In this study, ethanol was shown to enhance the oncolytic effect of dl355, and the combined use of ethanol and dl355 may lead to an unprecedented and powerful cancer therapy. However, it has been known that various reagents other than ethanol can affect the subcellular localization of HuR (Wang et al., 2013). For example, inflammatory cytokines such as TNF-α are known to increase the cytoplasmic HuR levels (Wang et al., 2013) and tumors are usually accompanied by inflammation. Thus, oncolytic viruses that can enhance the effects of inflammation are beneficial in the context of cancer treatment. We have also found that anti-cancer drugs such as cisplatin, enhance the cytoplasmic transport of HuR (data not shown). We expect that dl355 has higher combined effects with anticancer agents than oncolytic viruses developed so far.

Thus, it is considered that identifying more effective means among these various factors will lead to the development of more effective methods of cancer treatment.

Chapter 3

Enhancement of the activity of oncolytic adenovirus by augmenting HuR export of host cells by X-Ray

Introduction

For diagnosis purposes, the radiation is now widely used, such as X-Ray, CT scan, MRI, and some others but also for radiotherapy is one of the promising tools for cancer treatment. The common mechanism of radiation-induced tumor cells death is directly damaging DNA or indirectly through the oxygen radicals formation that disrupts the cellular pathway. Though the mechanism of cell death between oncolytic virus and radiation therapy is different but oncolytic viruses showed high potency with radiation combination. Many trials with different oncolytic viruses with radiation combinations previously performed. Especially the dl1520 showed the enhanced oncolytic effect with X-Ray combination (Geoerger et al., 2003). Though ethanol is not a common chemotherapeutic drug but in recent days low doses of ethanol are using as an anticancer agent in some primary level of hepatocellular carcinoma and thyroid and parathyroid carcinoma (Lencioni et al., 2005, Del Prete et al., 2004). In our previous investigation, we have found effective oncolytic activity by enhancing nuclear protein HuR by ethanol. It is also well documented that the nuclear protein HuR undergoes post-translational modification and regulates mRNA turnover under various stressful condition including ionizing radiation (Masuda et al., 2011). In this report, we hypothesized that treatment with X-Ray radiation on cancer cells enhances the cytoplasmic HuR level and also influences the oncolytic activity of dl355. We found cytoplasmic HuR level was upregulated with X-Ray radiation. Viral replication was tended comparatively higher with X-Ray radiated cell and showed potential cytolytic effect in cancer cells.

Material and Method

Cells and adenoviruses

In this study, we used cancer cells A549, HeLa and normal cell BJ. From the American Type Culture Collection (ATCC; USA), all cells were collected. W162 cells (a generous gift from T. Dobner) were used to generate the viruses. Cells were cultured at 37°C with 5% CO2 in Dulbecco's modified Eagle minimal essential medium (DMEM) (SIGMA-ALDRICH, D 5796, UK) containing 10% fetal bovine serum (BIOWEST, S-1650, Mexico) without antibiotics. E4orf6-deleted mutant (dl355) oncolytic adenovirus (Halbert et al., 1985) (generous gifts from Dr. T. Shenk) was used for virus infection.

The source of X-Ray

All cells were irradiated with X-Ray radiation with the presence of DMEM using CellRad X-ray generator (30 KV X-Rays), CellRad; Faxitron, Tucson, AZ, USA. After radiation, cells were incubated at 37°C and prepared for virus infection.

Western blotting

Cancer cells (A549, HeLa) were treated with Gy 1/min and Gy 2/min and incubated in 37°C for 2, 4 and 6 hours. Cytoplasmic and nuclear protein was separated using fractionation buffer (10 mM Tris HCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40 and protease inhibitor cocktail) followed by 5 minutes vigorous shaking and centrifugation at 12000 rpm for 30 sec. The supernatant was collected as a cytoplasmic protein and western blotting was performed using 10% SDS page gel.

To detect protein expression, anti-HuR 3A2 (sc-5261; Santa Cruz Biotechnology), anti-β-tubulin (cat. no. 05-661; EMD Millipore Corp., Darmstadt, Germany) antibodies were used. For secondary antibodies, HRP anti-mouse IgG (cat. no. 115-035-062; Jackson Immuno Research Laboratories) was used.

Virus proliferation assay

In 12-well plates, cells were seeded at a density of 1×10^5 cells/well for BJ cells and 5×10^4 cells/well for HeLa and A549 cells. After 4 hours of X-Ray radiation (1 Gy/min), cells were infected with dl355 at an MOI of 10 vp/cell. Cells were collected 48 hours after infection and lysed with three cycles of freezing and thawing. W162 cells were harvested in 24 well plates at a density of 2.5×10^5 cells/well and then infected with 3-fold serially diluted viruses. Viral titers (ifu/ml) were determined by using the Adeno-X^{TM} Rapid Titer kit (Cat no-632250, Clontech Laboratories, USA) according to the manufacturer's protocol.

Cytopathic effect assay (CPE assay) and cell viability assay (XTT assay)

The cells were cultured in 24-well plates (2.4×10^4 cells/well). After 24 hours, cells were radiated with 1Gy/min and incubated at 37°C for 4 hours. Cells were then infected with dl355

at an MOI of 100 (vp/cell), with and without radiation. After infection, cells were maintained for an additional 5 and 7 days and were stained with Coomassie brilliant blue.

To measure cell viability, cancer, and normal cells were seeded on 96-well plates at a density of 3.0×10^3 cells/well. XTT assays were performed using the Cell Proliferation KIT II (XTT) (Cat. No.11465015001, Roche, Germany). Cell viability was determined on days 0, 1, 3, 5 and 7.

Results

X-Ray enhanced HuR relocation to the cytoplasm

In order to identify the optimal radiation required to induce HuR translocation, we examined the levels of cytoplasmic HuR protein in cancer cells (A549, HeLa) at various X-Ray radiation (1 and 2 Gy/min) doses and incubated in 37°C for 2, 4 and 6 hours. We detected that 1 Gy/min of radiation resulted in observable HuR translocation after 4 hours in both cancer cells (A549, HeLa) (Fig. 1).

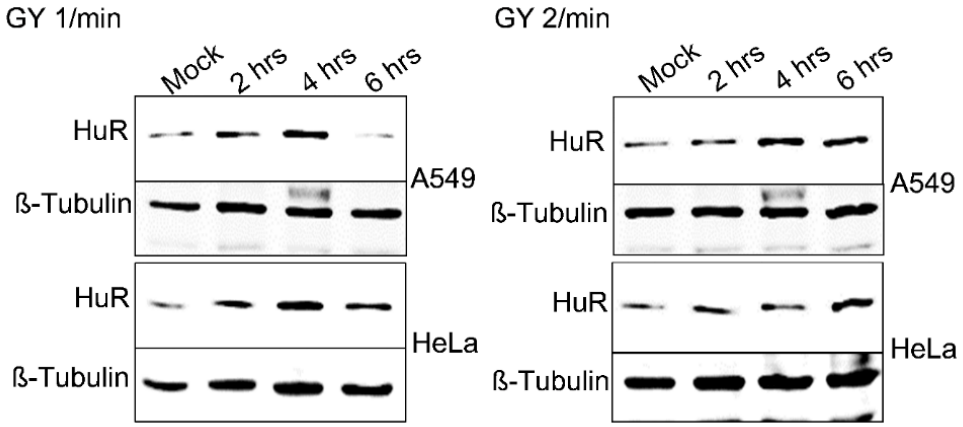


Fig. 1. Relocation of cytoplasmic HuR with X-Ray treatment.

