Oncolytic Urabe mumps virus: A promising virotherapy for triple-negative breast cancer

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Historically, the clinical utility of oncolytic virotherapy as a treatment for a wide range of cancer types was first demonstrated by three pilot human clinical trials conducted in Japan in the 1970s and 1980s using a wild-type Urabe mumps virus (MuV) clinical isolate. Using a sample of the actual original oncolytic Urabe MuV clinical trial virus stock (MuV-U-Japan) used in these Japanese clinical trials, we found that MuV-U-Japan consisted of a wide variety of very closely related Urabe MuVs that differed by an average of only three amino acids. Two MuV-U-Japan isolates, MuV-UA and MuV-UC, potently killed a panel of established human breast cancer cell lines in vitro, significantly extended survival of nude mice with human triple-negative breast cancer (TNBC) MDA-MB-231 tumor xenografts in vivo, and demonstrated significant killing activity against breast cancer patient-derived xenograft (PDx) cell lines grown as 3D organoids, including PDxes from patients resistant to anthracycline- and taxane-based chemotherapy. We also report success in developing a large-scale MuV-U production and purification process suitable for supporting Investigational New Drug applications for clinical trials. This study demonstrates the suitability of the MuV-UC virus for translation to modern clinical trials for treating patients with TNBC.

INTRODUCTION

Oncolytic virotherapy has emerged as an exciting and novel cancer treatment.1–4 Oncolytic viruses specifically kill tumor cells as they replicate, and the oncokilling effect and amplified by subsequent rounds of new virus production and infection, leading to destruction of additional tumor cells. Oncolytic virus killing triggers unique immune responses from the combination of virus-induced danger signals, heterogeneous antigenic variation of the cancer cells, and a novel mechanism of cancer cell destruction that generates a wide variety of immune targets. These unique mechanisms of tumor cell killing mediated by oncolytic virotherapies can synergize with current chemo- and radiotherapies as well as with novel antibody/protein immunotherapies to produce superior clinical responses of longer duration in a wider range of patients with cancer than any therapy alone. Historically, the potential of the field of oncolytic virotherapy for successfully treating a wide range of cancer types has been illustrated by positive clinical results and insights obtained from three pilot human clinical trials conducted in Japan in the 1970s and 1980s using a wild-type mumps virus clinical isolate that was circulating in 1967 Japan: Urabe mumps virus (MuV-U).3–7

Dr. Asada, the clinical investigator of the original oncolytic MuV-U clinical trial, theorized that utilizing a wild-type MuV would be most effective for treating cancers while still being safe because of the natural and specific preference of the virus for targeting cancer cells as a result of most patients having antibodies to MuV from pre-exposure, even a minimum amount, and because of the small risk of serious complications in adults with cancer even when never pre-exposed to MuV.7 These trials were done before the availability of MuV vaccines. These three clinical trials treated 18 types of cancer in 312 patients with cancer with minimal toxicity, and in one trial, tumors regressed completely or decreased to less than half of the initial size in a third of the patients treated, despite most patients having neutralizing antibodies before therapy.

We obtained a sample of the actual original oncolytic MuV-U clinical trial virus stock used in the Japanese clinical trials published in 19786 and 19887 from Dr. Koichi Yamanishi, the Director General of the Research Foundation for Microbial Diseases of Osaka University. Dr. Yamanishi was a clinical trial co-author of the publications of the 1978 and 1988 clinical trial using MuV-U virus to treat a variety of tumor types. This sample of the original MuV-U clinical trial stock (which we called MuV-U-Japan) used in these pioneering oncolytic virotherapy clinical trials provides an opportunity to further define the MuV-U-Japan product with modern techniques to aid effective translation of MuV-U into modern oncolytic virotherapy clinical trials for treating cancer. Unfortunately, a sample of the original MuV-U clinical isolate was not archived.

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Original Wild Type Urabe Mumps Virus Seed Stock

- Originally isolated in the late 1960’s from saliva of a patient with mumps symptoms and initially isolated using human embryonic kidney (HEK) cells and then passaged once using primary green monkey kidney (GMK) cells
- Large-Scale Amplification in Cultured Cells
- Amplification process development; Unknown number of passages using HEK cells and/or human amnion (AV3) cells

Supplied Three Oncolytic Virotherapy Human Clinical Trials

- Unknown number of additional passages using human embryonic kidney cells and/or human amnion cells
- Unknown number of different lots of the amplified Urabe Mumps Virus used in the clinical trials
- Actual clinical trial stock supplied to Dr. Federspiel from Dr. Koichi Yamanishi, an author on the published manuscripts describing two of the clinical trials
- Original Reported Titer: 2.0 × 10^4 TCID50/mL
- VVPL Titer: 2.2 × 10^5 TCID50/mL (after 40 years of storage)

Purify to Single Virus Isolates Using Limiting Dilution (3X) Vero Cells

Two cell killing phenotypes observed in wells with single virus:
1. Syncytia (fused cells, syncytium (SYN])
2. Cytopathic Effect (CPE)

12 Mumps Virus Clonal Isolates Characterized by Sequencing

Supplied Directed Attenuation Trials For Vaccine Development

- Serial passages in amniotic cavity of chick embryos followed by serial passages in quail and chicken fibroblasts cultures

Example Vaccine
Chiron SIPAR Mumps Virus Attenuated Vaccine Strain based on Urabe-AM9 mumps virus vaccine strain
Genbank AF314556.1

B

Day 5

Day 6

Uninfected Vero

MuAV SYN Phenotype

MuAV CPE Phenotype

C

| Gene | Base | Nucleotide Change | Amino Acid Change |
|------|------|-------------------|------------------|
| NP   | 16   | G to A            | non-coding       |
| NP   | 708  | A to C            | H to P           |
| NP   | 1433 | C to T            | H to Y           |
| NP   | 1443 | C to T            | T to I           |
| NP   | 1444 | C to T            | T to I           |
| NP   | 1465 | C to T            | silent           |
| NP   | 1474 | C to T            | silent           |
| NP   | 1483 | C to T            | silent           |
| NP   | 1496 | C to T            | L to F           |
| M    | 3670 | 2 T               | silent           |
| F    | 5129 | T to C            | L to P           |
| F    | 5281 | A to G            | F to S           |
| F    | 5584 | 3 C               | T to A           |
| F    | 5653 | 1 C to T          | silent           |
| F    | 5793 | C to G            | T to A           |
| F    | 8103 | G to A            | D to E           |
| F    | 8406 | C to T            | non-coding       |
| L    | 9749 | C to T            | R to Q           |
| L    | 9972 | C to T            | S to F           |
| L    | 14494| 2 G               | silent           |
The goal of this study was to identify and characterize the diversity of MuV-U subtypes contained in the MuV-U-Japan clinical trial stock sample in detail and then determine whether the MuV-U subtypes retained significant oncolytic activity. We found the MuV-U-Japan clinical trial stock sample consisted of a wide variety of very closely related Urabe MuVs that differed by an average of only three to five amino acids, but all were significantly different compared with an attenuated Urabe MuV vaccine strain. Representative MuV-U-Japan isolates also retained the ability to replicate to high titers in human cells at 37°C like a wild-type MuV, compared with the attenuated Urabe MuV vaccine strain, which was adapted to replicate to high titers only using chicken cells and required culture at 34°C. Because seven of the thirteen patients with breast cancer treated with MuV-U in the original pilot trials showed significant clinical responses, we assayed representative MuV-U subtypes for oncolytic killing of human breast cancer as a first disease indication. Two MuV-U-Japan isolates, MuV-UA and MuV-UC, potently killed a panel of established human breast cancer cell lines in vitro, significantly extended survival of nude mice with human triple-negative breast cancer (TNBC) MDA-MB-231 tumor xenografts in vivo, and demonstrated significant killing activity against breast cancer patient-derived xenograft (PDX) 3D organoids, including PDXs derived from patients with TNBC resistant to anthracycline- and taxane-based chemotherapy.

