Correlation between live attenuated measles viral load and growth inhibition percentage in non-small cell lung cancer cell line

Rasha Fadhel Obaid,[a] Younis A. Al-Khafaji,[b] Hamid N. Obied,[c] Salman A. AL-Jibouri,[a] Salam J. Mohammed,[d] and Rana F. Al-kilabi[a]

[a]Department of Medical Microbiology, College of Medicine, University of Kufa, Najaf, Iraq.
[b]Department of Medical Microbiology, College of Dentistry, University of Babylon, Babylon, Iraq.
[c]Department of Pharmacology, College of Medicine, University of Babylon, Babylon, Iraq.
[d]Department of Community Medicine, College of Medicine, University of Kufa, Najaf, Iraq.
Correspondence to Rasha Fadhel Obaid (email: rasha20_12@yahoo.com).
(*Submitted: 06 October 2017 – Revised version received: 17 October 2017 – Accepted: 13 November 2017 – Published online: 26 March 2018*)

Objective This study was performed to evaluate the anticancer effect of live attenuated measles virus (Edmonston strain) on human lung cancer cell line *in vitro*.

Methods Human lung cancer cell line (A549) was treated with different titers of the propagated live attenuated measles virus on Vero cells, including 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} of virus stock (8.860 $\log_{10}$ RNA copies/µl). The virus cytopathic effect (CPE) was studied at 24, 48, 72, and 96 hr. Oncolytic effects of the virus were assessed by cytotoxicity assay, apoptosis analysis, and proliferation marker Ki-67. The growth inhibition percentage was determined by Enzyme-Linked Immunosorbent Assay (ELISA) reader using crystal violet staining assay and compared with the viral load measured by quantitative real time-PCR.

Results The results of the study showed that measles virus had an inhibitory effect on the growth of human lung cancer cell line compared with untreated control group. There was highly significant positive correlation between viral load and PGI at 24, 48, 72, 96 hr ($P < 0.001$). The growth inhibition rate of a cell was associated with a decrement in the expression of the Ki-67 protein and increase in the ratio of early and late apoptotic cells in comparison with untreated control group.

Conclusion Live attenuated measles virus strain induced cytotoxic effect against human lung cancer cell line (A549) by induction of apoptosis as an important mechanism of anti-tumor activity, in addition, it indicates a correlation between the quantity of MV genomes and percentage of growth inhibition. This relation has proved that measles virus had anticancer effect.

Keywords Annexin-V, cytotoxicity, Ki-67 protein, lung cancer cell line (A549), measles virus, qRT-PCR

Introduction

Viral therapy is one of the most promising branches of medicine which detect viruses that can kill cancer cells. The detected viruses can be used as a method to treat malignant tumors, and this is done through the proliferation of the virus inside malignant tumor cells.[1,2] Cancer is one of the leading worldwide causes of death, and despite the considerable progress made by researchers in the diversity of cancer treatment methods, the mortality rate from malignant tumors are still alarmingly high, so discouraging cancer growth and reducing its spread is one of the major challenges facing modern medicine.[3] The current goal for developing new therapies for the treatment of cancer is to design or select therapeutic agents that have a high therapeutic index (i.e. high potency against malignant cells) with little or no toxicities to normal cells.[4] An oncolytic virus, is a type of viruses that are highly selective to tumor cells: it can replicate, spread and proliferated within the tumor cells without affecting normal tissue,[5] and genetically programmed to replicate within cancer cells and directly induce cytotoxic effect through cell lysis or apoptosis.[6] The use of replicating viruses for cancer therapy is attracting increasing interest. The advantages of virotherapy include the potential lack of cross-resistance with standard therapies and the ability to cause tumor destruction by numerous mechanisms.[7] A variety of viruses have been developed as oncolytic therapies, including adenovirus, vaccinia virus, herpesvirus, coxsackie A virus, Newcastle disease virus, and reovirus.[8] During the past decades, measles virus vaccine strains have emerged oncolytic platform that may not be compromised by pre-existing MV immunity.[9] Measles virus vaccine Edmonston or Schwarz strains uses mainly CD46 molecules to infect cells, during cancer development, tumor cells are often selected to express high levels of CD46 molecules, which inhibit the complement system.[10] This CD46 overexpression makes the tumor cells less sensitive to lysis by the complement but highly sensitive to measles vaccine virus infection.[11] This study was performed to evaluate the antitumor effect of live attenuated measles virus (Edmonton) vaccine strain on human lung tumor cell line *in vitro*, and determination of the correlation between the load of measles virus genomes and percentage of growth inhibition at different time intervals.

