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Intrinsic Oncolytic Activity of Hoshino Mumps Virus Vaccine Strain Against Human Fibrosarcoma and Cervical Cancer Cell Lines

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Abstract

Background: The use of oncolytic viruses as therapeutic agents is a promising treatment for various human cancers. Several viruses have been extensively examined to achieve tumor cell death.
Objectives: This study aimed at evaluating the natural oncolytic activity of mumps Hoshino vaccine strain against two human cancer cell lines, that is, HT1080 fibrosarcoma and HeLa cervical adenocarcinoma cell lines.
Methods: The cytolytic activity of the virus was evaluated using an MTT assay. Apoptosis was detected by Annexin-V/propidium iodide (PI) staining and analyzed via flow cytometry. To indicate viral replication in vivo, nude mice with HeLa heterografts were treated with the Hoshino strain of mumps virus.
Results: It was found that human fibrosarcoma and cervical cells were more sensitive to the mumps Hoshino strain, even at a very low multiplicity of infection (MOI) compared to normal human diploid cells. The results also showed that the Hoshino strain induced apoptosis in both cancer cells. A preliminary in vivo study revealed the significant suppression of tumor growth in the group treated with the mumps Hoshino strain compared to the control group.
Conclusions: The Hoshino vaccine strain of mumps virus showed promising oncolytic activities against human fibrosarcoma and cervical adenocarcinoma cells.

Keywords: Apoptosis, Mumps, Oncolytic Viruses, Oncolytic Virotherapy, Heterografts

1. Background

Currently, human cancers are the leading cause of disease-related mortality and continue to be major health problems. Although current therapeutic strategies in oncology, including the use of chemical anti-cancer drugs, radiation, surgical methods, or combinations of these modalities, are valuable tools for the treatment of some human tumors, other approaches appear to be ineffective. In recent years, many efforts have been made to find novel methods for the treatment of different human tumors. Viral therapy with oncolytic viruses (OVs) is one of the most important areas of cancer therapy. Evidence shows that various viruses, such as some members of the *Paramyxoviridae* family exhibit oncolytic activity (1-4).

The wild-type Urabe strain of mumps virus (*MuV*) was reported to exhibit significant oncolytic activities against different human cancers in the mid-1970's (5). The *MuV* belongs to the order *Mononegavirales*, the family *Paramyxoviridae*, the subfamily *Rubulavirinae*, and the genus *Orthorubulavirus*. Mumps, as one of several childhood infectious diseases, is a vaccine-preventable disease. However, the extensive use of attenuated vaccine strains has reduced the prevalence of this disease around the world (6). Although *MuV* has long been used as an anti-tumor agent in preclinical (7) and uncontrolled clinical trials (8-10). Limited studies have been recently conducted in this area. More recently, however, some natural and chimeric *MuV* strains have been studied as potential anti-cancer agents (11-16).

The attenuated Hoshino vaccine strain is one of the several commercial mumps vaccine strains, which is derived from a genotype-B wild-type *MuV* isolate and used as a vaccine in Japan, Korea, and Iran. This temperature-sensitive vaccine strain was developed by culturing on spe-

cific pathogen-free (SPF) chicken embryo cells at 32°C, using the plaque cloning technique (17). Experimental studies on infections in monkeys, as well as clinical trials (18), have shown that this vaccine strain is both safe and effective. It is also extensively used in Iran.

2. Objectives

To the best of our knowledge, no study has been yet published on the oncolytic activity of the Hoshino vaccine strain of *MuV*. Therefore, this study aimed at elucidating the natural oncolytic activity of this commercially available vaccine strain against two human cancer cell lines, that is, HT1080 fibrosarcoma and HeLa cervical adenocarcinoma cell lines.

