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ORIGINAL ARTICLE

Oncolytic measles virus-induced cell killing in radio-resistant and drug-resistant nasopharyngeal carcinoma

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Abstract

Introduction: The current first-line therapy for nasopharyngeal carcinoma (NPC) is often associated with long-term complications. Oncolytic measles virus (MV) therapy offers a promising alternative to cancer therapy. This study aims to investigate the efficacy of MV in killing NPC cells *in vitro*, both with or without resistance to radiation and drug therapy. *Materials and Methods*: NPC cell lines, CNE-1, CNE-2, HONE-1 and C666-1, were exposed to repeated cycles of gamma-irradiation and cisplatin to establish radio- and chemo-resistant cell lines, respectively. The expression of MV receptors, CD46 and nectin-4, were assessed with flow cytometer. To test the efficacy of viral infection, parental and both resistant NPC cells were infected with Measles-GFP-NIS *in vitro*. The progress of syncytia spread on NPC cells was monitored with fluorescence microscopy up to 60-hours post-infection (p.i.). MV-mediated killing was assessed using tetrazolium-based cell viability assay. *Results*: We established cisplatin-resistant (CR) NPC cell lines that exhibit more than two-fold shift in IC_{50} against cisplatin. Only CNE-2 and C666-1 acquired resistant traits after a cumulative 60-Gy gamma irradiation. All untreated parental and resistant NPCs expressed CD46 but not nectin-4 on their cell surface and were susceptible to MV infection. Syncytia were observable as early as 24 hours p.i. and cell loss was observable at 48-hours p.i. onwards. Interestingly, Measles-GFP-NIS shows higher infectivity in NPC with resistance phenotypes, except in CR-C666-1, and were killed more compared to their non-resistant counterparts. *Conclusion*: Measles-GFP-NIS demonstrated potential as an alternative treatment in relapse, recurrent, or advanced stage NPC which often exhibits resistance towards chemo- and radiotherapy.

Keywords: oncolytic measles virus, nasopharyngeal carcinoma, oncolytic virotherapy, radio-resistant oncotherapy, chemo-resistant oncotherapy

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is well-known to have high prevalence among the Chinese population which is one of the major ethnicities in South East Asian countries such as Malaysia, Taiwan, and China.[1] NPC affects males two times more than females, and Malaysian cancer statistics published in the year 2016 revealed that it is the fifth most common cancer with highest prevalence rate of 11 cases per 100,000 population in Chinese males. Radiotherapy is the first line treatment for primary NPC, followed by concurrent chemo-radiotherapy for advanced and recurrent patients.[1,2]

Although radiotherapy has significantly improved with the introduction of intensity modulated radiation therapy, myriad side effects associated to functional impairment and organ damages are inevitable, owing to the fact that the treatment relies on a straight-penetrating radioactive beam that also affect normal cells that are within the irradiation path.[3] Similarly, the use of cisplatin also causes broad ranges of

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late toxicity to patients.[4] Taken together, NPC survivors were left with great extent of functional impairment and discomfort affecting their quality of life, highlighting the destructiveness of current frontline NPC treatments.[5]

Oncolytic measles viruses have shown promises in specifically targeting tumor cells and have demonstrated efficient killing of chemo- or radio-resistant cancer cells.[6] Compared to chemo- and radiotherapy, side effect caused by oncolytic viruses are controllable and limited to short-term systemic response rather than permanent structural impairment and toxicities exemplified by radiotherapy or chemotherapy, respectively.[7] Early studies have demonstrated that MV, with sufficiently high cell surface CD46 density, is capable of selectively inducing cytopathic effects (CPE) known as syncytia in cancer cells, which in turn leads to the killing of infected cells, while leaving normal non-cancer cells unharmed.[8,9] With improvement over the past decades, MV to date is incorporated with sodium iodide symporter that transformed virotherapy into radiovirotherapy, specifically armed to further enhance MV-induced immunogenic cancer cell death, and improves oncolytic measles viral evasion from existing anti-measles neutralising antibody in vaccinated individual by pseudotyping measles H- and F-protein or polymeric coating of viral particle.[10-13] Clinical trials of MV to date reported no dose-limiting toxicity for viral dose as high as 10^{10} $TCID_{50}$ and recorded at least a doubling of the median of overall survival or complete regression of cancer in the best scenario highlighting that MV have the potential to treat advanced-stage or relapsed cancer.[14,15]

In this study, we infected NPC cell lines with the MV strain expressing green fluorescent protein (GFP) and sodium iodide symporter (NIS), Measles-GFP-NIS, to study the efficacy of the virus treatment *in vitro*. In addition, we extended the infectivity study onto cisplatin-resistant (CR) NPC cells as a model to evaluate the possibility of oncolytic measles virotherapy to treat recurrent NPC that survive secondary treatment. Thereon, we further evaluated MV on radio-resistant (RR) NPC cells harbouring the EBV genome as a model for clinical NPC that persist and resist primary NPC treatment.