Materials and Methods

Cell Culture

The human lung cancer cell line (A549) and Vero cell line were purchased from National Cell Bank of Iran-Pasteur Institute (NCBI). Cell line was maintained in Roswell Park Memorial Institute medium 1640 (RPMI-1640, Gibco-BRL), supplemented with 10% fetal bovine serum (Gibco) and 100 µl/ml penicillin/streptomycin (Sigma). Cells were incubated at 37°C in 5% CO_2 incubator for 24 hr or until a confluent monolayer formed. The cells were subcultured for several times and then seeded into 96-well microplates at a concentration of 4×10^5 cells per well with complete RPMI-1640 growth medium.[12]

Virus Preparation

Live attenuated measles virus (Edmonston) were obtained from Vaccine and Sera Institute in Baghdad, Iraq. Each vial vaccine contains 10 doses of lyophilized hyper attenuated measles virus, each dose contains at least 1000 CCID50. Virus prepared as recommended by manufacturing company by adding 5 ml of sterile deionized distilled water (DDW) to dissolve the lyophilized powder and shake well before use.

Propagation of Measles Virus Vaccine on Vero Cell Line

In this study, lives attenuated measles virus was propagated on Vero cell line after 90% confluence. Growth medium was decanted, cells washed with sterile PBS (pH 7.2) then inoculated with 2 ml of (diluted hyper attenuated measles virus strain), and incubated at 37°C for 2 hr with mild rolling of the flask every 10 min. After the end of incubation period, the remaining virus inoculum was discarded and the cells were maintained with 7 ml of serum-free RPMI-1640 medium. The cells were re-incubated at 37°C for 3–7 days, when syncytia reached 80–90% of infected culture. The supernatant medium from each flask was collected aseptically in sterile tubes and the remaining attached cells were scraped then underwent freezing and thawing for three times, and centrifugation of all harvested culture at 3000 rpm for 30 min at 4°C in cold centrifuge, and virus was propagated on Vero cell line from passage 1 till passage 8 and virus titer was assayed by real-time Polymerase Chain Reaction (RT-PCR) in each viral passage.[13–15]

Exposure Stage

Human lung cancer cell line (A549) were cultured in 96-well-flat bottom microtitration plates and incubated with serial titer dilutions of measles virus (concentrated inoculums = 8.860 $\log_{10}$). The plated cells were divided into two groups: Group-1 was the control group (untreated); group-2 cell line was treated with measles virus. Three replicates were used for each virus titer dilution, as well as the control group which was treated with serum-free medium only. The plates were incubated at 37°C in 5% CO_2 incubator for 24, 48, 72, and 96 hr.[16] Then viral load, Ki-67 expression, apoptosis, and cytotoxicity of measles virus were estimated.

Assessment of Measles Virus Cytotoxicity by Crystal Violet Assay

Crystal violet (CV) assay was used to determine the optical density of the cell growth in each well of the microtiter plate by using ELISA reader. It is a technique used in cell culture laboratories to detect remaining adherent cells that staining with crystal violet dye, which binds to proteins and DNA. This technique was performed according to method described by Castro-Garza et al.[12]

Assessment of Apoptosis by Annexin V-FITC

Annexin-V concentrations was determinated in cell culture supernatant by quantitative ELISA Kit (US biological): The test was performed according to the instructions of the kit manual of manufacturing company.