3. Methods

3.1. Cells and Virus

The HT1080 cell line was obtained from the Iranian Biological Resource Center (IBRC, Tehran, Iran). Normal human diploid (MRC-5) and HeLa cells were also obtained from the Human Viral Vaccine Department of Razi Vaccine and Serum Research Institute (RVSRI, Karaj, Iran). The cells were grown and passaged, using the Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), containing 10% (v/v) fetal bovine serum (FBS; Gibco, Waltham, MA, USA) at 37°C. The virus stock (Hoshino strain) was propagated in a primary SPF chicken embryo fibroblast (CEF) cell. The harvested virus was then extracted from the cell debris, using a 0.2-μm syringe filter (Sartorius, Goettingen, Germany). The infectivity titer (50% cell culture infective dose [CCID50]) was measured in the HeLa cell line, using a standard method, and virus titers were calculated according to the Spearman-Kärber method.

3.2. In Vitro Cell Viability Analysis

HeLa, HT1080, and MRC-5 cells (5,000 cells/well) were cultured in 100 μL of DMEM medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 5% (v/v) FBS (Gibco, Waltham, MA, USA) and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere in the air. To investigate the effects of viruses, the cells were infected with the Hoshino strain at a series of multiplicity of infection (MOI), including 0, 0.002, 0.02, 0.2, and 2 in 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium). After 96 hours, the medium was replaced with a fresh one, containing 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Life Science, St. Louis, MO, USA).

The plates were incubated for four hours at 37°C in a 5% CO_2 atmosphere in the air. The supernatants were then removed and 100 μL of dimethyl sulfoxide (DMSO; BDH Chemicals Ltd., Poole, England) was added to each well. Cell viability was measured at 570 and 630 nm, using an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynex MRX II, Chantilly, VA, USA). Virus infection was induced in triplicate. The results of cell viability are presented as the survival percentage of infected cells versus uninfected control cells, which were considered to have 100% viability. Data are presented as mean $\pm$ standard deviation (SD) of three measurements.

3.3. Apoptosis Measurements

Annexin-V/propidium iodide (PI) staining was performed for apoptosis analysis. The apoptotic cells were detected on day 4 post-infection, using the fluorescein isothiocyanate (FITC)-Annexin V apoptosis detection kit with PI (Biolegend, San Diego, CA, USA), according to the manufacturer's instructions. All flow cytometric measurements were performed at the Iranian Biological Resource Center (IBRC, Tehran, Iran). Data were analyzed, using the Flowing software V. 2.5.1. Differences in the median fluorescence intensity were compared, using paired *t*-test, and statistical significance was defined as $P < 0.05$.

3.4. In Vivo Analysis (Subcutaneous Tumor Heterograft Model)

In this preliminary experimental study, six-week-old male athymic B6 nude mice were obtained from Pasteur Institute of Iran (North Research Center). The mice were kept in separate ventilated cages and fed autoclaved standard laboratory chow and tap water ad libitum. The experiments and procedures were approved by the Ethics Committee of Razi Institute (approval number: RVSRI.REC.98.001). The HeLa cells (10^6), resuspended in 100 μL of phosphate-buffered saline (PBS), were subcutaneously implanted in the right flanks of mice, using an insulin syringe. Afterward, the animals were placed back into their cages and examined twice a week for tumor growth. The length and width of the tumors were measured with a Vernier caliper. The tumor volume (mm^3) was calculated as follows (19):

$$\text{Tumor volume} = \text{Length} \times \text{Width}^2 \times 0.5$$

When the tumors reached a diameter of 5 - 10 mm, the heterograft mice were divided into two groups as follows: (i) intratumoral administration of *MuV* Hoshino strain; and (ii) intratumoral administration of clarified cell lysates (mock). The treatment group received four doses of the virus at 2.5×10^5 $CCID_{50}$/dose twice a week for two weeks (total dose=1×10^6 $CCID_{50}$). The mice were euthanized as soon as they lost more than 20% of body weight,

or neurological deficits emerged, or the tumor diameter exceeded 2.5 cm.