MATERIALS AND METHODS

Cells and virus

NPC cell lines representing well differentiated carcinoma (CNE-1), poorly differentiated squamous carcinoma (CNE-2 and HONE-1), and undifferentiated carcinoma with constitutive expression of Epstein-Barr virus genome (C666-1) were cultured under standard cell culture condition (humidified incubator at 37°C with 5 % CO_2). These four cell lines were used as model to investigate the effect of MV infection in clinically relevant subtypes of NPCs.[16] Parental and resistant CNE-1 cell lines were cultured in Dubecco's DMEM media (HiMedia, India). Other parental and resistant NPCs (CNE-2, C666-1, and HONE-1) were cultured in RPMI media (HiMedia, India). All culture media were supplemented with 10 % foetal bovine serum (FBS, HyClone, GE Healthcare Life Sciences, Utah, USA) and 1% penicillin-streptomycin cocktail (HiMedia, India). Measles-GFP-NIS (Imanis Life Sciences, Minnesota, USA) were propagated with Vero cells and cryo-stocked at -80°C in serum-free OPTIMEM (Gibco, USA) media. Vero cells were grown in DMEM media supplemented with 5% FBS prior to virus infection. CNE-1, CNE-2, HONE-1, and C666-1 cell lines were kind gifts from Associate Professor Dr Yap Lee Fah (University Malaya Medical Centre, Malaysia) while Vero cell was from Associate Professor Dr Voon Gah Leong (International Medical University, Malaysia).

Establish radio- (RR) and cisplatin-resistant (CR) phenotypes

Resistant phenotypes were established by subjecting NPCs to repeated cycles of cytotoxic treatment and cell recovery. In brief, parental NPCs were seeded for 24 hours before the cells were either treated with cisplatin (479306, Sigma-Aldrich, USA) at inhibitory concentration that kills 10% of cell population (IC_{10}) for 48 hours under standard cell culture condition or irradiated with 2-Gy gamma radiation for 32 seconds (Gammacell 3000, Theratronics, Canada) per treatment. Dose of cisplatin were gradually increased to IC_{25}, IC_{50} and then IC_{75}, wherever applicable based on shift of cisplatin IC_{50} assessed with cisplatin sensitivity assay after every few cycles of cisplatin treatments. Culture media were replaced with fresh media after cytotoxic treatment and the cells were allowed to recover under cell culture conditions. The cycle of cytotoxic treatment and cell recovery was repeated until NPCs achieved more than 2-fold resistance (cisplatin resistance) or have completed a cumulative of 60-Gy irradiation with 6-days interval between irradiations. Cells

were deemed resistant if cell viability assessment shows cells exhibit higher fractions of surviving cells when exposed to higher dose of a series of gradually increasing dose of 2-, 4-, 8-, 16-, and 32-Gy irradiation or a series of gradually increasing dose of 2-, 4-, 8-, 16-, 32-, and 64 μg/mL cisplatin compared to respective parental cells. Resistant cells were up-scaled and cryo-stocked for downstream studies. Sensitivity of resistant cells were evaluated with MTT-based cell viability assay.

Cell surface protein marker identification and quantification

The presence of cell surface receptors for measles virus, CD46 and nectin-4, were quantified using FACSCanto II flow cytometer. In brief, cells were harvested with Accutase (Nacalai Tesque, Japan) and were stained independently with mouse anti-human CD46 (1:10 dilution, BD Biosciences, USA) and nectin-4 (1:100 dilution, R&D System, USA), isotype IgG2a, k (1:10 dilution, BD Biosciences, USA), and isotype IgG2b (1:100 dilution R&D Systems, USA) antibody for 30 minutes away from light and in ice. Cold 0.5% BSA in PBS was used to dilute the antibodies and perform post-staining washes. Cells were collected with 500 × g centrifugation for 5 minutes, washed three times and resuspended in 500 μL of cold 0.5% BSA in PBS for subsequent flow cytometry analysis.

Infection studies

3000 (CNE-1 and CNE-2) and 4000 (HONE-1 and C666-1) cells were seeded 24 hours prior to infection. CNE-1 and CNE-2 were seeded with lower cell numbers due to relatively faster doubling time compared to HONE-1 and C666-1. The titre of Measles-GFP-NIS was estimated to be 4.74×10^6 mL^{-1} with Reed-Muench method.[17] Cells were infected with Measles-GFP-NIS at a multiplicity of infection, MOI, of 0.2 (for microscopic monitoring of viral CPE across cell monolayer) or 1.0 (for flow cytometry of infection efficiency under the influence of fusion inhibitor peptide) in 200 μL OPTIMEM for 1 hour in cell culture condition. Growth media with or without fusion inhibitor peptide (20 μg/mL; Bachem, Switzerland) were added into the treated wells before incubating the plate further. Brightfield images were taken with NIKON Eclipse TS100 microscope at 40 × magnification while fluorescence images were taken with Axio Observer.A1 at 100 × magnification. The efficiency of Measles-GFP-NIS infection were determined using flow cytometer by measuring GFP signal using FITC setting.

