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A Novel Therapy for Melanoma and Prostate Cancer Using a Non-Replicating Sendai Virus Particle (HVJ-E)

Toshihiro Nakajima, Toshimitsu Itai, Hiroshi Wada, Toshie Yamauchi, Eiji Kiyohara and Yasufumi Kaneda

Additional information is available at the end of the chapter

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1. Introduction

The virotherapy approach to cancer therapy uses virus particles. It is based on the case reports since 1950s, which reported the regression of cancers including leukemia, Hodgkin's disease, and Burkitt's lymphoma after the infection of wild type viruses [1-11].

The earliest virotherapies involved injection of wild-type viruses and evaluation of their efficacies [1-3]. *Ex vivo* therapies using autologous irradiated tumors infected with oncolytic viruses were also investigated [12-18]. Deletion mutants of oncolytic viruses [19-24], and recombinant viruses carrying a therapeutic gene [25-32] that induce cancer apoptosis or cancer immunity have been developed and evaluated in the clinical setting.

Oncolytic viruses derived from adenovirus, poxvirus, reovirus, picornavirus, paramyxovirus, and herpes simplex virus are currently available and have been clinically evaluated [33-35]. In China, two adenovirus-based products (Gendicine and Ocorine) have been commercialized [36], while randomized phase III studies of two oncolytic viruses (reovirus and poxvirus) are ongoing in advanced countries [33-35]. Thus, virotherapy is expected to become available as a new approach for cancer treatment, and specific product approval is anticipated in the US, EU, and Japan [37].

The major drawback associated with virotherapy is safety since replicating viruses are used in this therapy. In order to reduce the toxicity to normal cells, oncolytic viruses with strict specificity for cancer cells were constructed [29, 38-42]. However, the use of these viruses is still considered to be high risk because it is theoretically possible that a virulent infection may occur after recombination with wild-type viruses [43].



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An alternative option to avoid such a risk is to use a non-replicating oncolytic virus [44]. We found that a non-replicating oncolytic virus (HVJ-E: hemagglutinating virus of Japanenvelope) is able to induce cancer cell-specific apoptosis and immunity [45]. The induction of apoptosis and activation of dendritic cells *in vitro*, and anti-tumor activity *in vivo* are similar to the wild-type hemagglutinating virus of Japan (also known as Sendai virus, HVJ) [45].

The hemagglutinating virus of Japan was discovered in Sendai, Japan, in the 1950s [46]. It is a paramyxovirus with a minus-strand RNA genome. The virus has fusogenic activity [47, 48], and is used to prepare hybridoma cells for the production of monoclonal antibodies, and heterokaryons for chromosome analysis [49-51].

The hemagglutinating virus of Japan-envelope is an inactivated HVJ particle [52]. It is manufactured by a process similar to that used for whole virus particle vaccines. Good manufacturing practice (GMP)-regulated processes have been established in their production for use in preclinical and clinical studies [53].

We conducted dose-setting efficacy studies for HVJ-E in a murine cancer model in which dosedependent anti-cancer activity was observed. We also conducted safety studies following good laboratory practices (GLP), including pharmacological safety studies and toxicokinetic (TK) studies in rats and monkeys, as part of an investigational new drug (IND) application.

Osaka University Hospital is currently conducting two investigational clinical studies with HVJ-E for the treatment of advanced melanoma and castration-resistant prostate cancer (CRPC) [54-56]. These clinical trials are the first human studies for HVJ-E and will reveal the safety and efficacy of the non-replicating virus (HVJ-E). Virotherapy with a non-replicating oncolytic virus is a new approach that is anticipated to provide a new strategy for cancer therapy.

2. A new strategy for cancer therapy

Most cancers are still incurable and new approaches are required to improve the efficacy of cancer treatments. However, conventional cancer therapies are problematic.

Chemotherapy with anti-cancer agents is useful in achieving tumor regression. However, the immune system, which is important in the removal of residual cancer cells, is also suppressed by these agents (Figure 1). Therefore, surviving cancer cells and cancer stem cells (CSC) eventually acquire drug resistance, resulting in tumor relapse (Figure 1) [57]. Thus, chemotherapy with cytotoxic drugs does not generally result in the necessary eradication of cancer cells required for long-term survival.