3.5. Statistical Analysis

Both in vitro and in vivo data are presented as mean ± standard deviation (SD). Datasets were compared by independent *t*-test in SPSS version 15. Statistical significance was set at $P < 0.05$.

4. Results

4.1. In Vitro Analysis

To determine the impact of viral dose (MOI) on viral cytotoxicity, MTT assays were performed on the HeLa, HT1080, and MRC-5 cell lines at different viral MOIs (Figure 1). Based on the results, the two cancer cell lines (HeLa and HT1080) showed almost the same sensitivity to this vaccine strain. Human fibrosarcoma and cervical cells were more sensitive to the *MuV* Hoshino strain, even at MOI of 0.02, compared to normal human diploid cells (MRC-5). Also, the overall mean effect of the virus at MOI of 2 was significant against non-cancerous cells ($P < 0.00001$).

4.2. Apoptosis Measurements

The effect of *MuV* Hoshino strain on the induction of apoptosis was assessed, using Annexin-V/PI staining. The flow cytometry results revealed that apoptosis occurred when cancer cells were treated with the *MuV* Hoshino strain (Figure 2). The left bottom quadrant of the cytograms indicates the total number of viable cells, while the left top quadrant represents non-viable (necrotic) cells with PI uptake. Also, the right quadrants represent the number of apoptotic cells. The apoptotic cell count was significantly higher in HeLa and HT1080 cells, infected with the *MuV* Hoshino strain ($P < 0.05$).

4.3. In Vivo Analysis

The HeLa cells were injected into the subcutaneous flank of male nude mice to determine the in vivo replication and oncolytic activity of Hoshino strain. For this purpose, *MuV* was administrated following tumor formation. Tumor nodules became visible within the first 10 days after implantation in 100% of inoculated mice. The groups of heterograft mice showed similar tumor volumes before treatment ($P = 0.1290$). All animals exhibited tumor growth for 30 days after receiving the first dose. On average, the *MuV*-treated animals showed a slower rate of tumor growth, compared to the control animals (Figure 3). The pairwise comparison of tumor volume revealed a significant delay in the tumor growth of the attenuated *MuV*-treated group, compared to the control group ($P = 0.01769$). This finding indicated that the attenuated *MuV* Hoshino strain exhibited effective oncolytic activity in vivo.

5. Discussion

Oncolytic virotherapy is a novel immunotherapeutic approach for the treatment of different cancers (20). To date, some oncolytic viruses have been approved for cancer treatment and some are being currently tested in different preclinical models and clinical trial phases (21). The use of paramyxovirus oncolytic viruses, such as *MuV*, for the treatment of human malignancies, is a promising approach to integrate current therapeutic strategies (1-3). The known receptor of *MuV*, sialic acid sugar, is overexpressed on the surface of different tumor cells (22). This feature makes *MuV* a desirable candidate for the treatment of various cancers. Accordingly, different strains of *MuV*, derived from different isolates, have been examined as OVs (5, 7-16).

The first clinical trials of Urabe *MuV* strain showed its oncolytic and immunotherapeutic potentials (5, 8-10). The results of preclinical studies have also shown that some natural and engineered *MuV* strains exhibit oncolytic activities against tumor cell lines in vitro and in vivo (7, 11-16). The present study supports previous studies, where the oncolytic properties of other natural *MuV* vaccine strains were demonstrated against different human cancer cell lines, including melanoma (12), ovarian cancer (13), different solid malignancies (14), fibrosarcoma, adenocarcinoma (15), leukemia, and lymphoma (16).