RESULTS

MuVs

MuV is a member of the Paramyxoviridae family, genus Orthorubulavirus. MuVs are enveloped, with negative-strand RNA genomes (15,384 bases) containing 7 transcription units encoding 9 viral proteins: nucleocapsid-associated protein (NP), phosphoprotein (P), V protein, I protein, matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L). The NP protein binds with the viral genomic RNA to form the template for RNA synthesis. The P and L proteins form the RNA-dependent RNA polymerase. The V protein helps the virus evade the cell's innate immunity by blocking interferon signaling. The functions of the I and SH proteins are currently unknown. The M protein is believed to orchestrate the viral assembly process linking the nucleocapsid complex with the F and HN proteins in the membrane. HN and F are viral glycoproteins that enable receptor binding (sialic acid) and subsequent membrane fusion required for virus entry.

The genomic sequences of MuVs are very diverse, but all belong to only one serotype. MuVs are divided into 12 different genotypes (A, B, C, D, F, G, H, I, J, K, L, and N), mainly based on the nucleotide sequence differences of the 300-base SH gene. Intrageneotype MuV nucleotide sequence variation can be as high as 4% (~600 nucleotide differences in the 15,384-base genome), whereas intergenotype variation ranges from 8%–19% (1,200–2,900 nucleotide differences). The vaccines against MuV were developed before molecular biology techniques, including nucleotide sequencing, were available to document this sequence diversity. Several effective and safe MuVs vaccines were produced using different MuV genotypes. The MuV vaccine currently used in the United States is based on the Jeryl-Lynn strain, a genotype A MuV that was subsequently sequenced and found to be a mixture of two distinct but related genotype A MuVs that differ by 414 nucleotides (2.7%), of which 84 are amino acid coding changes. Additional examples of the diversity of MuVs are provided in Table S1.

The original MuV-U strain used in the Japan oncolytic virotherapy pilot clinical trials was derived in 1967 from a clinical isolate from a child named Urabe, who showed typical mumps symptoms. The virus was initially cultured using primary human embryonic kidney (HEK) cells, amplified for one passage using primary green monkey kidney (GMK) cells, and designated MuV-U strain seed stock (Figure 1A). This work was done in the Department of Virology, Research Institute of Microbial Diseases, Osaka University, Osaka, Japan. This initial MuV-U seed stock was then amplified in cultured HEK cells and/or human amnion (AV3) cells in large-scale process development studies at this institute. The amplified MuV-U seed stock was used for two very different programs; one program explored use of the wild-type MuV-U directly as an oncolytic virotherapy for treating cancer in human clinical trials, and the second program sought to generate a live but attenuated Urabe-based MuV vaccine for a national vaccine program. Japan developed an attenuated vaccine strain from the genotype B MuV-U seed stock; an example of this vaccine strain is the Urabe-AM9 Chiron SIPAR strain.

Figure 1. Initial characterization of the oncolytic Urabe Mumps Virus clinical trial stock (MuV-U-Japan)

(A) Partial history of the derivation of the original oncolytic MuV-U used in three human clinical trials conducted in Japan in the 1970s and 1980s, followed by the lineage derivation of clonal isolates from this study. (B) Representative pictures of the two major cytopathic effect phenotypes caused by MuV-U-Japan infection of Vero cells by single virus isolates. (C) The nucleotide sequence differences observed by Sanger sequencing of the MuV-U-Japan genome compared with the reported genome of the attenuated MuV-U vaccine SIPAR (GenBank: AF314558.1). The nucleotide sequence at three of the positions, bases 3,670, 5,584, and 14,494, displayed a mixture of two bases.
| Gene | Base | Nucleotide Change | Amino Acid Change | Origin | Isolate A | Isolate B | Isolate C | Isolate D | Isolate E | Isolate F | Isolate G | Isolate H | Isolate I | Isolate J | Isolate K | Isolate L |
|------|------|------------------|-------------------|--------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| NP   | 16   | G to A           | noncoding         | Japan   | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 708  | A to C           | H to P            |         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1359 | G to A           | R to K            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1433 | C to T           | H to Y            |         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1443 | C to T           | T to I            |         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1444 | C to T           | silent            |         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1465 | C to T           | silent            |         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1474 | C to T           | silent            |         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1483 | C to T           | silent            |         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1496 | C to T           | L to F            |         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1547 | T to C           | F to P            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1548 | T to C           |                  |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1554 | T to C           | V to A            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1563 | T to C           | L to P            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1588 | T to C           | silent            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| NP   | 1599 | T to C           | L to S            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| PIV1 | 2348 | G to A           | R to G            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| PIV1 | 2585 | C to T           | H to Y            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| M    | 3670 | T to C           | L to P            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| M    | 3722 | T to C           | silent            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| M    | 4275 | C to A           | L to I            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| F    | 5129 | T to C           | F to S            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| F    | 5281 | A to G           | T to A            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| F    | 5584 | C to T           | silent            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| F    | 5663 | A to G           | T to A            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| F    | 5703 | C to G           | D to E            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| F    | 5634 | A to C           | Y to S            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| F    | 6110 | C to T           | T to I            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| F    | 6137 | G to A           | S to N            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| SH   | 6271 | C to T           | P to S            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| HN   | 8682 | G to T           | A to V            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| HN   | 7141 | T to C           | silent            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| HN   | 7605 | C to A           | T to K            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| HN   | 7804 | C to T           | silent            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| HN   | 8103 | G to A           | R to Q            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| HN   | 8177 | C to A           | L to I            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| HN   | 8189 | A to G           | K to E            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| HN   | 8406 | C to T           | noncoding         |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| L    | 9634 | G to A           | R to K            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| L    | 9749 | C to T           | H to Y            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| L    | 9972 | C to T           | S to F            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| L    | 13328| A to C           | N to H            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| L    | 13540| A to G           | silent            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| L    | 14494| G to A           | silent            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| L    | 14530| G to A           | silent            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| L    | 14063| A to C           | silent            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |
| L    | 15264| T to C           | H to T            |         | ✓         |          | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         | ✓         |

SYN: syncytia  CPE: cytopathic effect

(legend on next page)
contained virus quasispecies with amino acid differences that may account for the different cytopathic phenotypes.