Immune therapies for cancer offer a new approach to cancer treatment, and several products, including sipuleucel-T, are currently approved in advanced countries [58, 59]. The aim of these therapies is the removal of cancers by the immune system. Numerous cancer immune therapies are currently under evaluation in clinical studies. However, these agents are not potent because of lack of cytotoxic effect on cancer cells (Figure 1).

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Figure 1. Problems with conventional therapies and a 2-step strategy for cancer treatment. Conventional anticancer therapies are problematic and a 2-step therapeutic strategy is proposed for effective cancer treatment. Virotherapy possesses ideal characteristics for such a 2-step therapy. Problems associated with conventional anticancer drugs and immune therapies, including cancer vaccines, are shown.

These observations suggest that a two-step strategy is necessary for the eradication of cancer cells (Figure 1).

During the first step, the direct killing of cancer cells is necessary for reduction of tumor volume. CSCs are usually resistant to conventional anti-cancer drugs and continue to proliferate during chemotherapy. Therefore, an agent that targets and kills CSCs is required for effective cancer treatment.

During the second step, the removal of residual cancer cells (and CSCs) from the body by a cancer-specific immune response is necessary to avoid relapse of the condition [57]. However, it is difficult for immune cells to recognize and remove CSCs because they exist as a minority population within the tumor, and possess a lower antigenicity than differentiated cancer cells [57]. Oncolytic viruses have the capability of both directly killing cancer cells and inducing cancer immunity.

It has been reported that several oncolytic viruses have the capability to kill CSCs [60-67]. The reovirus-based oncolytic virus [61], telomerase-specific oncolytic adenovirus [62], and herpes simplex virus-based oncolytic viruses (G47Delta and Delta 68H-6) [63, 64] reduced CSCs in murine models of breast cancer, esophageal cancer, and malignant glioma, respectively. Thus, a virotherapy approach in patients is expected to kill cancer cells and eradicate cancer cells including CSCs (Figure 1).

3. Non-replicating virus particles as anti-cancer agents

The use of non-replicating virus particles is a new approach in virotherapy.

A non-replicating virus particle, HVJ-E, is currently being developed as a potential new agent for the treatment of advanced melanoma and CRPC [44, 55, 56]. It is derived from HVJ, a

member of the paramyxovirus family (Figure 2). The HVJ-E particle is prepared by inactivating the wild-type virus (HVJ) by treatment with an alkylating agent and UV irradiation [52, 53]. HVJ-E was originally developed as a drug delivery system (vector) for various biopharmaceuticals such as plasmid DNAs, siRNAs, decoy oligonucleotides, antibody proteins, and anticancer drugs [52, 68-75].

Kurooka and Kaneda discovered that the HVJ-E particle itself displayed anti-cancer effects in a murine model of colon cancer [45]. Similar to the live (replicating) virus, HVJ-E induced maturation and differentiation of human and murine dendritic cells (DCs). It also induced infiltration of immune cells into the tumor tissue followed by activation of cancer cell-specific cytotoxic T cells. Furthermore, HVJ-E suppressed the function of regulatory T cells (Treg), which have been reported to be negative regulators of cancer immunity. Thus, HVJ-E activates cancer immunity, and simultaneously suppresses Treg [45].

- 1. Structure
 - a. Spherical particle of diameter 200–300 nm
 - b. Contains single strand RNA (ssRNA)
 - c. Contains proteins+lipid for delivery
 - (functions as a natural DDS for nucleic acid)
- 2. Mode of action
 - a. The RNA in the particle acts as an RIG-I agonist and induces an induces cancer cell apoptosis
 - b. Activates the RIG-I/MAVS pathway and also induces anticancer immune responses
- 3. Manufacture
 - a. Process adapted to GMP guidelines, and pilot plant for clinical trial is available
 - b. Freeze-dried formulation is stable for over 21 months in refrigerator.