Cell killing quantification

NPC cells were infected with Measles-GFP-NIS (MOI=0.2) in OPTI-MEM for 1 hour under standard cell culture conditions. Fresh media were added and infected cells were cultured under standard cell culture conditions. Cell viability assay was performed with Cell Counting reagent (Nacalai Tesque, Japan) according to the manufacturer's instructions at 60 hours post-infection. Absorbance at 450 nm was measured with Infinite 200 Pro culture plate spectrophotometer (TECAN, Switzerland). The experiment was repeated twice and performed in triplicate. Cell killing was presented as fraction of dead cells calculated using the following equations:

$$\textit{Fraction of dead cells} = 1.0 - \textit{Fraction of viable cells}$$

$$\textit{Fraction of viable cells} = \frac{A_{450nm}\ \textit{of infected cell}}{A_{450nm}\ \textit{of mock infectd cell}}$$

Statistical analysis

The significant difference between control, treatment, and parental cell lines was calculated using GraphPad Prism 9. Parametric t-test with Welch's correction with an assumption of unequal SD between groups was used to calculate the significance of data between two groups. One-way parametric ANOVA with Welch's t-test assuming unequal SD between groups was used to calculate the significance between data across three groups.

RESULTS

NPC cell lines with cisplatin-resistance and radio-resistance

Platinum-based drug is the primary choice for chemotherapy in the treatment of advance, recurrent and metastasised NPC, as recommended by clinical practice guidelines in Malaysia and other nations.[2,16] Thus, cisplatin resistance in NPC patients lead to poor survival outcomes.[18] We established here cisplatin resistant cell lines that can tolerate a 2.51-to-4.42-fold increase in the IC_{50} dose of cisplatin (TABLE 1) for the subsequent study on the infectivity of MV in NPC, which could potentially serve as a treatment option for patients with recurrent NPC.

While cisplatin resistance was successfully established in all NPC cell lines, not all NPC cells exhibited resistance to gamma-radiation

TABLE 1: Fold shift of cisplatin IC_{50} in NPCs over repeated cycles of cytotoxic treatment and recovery

Cell lines	n	IC_{50} (μg/mL)	Fold change
CNE-1			
Untreated (parental line)	6	3.27 ± 1.21	
Treated (cisplatin-resistant)	6	14.44 ± 1.46	4.42
CNE-2			
Untreated (parental line)	6	5.08 ± 0.37	
Treated (cisplatin-resistant)	6	12.73 ± 5.23	2.51
C666-1			
Untreated (parental line)	6	5.22 ± 1.40	
Treated (cisplatin-resistant)	6	14.04 ± 1.27	2.69
HONE-1			
Untreated (parental line)	6	3.93 ± 1.93	
Treated (cisplatin-resistant)	6	10.16 ± 0.17	2.59

following a cumulative of 60-Gy radiation treatment *in vitro*. Out of all four NPC cells that completed cumulative 60-Gy gamma radiation treatment, only CNE-2 and C666-1 cell lines (FIG 1) showed increased cell survival when exposed to a series of increasing dose (2- to 32-Gy) of irradiation. Increased cell survival indicates resistance to radiation, with C666-1 showing clear resistance that tolerates up to 32-Gy gamma radiation, while CNE-2 shows resistance up to 16-Gy irradiation. Subsequent experiments involving radio-resistant cells were done only on RR-CNE-2 and RR-C666-1 because only these two cells exhibit resistant phenotype after completing cumulative 60-Gy of gamma radiation.