Estimation Human Ki-67 Protein

Ki-67 protein concentrations was determinated in cell culture supernatant by quantitative ELISA Kit (Elabscience): The test was performed according to instructions of the kit manual of manufacturing company.

Molecular Test for Detection Viral Load of Measles Virus in Cell Culture (Vero and Lung Cell Lines) Supernatant by qRT-PCR

Primers and probe

The primers and probe were designed by using the complete sequence of nucleoprotein in measles virus using NCBI GeneBank and Primer3 online and these primers were provided by Bioneer Company, Korea as

Viral RNA Extraction

Viral RNA was extracted from frozen cell culture samples by using AccuZol TM Total RNA extraction kit (Bioneer, Korea) The extraction process was proceeded according to the instructions of the manufacturing company.

Reverse Transcription Real-Time PCR

Real-time-PCR was performed for detection and quantitation of measles virus in cell culture experimental samples based on specific primers and probe for nucleoprotein gene in measles virus and this technique was performed according to the method described by Hummel et al.[17]

Statistical Analyses

Statistical analysis was done by using SPSS version 20 in which, mean and standard deviation were used as descriptive statistics and analysis of variance with LSD for comparison between groups. P value ≤ 0.05 regarded significant.

Results

Detection of Measles Virus Load in Supernatant of Infected Vero Line by qRT-PCR

The viral titer was determined by the quantitative RT-PCR (qRT-PCR) assay. This assay was used to detect the MV N gene of RNA genome, starting from the first passage up to eighth passage. The titer of propagated human measles virus on Vero cell was increased gradually with the subsequent passage number until it reached maximum titer at the eighth passage which it was 8.860 $\log_{10}$. Table 1 and Fig. 1 reflected a significant increase in MV viral load as detected by qRT-PCR, which reached its maximum viral load at passage 8 ($P < 0.05$).

Figure 1 revealed that the threshold cycle C_t value of RT-PCR test as an indicator of the viral load detected in supernatant infected Vero cell culture. The lowest the threshold cycle C_t values indicated the highest MV RNA load were obtained.

Table 1. **The primers and probe**

Primer	Sequence		Product size
Measles N primer	*F	AGACATTGACACTG-CATCGG	89 bp
	*R	AAGATTCCTGCCAT-GGCTTG	
Measles N probe		FAM-AGGTCAGCTGACGCCCTGCT-BHQ1	

NCBI reference code: measles virus N gene for nucleoprotein, partial CDS, strain: MVi/Tokyo.JPN/38.00(KO) GenBank: AB095426.1. *F, Forward primer; *R, Reverse primer.

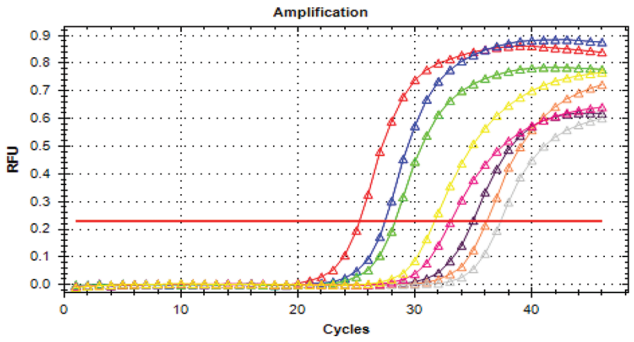

Fig 1. **Real-time PCR amplification plot of Measles virus vaccine (MVV) nucleoprotein gene in supernatant of Vero cell culture at different passages. The amplification plots were shown as red plot (P8), blue plot (P7), green plot (P6), yellow plot (P5), pink plot (P4), black plot (P3), orange plot (P2), and the gray plot (P1).**