Fujiwara and Kaneda *et al.* reported that HVJ-E induced innate immunity [76]. Intratumoral injection of HVJ-E promoted infiltration and activation of natural killer (NK) cells by the induction of C-X-C motif chemokine 10 (CXCL10) and type I interferons. When HVJ-E was injected into the tumor of a murine model of renal cell carcinoma (RCC), NK cells exhibited cytotoxic activity against the RCC cell line *in vivo* [76]. The involvement of NK cells in the anti-tumor effect was also confirmed by showing the depletion of NK cells using an asialo-GM1 antibody [76]. Activated NK cells produced interferon- γ , which induces cancer-specific cytotoxic T cells [76]. These results indicated that HVJ-E is able to induce both innate and adaptive immunities.

In addition to the induction of cancer immunities, HVJ-E has the capability to induce cancer cell-specific apoptosis. Kawaguchi and Kaneda *et al.* reported that HVJ-E showed a dose-dependent, direct killing effect on human prostate cancer cell lines. In contrast, it showed no suppression of normal human prostate epithelium proliferation [77].

HVJ-E also induced apoptosis of prostate cancer cells *in vivo* because it showed an anti-tumor effect in severe combined immunodeficiency (SCID) mice that lack B lymphocyte- and T lymphocyte-mediated immunities [76, 77]. When NK cells were depleted from SCID mice by injection of the asialo-GM1 antibody, intratumoral injection of HVJ-E still showed an anti-tumor effect in a murine model of CRPC [77]. This result suggested that HVJ-E showed a direct killing activity *in vivo*. HVJ-E-mediated apoptosis of cancer cells was further confirmed in a murine model of prostate cancer using a NOD/SCID mouse, which lacks both innate (NK cell-mediated) and adaptive (antibody and CTL-mediated) immunities [54].

Numerous studies have revealed that the non-replicating HVJ-E particle shows anti-tumor effects in murine models of renal cell carcinoma, glioma, colon, bladder and CRPCs [45, 76-79].

In contrast, previous studies have reported that the non-replicating oncolytic Newcastle disease virus (NDV) failed to show an anti-tumor effect *in vivo* [80, 81]. These results are inconsistent with results obtained with HVJ-E [45, 76, 77, 79], and the putative reason is the difference between the number of particles used in the studies. In studies with NDV, 5×10^9 PFU of oncolytic virus were systemically or locally administrated [81], whereas the number of HVJ-E particles administered was estimated to be higher.

Another possibility is the difference in the capability of the virus to deliver its RNA fragments to target cancer cells. The Z strain-derived HVJ-E used in our studies has the highest level of membrane fusion activity [52]. Therefore, it is possible that HVJ-E has the ability to deliver more RNA component to the target cancer cells than the UV-inactivated NDV particle. The difference in process of inactivation may be responsible for the activity of non-replicating oncolytic viruses. The Newcastle disease virus was inactivated by UV irradiation, whereas HVJ-E was inactivated by a combination of treatment with an alkylating agent and UV irradiation [53]. Inactivation conditions affect the efficiency of delivery, and strictly regulated processes are necessary to obtain suitable performance [53].

4. Mode of actions

The major target cells of HVJ-E are cancer cells and dendritic cells (DCs) (Figure 3A) [44, 45, 76].

Treatment of cancer cells with HVJ-E enhanced the expression and activation (cleavage) of caspases 3, 8, and 9 (Figure 4A) [77], and induced dose-dependent apoptosis of melanoma, prostate, and other cancer cell lines *in vitro* (Figure 4B) [77]. Interestingly, no apoptotic effects were observed on normal epithelial cells derived from murine prostate [54, 77]. Thus, the apoptotic activity of HVJ-E is considered to be specific to cancer cells [54, 77].



Figure 3. The mechanism of anti-cancer effects of HVJ-E/GEN0101. A novel type of virotherapy agent (HVJ-E/GEN0101) has a multi-mode of action that is ideal for 2-step cancer treatment. **(A)** Target cells and sequential anticancer effects of HVJ-E/GEN0101. **(B)** Signaling pathway induced by stimulation with HVJ-E/GEN0101. The RIG-I/ MAVS pathway is the major pathway involved. RIG-I is a cytosolic receptor for nucleic acids: it usually functions as a sensor to recognize viral infection. The nucleic acids in the HVJ-E/GEN0101 particle act as an agonist for RIG-I and induce cancer cell-specific gene expression followed by the induction of apoptosis.