The initial consensus nucleotide sequence of the viral genome of an aliquot of the MuV-U-Japan clinical trial sample was determined by first isolating viral RNA and then synthesizing cDNA from the viral RNA using reverse transcriptase. A series of PCR amplifications was used to produce overlapping 1- to 2-kb double-stranded DNA fragments from the MuV-U-Japan cDNA using oligonucleotide primer sets developed using the attenuated MuV-U SIPAR vaccine nucleotide sequence reference. The complete nucleotide sequences of both strands of each purified PCR fragment population were determined using Sanger sequencing. A consensus MuV-U-Japan nucleotide sequence was assembled from the PCR fragment consensus sequences using the Sequencer program and then compared with the Urabe SIPAR vaccine sequence using the ClustalW pairwise alignment function in the MacVector 14.5.3 program. Of the 15,384 nucleotides in the MuV genome, 20 nucleotide differences were found between the Urabe SIPAR vaccine genome and the assembled MuV-U-Japan consensus genome, with 13 of these changes resulting in an amino acid change (Figure 1C). A mixture of two bases was observed at genome positions 3,670, 5,584, and 14,494 in the assembled MuV-U-Japan genome, another indication that the MuV-U-Japan stock was a mixture of viral quasispecies.

To characterize the extent of virus quasispecies contained in the original MuV-U-Japan sample, 12 clonal virus isolates were generated using three rounds of limiting dilution using Vero cells and named isolates A–L: six MuV-U isolates produced the syncytium SYN phenotype (A, D, and G–J), and six isolates produced the cell-rounding CPE phenotype (B, C, E, F, K, and L). A consensus nucleotide sequence for each clone was generated by RT-PCR, as described above, and compared with the Urabe SIPAR vaccine and the assembled MuV-U-Japan genome sequences. Although each of the nucleotide sequences of the clonal isolates contain most of nucleotide differences observed between the Urabe SIPAR and MuV-U-Japan (origin Japan) genomes, each clonal isolate genome also contained unique additional nucleotide and coding differences resulting in 12 MuV-U virus isolates (Figure 2). The number of unique differences was relatively small, averaging 5 genomic differences per isolate. However, we did not observe an obvious pattern of nucleotide/coding changes that characterized the SYN isolates from the CPE isolates.

**Replication characteristics of the MuV-U isolates compared with a MuV-U-based attenuated vaccine, Urabe SIPAR**

The goal of the Urabe MuV vaccine lineage was to select a safe, attenuated virus strain suitable for use as a vaccine for children (Figure 1A). This was accomplished by using serial passages of the original Urabe MUV seed stock in normally non-susceptible avian embryos and then avian fibroblast cultures that resulted in significant attenuation, including replication to higher titers in avian versus mammalian cells, and acquisition of a temperature-sensitive phenotype.12,13 We purchased a virus stock of the attenuated Urabe SIPAR vaccine from the ATCC that was produced by infecting chicken embryo fibroblasts (CEF) incubated at 34°C for 3–4 days, and the virus stock was prepared from the supernatant and cell lysate of the infected culture (Figure 3A).

Four representative MuV-U clonal isolates, two SYN phenotype viruses (isolate A and Isolate I), and two CPE phenotype viruses (isolate B and isolate C), were chosen to compare their viral replication kinetics in susceptible cell lines with the attenuated Urabe SIPAR vaccine virus. Cultures of Vero cells were infected at an MOI of 0.1 with the four MuV-U isolates and the ATCC Urabe SIPAR attenuated vaccine stock and incubated at 37°C. A second Urabe SIPAR virus-infected culture was incubated at 34°C. Samples of the infected culture supernatants were collected daily, and the infectious titer was quantitated with a qualified TCID50 assay using Vero cells (Figure 3A). The attenuation process of the Urabe SIPAR vaccine virus included optimized replication in chicken embryo fibroblasts that resulted in mutation that required a production temperature of 34°C to produce a maximum titer 3–4 days after infection; the ATCC virus stock produced in this way reached a titer of $1.6 \times 10^6$ TCID50/mL. The replication of the SIPAR virus at 34°C using Vero cells produced a titer ~10-fold lower compared with the ATCC CEF culture; however, titers of less than $10^5$ TCID50/mL were produced using Vero cells incubated at 37°C, demonstrating the temperature sensitivity of the attenuated SIPAR virus. Maximum titers of the four MuV-U isolates were produced using Vero cells by day 3, with isolate C producing the highest titer of $2.0 \times 10^5$ TCID50/mL and isolate A producing the lowest titer of the four viruses, ~50-lower at $4.3 \times 10^5$ TCID50/mL.

To characterize the replication of these four MuV-U isolates, a human tumor cell line was tested that may support higher replication compared with Vero cells. Duplicate small shake flask cultures (30 mL, 106 cells/mL) of the VVPL production HeLa S3 suspension cell line were infected at an MOI of 0.1 and incubated at 37°C for 4 days. Daily samples of the infected culture supernatants were collected, and the infectious titer was quantitated (Figure 3B). All four isolates produced titers 5- to 10-fold higher when replicating in the human HeLa S3 cells compared with Vero cells, although the relationship of infectious titers of the four isolates C > B, I > A was maintained. Isolate C (the cell-rounding CPE phenotype in Vero cells) consistently produced the highest titer. The syncytium phenotype of isolate A is especially robust in Vero cells, which may limit the maximum titer that can be attained by this isolate before the cells are killed.
A

| Virus                    | Temp °C | Cell Type | Harvest Material | Day of Harvest | Titer TCID₅₀/mL |
|-------------------------|---------|-----------|------------------|----------------|----------------|
| Urabe SIPAR ATCC        | 34      | CEF       | Supernatant      | 3              | 1.6 x 10⁶      |
| Urabe SIPAR ATCC        | 34      | Vero      | Supernatant      | 4              | 3.1 x 10⁵      |
| Urabe SIPAR ATCC        | 34      | Vero      | Supernatant      | 5              | 1.7 x 10⁵      |
| Urabe SIPAR ATCC        | 34      | Vero      | Supernatant      | 6              | 1.8 x 10⁵      |
| Urabe SIPAR ATCC        | 34      | Vero      | Supernatant      | 7              | 5.0 x 10⁵      |
| Urabe Mumps Isolate A   | 37      | Vero      | Supernatant      | 3              | 4.1 x 10⁵      |
| Urabe Mumps Isolate B   | 37      | Vero      | Supernatant      | 4              | < 10²          |
| Urabe Mumps Isolate B   | 37      | Vero      | Supernatant      | 5              | < 10²          |
| Urabe Mumps Isolate C   | 37      | Vero      | Supernatant      | 6              | < 10²          |
| Urabe Mumps Isolate C   | 37      | Vero      | Supernatant      | 7              | < 10²          |
| Urabe Mumps Isolate I   | 37      | Vero      | Supernatant      | 3              | 4.3 x 10⁵      |
| Urabe Mumps Isolate I   | 37      | Vero      | Supernatant      | 3              | 5.0 x 10⁵      |
| Urabe Mumps Isolate I   | 37      | Vero      | Supernatant      | 3              | 2.0 x 10⁸      |
| Urabe Mumps Isolate I   | 37      | Vero      | Supernatant      | 3              | 2.1 x 10⁷      |