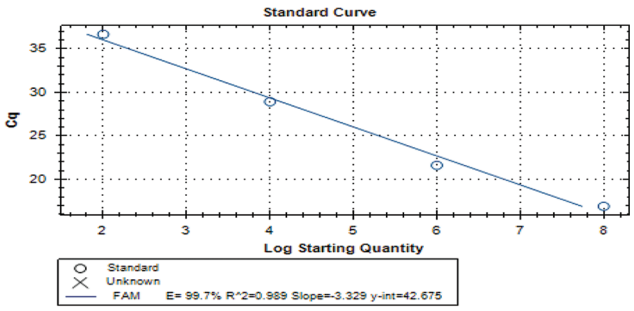

Fig 2. **Real-time PCR standard curve of MVV nucleoprotein gene in supernatant Vero cell culture at different passages, the log starting quantity curve was shown at RT-PCR efficiency 99.7%.**

Standard curve shows that the threshold cycle (C_t) on the *y*-axis and the starting quantity of RNA target on the *x*-axis. Slope, *y*-intercept, and correlation coefficient values are used to provide information about the performance of the reaction. The standard curve demonstrated a high linear correlation (R^2) = 0.989, *y*-intercept = 42.675, the regression slope was −3.329, and efficiency was 99.7% (Fig. 2).

Oncolytic Effect of Measles Virus on Human Lung A549

Cytotoxic effect of measles virus was investigated in human lung adenocarcinoma cell line exposed to different titers of measles virus (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) for 24, 48, 72, 96 hr, and control group (untreated), then stained with crystal violet dye. The optical density for cell line cell was measured at 459 nm by ELISA reader.

The result of this study showed that measles virus had a growth inhibition effect on lung cell line in comparison with control group and the cytotoxic effect was increased proportionally with the viral load and time. Figure 3 showed that the growth inhibition percentage of measles virus on human lung cell line at high titer (10^{-1}) was 39.015, 70.642, 77.076, and 90.959% for 24, 48, 72, and 96 hr, respectively. While the growth inhibition percentage of measles virus at low titer (10^{-5}) was 11.20, 19.843, 26.678, and 52.827% after 24, 48, 72, and 96 hr, respectively. However, Fig. 3 reflected high significant differences in growth inhibition percentage between cell that was treated with measles virus and untreated control group ($P < 0.001$).

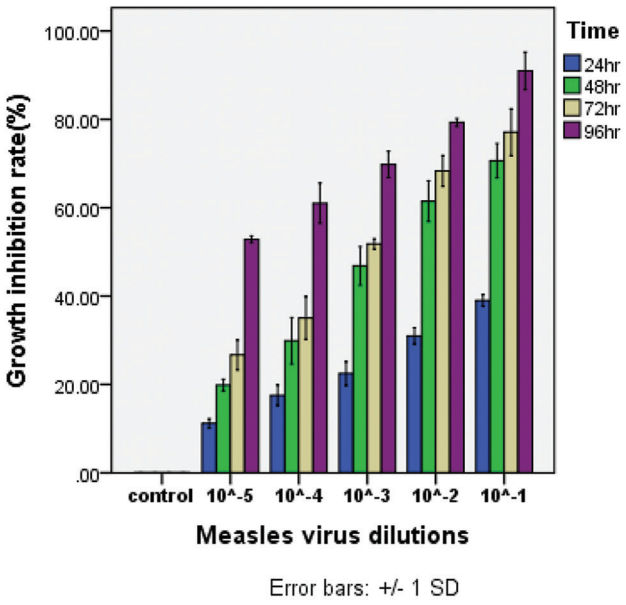

Fig 3. **Cytotoxic effect of measles virus vaccine on lung A549 cell line after 24, 48, 72, and 96 hr of exposure as evidenced by crystal violet assay ($P < 0.001$).**