Figure 4. HVJ-E/GEN0101 induces apoptosis of human and murine melanoma cells. (A) Induction of caspase activity after treatment of melanoma cells with HVJ-E/GEN0101. Human (A375) and murine (B16/BL6) melanoma cells were treated with various amounts of HVJ-E/GEN0101, and caspase activities were measured 24 hours later. A dose-dependent activation was observed. **(B)** Survival of melanoma cells after treatment with various amounts of HVJ-E/GEN0101. Human (A375) and murine (B16/BL6) melanoma cells after treatment with various amounts of HVJ-E/GEN0101. Human (A375) and murine (B16/BL6) melanoma cells were treated with various amounts of HVJ-E/GEN0101. Human (A375) and murine (B16/BL6) melanoma cells were treated with various amounts of HVJ-E/GEN0101 and cell survival was measured by WST-8 assay 72 hours later.

Matsushima and Kaneda *et al.* conducted an investigation to determine the active component for anti-cancer effects, and identified RNA fragments within the particle [54]. Moreover, Kaneda *et al.* analyzed the signaling pathway involved and revealed that retinoic acid inducible gene-I (RIG-I) is a key factor for signal transduction [44, 54, 77]. Retinoic acid inducible gene-I is a cytosolic nucleic acid receptor and was originally identified as a sensor that recognizes infection by single strand RNA viruses [82, 83]. Thus, RIG-I has been recognized as an inducer of immune response against infected viruses [82-84].

The RNA fragments delivered by HVJ-E bind the helicase domain of RIG-I in the cytoplasm and change the conformation to unmask the caspase activation and recruitment domain (CARD) (Figure 3B). After binding with the RNA fragments, RIG-I interacts with the mitochondrial antiviral signaling (MAVS) protein on the mitochondrial membrane (Figure 3B) [84]. The mitochondrial antiviral signaling protein forms a complex with an adaptor protein (TRAF3/5), stimulates transcription factors (IRF3 and IRF7), and promotes the expression of interferon- α and β (Figure 3B) [85, 86]. It also stimulates kinases of regulator protein of transcription factor NK- κ B, resulting in increased expression of cytokines, chemokines, and other genes (Figure 3B) [45, 54, 76, 77].

It has been reported that the RIG-I/MAVS pathway is activated after cancer cells are treated with HVJ-E [54]. Transfection of isolated RNA fragments from HVJ-E particle also induced apoptosis of cancer cells *in vitro* [54]. Thus, HVJ-E uses a natural oligonucleotide (RNA fragment from the inactivated virus genome) as an active ingredient, and a natural virus particle (envelope of HVJ) as a delivery system for a nucleic acid medicine (RNA fragments). The apoptosis induced by HVJ-E was cancer cell-specific because normal endothelial cells showed no apoptosis after the treatment with HVJ-E [54, 77].

Involvement of RIG-I in cancer cell-specific apoptosis has also been indicated in ovarian cancers and melanoma. Kübler and Barchet et al. reported that the RIG-I agonist (Poly(dAdT)) induced apoptosis and expression of MHC class I molecules in ovarian cancer cells [87, 88]. Van and Bell et al. also reported apoptosis of ovarian cancer cells after treatment with dsRNA [89]. Analysis by shRNA-mediated knockdown revealed that RIG-I and other receptors for dsRNA (TLR3 and MDA-5) were involved in the caspase 8/9mediated apoptosis of cancer cells. Similar to sensitivity of HVJ-E, epithelial cells derived from ovarian surface was resistant to apoptosis mediated by RIG-I signal pathway. When combined with conventional chemotherapy (carboplatin/paclitaxel), treatment with dsRNA showed a synergistic suppression of ovarian cancer cell viability [89]. Besch et al. reported that stimulation of RIG-I and MDA-5 induced apoptosis of human melanoma cells [90]. The authors used pppRNA and poly(I:C) as ligands for RIG-I and MDA-5, and showed the involvement of caspase-9 and Apaf-1 during apoptosis. They also reported the reduction of lung metastasis by treatment with ligands for RIG-I and MDA-5 in the NOD/SCID mouse [90]. Details of the underlying pathways are currently being analyzed using siRNAs of apoptosis-related factors [54]. It has been suggested that differences in the expression of apoptotic genes such as Noxa, TRAIL, and TRAIL receptors in cancer cells and normal cells determine the specificity of apoptosis induced by HVJ-E (Figure 3B) [54].