Cytoplasmic HuR protein abundance of X-Ray irradiated cells were determined by western blotting with β-tubulin as an internal control.

X-Ray radiation up-regulates dl355 replication

To identify the viral replication with X-Ray radiation cancer cells A549, HeLa and normal cells BJ were used (Fig. 2). Viral propagation in A549 cells was higher than HeLa cells in both combination and virus-only treatments. In A549 cells, virus production was 3×10^8 ifu/ml in the combination treatment condition and 2.2×10^8 in dl355 infection only. Besides, virus production in HeLa cells infected with dl355 after radiation treatment was 2.4×10^8 ifu/ml, and 1.3×10^8 ifu/ml with dl355 infection alone. In normal cell line BJ, the virus production of combination with radiation and dl355 infected alone were 2×10^7 and 9×10^6. Together, these results indicate that X-Ray radiation influences dl355 replication both in cancer and normal cells.

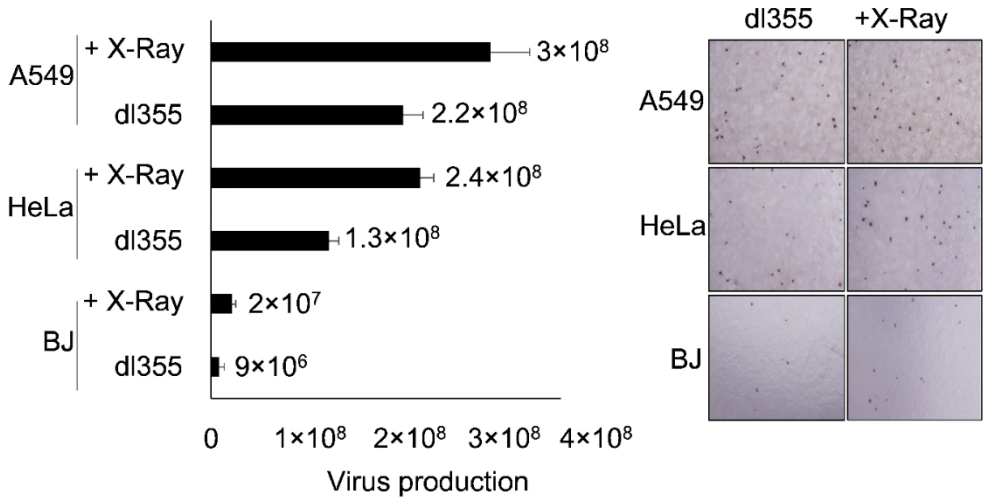


Fig. 2. X-Ray treatment upregulates viral replication.

Virus production was examined in 48 hours after infection in both cancer and normal cells infected with dl355 alone, or infected with dl355. 4 hours after X-Ray radiation. Each bar on the graph represents the virus production (ifu/ml) (left). W162 cells were used to hexon staining to determine viral load (right panel).

X-Ray increases the Cytolytic tendency of dl355

Cytolytic activity was analyzed using a cytopathic effects (CPE) assay. Cancer (A549 and HeLa) and normal (BJ) cells were either treated with X-Ray radiation alone, infected with dl355 (MOI 100 vp/cell), or infected with dl355 after 4 hours of radiation. Cells were stained

by Coomassie brilliant blue on 5 and 7 days after infection (Fig. 3A). Cytotoxicity effect was shown in both dl355 infected radiated and also only virus-infected cancer cells. But, the cytotoxic effect was comparatively higher in dl355 infected radiated cancer cells, especially in HeLa cell. Moreover, most of the normal cells persisted in both treatments (dl355 infection alone or dl355 infection after radiation), and radiation treatment alone.

Then, cell viability was checked after each treatment by XTT assay. A549, HeLa, and BJ cells were treated with the same conditions as in Fig. 3A and then XTT assay was executed. The viability of both cancer cell lines showed a downregulate tendency with dl355 infection in the radiated cells compared to the cell viability with dl355 infection alone (Fig. 3B). Cell viabilities were unaffected in uninfected cells and also both cancer and normal cells, treated with radiation only. In addition, most of normal cells were survived with dl355, combination and only X-ray radiation treatment. Together, these results give a hint that HuR augmented with X-Ray radiation can inspire the cytolytic activity of dl355.