Parental and resistant NPC cell lines express CD46 but not nectin-4

MV haemagglutinin (H) protein requires either

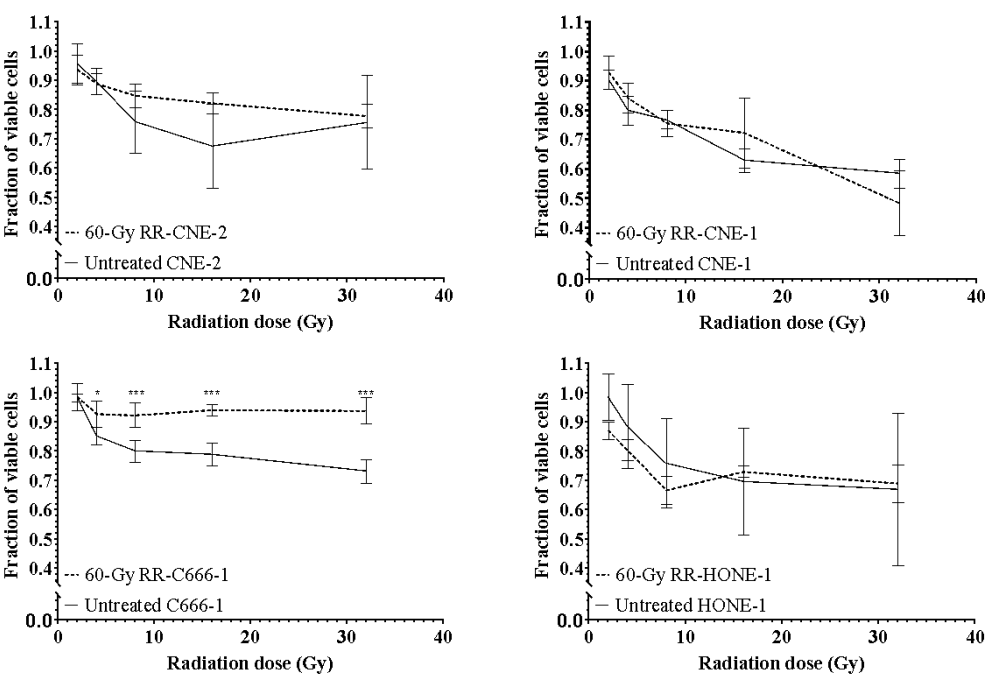


FIG. 1. All NPC cells were treated with cumulative dose of 60-Gy gamma radiation and resistance was validated by exposing the treated and Parental cells to 2-, 4-, 8-, 16-, and 32-Gy gamma radiation. Only CNE-2 and C666-1 NPC exhibit resistance. P-value ≤ 0.05 (*) and < 0.001 (***).

CD46 or nectin-4 receptor on the host cell for viral particle to anchor followed by viral particle-host cell fusion initiated by MV fusion (F) protein.[12] Figure 2 demonstrated that all NPC in our study, with and without resistant phenotypes, expresses CD46 but not nectin-4. Only CR-CNE-1 and -HONE-1 show almost 50% reduction in CD46 expression compared to respective parental cells after acquiring resistance.

Measles-GFP-NIS efficiently infects and kills parental, chemo- and radio-resistant NPC cells

Measles-GFP-NIS successfully infects parental, CR-, and RR-NPC (FIG 3A). All CR-NPC cells show a higher number of GFP-positive cells than their respective parental cells, albeit no significant difference among the treatment group (FIG 3B). CR-C666-1 has 1.3 times more GFP positive cells compared to its parental counterpart ($p>0.05$, one-way ANOVA with Welch's t-test), followed by 1.6 times for CR-CNE-2 pair ($p>0.05$, one way ANOVA with Welch's t-test), 1.9 times for CR-HONE-1 pair ($p>0.05$, Welch's t-test), and 4.1 times for CR-CNE-1 pairs ($p>0.05$, Welch's t-test). However, only RR-CNE-2 has 0.7 times lower GFP positive cells compared to parental CNE-2 ($p>0.05$, one-way ANOVA with Welch's t-test) which may suggest radio-resistance associated resistance to Measles-GFP-NIS infection. On the other hand, RR-C666-1 shows 1.9 times more GFP-positive cells than parental C666-1 ($p>0.05$, one-way ANOVA with Welch's t-test), similar to results among CR-NPCs. This data suggests that cisplatin and gamma-radiation treatment of cells potentially enhances measles-GFP-NIS infection. Measles-GFP-NIS replicated and spread in all NPC cell lines in this study as evidenced by the CPE (FIG 4), and widespread GFP signals (FIG 5). Formation of syncytia (FIG 4), a cytopathic feature of MV infection, was notable from 36 up to 60 hours post-infection. Among the NPCs, development of syncytia was noted earliest at 36 hours post-infection in parental- and CR-HONE-1 (FIG 4 and FIG 5). Thus, the infected cells were killed earlier (< 48 hours post-infection) compared to other NPCs. Notably, clumps of cell bodies are extensively observed at 60 hours post-infection in all NPC cells (FIG 4 and FIG 5). Cell viability assay result in FIG 6 shows Measles-GFP-NIS generally kill CR-NPC better than parental NPC cell. With CR-CNE-1 showing 1.6 times higher killing than parental CNE-1 ($p < 0.01$, One-way ANOVA with Welch's t-test), followed by 1.21 times higher killing for CR-HONE-1 pair ($p < 0.001$, One-way ANOVA with Welch's t-test), 1.08 times higher killing for CR-CNE-2 pair ($p > 0.05$, One-way ANOVA with Welch's t-test) and lastly 0.9 times lower killing