B

C

| Virus                    | HeLaS3 Cells 1.6 L Prep # | Harvest Titer TCID₅₀/mL | Purified Titer TCID₅₀/mL | Final Volume |
|-------------------------|---------------------------|-------------------------|--------------------------|--------------|
| MuV-UA (Urabe Mumps Isolate A) | 2014-05                  | 2.0 x 10⁸                | 4.5 x 10⁸                | 237 mL       |
| MuV-UC (Urabe Mumps Isolate C) | 2014-08                  | 1.0 x 10⁸                | 1.5 x 10⁸                | 252 mL       |

D

| Virus       | LaS 293 HEK 25 L Prep # | Harvest Titer TCID₅₀/mL | Purified Titer TCID₅₀/mL | Purification Protein/DNA | Final Volume | Infectivity to NP RNA |
|-------------|-------------------------|-------------------------|--------------------------|--------------------------|--------------|------------------------|
| MuV-U-Japan | 2017-01                 | 3.8 x 10⁹                | 1.8 x 10¹⁰               | 99%/99%                  | 200 mL       | 1:76                   |
| rMuV-UC-GFP | 2017-02                 | 7.9 x 10⁹                | 2.7 x 10¹⁰               | 98%/99%                  | 220 mL       | 1:59                   |
| MuV-UA      | 2017-05                 | 3.4 x 10⁹                | 3.1 x 10¹⁰               | 98%/99%                  | 153 mL       | 1:742                  |
| MuV-UA      | 2018-01                 | 4.8 x 10⁹                | 9.9 x 10¹⁰               | 98%/99%                  | 210 mL       | 1:212                  |
| MuV-UC      | 2017-04                 | 7.4 x 10⁹                | 6.1 x 10¹⁰               | 98%/99%                  | 224 mL       | 1:130                  |
| MuV-UC      | 2019-01                 | 5.0 x 10⁹                | 3.0 x 10¹⁰               | 99%/98%                  | 256 mL       | 1:17                   |
Isolate C with the CPE phenotype, now called MuV-UC, and isolate A with the SYN phenotype, now called MuV-UA, were chosen for further study because they represent the spectrum of possible oncolytic activity contained in the MuV-U-Japan clinical trial stock. Initial, small-scale, partially purified preclinical virus stocks were produced using suspension HeLaS3 cells (2 × 10⁶/mL) infected at an MOI of 0.1 and incubated at 37°C for 2 days (MuV-UC) or 3 days (MuV-UA). The supernatants of the infected cells were harvested (~1.6 L), clarified by filtration, and then treated overnight with Benzonase (10 units/mL) at 4°C. Using a hollow-fiber, tangential flow filtration (TFF), 750-kDa pore size cartridge, the processed bulk product was partially concentrated by ultrafiltration and then purified by diafiltration into a sucrose storage buffer (5% sucrose, 50 mM Tris [pH 7.4], and 2 mM MgCl₂). The purified product was then polished by filtration, aliquoted, and stored at a temperature lower than −60°C. At this small scale, the final titers of the purified virus stocks were only slightly higher compared with supernatants (Figure 3C).

The MuV-UA and MuV-UC isolates efficiently killed a panel of established human breast cancer cell lines in vitro

To follow up on the partial success of treating patients with breast cancer in the original MuV-U clinical trials, and because new treatments for patients with breast cancer are still needed, especially for those with estrogen receptor-negative, progesterone receptor-negative, or HER2-negative or TNBC, the MuV-Us were initially assayed for oncolytic killing efficacy against breast cancer. Initially, we surveyed the ability of MuV-UA and MuV-UC to kill a panel of 9 different established human breast cancer cell lines that represents the wide range of phenotypes and genotypes in human breast cancers.15,16 Human breast cancers and their derived continuous cell lines have a wide range of genetic and physiological defects that complicate treatment. A related oncolytic paramyxovirus, measles virus with a sodium iodide symporter transgene, MV-NIS, which has been shown previously to have oncolytic activity against a variety of human cancer cell lines, including breast cancer, was included as a comparative control.17

Triplicate cultures of tumor cells were left uninfected (MOI 0) or infected with HeLaS3-produced virus stock at an MOI of 0.1, 1, and 10, and the cultures were incubated to allow virus replication and killing over 5 days. MTS cell proliferation assays were performed on day 3 and day 5 after infection, and the results were graphed as percentage of the uninfected control cultures (Figure 4). Both MuV-U isolates were very potent at killing all nine breast cancer cell lines tested, including triple-negative hormone receptor breast cancer cell lines, at killing efficiencies equal to or more potent than MV-NIS. Control Vero cell cultures were also infected with each experiment to verify the integrity of virus stocks; a consistent difference in killing Vero cells was observed between MuV-UA and MuV-UC.

An initial large-scale virus manufacturing process using LaSt 293 HEK suspension cells enabled production of purified high-titer MuV-U virus stocks

Because we expected that high doses of MuV-U viruses would be needed to treat human tumor xenografts in mouse models by intravenous administration, the Mayo Clinic VVPL developed large-scale virus production and purification methods to manufacture purified MuV-U virus stocks with high titers. The LaSt 293 HEK suspension cell line was used for large-scale MuV-U virus production. The LaSt 293 HEK cell line is a robust suspension HEK293 cell line generated by the Mayo Clinic VVPL by adapting a GMP HEK293 adherent cell line to serum-free growth conditions. For this experiment, a portion of the original MuV-U-Japan clinical trial stock and the rMuV-U-GFP stock described previously were amplified for one passage using HeLaS3 cells, concentrated, and purified as the MuV-UA and MuV-UC isolate HeLaS3 stocks described above.