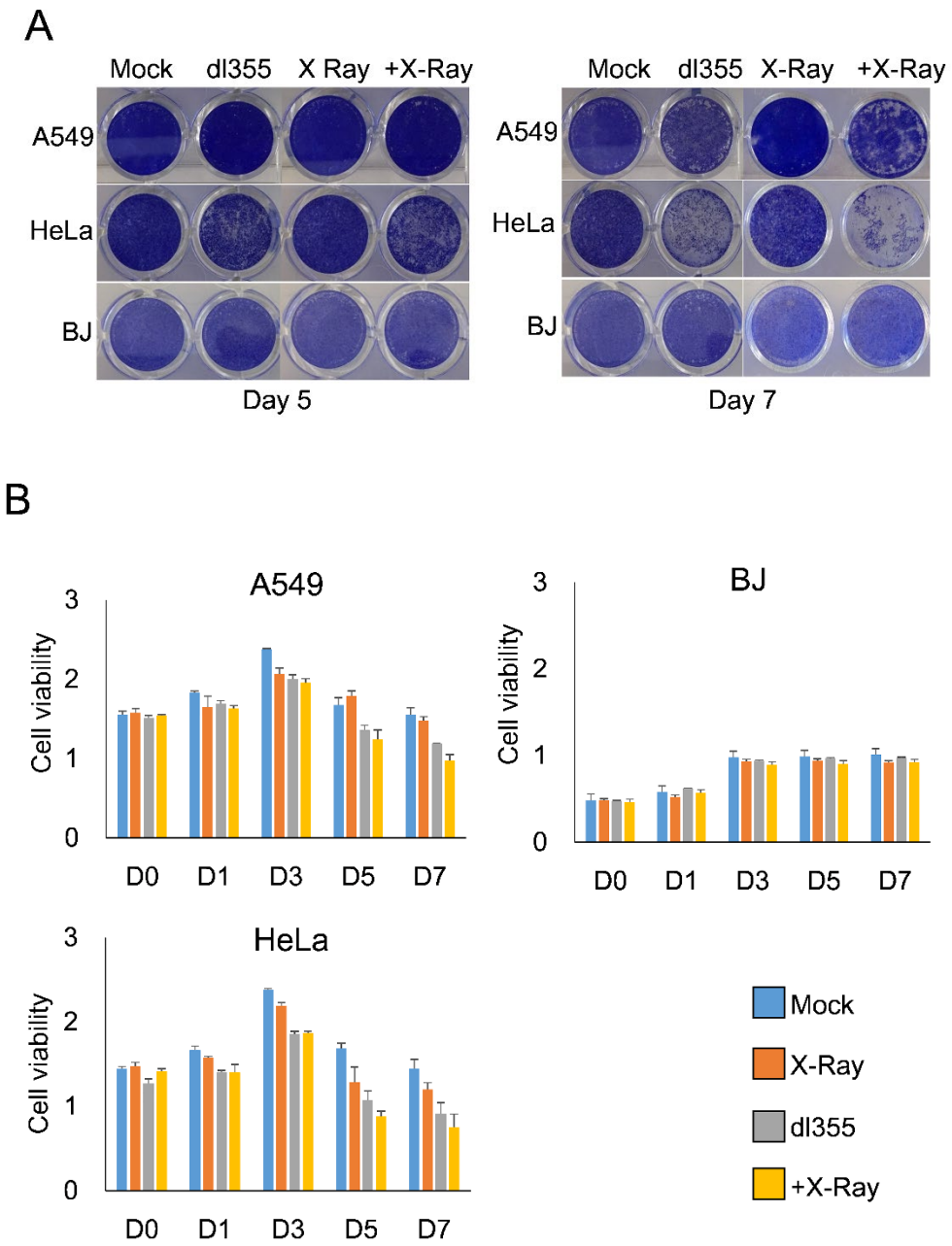


Fig. 3. The effect of X-Ray for the cytolytic activity of dl355.

(A) Cells were treated with X-Ray radiation or dl355 infection after X-Ray radiation and dl355 alone. Cytotoxicity was evaluated by Coomassie brilliant blue stain 5 and 7 days after infection. (B) With same treatment as A, cell viability was checked by XTT assay. Data are representation of three individual experiments.

Discussion

In our present studies, with X-Ray radiation, HuR was exported to the cytoplasm in both cancer cells (Fig. 1). In our other study, in cancer cells, dl355 showed enhanced virus production by HuR upregulation with ethanol treatment (Chapter 2). Nevertheless, an increased amount of HuR with X-Ray radiation showed an enhanced tendency of virus production but not as we expected in both cancer cells (Fig. 2). Moreover, the aptitude of cytolytic activity was observed in combination treatment when compared with dl355 alone and affinity to decrease cell viability was observed in both cancer cells with radiation combination in 7 days. However, the radiation can kill cells usually by changes the morphology including loss of normal nuclear structure and degradation of DNA. But our study did not reveal cytotoxic or killing effect with only X-Ray radiation treatment till day 7 (Fig. 3A) in both cancer and normal cell. And also cell viability was unchanged in only radiated cells (Fig. 3B). These results indicated that one time 1 Gy/min radiation upregulates HuR without any harmful effect, though the cytolytic effect of dl355 was not significantly exacerbated with radiation. Virus replication and cell killing depend on various mechanisms. We have not checked yet the HuR induced ARE-mRNA stabilization with X-Ray radiation. These studies have been under consideration to find out the effectiveness of dl355 activity with HuR augmentation by X-Ray radiation.

Collectively, these results demonstrated that in cancer cells with X-Ray radiation, dl355 showed an enhanced appetite for oncolytic activity when compared with dl355 infection alone. Thus, it is considered that X-Ray radiation would be one of the combination options with dl355 virotherapy.

Chapter 4

Enhancement of the activity of oncolytic adenovirus by augmenting HuR export of host cells by Ultra-Violet Radiation

Introduction

Ultraviolet radiation (UV radiation) is one of the leading environmental risk factors for various diseases. Acute exposer of UV radiation into the eye can cause photokeratitis or snow blindness and chronic exposer, resulting in an increased incidence of cataracts (Sliney et al., 1997). The UV radiation also responsible for collagen damage, decreasing skin elasticity and increasing advanced aging and wrinkling (Diffey et al., 1980, Fisher et al., 1997). Overexposure to UV radiation also the main reason for skin cancers like basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and malignant melanoma (Diffey et al., 1997). Though UV radiation can be damaging to DNA integrity and has a potentially severe side effect for the host, it is a greatly effective treatment for some chronic skin conditions like psoriasis, eczema, vitiligo, Polymorphic light eruption (PMLE) (Grims et al., 2015). Ultraviolet A (UVA) with a skin sensitizing psoralen (PUVA) is very effective (Grims et al., 2015) for these skin lesions. Current research exhibits that Ultraviolet B (UVB) treatments are less carcinogenic than conventional UVA method due to indirect DNA damage (Grims et al., 2015). In our previous investigation (Chapter 3), we evaluated the higher cytolytic tendency of dl355 by HuR augmentation with X-Ray radiation in the cancer cells.

Though UV radiation has various adverse effect, but it was reported that ultraviolet radiation plays a critical role for HuR upregulation (Wang et al., 2013) in cytoplasm though, the signaling mechanisms engaged in stabilization of mRNA with UV response have not been identified, nor has the UV induced transcript selectivity stabilization been defined. In this current report, we hypothesized that like X-Ray radiation, the up-regulation of HuR induced by UV radiation exposer in cancer cells will also enhance the oncolytic activity of dl355.

Material and Method

Cells and adenoviruses

For this experiment, cancer cells HeLa (human cervical carcinoma cell), A549 (human lung carcinoma cell), and normal cells BJ (human normal fibroblast cell) were used. All cells were taken from the American Type Culture Collection (ATCC; USA). Virus was generated by usingW162 cells (generous gift from T. Dobner). Dulbecco's modified Eagle minimal essential medium (DMEM) (SIGMA-ALDRICH, D 5796, UK) containing 10% fetal bovine serum (BIOWEST, S-1650, Mexico) was used for cells culture and was incubated at 37°C without antibiotics. For virus infection, oncolytic adenovirus dl355 was used (generous gifts from Dr. T. Shenk).

The source of UV radiation

The cells were radiated with UV radiation in the presence of DMEM. For Radiation, UVP CL-1000 Ultraviolet Crosslinker, Upland, CA 91786 USA was used. After UV radiation cells were incubated at 37°C.

Western blotting

To check cytoplasmic HuR exportation with UV radiation, cancer cells (A549, HeLa) were treated with 10 J/m^2 and 15 J/m^2. After UV radiation, cells were incubated 2, 4 and 6 hours. Cytoplasmic and nuclear protein was separated using fractionation buffer (10 mM Tris HCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40 and protease inhibitor cocktail) followed by 5 minutes vigorous shaking and centrifugation at 12000 rpm for 30 sec. The supernatant was collected as a cytoplasmic protein.