The most important advantage of using attenuated vaccine strains, such as Hoshino strain, in oncolytic therapy is that it is both well-characterized and safe (commercially licensed for application). In the current study, an accepted and standard MTT assay was performed to evaluate the effects of *MuV* Hoshino strain on the viability of cells. Based on the Figure 1, the *MuV* Hoshino strain, even at very low MOIs, exhibited significant oncolytic activity against fibrosarcoma and cervical cancer cell lines. However, at an MOI of 2, the results were significantly different from non-cancerous cells ($P < 0.00001$). This suggests that the *MuV* Hoshino vaccine strain has greater oncolytic activities against fibrosarcoma and cervical cancer cell lines, compared to non-cancerous fibroblast-like MRC-5 cells. In other words, the results revealed the selectivity of *MuV* Hoshino strain for the lysis of these cancer cells.

Annexin-V/PI staining is one of the most widely used techniques for evaluating apoptosis quantitatively (23). This assay was used in the present study to analyze the effect of *MuV* Hoshino strain on apoptosis induction in the infected cells. The results revealed that the apoptotic and necrotic cell population significantly increased in both

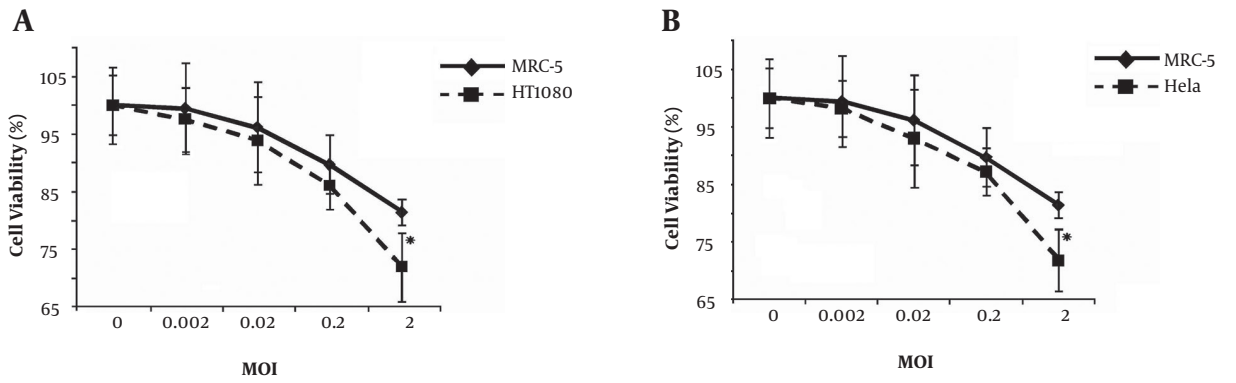

Figure 1. The oncolytic effects of *MuV* Hoshino strain on human tumor cell lines versus non-cancerous control cells in vitro. A, Viability of the HT1080 cell line and human diploid MRC-5 cells, infected with the *MuV* Hoshino strain at different MOIs after 96 hours. B, Viability of HeLa cell line and human diploid MRC-5 cells infected with *MuV* at different MOIs after 96 hours. Asterisks indicate statistical significance ($P < 0.05$) against non-cancerous human diploid MRC-5 cells at the corresponding time point. Error bars indicate standard errors of the means.