Large-scale, preclinical virus stocks were generated from 25-L cultures of LaSt 293 HEK cells (2 × 10⁶ cells/mL) in 50-L WAVE bioreactors incubated at 37°C and infected with an MOI of 0.1 with HeLaS3 preclinical stocks of MuV-U-Japan, rMuV-UC-GFP, MuV-UA, or MuV-UC, and the infected cell supernatants were harvested at ~48 h (MuV-UA was harvested at 69 h because of the slower replication rate). The harvested supernatants were clarified and treated with Benzonase, and the treated bulk harvests were then concentrated ~10-fold, purified by diafiltration with sucrose-based storage buffer, followed by a final ~5-fold concentration using two rounds of hollow-fiber TFF. The products were then polished using a depth filter, aliquoted, and stored at a temperature lower than −60°C. The entire production and purification process was performed by qualified Mayo Clinic VVPL personnel, using aseptic conditions to ensure sterility. The manufacturing process produced virus stocks with infectious virus titers 50- to 100-fold higher than the smaller-scale HeLaS3 stocks and removed more than 98% of residual protein and cellular DNA in the final products compared with the starting supernatants (Figure 3D).

The initial 2017-04 production of MuV-UC LaSt virus stock resulted in a lower-than-expected final infectious virus titer and a lower-than-expected infectivity-to-MuV NP RNA ratio. Subsequent experiments point to the time when the clarified and Benzonase-treated MuV-UC supernatant was stored at 4°C before TFF diafiltration into the sucrose storage buffer as the likely reason for the loss of infectivity. The 2017-04 MuV-UC preparation was stored for a total of 4 days at 4°C (over a weekend) before the buffer was changed to the sucrose...
| Cell Line   | ATCC #  | Molecular Classification | Hormone Receptor Status |
|------------|---------|--------------------------|------------------------|
|            |         | Subtype | Subgroup | ER | PR | Her2 |
| MDA-MB-468 | HTB-132 | TNBC    | Basal A  | -  | -  | -    |
| MDA-MB-231 | HTB-26  | TNBC    | Basal B  | -  | -  | -    |
| BT549      | HTB-122 | TNBC    | Basal B  | -  | -  | -    |
| Hs578T     | HTB-126 | TNBC    | Basal B  | -  | -  | -    |
| MCF7       | HTB-22  | ER+     | Luminal A| +  | +/-| -    |
| T47D       | HTB-133 | ER+     | Luminal A| +  | +/-| -    |
| ZR-75-1    | CRL-1500| ER+     | Luminal A| +  | +/-| -    |
| BT474      | HTB-20  | HER2+   | HER2    | +  | +  | +    |
| SKBR3      | HTB-30  | HER2+   | HER2    | -  | -  | +    |

![Graphs and images](legend on next page)
storage buffer, whereas the 2019-01 MuV-UC preparation was stored only for 1 day at 4°C and retained a higher infectious virus titer and ratio to MuV NP RNA. We also tested harvesting the MuV-UA virus at 48 h (2018-01 preparation), which resulted in a high-titer stock with a slightly lower infectious titer but a much higher infectious titer-to-MuV NP RNA ratio compared with the 2017-05 preparation harvested at 69 h.

Significant increases in survival of nude mice with human TNBC MDA-MB-231 tumor xenografts after treatment with MuV-U viruses
MuV is a human virus with no representative animal models that allow effective viral replication or produce pathogenesis similar to disease in human patients. The well-established immunocompromised xenograft breast cancer model of TNBC MDA-MB-231 cell line xenografts in athymic nude mice was used to assess the oncolytic killing of MuVs in vivo.\textsuperscript{13,14} Nude mice with established MDA-MB-231 xenografts in the left leg flank (average, 50 mm\textsuperscript{3}) were divided into experimental groups (n = 10) and treated with one or three doses delivered by intratumoral (IT) or intravenous (i.v.) tail vein injection(s) (50-μL volume) with vehicle control buffer or a LaSt 2017 MuV-U virus stock (Figure 3D): MuV-UA, MuV-UC, a 50:50 combination of both isolates (MuV-UA+UC), MuV-U-Japan, or rMuV-UC-GFP. We tested the MuV-UA+UC combination in a mimic of the original MuV-U-Japan stock was 50:50 SYN and CPE phenotypes. For the three dose IT treatment, animals were rotated by 120° for each dose (day 0, 0°; day 7, 120°; day 14, 240°) to distribute the doses more efficiently to different areas of the tumor. The survival, tumor volume, and weight of each mouse were measured at least 3 times a week over the course of the experiment.

IT MuV delivery
In one experiment, delivery of one dose of 1 × 10\textsuperscript{5} TCID\textsubscript{50} by IT injection of xenografts on day 0 resulted in a significant increase in survival of mice in groups treated with MuV-U viruses compared with vehicle (Figure 5A). The growth of most of the MuV-U treated xenograft tumor volumes regressed in size considerably over the course of the experiment (Figure S1A).

A MuV-U qRT-PCR assay was developed to quantitate levels of NP RNA isolated from harvested tumors or blood. The MDA-MB-231 xenografts were injected with vehicle or 1 × 10\textsuperscript{6} TCID\textsubscript{50} of rMuV-UC-GFP by IT delivery, and infected tumors were harvested on days 1, 2, 3, 5, 7, 10, and 15. RNA was extracted from each tumor, and the levels of MuV NP RNA were quantitated. More than 10\textsuperscript{4} copies of MuV NP RNA per microgram of tumor RNA was present already on day 1 and even higher levels of MuV NP RNA on subsequent days (Figure 5B).

In a follow-up experiment, tumor xenografts were treated with three doses of 1 × 10\textsuperscript{5} TCID\textsubscript{50} by IT injection on days 0, 7, and 14 to more efficiently deliver virus to more tumor area. Again, the experimental groups treated with MuV-U viruses showed a significant survival advantage compared with the vehicle group (Figure 5C), but this was not significantly different compared with the one-dose experiment. However, the tumor volumes of mice treated with three doses of an MuV-U regressed to barely or not detectable sizes, and the regression was maintained in almost all mice for the duration of the experiment (Figure S1B). To assess possible MuV in the bloodstream of IT-treated animals, RNA was extracted from blood samples collected on days 1, 3, 10, and 21 from the three-dose IT experiment (Figure 5C, right panel). Very little, if any, MuV NP RNA copies were detected in the blood of IT MuV-U-treated animals (Figure 5C, left panel).

A third experiment explored the minimum effective dose of only MuV-UC for treating TNBC MDA-MB-231 xenografts compared with vehicle. Experimental groups of mice were treated with one dose (day 0) or three doses (days 0, 7, and 14) with vehicle or with MuV-U at 1 × 10\textsuperscript{3}, 1 × 10\textsuperscript{4}, or 1 × 10\textsuperscript{5} TCID\textsubscript{50} per dose, with all 8 experimental groups studied simultaneously. The survival curves are presented as two graphs: the one-dose-treated groups (Figure 5D, left panel) and the three-dose-treated groups (Figure 5D, right panel). Significant increases in survival were obtained in 5 of 6 MuV-UC-treated groups compared with the vehicle control groups; only the one dose of 1 × 10\textsuperscript{3} TCID\textsubscript{50} MuV-UC did not result in an increase in survival. Even treatment with just one dose of 1 × 10\textsuperscript{3} TCID\textsubscript{50} MuV-UC resulted in survival of 50% of the animals. The tumor volumes of each animal in the experiment are shown in Figure S2.