To detect HuR protein expression, anti-HuR 3A2 (sc-5261; Santa Cruz Biotechnology), anti-β-tubulin (cat. no. 05-661; EMD Millipore Corp., Darmstadt, Germany), antibodies were used. For secondary antibodies, HRP anti-mouse IgG (cat. no. 115-035-062; Jackson Immuno Research Laboratories) was used.

Virus proliferation assay

With a density of 1×10^5 cells/well for BJ cells and 5×10^4 cells/well for HeLa and A549 cells were seeded in 12 well plates. After 24 hours, cells were radiated with UV radiation (10 J/m^2). Then after 6 hours, cells were infected with dl355 at an MOI of 10 (vp/cell). 48 hours after infection, cells were collected and lysed with three cycles of freezing and thawing. W162 cells were seeded at a density of 2.5×10^5 cells/well in 24-well plates and then infected with 3-fold serially diluted viruses. Viral titers (ifu/ml) were surveyed by using the Adeno-X^{TM} Rapid Titer kit (Cat no-632250, Clontech Laboratories, USA) according to the manufacturer's protocol.

Cytopathic effect assay (CPE assay) and cell viability assay (XTT assay)

Cells were cultured in 24-well plates (2.4×10^4 cells/well) and were incubated for 24 hours in 37°C. After that, cells were radiated with 10 J/m^2 and incubated for 6 hours. Cells were then infected with dl355 at an MOI of 100 (vp/cell), with and without radiation. After infection, cells were incubated for 5 and 7 days and were stained with Coomassie brilliant blue.

To acquire the cell viability condition, cancer, and normal cells were harvested on 96-well plates at a density of 3.0×10^3 cells/well. XTT assays were carried out using the Cell Proliferation KIT II (XTT) (Cat. No.11465015001, Roche, Germany). Cell viability was determined on days 0, 1, 3, 5 and 7.

Results

UV radiation enhanced HuR expression in cancer cell

In order to recognize the best radiation required to encourage HuR translocation, we inspected the levels of cytoplasmic HuR protein in cancer cells (A549, HeLa) at several UV radiation (10 J/m^2 and 15 J/m^2) doses (Fig. 1). We identified that with both 10 and 15 J/m^2 of radiation resulted in observable HuR translocation. 15 J/m^2 UV radiation showed enhanced HuR expression in both 4 and 6 hours in A549 cancer cell. But with 10 J/m^2 radiation, HuR expression was high level after 6 hours in A549. On the other hands, in HeLa cell, HuR expression was not enhanced in both 10 and 15 J/m^2 UV radiation. Interestingly, HuR expression was suddenly downregulated with both 10 and 15 J/m^2 of UV in A549 cells in 2 hours after radiation. In the case of HeLa cells, HuR expression was diminished in both after 2 hours of 15 J/m^2 radiation and slightly increased in 4 and 6 hours of radiation. With 10 J/m^2 of radiation no changes of HuR expression were detected.

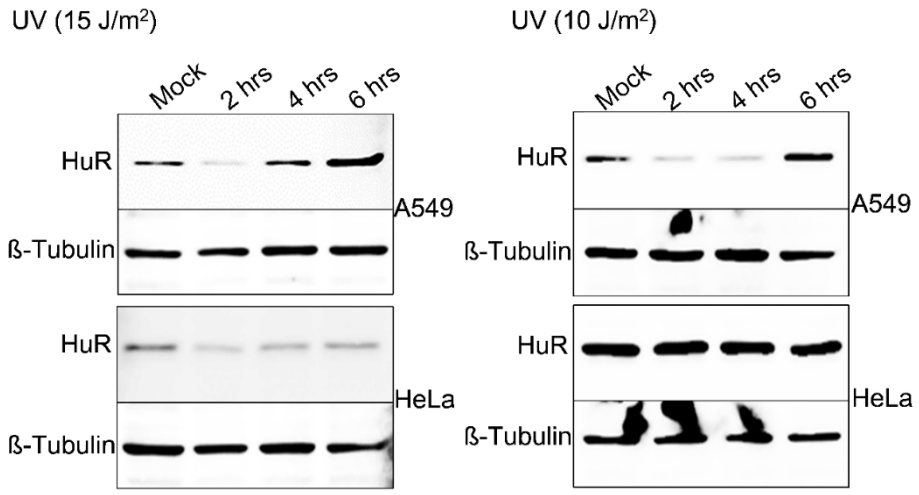


Fig. 1. Relocation of cytoplasmic HuR with UV treatment.

A549 and HeLa were radiated with 10 and 15 J/m^2. Cytoplasmic HuR expression was checked in 2, 4, and 6 hours after UV radiation by western blotting with β-tubulin as an internal control.

dl355 replication was not influenced by UV radiation

To know whether the amplified cytoplasmic HuR levels with the UV radiation affects the replication efficiency of dl355, virus titer assay was performed. As a consideration of lower dose, cancer and normal cells were treated with 10 J/m^2. dl355 was infected at an MOI 10 vp/cell after 6 hours of radiation and its replication was assessed using two cancer cells, HeLa, A549 and also normal cells BJ. In both cell types, unfortunately, no inequality was detected between radiated and only virus treated cells (Fig. 2). Virus propagation was least higher in combination treatment but almost similar.

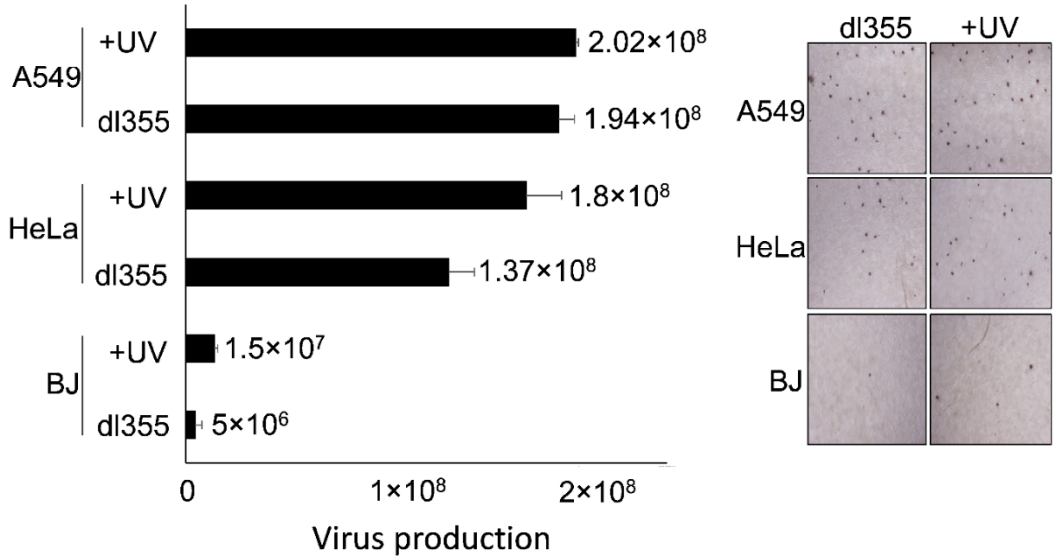


Fig. 2. UV treatment upregulates viral replication.