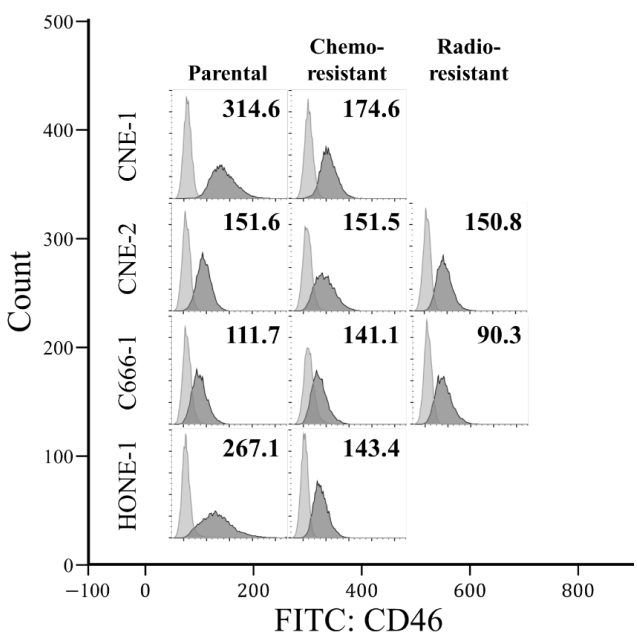
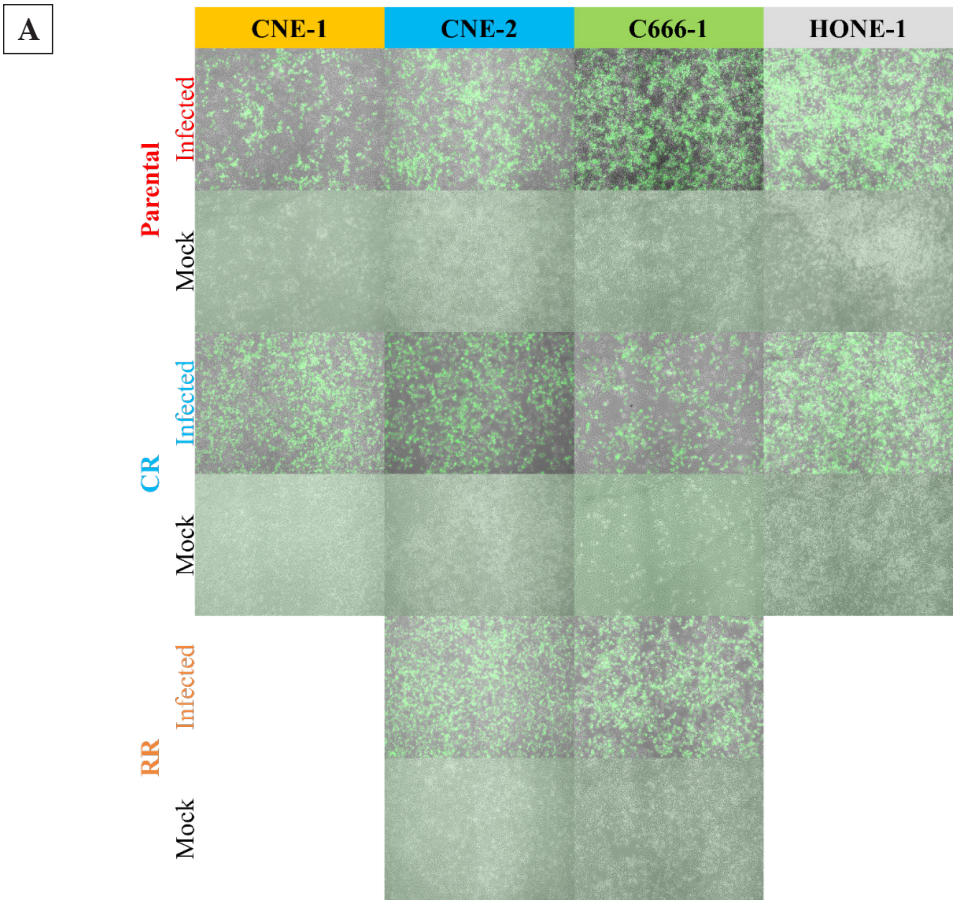


FIG. 2. Representative result of CD46 expression of parental, cisplatin- and radio-resistant NPCs. Parental, CR-, and RR- NPCs consistently expressed CD46. Only CR-CNE-1 and -HONE-1 show almost 50 % reduction in CD46 expression compared to respective parental cells after acquiring resistance.



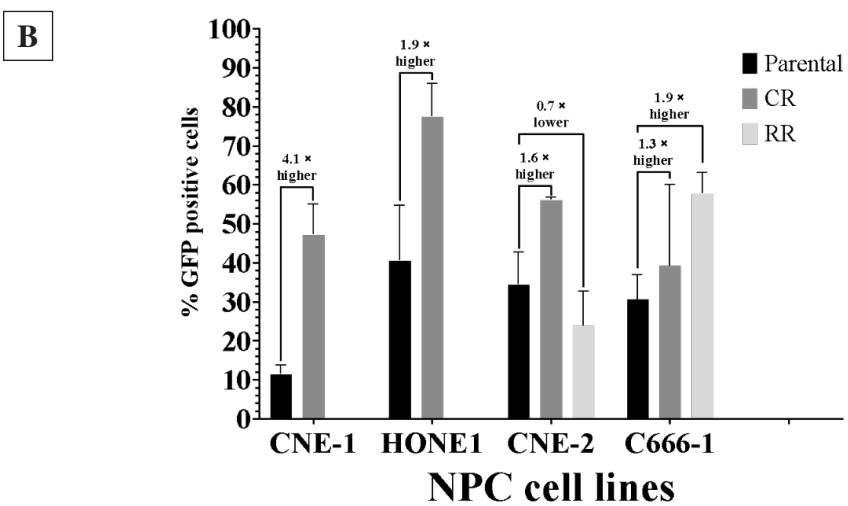


FIG. 3. (A) Parental, CR-, and RR-NPC infected with Measles-GFP-NIS express green signal under fluorescence microscopy (B) CR-NPC shows a higher count of green fluorescence signal than its parental counterparts, albeit no significance difference among treatment group. Two biological replicate measurements were taken for each NPC cell. No statistically significant changes in the infectivity (One-way ANOVA with Welch's t-test). Inset: Number represents fold change of GFP positive cells in resistant NPCs compared to respective parental cells.