IV MuV delivery
In one experiment, delivery of one dose of 2 × 10\textsuperscript{7} TCID\textsubscript{50} by i.v. injection on day 0 resulted in a significant increase in survival of mice in all groups treated with MuV-U viruses compared with vehicle (Figure 6A, top panel). The tumor volumes of each animal in the experiment are shown in Figure S3A. Blood samples were collected from 3 mice in each experimental group on days 1, 3, 7, and 15. RNA was extracted and assayed for MuV-U NP RNA copies by qRT-PCR. Detectable levels of MuV-U NP RNA were detected in the blood of MuV-U-treated animals through at least day 7 (Figure 6A, bottom panel). The circulating levels of rMuV-UC-GFP were much lower compared with animals treated with the other MuV-U viruses, at least as measured by MuV-U NP RNA copies.

In a follow-up experiment, tumor xenografts were treated with three doses of 2 × 10\textsuperscript{7} TCID\textsubscript{50} delivered by i.v. injection on days 0, 4, and 8. Treatment with the MuV-U viruses significantly increased survival.
compared with vehicle controls (Figure 6B, top panel). The tumor volumes of each animal in the experiment are shown (Figure S3B). Blood samples were collected from 5 mice in each experimental group on days 10, 15, 20, and 24. RNA was extracted and assayed for MuV-U NP RNA copies by qRT-PCR. As in the one-dose-treated animals, levels of MuV-U NP RNA were detected in the blood of MuV-U-treated animals through at least day 20 (Figure 6B, bottom panel).

With three doses, MuV-UC and the MuV-UA+UC-treated animals had significantly higher levels of circulating MuV-U NP RNA copies over time compared with the other MuV-U-treated groups. The
circulating levels of rMuV-UC-GFP were similar to MuV-UA- and MuV-U-Japan-treated animals, as measured by MuV-U NP RNA copies.

MuV-Us efficiently kill breast cancer PDX cell lines grown as 3D organoids in culture, including from TNBC tumors that were resistant to standard chemotherapy

PDX breast cancer cell lines grown as 3D organoids in culture have been shown to more closely recapitulate the structural organization, heterogeneity, and biology of breast tumors compared with 2D cultures and immortalized tumor cell lines.\textsuperscript{18} Previously, pretreatment PDX breast cancer lines were established from patients with primary breast cancer who participated in a prospective neoadjuvant study of a standard chemotherapy, The Breast Cancer Genome Guided Therapy Study (BEAUTY; ClinicalTrials.gov: NCT02022202).\textsuperscript{19,20} These patients were treated with standard taxane-based chemotherapy for 12 weeks, followed by anthracycline-based chemotherapy for 8 weeks, and their response to the chemotherapy treatment was recorded.
Seven PDX breast cancer lines from the BEAUTY study were tested for MuV-U susceptibility in this study, including PDX lines derived from two patients who did not respond to the standard chemotherapy treatment, MC-BR-BTY-0014 and MC-BR-BTY-0009, and another PDX line, MC-BR-BTY-0006, derived from a patient who only had a partial response. The PDX lines were grown as 3D organoid models because this approach best recapitulated the results of the patient response to chemotherapy treatment in a culture system. The PDX 3D organoid cultures were infected with different levels of MuV-U viruses as well as MV-NIS to quantitate their susceptibility to these oncolytic virotherapies. Duplicate cultures of the PDX 3D organoids were left uninfected (MOI 0) or infected with virus at an MOI of 0.1, 1, and 10, and the cultures were incubated to allow virus replication and killing over 5 days. CellTiter-Glo 3D cell viability assays were performed on day 5 after infection, and the results were graphed as the percentage of the uninfected control cultures (Figure 7). The MDA-MB-231 TNBC cell line was included in every experiment as a control to demonstrate and verify oncolytic activity of the virus stocks.

All seven PDX 3D organoid cultures were susceptible to killing by MuV-UA, MuV-UC, and rMuV-UC-GFP, and six of seven PDX 3D organoid cultures were susceptible to the amplified original MuV-U-Japan mixture, including PDX lines derived from the chemotherapy non-responders and partial responder. There was some variability in the overall cell survival profiles versus virus dose, with some cultures very susceptible to MuV-U killing even at low virus levels (for example, MC-BR-BTY-0014 and MC-BR-BTY-0006), whereas other PDX 3D cultures required significantly higher virus doses to achieve 30%–60% killing (for example, MC-BR-BTY-0004). But overall, the four different MuV-U viruses had similar levels of oncolytic activity within a PDX line. Five of seven PDX 3D organoid cultures were susceptible to MV-NIS oncolytic killing, and two PDX lines were not susceptible to MV-NIS killing at any virus level (MC-BR-BTY-0014 and MC-BR-BTY-0004). Five of the seven PDX 3D cultures were similarly susceptible to oncolytic killing by MuV-U and MV-NIS. However, two PDX lines, MC-BR-BTY-0004 and MC-BR-BTY-0006, were much more susceptible to killing by MuV-U compared with little or no killing by MV-NIS.

MuV-Us efficiently kill the pancreatic tumor cell line BxPC3 and the lung tumor cell line A549 in vitro

The MuV-U oncolytic virotherapy pilot clinical trials in Japan produced clinical responses in patients with different tumor types. Here showed that the newly isolated MuV-Us have retained significant oncolytic killing of breast cancer models as a first clinical indication for translation to the clinic. Initial pilot in vitro experiments were done, assaying representative established cell lines of two other solid tumor types: the pancreatic tumor cell line BxPC3 and the lung tumor cell line A549. MDA-MB-231 breast tumor cell cultures were also infected as controls to verify the integrity of the virus stocks.

Triplicate cultures of tumor cells were left uninfected (MOI 0) or infected with virus stocks at an MOI of 0.1, 1, and 10, and the cultures were incubated to allow virus replication and killing over 5 days. Killing by the MuV-Us MuV-UA, MuV-UC, rMuV-UC-GFP, and MuV-U-Japan and the oncolytic measles viruses MV-NIS and MV-GFP was compared using CellTiter-Glo Cell viability assays performed on day 3 and day 5 after infection, and the results were graphed as the percentage of the uninfected control cultures (Figure 8). All oncolytic MuV-U efficiently killed all three tumor cell lines in this assay but were especially potent at killing BxCP3 tumor cells. The MuV-U killed A549 tumor cells as well as the previously characterized MDA-MB-231 breast tumor cells in vitro. Both oncolytic MV viruses efficiently killed all three tumor cell lines in this assay but were much less potent at killing BxPC3 tumor cells compared with MuV-U.