Virus production was evaluated in 48 hours after infection in both cancer and normal cells with dl355 alone and with UV radiation. Virus production (ifu/ml) indicates each bar of the graph (left). Hexon staining of W162 cells was used to determine viral load (right).

No Cytolytic disparity was detected with UV radiation

Cytopathic effect assay (CPE) was executed to find out the cytolytic effect of dl355 augmented by UV radiation. Cancer (A549 and HeLa) and normal (BJ) cells were either treated with UV radiation alone, infected with dl355 (MOI 100 vp/cell), or infected with dl355 after 6 hours of UV radiation. The living cells were stained with Coomassie brilliant blue on 5 and 7

days after infection (Fig. 3A). Cytotoxic effect was shown in both UV radiation with or without dl355 infected, UV radiated and non-radiated cancer cells. But, cytotoxicity tendency was almost same between radiated and non-radiated dl355 infected cancer cells. Furthermore, most of the normal cells persisted in both treatments with dl355 (dl355 infection alone or dl355 infection after radiation). And radiation treatment alone showed no cytotoxic effects in either the cancer or normal cell lines.

Then, cell viability after each treatment was assessed by XTT assay. A549, HeLa, and BJ cells were treated with the same conditions as CPE assay, and XTT assay was implemented on day 0, 1 ,3, 5 and 7 days after treatment. The viability of both cancer cell lines were decreased in radiation combination and in only infected with dl355 (Fig. 3B). There was no dissimilation observed between two treatments option. Cell viabilities were unchanged in uninfected cells, both cancer and normal, treated with radiation only. And 7 days after dl355 infection alone, or in combination with radiation, most normal cells were survived. This result suggested that MOI 100 vp/cell infection with UV radiation (10 J/m^2 and 15 J/m^2) is safer dose for normal cells.

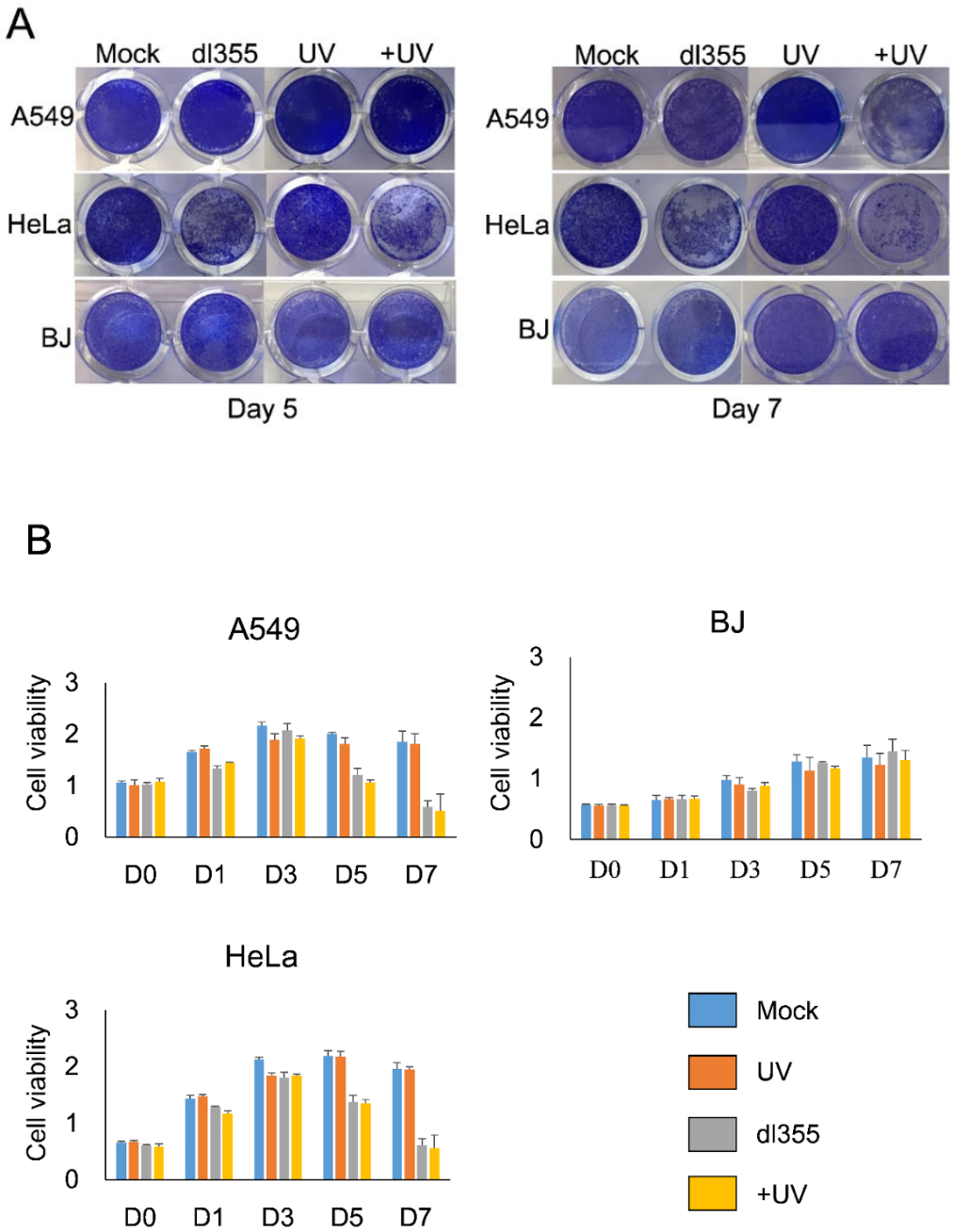


Fig. 3. The effect of UV for the cytolytic activity of dl355.

(A) Cytolytic effect with UV radiation, dl355 and dl355 infection after UV radiation was evaluated by CPE assay. (B) Cytolytic effect with the same treatments were evaluated by XTT assay. Data are representation of three individual experiments.

Discussion

In this study, we found that cytoplasmic HuR level was increased by low levels of UV irradiation in cancer cells (Fig. 1). This result suggests that HuR might bind to ARE-mRNA in a UV-dependent manner, which might lead to viral replication. To proof that, we checked the viral production and found an enhanced tendency of virus production with UV radiation but not as our expectation in both cancer cells (Fig. 2). In our previous investigation also, we found dl355 enhanced virus production with ethanol and X-ray treatment in the cancer cells (Chapter 2 and 3).