HVJ-E also induced differentiation and maturation of murine and human DCs [45]. It induced the expression of surface markers on mature DCs, and the production of various cytokines and chemokines from DCs [45, 76, 77]. Activated DCs induce both innate (NK cell-mediated) and adaptive (cytotoxic T cell-mediated) immunities (Figure 3A) [45, 76]. It has been reported that an RIG-I agonist (Poly(dAdT)) induced the production of cytokines (IL-6 and TNF- α) and chemokines (CXCL1-and CCL5/RANTES) [87, 88] in human ovarian cancer cells.

5. Efficacy in preclinical studies

The efficacy of the non-replicating oncolytic virus (HVJ-E/GEN0101) was examined in murine models of melanoma and prostate cancer (Figure 5A). GEN0101 is the identification code for the agent. The data have indicated that the anti-tumor effect is dose-depend-

ent similar to other non-viral anti-cancer agents (Figure 5B). This feature is important for the development of non-replicating oncolytic virus as a novel therapeutic agent; identification of the optimal dose for non-replicating oncolytic viruses is easier than determining the optimal dose for replicating oncolytic viruses as the latter are subject to change (increase) resulting from replication in target cancer cells. The effects of administration in a xenograft model of human CRPC were also examined (Figure 5A). Efficacy after subcutaneous administration was revealed (Figure 5C). It is known that the SCID mice lacks B lymphocyte- and T lymphocyte-mediated immunities such as antibody production, and induction of cytotoxic T cells but retains monocytes and NK cells important for innate immunity. Thus, the non-replicating virus (HVJ-E/GEN0101) is able to induce innate immunity, and show anti-cancer activity in vivo even in the absence of direct killing of cancer cells. Efficacy by subcutaneous administration is important for the development of a non-replicating virus because subcutaneous administration is more common than intratumoral administration. Intratumoral administration kills cancer cells directly and promotes the release of tumor antigens, which are recognized by immune cells. Subcutaneous administration is expected to enhance and sustain the immune response. Therefore, the combination of intratumoral and subcutaneous administration is suggested as a suitable regimen in the clinical setting.

In summary, the results of efficacy studies have indicated that the anti-cancer effect of the non-replicating oncolytic virus HVJ-E/GEN0101 is dose-dependent similar to conventional anti-cancer drugs, and subcutaneous administration may be the preferred administration route for the viral and non-replicating viral agents.

6. Safety studies

Safety of the non-replicating oncolytic virus (HVJ-E/GEN0101) has been confirmed in nonprimate (rat) and primate (Cynomolgus monkey) animals. Lists of the studies that have been conducted are shown in Table 1A and B.

Results from single dose general toxicity studies revealed that no death or severe finding was observed even in the maximum dosage groups. Similar to the single dose studies, no severe finding was observed in repeated dose, general toxicity studies (Table 1A).

Results from immunological and genetic toxicity studies in rat and monkeys revealed that no abnormal symptoms related to the test agent were observed. The levels of IL-6 and IFN- γ in monkey serum were analyzed after subcutaneous injection of HVJ-E/GEN0101, and the levels of both cytokines were determined to be within the normal range (data not shown). A core battery of safety pharmaceutical studies was performed to determine the effects on major organs (central nervous, respiratory, and cardiovascular systems); no abnormal effect was observed with the exception of transient and non-severe pyrexia (Table 1B).