**DISCUSSION**

Here we report the unique ability to perform a modern characterization and assessment of the antitumor activity of an actual clinical trial virus stock of essentially the wild-type Urabe MuV and comparison with the related attenuated Urabe SIPAR vaccine strain. The clinical MuV isolates that were the basis for all modern attenuated MuV vaccines were not archived and therefore unavailable for a comparison and characterization by current advanced molecular techniques. Although a detailed manufacturing history of the MuV-U-Japan clinical trial sample is lacking, the comparison of genomic and virus replication differences between MuV-U-Japan isolates and the attenuated Urabe SIPAR vaccine strain in this study indicate that MuV-U-Japan is likely representative of the original wild-type MuV-U clinical isolate.

The observation that the MuV-U-Japan virus stock was a mixture of very closely related quasispecies was not unexpected, given that the normal levels of intragenic MuV variation can reach 4% differences. Although all 12 MuV-U isolates were unique, the sequence differences only averages 3–5 coding differences between isolates. The coding differences between MuV-U isolates resulted in replicative differences with up to 50-fold differences in infectious titer. However, the two different cytotoxic killing phenotypes observed when Vero cells were infected with the MuV-U isolates did not correlate to specific nucleotide differences. It may be that the rate of replication and/or maximum infectious titer achieved could explain the SYN cell fusion phenotype compared with the CPE cell-rounding phenotype in Vero cells. MuV-U replication was significantly higher in human tumor cells compared with Vero cells, and the lower replication rate in Vero cells may have enhanced the cytotoxic phenotypes.
The risk versus benefit analysis of using an essentially wild-type MuV for cancer treatment was originally articulated by Dr. Asada, the principal investigator of the first MuV-U oncolytic clinical trial publication. He theorized that wild-type MuV would be safe because of the virus’ natural and specific preference for targeting cancer cells, because of most patients having antibodies to MuV, even a minimal amount; because of the small risk of serious complications in adults with cancer, even when never exposed to MuV; and because the major morbidity of mumps is age and sex specific, with the highest risk in postpubertal boys, primarily from complications of meningitis, encephalitis, and orchitis. Patients in the Japanese clinical trials were safely treated i.v. and IT with multiple doses (as many as 15 doses) of the original Japan MuV-U clinical product, with each dose as high as $10^9$ TCID50.

In the MuV-U oncolytic virotherapy pilot clinical trials in Japan that treated over 300 patients with 18 different tumor types, significant clinical responses were obtained, including in 7 of the 13 breast cancer patients treated with MuV-U-Japan. In this study, we focused on characterizing whether the current MuV-U-Japan virus stock and the isolates MuV-UA and MuV-UC as well as a recombinant MuV-UC rMuV-UC-GFP had oncolytic efficacy against human breast cancer models as a possible first clinical disease indication candidate. All MuV-U viruses efficiently killed a panel of human breast cancer cell lines, including MDA-MB-231, BxPC3, and A549.

Figure 8. Oncolytic killing activity of MuV-U and MV viruses of representative pancreatic and lung solid tumor cell lines
Cultures of each cancer cell line were left uninfected (MOI 0) or infected at MOIs of 0.1, 1.0, and 10 with an oncolytic virus. CellTiter-Glo cell viability assays were performed on triplicate samples collected on days 3 and 5 after infection. The results are the average and standard deviation of the percentage of cell survival compared with the uninfected control.
breast cancer cell lines in vitro and efficiently extended the survival of athymic nude mice with TNBC MDA-MB-231 tumor xenografts. IT delivery of even relatively low-dose levels of MuV-UC effectively increased survival and controlled tumor growth using this model. The maximum MuV-U dose so far tested, delivered i.v., increased survival to an average of 50%. In the original trials, clinical responses were observed, using i.v. delivery despite most patients having pre-existing antibodies to MuV from previous exposure; therefore, the fact that modern patients will now have been vaccinated against MuV may not be a hindrance.

We and others have hypothesized that patients with cancer resistant to traditional chemotherapies may respond to treatment with oncolytic viruses, which have very different mechanisms of action compared with chemotherapy and, therefore, could be viable treatment options for these patients. In this study, we showed that human PDX breast cancer lines grown as 3D organoids in culture, including lines from patients who did not respond to standard chemotherapy treatments, were efficiently killed by MuV-U treatment, supporting this hypothesis. We did observe that the established oncolytic measles virus MV-NIS efficiently killed 5 of the 7 PDX lines tested, but 2 lines were resistant to MV-NIS killing. Just like the success or failure of each developed chemotherapy in treating different cancer types and different patient populations, each oncolytic virotherapy will have its own efficacy/patient success profile; therefore, multiple available virus platforms will be needed to have the greatest effect. A patient treated with a virotherapy will produce an immune response that makes additional treatment with the same virus problematic, but treatment with a second different oncolytic virotherapy will not be affected because it will be based on a different virus platform.

TNBC accounts for 15%–20% of all breast cancers and is the most aggressive disease subtype. Clinical outcomes for patients with TNBC remain inferior compared with other breast cancer subtypes, with earlier disease relapse and worse survival, highlighting the critical need for development of novel effective therapeutic approaches. This study demonstrates that the MuV-U isolates have retained wild-type MuV characteristics, which likely lead to the significant efficacy in treating human breast cancer models. We also report success in developing a large-scale MuV-U production and purification process suitable for supporting IND applications and GMP virus production for clinical trials. This study demonstrates the suitability of MuV-UC for translation to modern clinical trials for treating patients with TNBC.

MATERIALS AND METHODS

Cells

The Vero cells used in this study were from a Mayo Clinic VVPL cGMP cell bank grown in DMEM supplemented with 5% fetal bovine serum. The suspension HeLaS3 cells used in this study were from a Mayo Clinic VVPL cell bank and adapted to serum-free medium 293 SFM II (Gibco Invitrogen) supplemented with 4 mM GlutaMAX-I (Gibco) and 0.1% Pluronic F68 (Thermo Fisher Scientific, Waltham, MA) and grown in shake flasks.

The LaSt 293 HEK cell line used in this study is a robust suspension HEK293 cell line generated by the Mayo Clinic VVPL by adapting a GMP HEK293 adherent cell line to serum-free growth conditions using serum-free protein expression medium (PEM; Gibco Invitrogen) supplemented with 4 mM GlutaMAX-I (Gibco) and 0.1% Pluronic F68 (Thermo Fisher Scientific, Waltham, MA) and grown in shake flasks and WAVE bioreactors.