In this study, we observed the cytolytic activity in combination of dl355 and UV radiation by CPE assay. We found that, low dose of UV radiation, like 10 J/m^2, treatment has no cytotoxic effect on both cancer and normal cells (Fig. 3A). And the cell viability also showed the same tendency (Fig. 3B) with CPE assay. Taken together of these results indicated that, such dose of UV radiation can upregulates cytoplasmic HuR but the cytolytic effect was not significantly exacerbated even in combination with dl355. We need to focus on to fix an effective UV radiation dose and time courses for the best combination treatment.

It is still unclear the relationship of ARE-mRNA stabilization by UV radiation. Previous reports showed the binding of HuR with ARE-mRNAs in normal cells to promote their stabilization (Wang et al., 2013) but no studies were addressed in between HuR mediated stabilization via UV radiation. In these studies, we focused to find out the effectiveness of dl355 activity with HuR augmentation by UV radiation. Further studies are required to get answer of these mechanism.

Collectively, our results demonstrated that UV radiation and dl355 combination treatment showed a tendency to enhance oncolytic activity in cancer cells compared dl355 infection alone. Thus, if we could answer all those above, it will be considered that UV radiation would be one of the combination options with dl355 virotherapy.

Conclusion and future prospect

In this study, we found that several HuR export enhancers can up-regulate the activity of oncolytic adenovirus dl355. dl355 has deletion in E4orf6 gene, which has potential to export HuR to the cytoplasm of cells and stabilize ARE-mRNA. Since ARE-mRNA stabilization is required for adenovirus replication, E4orf6-deleted dl355 fail to replicate in normal cells. However, ARE-mRNA is constitutively stabilized in cancer cells, E4orf6 is not necessary for adenovirus replication. That is the reason why dl355 can replicate in cancer cells but not in normal cells.

Ethanol was able to enhance the relocalization of HuR to the cytoplasm and up-regulated dl355 replication and its cytolytic activities. X-Ray also facilitated the export of HuR to the nucleus and slightly increased the activity of dl355. In the case of UV, however, the activity of dl355 could not be increased despite a slight increase in the export of HuR. At present, there is no answer to this reason, but we think it will be necessary to consider the conditions such as UV irradiation in the future.

The effect of dl355 has been proven in vivo experiments using animals. In the future, it will be necessary to study the combined effects of ethanol and x-rays in the same experimental system. Furthermore, it is necessary to add a study aiming at clinical research and develop a treatment method using this virus into a cancer treatment method that can contribute to humankind.

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References:

Aghi, M., Hochberg, F., Breakefield, X. O. (2000). Prodrug activation enzymes in cancer gene therapy. J Gene Med. 2, 148–164.

Berk, A. J. (2013). Adenoviridae, Fields virology, D.M Knipe, P.M. Howley Eds. (Philadelphia, PA, USA. : Lippicott Williams & Wilkins Ltd), pp. 1704-1731.

Berk, A.J. (2006). Adenoviridae: the viruse and their replication, Fields virology, In: HowleyDMKP ed. (Lippincott Williams & Wilkins, Philadelphia), pp 2354-2394.

Ben-Israel, H., Kleinberger, T. (2002). Adenovirus and cell cycle control. Front Biosci. 7, d1369-1395.

Barker, D.D., and Berk, A. J. (1987). Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. Virology. 156, 107-121.

Baker, A.T., Aguirre-Hernández, C., Halldén, G., & Parker, A.L. (2018). Designer oncolytic adenovirus: Coming of age. Cancers. 10(6), 201.

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

Bridge, E., and Ketner, G. (1989). Redundant control of adenovirus late gene expression by early region 4. J. Virol. 63(2), 631-638.

Brennan, C. M., Gallouzi, I. E., Steitz, J. A. (2000). Protein ligands to HuR modulate its interaction with target mRNAs in vivo. J. Cell Biol. 151, 1–14.

Brennan, C. M., Steitz., J. A. (2001). HuR and mRNA stability. Cell Mol Life Sci. 58, 266-277.

Bressy, C., Benihoud, K. (2014). Association of oncolytic adenoviruses with chemotherapies: An overview and future directions. Biochem Pharmacol. 90, 97-106.

Blanchette, P., Kindsmuller, K., Groitl, P., Dallaire, F., Speiseder, T., Branton, P. E., Dobner, T. (2008). Control of mRNA export by adenovirus E4orf6 and E1B55K proteins during productive infection requires E4orf6 ubiquitin ligase activity. J Virol. 82, 2642-2651.

Cuconati, A., Degenhardt, K., Sundararajan, R., Anschel, A., White, E. (2002). Bak and Bax function to limit adenovirus replication through apoptosis induction. J Virol. 76 (9), 4547-4558.

Chen, Y., DeWeese, T., Dilley, J., Zhang, Y., Li, Y., Ramesh, N., Lee, J., Pennathur-Das, R., Radzyminski, J., Wypych, J., Brignetti, D., Scott, S., Stephens, J., Karpf, D. B., Henderson, D. R., Yu, D. C. (2001). CV706, a prostate cancer-specific adenovirus variant, in combination with radiotherapy produces synergistic antitumor efficacy without increasing toxicity. Cancer Res. 61, 5453–5460.

Chu, R.L., Post. D. E., Khuri, F. R., Meir, E. G. V. (2004). Use of Replicating Oncolytic Adenoviruses in Combination Therapy for Cancer. Clinical Cancer Research. 10, 5299-5312.

Diffey, B. L. (1980). Ultraviolet radiation physics and the skin. Phys Med Biol. 25(3), 405–426.

Dobner, T., Kzhyshowska, J. (2001). Nuclear export of adenovirus RNA. Curr. Top. Microbiol. Immunol. 259, 25-54.

Diffey, B. L., Hart, G. C. (1997). Ultraviolet and Blue-light Phototherapy: Principles, Sources, Dosimetry and Safety. Report 76, York: IPEM, 27.

Debbas, M., White, E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. 7, 546-554.

Dalmau, J., Furneaux, H.M., Cordon-Cardo, C., Posner, J.B. (1992). The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues. Am J Pathol. 141 (4), 881–886.

Del Prete, S., Caraglia, M., Russo, D., Vitale, G., Giuberti, G., Marra, M., D'Alessandro, A. M., Lupoli, G., Addeo, R., Facchini, G., Rossiello, R., Abbruzzese, A., Capasso, E. (2004). Percutaneous ethanol injection efficacy in the treatment of large symptomatic thyroid cystic nodules: ten-year follow-up of a large series. Thyroid. 12(9), 815-821.

Esclatine, A., Taddeo, B., Evans, L., Roizman, B. (2004). The herpes simplex virus 1 U_L41 gene-dependent destabilization of cellular RNAs is selective and may be sequence-specific. PNAS. 101, 3603–3608.