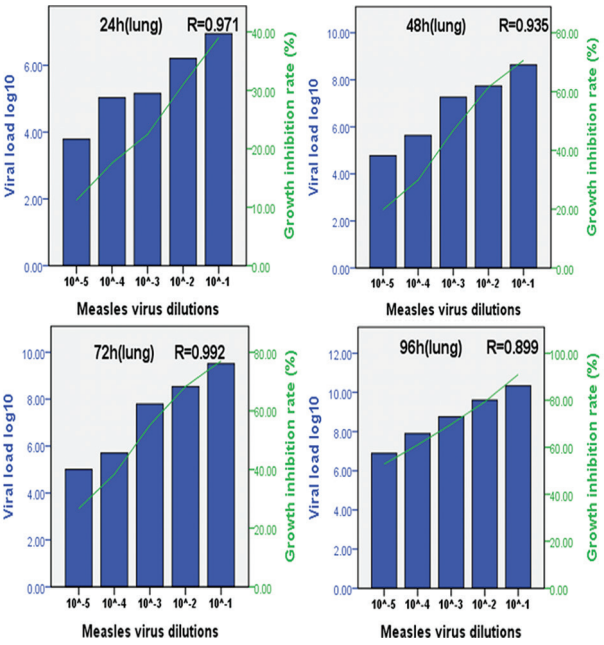

Fig 4A. **Correlation between viral load and percentage growth inhibition in lung cell line at 24, 48, 72, and 96 hr. Viral load— ▮, PGI— —.**

Correlation Between the Number of Viral Loads and Percentage Growth Inhibition in Lung Cancer Cell Line

To determine the effect of measles virus vaccine on cancer cells, we studied correlation between the number of viral genomes load and percentage growth inhibition. The growth inhibition percentage in lung (A549) cancer cell line was determined by ELISA reader and compared with the viral load measured by qRT-PCR. Cells were exposed to different titer of measles virus (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) for 24, 48, 72, and 96 hr.

Figure 4A shows measles virus load in lung cell line treated with (10^{-1}) virus was (6.94 $\log_{10}$) at 24 hr, and increased gradually until it reached maximum titer (10.336) at 96 hr. Also, the percentage of growth inhibition of lung cancer cell line treated with (10^{-5}) measles virus, was (39.015) at 24 hr, then increased gradually reaching it's high mean value (90.959 $\log_{10}$) at 96 hr.

Fig. 4B revealed that the threshold cycle C_t value of RT-PCR test as an indicator of the viral load which was detected in supernatant of infected lung cancer cell line culture at different durations of exposure. The lowest the threshold cycle C_t values, indicate that higher MV RNA load were obtained at dilution (10^{-1}). Growth inhibition rate at various time durations was increased proportionally with viral load in lung cell lines culture. The result demonstrated that there was a significant correlation between viral load measured by qRT-PCR and percentage of growth inhibition determined by ELISA reader. This correlation proved that measles virus had anticancer cytotoxic effect.

There is a significant strong positive correlation between viral load and PGI at 24, 48, 72, and 96 hr ($P < 0.001$).

Effect of Measles Virus on Proliferation and Apoptosis in Lung Cancer Cell

The result showed that measles virus-induced apoptosis and growth inhibition of cancer cells. The inhibition of cell growth was associated with a decrement in the expression of Ki-67 protein and increased in the ratio of early and late apoptosis compared with control group. Induction of cell apoptosis is an important mechanism of anti-tumor activity of measles virus.