The established cancer cell lines used in this work were purchased from the ATCC (Manassas, VA) and were maintained in medium recommended by the ATCC at 37°C and 5% CO2: the breast cancer cell lines MDA-MB-468, MDA-MB-231, BT549, Hs578T, MCF7, T47D, ZR-75-1, BT474, and SKBR3; the pancreatic cancer cell line BxPC3; and the lung cancer cell line A549.

The generation of breast cancer PDX cell lines and growth of these breast cancer PDX lines as 3D organoids have been described previously.19–23

Viruses

Construction and rescue of the recombinant MuV-U with the GFP reporter gene rMuV-UC-GFP have been described previously.24 A virus stock of the attenuated MuV-U vaccine strain SIPAR 02 (GenBank: AF314558.1) was purchased from the ATCC. Construction and rescue of MV-NIS and MV-GFP have been described previously.17

MuV-U replication and purification

Cells were routinely infected with an MuV-U at an MOI of 0.1, and the infected cell supernatants were collected 2–3 days later, depending on the MuV-U isolate. The supernatants from small, infected cultures were clarified by centrifugation, 800 × g for 10 min at 20°C, to remove any intact cells and large debris. Large culture supernatants were using a 1.2-µm filter (e.g., Sartapure GF Plus capsule filter, Sartorius, Gottingen, Germany). The clarified supernatant was then treated with Benzonase endonuclease (10 units/mL) overnight at 4°C to digest cellular nucleic acid. The processed bulk MuV-U was then purified using hollow-fiber TFF cartridges (e.g., sterile ReadyToProcess hollow fiber cartridge, 750 kD, 0.5 or 1 mm i.d. fiber, Cytiva, Marlborough, MA), first concentrating the bulk by ultrafiltration to reduce volume and then purifying by diafiltration with 6 volumes of sucrose buffer (5% sucrose, 50 mM Tris [pH 7.4], 2 mM MgCl2). For large preparations (>10 L), a second ultrafiltration concentration step can be done to a final total concentration of ~50-fold. A final polishing filtration step (e.g., Mini Profile Star 1.5-micron capsule filter, Pall, Port Washington, NY) was done to remove any aggregates that may have formed from TFF. The final purified MuV-U product was vialled and stored at a temperature lower than ~60°C.
**TCID\textsubscript{50} infectious virus titer assay**

The infectious virus titers were determined using the TCID\textsubscript{50} method, Vero cells, and a 5-fold dilution series for the samples and calculated using the Spearman-Karber method. 25,26

**Viral genome nucleotide sequencing**

In general, total RNA was extracted from virus stocks using the QIAamp Viral RNA Kit (QIAGEN, Valencia, CA), followed by the RNA being transcribed to cDNA using reverse transcriptase. A set of forward and reverse oligonucleotide primers was designed to synthesize overlapping 1- to 2-kb dsDNA fragments using the reported attenuated MuV-U vaccine strain SIPAR 02 (GenBank: AF314558.1). The nucleotide sequence was determined from both strands of the purified PCR products by Sanger sequencing. The nucleotide sequence of both strands was assembled using the Sequencher DNA sequence analysis software (Gene Codes, Ann Arbor, MI). The nucleotide sequences of the oligonucleotide primers used in this study are available upon request.

**Cell viability assays**

Three different cell viability assays were used to quantitate cell killing by oncolytic viruses. The CellTiter 96 AQueous One Solution MTS cell proliferation assay (Promega, Madison, WI), the CellTiter-Glo 3D cell viability assay (Promega, Madison, WI), and the CellTiter-Glo cell viability assay (Promega, Madison, WI) were used, following the manufacturer’s recommendations.

**Total protein assay**

Total protein levels in virus preparations were quantitated using a bicinchoninic acid protein assay kit (Sigma-Aldrich, St. Louis, MO), following the manufacturer’s recommendations.

**Residual cellular DNA assay**

Residual cellular DNA in MuV-U preparations was quantitated using the PicoGreen dsDNA Quantitation Assay Kit (Molecular Probes, Eugene, OR), following the manufacturer’s recommendations.

**MuV-U NP gene RNA qRT-PCR assay**

Total RNA was extracted from purified virus stocks using the QIAamp Viral RNA Kit (QIAGEN, Valencia, CA), tumor tissue using the RNeasy Fibrous Tissue Kit (QIAGEN, Valencia, CA), or mouse blood using the RNeasy Protect Animal Blood Kit (QIAGEN, Valencia, CA), following the manufacturer’s recommendations.

A qRT-PCR assay was developed and qualified for detection and quantitation of MuV-U NP gene RNA. The oligonucleotides and probe were synthesized and purified by Integrated DNA Technologies (Coralville, IA). The MuV-U forward primer was 5’-CGG AGG CTA CCC ATT GAT ATT C-3’. The MuV-U reverse primer was 5’-TAG GAA AGG TCG TGC ATA AGT G-3’. The probe 5’-TGC TAT GGG AGT CGG TAC AGT CCT-3’ has the 5’ end modified with 6-FAM (carboxylfluorescein), and the probe is modified with two quenchers: internally with ZEN and at the 3’-end with 3IABkFQ (3’ Iowa Black fluorescence quencher). The Roche LightCycler 480 RNA Master Hydrolax Probes were used in a reaction volume of 25 μL. A Roche LightCycler 480 II real-time PCR instrument was used with a program of 45 cycles of 95°C/15 s, 60°C/45 s. LightCycler 480 software was used to analyze the data.

**In vivo experiments**

All animal protocols were reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee. Outbred athymic nude mice, Crl:NU(NCr)-Foxn1nu, were purchased from Charles River Laboratories (Wilmington, MA). Mice were implanted with 2 × 10⁷ MDA-MB-231 cells in the left flank at 5–7 of age, when body weights were 20–28 g at the time of implantation, and the mice were treated when the tumors were averaging ~50 mm³. Tumor volumes were measured by handheld calipers. The mice were monitored daily until the end of the experiment or when they reached the euthanasia criteria: clinical signs of neurotoxicity, tumor ulceration, tumor volume greater than 1,000 mm³, weight loss greater than 10%, or inability to gain access to food and water.

**Statistical analyses**

Survival curves and analysis were done using Prism 9 for macOS (GraphPad Software, San Diego, CA). The significance p values were determined using the Mantel-Cox log rank test.

**Data availability statement**

All raw data are available upon request.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2022.11.002.

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**AUTHOR CONTRIBUTIONS**

M.D.B. led in vitro and in vivo testing. R.J.S. led qRT-PCR assay development and sample testing and assisted with in vitro and in vivo testing. G.M.P. led analysis of genomic sequences and assisted with in vitro testing. L.A.S. led production of mumps virus stocks. Y.Z. and L.W. provided established PDX cultures growing as 3D organoids. J.Y. developed/generated PDX lines. J.C.B. and M.P.G. co-led the clinical study that led to development of the PDX models. M.J.F. designed and oversaw project experiments and analyses and wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.
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