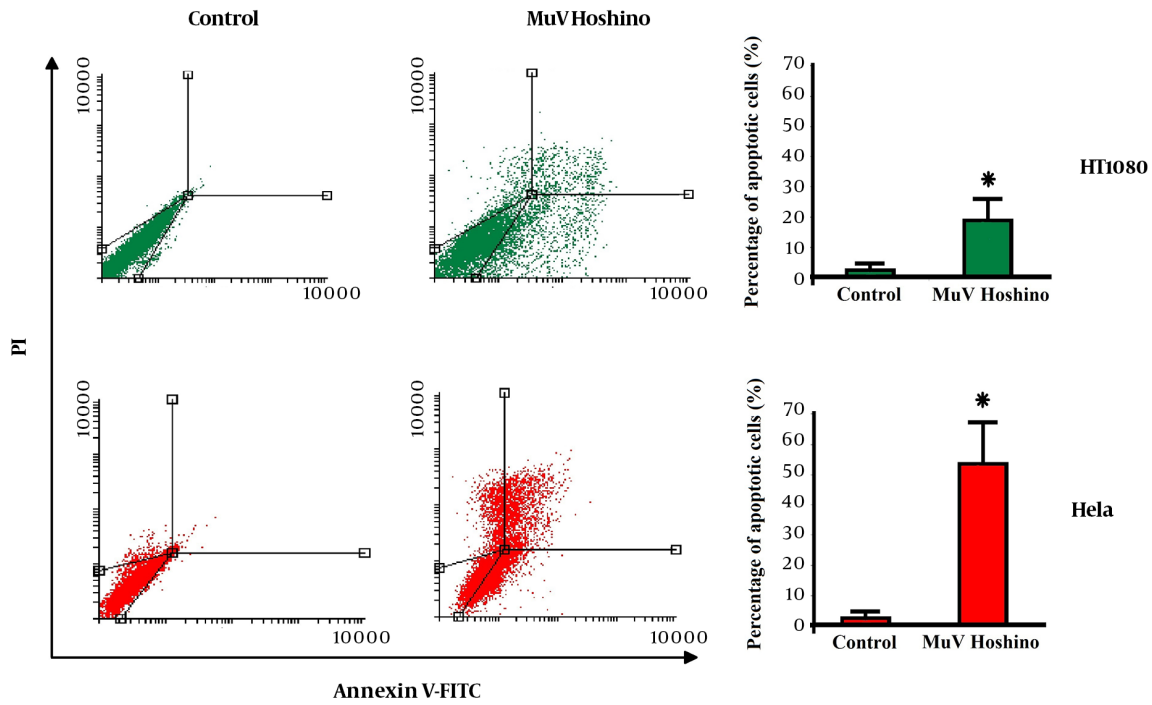

Figure 2. Apoptosis induction by the *MuV* Hoshino strain on HT1080 and HeLa cells (one of the three experiments is shown here). Bar graphs illustrate the percentage of apoptotic cells in the three experiments. Data are presented as mean ± SD. Asterisks indicate statistical significance versus the control ($P < 0.05$).

treated cancerous cells. Similar results have been observed regarding other *MuV* strains (14-16, 24). According to previous studies, *MuV* induces cellular apoptosis by upregulation of interleukin-1 beta (IL-1β) (24), or Fas/Fas ligand (FasL), or both (16).

Although previous studies have indicated that *MuV* SH and V proteins can have anti-apoptotic functions (25-28), apoptosis may be the predominant mechanism in the oncolytic activity of the *MuV* Hoshino strain, possibly due to the altered expression of some apoptotic receptors (29, 30), besides changes in apoptotic signaling pathways in these cancer cells. Moreover, HeLa is one of the cancer cell lines,