Freytag, S. O., Stricker, H., Pegg, J., Paielli, D., Pradhan, D. G., Peabody, J., DePeralta-Venturina, M., Xia, X., Brown, S., Lu, M., Kim, J. H. (2003). Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate-to high-risk prostate cancer. Cancer Res, 63, 7497–7506.

Fisher, G. J., Wang, Z. Q., Datta, S. C., Varani, J., Kang, S., and Voorhees, J. J. (1997). Pathophysiology of premature skin aging induced by ultraviolet light. N Engl J Med. 337(20), 1419–1428.

Grimes, D. R. (2015). Ultraviolet radiation therapy and UVR dose model. Med Phys. 42 (1), 440-455.

Guo, ZS., Thorne, SH., Bartlett, DL. (2008). Oncolytic virotherapy: Molecular targets in tumor-selective replication and carrier-cell mediated delivery of oncolytic viruses. Biochim Biophys Acta. 1785, 217-31.

Geoerger, B., Grill, J., Opolon, P., Morizet, J., Aubert, G., Lecluse, Y., Beusechem, V.W.V, Gerritsen W. R., Kirn, D. H., Vassal, G. (2003). Potentiation of radiation therapy by the oncolytic adenovirus dl1520 (ONYX-015) in human malignant glioma xenografts. Br J Cancer, 89, 577–584.

Gallouzi, I. E., Steitz, J. A. (2001). Delineation of mRNA export pathways by the use of cell-permeable peptides. Science. 294, 1895-1901.

Halbert, D. N., Cutt, J. R., Shenk, T. (1985). Adenovirus Early Region 4 Encodes Functions Required for Efficient DNA Replication, Late Gene Expression, and Host Cell Shutoff. J Virol. 56(1), 250-257.

Higashino, F., Aoyagi, M., Takahashi, A., Ishino, M., Taoka, M., Isobe, T., Kobayashi, M., Totsuka, Y., Kohgo, T., Shindoh, M. (2005). Adenovirus E4orf6 targets pp32/LANP to control the fate of ARE-containing mRNAs by perturbing the CRM1-dependent mechanism. J Cell Biol. 170(1), 15-20.

Herrmann, C.H., Dery, C. V., Mathews, M .B. (1987). Transactivation of host and viral genes by the adenovirus E1B 19K tumor antigen. Oncogene. 2(1), 25-35.

Harada, J.N., Shevchenko, A., Shevchenko, A., Pallas, D. C. and Berk, A. J. (2002). Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. J. Virol. 76, 9194–9206.

Hinman, M. N., Lou, H. (2008). Diverse molecular functions of Hu proteins. Cell Mol Life Sci. 65(20), 3168-3181.

Jehung, J. P., Kitamura, T., Matsuda, A. Y., Kuroshima, T., Towfik, A., Yasuda, M., Sano, H., Kitagawa, Y., Minowa, K., Shindoh, M., Higashino, F., (2018). Adenovirus infection induces HuR relocalization to facilitate virus replication. Biochem. Biophys. Res. Commun. 495, 1795-1800.

Kruyt, FA., Curiel, DT. (2002). Toward a new generation of conditionally replicating adenoviruses: pairing tumor selectivity with maximal oncolysis. Hum Gene Ther. 13, 485-495.

Kim, J., Lee, B., Kim, J. S., Yun, C. O., Kim, J. H., Lee, Y. J., Joo, C. H., Lee, H. (2002). Antitumoral effects of recombinant adenovirus YKL-1001, conditionally replicating in α-fetoprotein-producing human liver cancer cells. Cancer Lett. 180(1), 23–32.

Khuri, F.R., Nemunaitis, J., Ganly, I., Arseneau, J., Tannock, I. F., Romel, L., Gore, M., Ironside, J., MacDougall, R. H., Heise, C., Randlev, B., Gillenwater, A. M., Bruso, P., Kaye, S. B., Hong, W. K., Kirn, D. H. (2000). A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. Nat Med. 6, 879–885.

King, P.H., Dropcho, E.J. (1996). Expression of Hel-N1 and Hel-N2 in small-cell lung carcinoma. Ann. Neurol. 39, 679–681.

King, P.H. (1997). Differential expression of the neuroendocrine genes Hel-N1 and HuD in small-cell lung carcinoma: Evidence for down-regulation of HuD in the variant phenotype. Int. J. Cancer. 74, 378–382.

Kelly, E., Russell, SJ. (2007). History of Oncolytic Viruses: Genesis to Genetic Engineering, Molecular Therapy. 15, 651–659.

Kuroshima, T., Aoyagi, M., Yasuda, M., Kitamura, T., Jehung, J. P., Ishikawa, M., Kitagawa, Y., Totsuka, Y., Shindoh, M., Higashino, F. (2011). Viral mediated stabilization of AU-rich element containing mRNA contributes to cell transformation. Oncogene. 30, 2912-2920.

Lopez de Silanes, I., Lal, A., Gorospe, M. (2005). HuR: post-transcriptional paths to malignancy. RNA Biol. 2, 11-13.

Lowe, S.W. and Ruley, H. E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. 7(4), 535-545.

Li, Y., Chen, Y., Dilley, J., Arroyo, T., Ko, D., Working, P., & Yu, D. C. (2003). Carcinoembryonic antigen-producing cell-specific oncolytic adenovirus, OV798, for colorectal cancer therapy. Mol Cancer Ther. 2, 1003–1009.

Lamont, J. P., Nemunaitis, J., Kuhn, J. A., Landers, S. A., McCarty, T. M. (2000). A prospective phase II trial of ONYX-015 adenovirus and chemotherapy in recurrent squamous cell carcinoma of the head and neck (the baylor experience). Ann Surg Oncol. 7, 588–592.

Lamfers, M. L. M, Grill, J., Dirven, C. M. F, Beusechem, V. W. V, Geoerger, B., Berg, J. V. D, Alemany, R., Fueyo, J., Curiel, D. T., Vassal, G., Pinedo, H. M., Vandertop, W. P., Gerritsen, W. R. (2002). Potential of the conditionally replicative adenovirus Ad5-Delta24RGD in the treatment of malignant gliomas and its enhanced effect with radiotherapy. Cancer Res. 62, 5736–5742.

Leppard, K. N. (1997). E4 gene function in adenovirus, adenovirus vector and adeno-associated virus infections. J. Gen. Virol. 78, 2131-2138.

Lencioni, R., Llovet, J. M. (2005). Percutaneous ethanol injection for hepatocellular carcinoma: Alive or dead?, J Hepatol. 43, 377-380.

Livraghi, T., Paracchi, A., Ferrari, C., Reschini, E., Macchi, R. M., Bonifacino, A. (1994). Treatment of autonomous thyroid nodules with percutaneous ethanol injection: 4-year experience. Radiology. 190(2), 529-533.