Figure 5. Efficacy studies of HVJ-E/GEN0101 in a murine model of melanoma and prostate cancer.(A) Protocols for the efficacy study using murine models of melanoma and prostate cancer. Left: C57/BL6 mice were transplanted with B16/BL6 cells and 3 intratumoral injections of HVJ-E/GEN0101 were administered after tumor formation (4 days after transplant). A time course study of change in tumor volume was performed, and the difference in tumor volume among the three groups was statistically analyzed at the end of the study. Significant differences were determined by Dunnett's multigroup test, and significant differences to the medium (control) group were observed (p < 0.01). The survival rate after the injection of HVJ-E/GEN0101 was also monitored in the melanoma model. A survival study was performed and the difference among the three groups was statistically analysis. Significant differences to the medium group were observed (p < 0.001). Right: Protocol for the efficacy study in a murine model of prostate cancer. Human CRPC (PC-3)-bearing mice were used for the study. A summary of the study protocol is shown. The anti-tumor effect of HVJ-E/GEN0101 for each administration route was examined. Severe combined immunodeficiency mice were transplanted with PC-3 cells. Two intratumoral injections or 6 subcutaneous injections of HVJ-E/GEN0101 were performed after tumor formation (4

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days after transplant). A time course study of change in tumor volume was performed and differences in tumor volume between the two groups were statistically analyzed at the end of study. Significant differences were determined by t-test and significant differences to the medium were observed in both routes (p < 0.01 and p < 0.05). (B) Efficacy study in a murine model of melanoma. Dose-dependency of the anti-tumor effect of HVJ-E/GEN0101 was revealed. (C) Efficacy study in a murine model of prostate cancer. A time course study of change in tumor volume was performed and differences in tumor volume between the two groups were statistically analyzed at the end of the study. Significant differences were determined by t-test, and significant differences to the medium were observed for both administration routes (p < 0.01 and p < 0.05).

a) Toxicological study (1)	: General toxicity study			
Dosing Regimen	Specie	es	Route	Dose
Single Dose Repeated Dose	Rat		iv	single
	Rat		SC	single
	Cynomolgus monkey		iv	single
	Cynomolgus monkey		SC	single
	Rat		iv	7 days
	Rat		SC	6 times in 2 weeks
	Cynomolgus monkey		SC	6 times in 2 weeks
b) Toxicological study (2)	: Safety pharmacology	and other studies		
Study	Method	Species	Route	Dose
- Safety pharmacology -	Rat FOB	Rat	SC	single
	Respiratory	Rat	SC	single
	Cardiovascular	Cynomolgus monkey	SC	single
TK	Q-PCR	Rat	SC	6 times 2 weeks
Genetic toxicity	Micronucleus	Rat	SC	single
Antibody production	ELISA	Rat	SC	6 times 2 weeks

Table 1. Summary of toxicological studies

7. Clinical studies

Two clinical studies using the non-replicating oncolytic virus (HVJ-E/GEN0101) are currently being conducted in Osaka University Hospital. The target diseases are advanced melanoma (stage IIIC and stage IV) and CRPC [55, 56]. These proof-of-concept studies in melanoma and CRPC using the non-replicating oncolytic virus were initiated in July 2009 and July 2011, respectively [55, 56]. The respective summaries of both studies are shown in Table 2A and B. The primary endpoints of these studies were safety and tolerability based on the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0, whereas the secondary endpoints were efficacy and confirmation of the mode of action. The major difference between the regimens for the melanoma and CRPC studies was the route of administration and number of administrations. A combination of intratumoral and subcutaneous routes of administration (one intratumoral and three subcutaneous injections) was adopted in the CRPC study. In addition, a new injection system developed by Okayama University was used in the CRPC

study. This system permits stable refrigerated storage of the test article, and accurate injection into the prostate [91].

anoma			
Phase I/II investigational clinical study of inactivated HVJ-E administration for advanced			
malignant melanoma patients			
Malignant melanoma (AJCC stage IIIC or stage IV)			
Masking: Open Label			
Allocation: Non-Randomized			
Primary endpoint: Safety and tolerability			
Secondary endpoint: Anti-tumor immunity and validity			
Target sample size: 6–12 patients			
Route: Intratumoral			
Dose: 6 times in 2 weeks/cycle, 2 cycles			
Osaka University Graduate School of Medicine (Osaka University Hospital)			
https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&type			
=summary&recptno=R000002889&language=E			
stant prostate cancer			
Phase I/II investigational clinical study to assess safety and efficacy of intratumoral and			
subcutaneous injection of HVJ-E to castration-resistant prostate cancer patients			
Castration resistant prostate cancer (CRPC)			
Masking: Open Label			
Allocation: Non-Randomized			
Primary endpoint: Safety and tolerability			
Secondary endpoint: Anti-tumor immunity and validity			
Target sample size: 6–12 patients			
Route: Intratumoral × 1 then SC × 3			
Dose: 4 times in 2 weeks/cycle, 2 cycles			
Osaka University Graduate School of Medicine (Osaka University Hospital)			
https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&type			