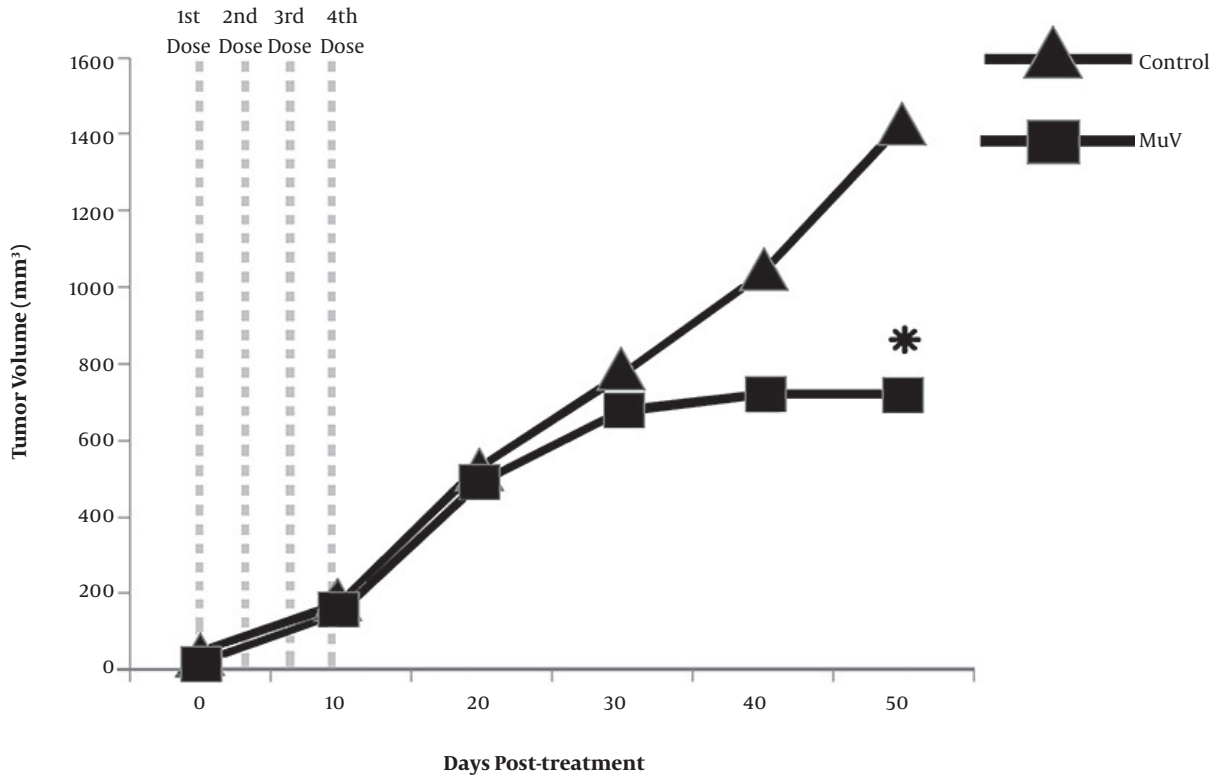

Figure 3. Therapeutic effects of the attenuated *MuV* Hoshino strain in the HeLa heterograft model. Asterisks indicate statistical significance versus the control group ($P < 0.05$) at the corresponding time point.

which contains wild-type Kirsten rat sarcoma viral oncogene homolog K-RAS (31). A previous study showed that K-RAS mutations resulted in resistance to apoptosis (32). Therefore, the higher level of apoptosis in the HeLa cell line may be related to the increased apoptosis by K-RAS expression in response to viral replication.

Moreover, the treatment of heterograft mice with the *MuV* Hoshino strain at typical doses for vaccination revealed significant oncolytic activities following intratumoral therapy. The *MuV* virotherapy is a suitable candidate for cervical and fibrosarcoma cancers, because the tumors are solid. This method allows for direct intratumoral injection into the tumor site and minimizes virus inactivation by specific anti-virus neutralizing antibodies in the circulation system (33). Also, direct injection into solid tumors increases its spread in cancerous cells and decreases normal tissue damage (34). One of the possible accelerators of cell sensitivity to *MuV* is viral attachment (with high affinity) due to the high expression of *MuV* receptors (sialic acid residues) on the cell membrane.

5.1. Conclusion

Although the oncolytic activity of the *MuV* Hoshino strain needs to be confirmed in future preclinical studies, the primary findings of this study indicated that this commercially available attenuated *MuV* vaccine strain might be potentially used as an oncolytic therapeutic agent to treat fibrosarcoma and cervical cancers. Further research is necessary to explain the potential oncolytic activity of this strain against other cancer cell lines.

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Footnotes

Authors' Contribution: B.A.: Carried out all experimental work, study design, data and statistical analysis, figures preparation and manuscript writing. A.M. and A.R.K.: Scientific supervisors of the project and finalized manuscript. A.G.L.: Participated in evaluation and manuscript editing. R.F.: Participated in in vivo analysis and manuscript editing. All authors read and approved the final version of this manuscript.

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