Matsuda, A. Y., Mikawa, Y., Habiba, U., Kitamura, T., Yasuda, M., Alam, M. T., Kitagawa, Y., Miowa, K., Shindoh, M., and Higashino, F. (2019). Oncolytic potential

of an E4-deficient adenovirus that can recognize the stabilization of AU-rich element containing mRNA in cancer cells. Oncol Rep. 41(2), 954-960.

Maroun, J., Muñoz-Alía, M., Ammayappan, A., Schulze, A., Peng, K. W. & Russell, S. (2017). Designing and building oncolytic viruses. Future Virol. 12(4), 193-213.

McCormick, C., Ganem, D. (2005). The kaposin B protein of KSHV activates the p38/MK_2 pathway and stabilizes cytokine mRNAs. Science. 307, 739–741.

Masuda, K., Abdelmohsen, K., Kim, M. M., Srikantan, S., Lee, E. K., Tominaga, K., Selimyan, R., Martindale, J. L., Yang, X., Lehrmann, E., Zhang, Y., Becker, K. G., Wang, J. Y., Kim, H. H., and Gorospe, M. (2011). Global dissociation of HuR-mRNA complexes promotes cell survival after ionizing radiation. EMBO J. 30(6), 1040–1053.

Pack, GT. (1950). Note on the experimental use of rabies vaccine for melanomatosis. AMA Arch Derm Syphilol. 62, 694-5.

Querido, E., Blanchette, P., Yan, Q., Kamura, T., Morrison, M., Boivin, D., Kaelin, W. G., Conaway, R. C., Conaway, J. W., Branton, P. E. (2001). Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev. 15(23), 3104-3117.

Rebane, A., Aab, A., Steitz, J. A. (2004). Transportins 1 and 2 are redundant nuclear import factors for hnRNP A1 and HuR. RNA.10 (4), 590-599.

Rodriguez, R., Schuur, E. R., Lim, H. Y., Henderson, G. A., Simons, J. W., & Henderson, D. R. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: A selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Research. 57, 2559–2563.

Rogulski, K. R., Freytag, S. O., Zhang, K., Gilbert, J. D., Paielli, D. L., Kim, J. H., Heise, C. C., Kirn, D. H. (2000). In vivo antitumor activity of ONYX-015 is influenced by p53 status and is augmented by radiotherapy. Cancer Res. 60, 1193–1196.

Ring, CJA. (2002). Cytolitic viruses as potential anti-cancer agents. J Gen Virology. 83, 491-502.

Russell, W. C. (2000). Update on adenovirus and its vectors. J Gene Virology. 81, 2573–2604.

Ravindra, PV., Tiwari, AK., Ratta, B., Bais, MV., Chaturvedi, U., Palia, SK., Sharma, B., Chauhan, RS. (2009). Time course of Newcastle disease virus-induced apoptotic pathways. Virus Res. 144, 350-354.

Rao, XM., Zheng, X., Waigel, S., Zacharias, W., McMasters, KM., Zhou, HS. (2006). Gene expression profiles of normal human lung cells affected by adenoviral E1B. Virology. 350 (2), 418-428.

Ries, S., & Korn, W. M. (2002). ONYX-015: mechanisms of action and clinical potential of a replication-selective adenovirus. British Journal of Cancer. 86, 5-11.

Sliney, D. H. (1997). Ultraviolet radiation effects upon the eye: Problems of dosimetry. Radiat Prot Dosim. 72, 197–206.

Seth, P. and Higginbotham, J. (2000). Advantages and disadvantages of multiple different methods of adenoviral vector construction. Methods MOl Med. 45, 189-198.

Stillman, B. (1986). Functions of the adenovirus E1B tumor antigens. Cancer Surv. 5(2), 389-404.

Sung, J. Y., Baek, J. H., Kim, Y. S., Jeong, H. J., Kwak, M. S., Lee, D., Moon, W. J. (2008). One-step ethanol ablation of viscous cystic thyroid nodules. AJR. 191, 1730-1733.

Sarnow, P., Hearing, P., Anderson, C. W., Halbert, D. N., Shenk, T., Levine, A. J. (1984). Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. J Virol. 49(3), 692-700.

Vakalopoulou, E., Schaack, J., Shenk, T. (1991). A 32-kilodalton protein binds to AU-rich domains in the 3' untranslated regions of rapidly degraded mRNAs. Mol Cell Biol. 11(6), 3355-3364.

Veldman, M. W., Reading, C. C., Farrell, M. A., Mullan, B. P.,Wermers, R. A., Grant, C. S.,Thompson, G. B. (2008). Percutaneous parathyroid ethanol ablation in patients with multiple endocrine neoplasia type 1. AJR. 191(6), 1740-1744.

Vaha-Koskela, MJV., Heikkila, JE., Hinkkanen, AE. (2007). Oncolytic viruses in cancer therapy. Cancer Lett. 254, 178-216.

Wang, J., Guo, Y., Chu, H., Guan, Y., Bi, J., Wang, B. (2013). Multiple Functions of the RNA-Binding Protein HuR in cancer progression, Treatment Responses and Prognosis. Int. J. Mol. Sci. 14 (5), 10015-10041.

Woo, J. L., Berk, A. J. (2007). Adenovirus ubiquitin-protein ligase stimulates viral late mRNA nuclear export. J Virol. 81(2), 575-587.

Wildner, O., Blaese, R. M., Morris, J. C. (1999). Therapy of colon cancer with oncolytic adenovirus is enhanced by the addition of herpes simplex virus-thymidine kinase. Cancer Res. 59, 410–413.

Wang, W., Furneaux, H., Cheng, H., Caldwell, M. C., Hutter, D., Liu, Y., Holbrook, N. & Gorospe, M. (2000). HuR regulates p21 mRNA stabilization by UV light. Mol. Cell. Biol. 20 (3), 760–769.

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

Zaigham, A., and Rehman, S. (2018). An Overview of Cancer Treatment Modalities. http://dx.doi.org/10.5772/intechopen.76558.

Zheng, X., Rao, X. M., Snodgrass, C. L., McMasters, K. M., and Zhou, H. S. (2006). Selective replication of E1B55K-deleted adenoviruses depends on enhanced E1A expression in cancer cells. Cancer Gene Ther. 13(6), 572-583.

Zheng, X., Rao, X. M., Gomez-Gutierrez, J. G., Hao, H., McMasters, K. M., and Zhou. H. S. (2008). Adenovirus E1B55K region is required to enhance cyclin E expression for efficient viral DNA replication. J Virol. 82(7), 3415-3427.

Zhang, L., Akbulut, H., Tang, Y., Peng, X., Pizzorno, G., Sapi, E., Manegold, S., Deisseroth, A. (2002). Adenoviral vectors with E1A regulated by tumor-specific promoters are selectively cytolytic for breast cancer and melanoma. Mol Ther. 6(3), 386–393.