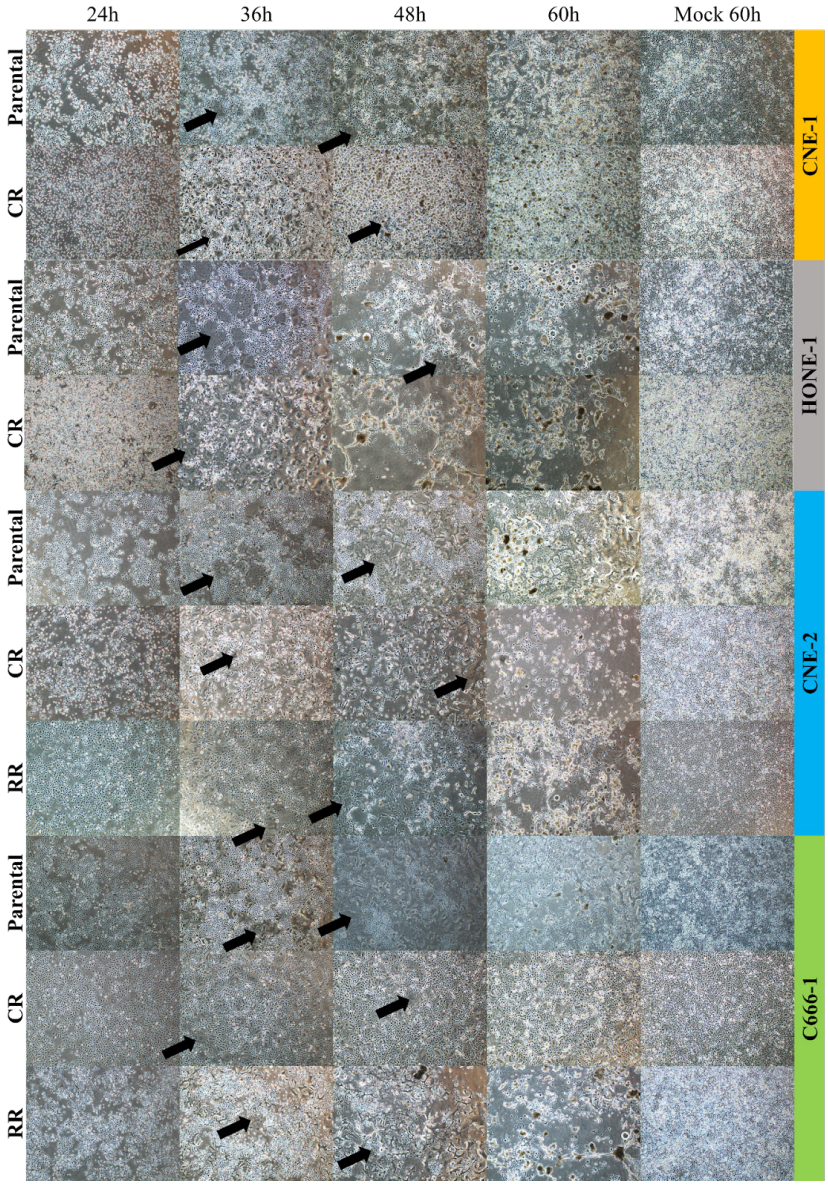


FIG. 4. Brightfield microscopic monitoring of CPE at 24-, 36-, 48-, and 60-hours post-infection in NPCs. Arrowhead showing patch of syncytia, CPE hallmark of MV infection, in NPC monolayer cells.

in CR-C666-1 pair ($p > 0.05$, One-way ANOVA with Welch's t-test). Killing in RR-NPCs are either equal or higher than in parental cells, with RR-CNE-2 shows 1.06 times higher killing than parental CNE-2 ($p > 0.05$, One-way ANOVA with Welch's t-test), and RR-C666-1 shows 1.28 times higher killing than parental C666-1 ($p < 0.05$, One-way ANOVA with Welch's t-test). Collectively, clumps of cell bodies observed in FIG 4 and 5 together with cell killing data in FIG 6 show clear evidence of effective killing of NPC regardless of chemo- or radio-resistant status.

DISCUSSION

Our data demonstrated that Measles-GFP-NIS is efficient in infecting and killing NPC cells *in vitro*. Here, we observed resistance to cisplatin and gamma-radiation did not affect the virus-mediated killing in these cells. Higher percentage of RR-CNE-2 (55% RR vs 34% P vs 56% CR) and RR-C6661 (58% RR vs 30% P vs 39% CR) were infected by the Measles-GFP-NIS, albeit not significantly different, compared to their CR- and parental counterparts, suggesting that changes to cellular expression in response