 Table 2. Design of investigational clinical studies

8. Discussion and conclusion

The major disadvantages associated with oncolytic viruses are safety concerns because viral replication could theoretically cause the emergence of new pathogenic viruses [43]. The use of non-replicating oncolytic viruses is expected to resolve the safety issues associated with conventional oncolytic viruses because they are unable to replicate in target cells.

The modes of action underlying the anti-cancer effects of non-replicating virus on cancer cells and immune cells have been analyzed. One major signaling pathway is the RIG-I/ MAVS pathway [54]. RIG-I is a cytosolic nucleic acid receptor that acts as a sensor that detects virus infection [82-84]. Similar to wild-type RNA viruses, the non-replicating virus (HVJ-E) is able to activate the RIG-I/MAVS pathway in DCs and induce both innate and adapted immunities. The non-replicating virus also activates the RIG-I/MAVS pathway in cancer cells and induces cancer cell-specific apoptosis. Genetic analyses suggest that differences in the expression of apoptosis-related genes define the sensitivity to the treatment with a non-replicating virus (HVJ-E) [54]. Furthermore, it is suggested that methylation of the respective enhancer/promoter regions underlies differences in transcription of apoptosis-related genes [54].

RIG-I, TLR3, and MDA-5 signaling pathways are involved in the apoptosis of ovarian cancers [87-89] and melanoma [90]. Thus, the RIG-I/MAVS pathway is likely to emerge as a new target for the development of drugs that induce cancer cell-specific apoptosis.

The non-replicating virus (HVJ-E) activates DCs to produce IL-6, which suppress the function of Treg [45]. This effect is expected to maintain the induced cancer immunity because Treg is known to be a negative regulator of immune responses [92-94]. It has been reported that cancer cells escape cancer immunity by the recruitment and activation of Treg. Therefore, it will be important to control the function of Treg for long-term effective induction and maintenance of cancer immunities. Suzuki and Kaneda et al. reported that the RIG-I/MAVS pathway was not required to induce the expression of IL-6 [95]. The attachment of the HVJ-E particle to the surface of DCs was sufficient for the production of IL-6, suggesting that the RNA fragments are unnecessary for the induction of this cytokine [95]. Detailed analyses identified that the F protein on the surface of HVJ-E is involved in the production of IL-6 [95]. Binding of the F protein to target cells requires expression of the HN protein [96]. Several gangliosides, such as GD1a and sialyl paragloboside, are implicated in the association of the HVJ-E particle and cancer cells because the HN protein binds the sialic acids of gangliosides. The receptor for the F protein remains unidentified to date. Taken together, the RIG-I/MAVS signal pathway, and a second pathway that induces the production of IL-6 may cooperate in the activation and sustainment of cancer immunity induced by the non-replicating virus (HVJ-E).

The development of a non-replicating oncolytic virus other than HVJ-E is possible because the manufacturing process for such a particle is similar to that of whole particle viral vaccines. In case of HVJ-E, the virus is inactivated by treatment with an alkylating agent and UV irradiation, a process used for the production of vaccines against viral diseases. Thus, the development of oncolytic viruses could be converted to the development of non-replicating oncolytic particles by similar manufacturing processes.

A disadvantage associated with non-replicating oncolytic viruses may be the defect in transmission ability. Therefore, it is possible that a greater amount of non-replicating oncolytic virus may be required for effective treatment compared with a live oncolytic virus. Alternatively, more frequent injections may be necessary for complete tumor eradication compared with the use of live oncolytic viruses. However, it is important to achieve a balance between

the risks and benefits associated with the therapy. In our opinion, repeated administration of the non-replicating virus should be tolerable because no severe finding was observed during our safety studies.