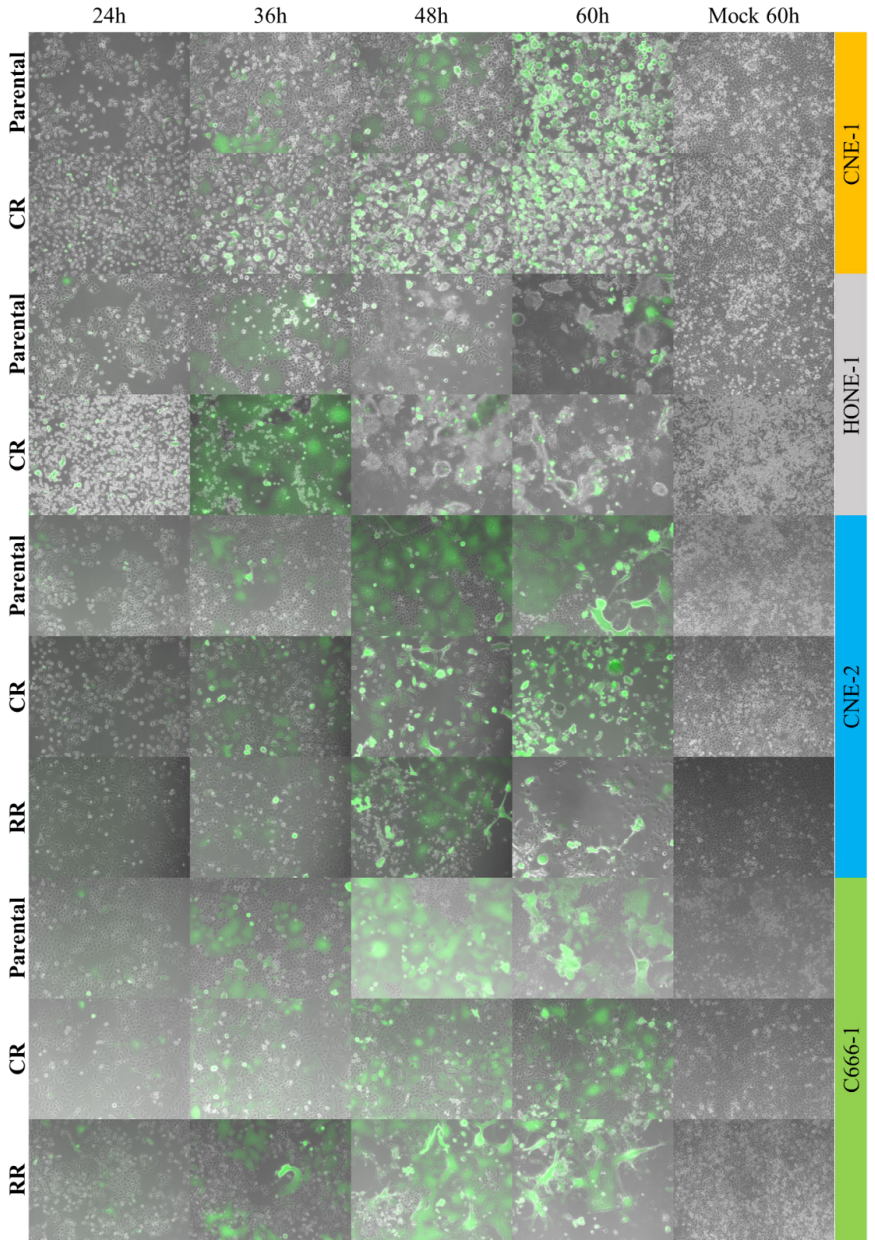


FIG. 5. Fluorescence microscopic monitoring of CPE at 24-, 36-, 48-, and 60-hours post-infection in NPCs.

to long-term irradiation exposure sensitized RR-NPC cells to infection. Notably, FIG 6 demonstrated significantly higher killing in CR-CNE-1, CR-CNE-2 and RR-C666-1 cells. Radiation resistance did not seem to impair virus-induced cell killing and RR-NPC are more susceptible than resistant to Measles-GFP-NIS infection. In addition, despite the almost 50% reduction (FIG 2) in CD46 expression observed in CR-CNE-1 and CR-HONE-1 compared to their respective parental counterparts, more than 90% of infected cells were killed in both CR cell lines (FIG 6). A previous study performed on Chinese hamster ovary cells has demonstrated that the CPE induced by MV is dramatic only when the number of CD46 receptors is above a certain threshold.[8] Therefore, it is possible that despite the reduction of CD46 expression on CR-CNE1 and CR-HONE1 compared to their respective parental counterparts, the number of CD46 receptors in these resistant cells remains above the required threshold for significant CPE

and effective viral entry. Based on our data, pre-exposure to cytotoxic elements such as cisplatin seems to enhance cell killing induced by Measles-GFP-NIS although the observed synergy may be cell-type specific. Notably, previous studies had demonstrated successes in combining MV with various chemotherapy drug both in animal model and clinical trials.[10,19] Our data agrees that ionizing radiation and chemotherapy could sensitize tumour cell and improve oncolytic capacity of oncolytic virotherapy as adjuvant cancer therapy.[20-23]

Our data demonstrated that MV can kill C666-1 and other NPC cells efficiently, including cells exhibiting resistance phenotype towards cisplatin and radiation. The C666-1 cell line is the primary *in vitro* undifferentiated carcinoma model for clinical NPC in South-East Asia because it persistently harbours Epstein-Barr virus genome, the primary oncogenic driver of NPC in the region.[24,25] Growing number of studies have demonstrated MV efficacy across different types of solid tumours, including head and neck squamous cell carcinoma, in clinical trials.[26,27] *In vitro*, MV has been tested in laryngeal carcinoma, Hep2 cell line, in combination with anti-EGFR antibody, Nimotuzumab.[28] To date, our study is the first that tested MV in NPC cells.