In conclusion, non-replicating virus particles such as HVJ-E may resolve the safety issue of conventional virotherapy and provide a new strategy in cancer treatment.

9. Future perspectives

The first non-replicating oncolytic virus (HVJ-E) is currently under evaluation in clinical studies. Proof-of-concept data for non-replicating viruses in both clinical and non-clinical studies are necessary for further development of this approach. Osaka University Hospital is currently conducting two phase I/IIa studies: one for advanced melanoma and another for CRPC [55, 56]. The results of these studies will reveal the safety, efficacy, and optimal dosage regimen necessary for phase II study or randomized, double blind phase III study.

Combination treatment may be an effective approach to increase efficacy [97]. Indeed, an increase in therapeutic efficacy has been reported for virotherapies combined with photodynamic therapy [98, 99], radiotherapy [100], chemotherapy [101, 102], or gene therapy [103]. Kiyohara and Kaneda reported that combination of the non-replicating virus (HVJ-E) and gene therapy (IL-12) increased efficacy in a murine model of melanoma [104]. Furthermore, it was reported that a combination of non-replicating virus (HVJ-E) and chemotherapy [bleomycin or cis-diamminedichloroplatinum (CDDP)] increased efficacy in murine models of colon and bladder cancers [78, 105].

Technologies for systemic administration and targeting for HVJ-E are under development. The HN protein of HVJ-E has hemagglutinating activity and causes agglutination and lysis of erythrocytes *in vitro*. Currently, inactivation of the HN protein, decreased expression of the HN protein, and "masking" with platelets is being developed for intravenous injection of HVJ-E. Targeting after the intravenous injection is also important for systemic delivery. The addition of transferrin, a single chain antibody, or platelets have been suggested as suitable modifiers for HVJ-E.

The selection of viruses, or viral strains for the preparation of non-replicating oncolytic viruses is also important for obtaining higher efficacy because the level of immune response is dependent on the selection of virus strains [106]. A number of replicating oncolytic viruses are currently under clinical development [35, 97]. Therefore, it may be feasible to select a suitable virus for therapeutic application from the oncolytic viruses that have been developed [106, 107]. The tropism and complement resistance features of each virus should be considered for targeting and stabilization in serum.

HVJ-E is the only non-replicating oncolytic virus currently undergoing clinical investigation. These studies establish a new strategy for the virotherapy and gene therapy fields. The primary goal is to provide a novel approach for improving cancer therapy.

Summary

Conventional cancer therapies suffer from one paradox: although chemotherapeutic agents strongly kill cancer cells and decrease tumor volume, they simultaneously suppress the immune system. Chemotherapy frequently results in tumor relapse because residual cancer cells and cancer stem cells escape immune responses. In contrast, immune therapies including therapeutic cancer vaccines, effectively induce cancer immunity, but possess weak cytotoxic activity against cancer cells. Therefore, these treatments usually show weak efficacy. It has been reported that cancer is able to progress even after the activation and proliferation of cancer-specific cytotoxic T lymphocytes.

Virotherapy is predicted to become an alternative approach to obtain a model cancer therapy because it generally displays both oncolytic and immunostimulatory activities. However, the major drawback associated with current virotherapy is safety concerns. Virotherapy using a non-replicating virus is a new approach aimed at resolving safety issues. Thus, it is expected to become a novel concept for cancer therapy in the near future.

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Author details

Toshihiro Nakajima¹, Toshimitsu Itai¹, Hiroshi Wada¹, Toshie Yamauchi¹, Eiji Kiyohara^{2,3} and Yasufumi Kaneda²

1 GenomIdea, Inc., Midorigaoka, Ikeda, Osaka, Japan

2 Division of Gene Therapy Science, Department of Molecular Therapeutics, Graduate School of Medicine, Osaka University, Yamada-oka, Suita, Osaka, Japan

3 Department of Dermatology, Graduate School of Medicine, Osaka University, Yamadaoka, Suita, Osaka, Japan

T. Nakajima is a CEO of GenomIdea. T. Itai, H. Wada, and T. Yamauchi are employees of GenomIdea. The remaining authors have no conflicts of interest.

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