MV confers three important advantages over other oncolytic viruses. Firstly, MV entry protein, CD46 is naturally expressed in human differentiated cells as a key cell surface protein to deter self-immunity attack by complement.[29] High doses of up to 10^9 $TCID_{50}$ of MV administered in clinical trials have shown no dose-limiting toxicity with only common low-grade adverse effects of fever and abdominal discomfort.[14] Notably, the protein is overexpressed in many tumour cells to favour tumour cell survival by escaping immune-mediated killing.[30] Thus, MV are naturally suited to target most of tumour cells, both solid and liquid, in any part of human body and has demonstrated clinical efficacy in targeting multiple myeloma localised in the brain.[31] NPC are commonly diagnosed late, and the disease often relapse after recovery from advanced stage of the disease.[32] Secondly, the latest generation of MV variant is genetically engineered with sodium iodide symporter, making the virotherapy into targeted viro-radiotherapy.[33] Thirdly, MV is well known to cause immunogenic cell death which further enhances treatment efficacy via "cancer vaccine" pathway.[33,34] With the newly developed MeV-Stealth that is able to evade existing anti-measles immunity in vaccinated individual, MV is gaining momentum as a promising wide-spectrum anticancer vector that can overcome the therapeutic challenges posed by metastatic and advanced-stage cancers.[29]

Our study did not investigate in detail the molecular pathway induced by Measles-GFP-NIS leading to the formation of syncytia and regulated cell-death pathways. Future studies addressing this gap of knowledge will be insightful to understand better the biomarkers behind formation of syncytia and its role in driving oncolysis by MV.

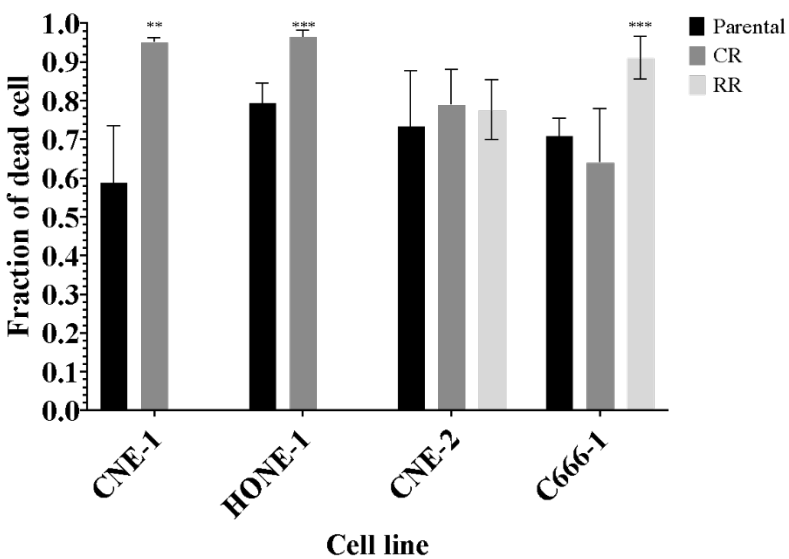


FIG. 6. Measles-GFP-NIS killed at least 59 % of NPC cells. P-value ≤ 0.05 (*), ≤ 0.01 (**) and < 0.001 (***).

CONCLUSION

Our result demonstrated that the oncolytic measles virus efficiently kills NPC cell lines, with and without resistance phenotypes *in vitro*. The contradictory result observed in CR-C666-1 hints that CR associated to EBV in NPC could yield lower therapeutic efficiency in clinical application. Involvement of EBV in CR and the collective effect of EBV-associated CR onto oncolytic measles virus secondary infection require in-depth investigation. Further study is necessary to validate whether systemic immune response against syncytia is adequate to compensate the lack of cell killing observed in infected CR-C666-1. The inclusion of non-carcinoma cells as safety control would be assuring should the MV make it to clinical application for treatment of NPC. In summary, oncolytic virotherapy is effective in killing RR- than CR-NPC cells and could be adapted to radio-virotherapy or standalone primary treatment in treating NPC patients.

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Informed Consent Statement: Not applicable as the study involved only the use of established cell lines and does not involve human subjects.

Authors' contributions: All authors have read and agreed to the published version of the manuscript. LHK data acquisition, formal analysis, writing, review and editing. OHT conceptualisation, formal analysis, writing, review and editing, supervision, funding acquisition, final approval of the manuscript. NYF review and editing, supervision. KLV conceptualisation, review and editing. CLY conceptualisation, review and editing.

Conflicts of Interest: The authors declare no conflict of interest.

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