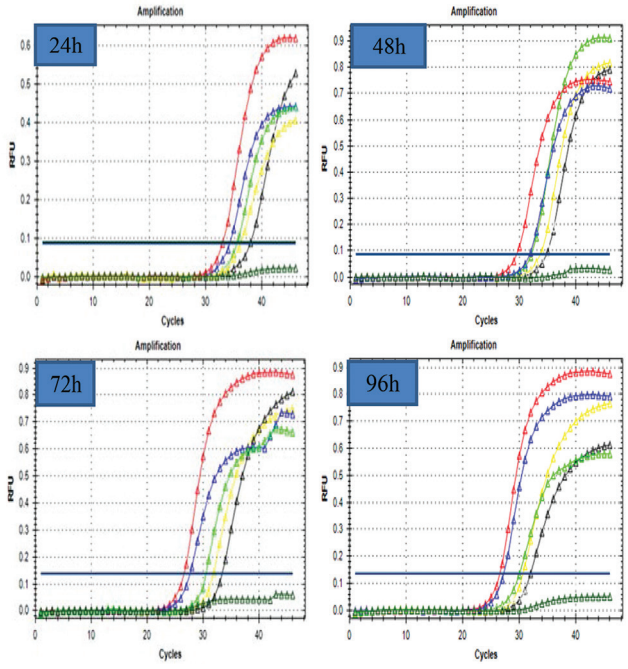

Fig 4B. **Real-time PCR amplification plot of nucleoprotein gene of MVV in supernatant lung cell line at 24, 48, 72, 96 hr incubation. The positive viral amplification was shown to cross-up the threshold cycle line. The amplification plots were shown as red plot for virus dilution (10^{-1}), blue plot for virus dilution (10^{-2}), green plot for virus dilution (10^{-3}), yellow plot for virus dilution (10^{-4}), black plot for virus dilution (10^{-5}), and the line plot under threshold line as negative control (untreated).**

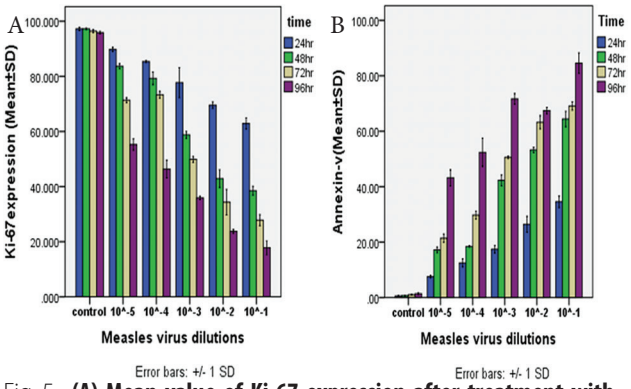

Fig 5. **(A) Mean value of Ki-67 expression after treatment with measles virus at different durations ($P < 0.001$). (B) Mean value of Annexin-V after treatment with measles virus at different durations ($P < 0.001$).**

Table 2. **Viral load detection by qRT-PCR. Viral RNA copies/µl in supernatant of Vero cell culture infected with measles virus vaccine strain at different passages. Mean ± SD for three replicates ($P < 0.05$)**

Passage number	Viral load $\log_{10}$ (RNA copies/µl) Mean ± SD
P1	4.3300 ± 0.24434
P2	4.5967 ± 0.24007
P3	5.2067 ± 0.15044
P4	5.7467 ± 0.09292
P5	6.2600 ± 0.14177
P6	7.6967 ± 0.13868
P7	8.5067 ± 0.07024
P8	8.8600 ± 0.13528

However, Fig. 5A and B reflected a significant difference between cell that was treated with measles virus in comparison with control group ($P < 0.001$).

Discussion

Live attenuated measles virus Edmonston strain was propagated on Vero cell line and used as oncolytic virus for human non-small cell lung cancer cell line. qRT-PCR technique was used as rapid quantitative method for estimating of the titer of propagated measles virus vaccine. It is a highly sensitive and specific diagnostic tool. The titer of propagated measles virus vaccine gradually increased with subsequent passage number due to rapid adaptation of the virus on Vero cell line, started with 4.33 $\log_{10}$ copies/µl, with mild increment in the second passage to 4.596 copies/µl and reached its maximum titer in eighth passage which was 8.86 (Table 2 and Fig. 1). The cycle threshold value measured by RT-PCR which was used as an indicator of the viral load found in the supernatant of cell culture.

These results are in agreement with many other previous studies El Mubarak et al.[18] who documented that qRT-PCR is efficient rapid technique for detecting measles infection or vaccination. Also, Ammour et al.,[19] reported that qRT-PCR was successfully used for estimating the titers of measles,

mumps and rubella viruses in infected cell culture supernatants, and this technique is cheaper and faster than titration methods that used in cell culture techniques. The present study indicated that both tissue culture and RT-PCR-technique are efficient technique for estimation of successful propagation of measles virus on Vero cell.

This study showed that measles virus had greatest growth inhibition effect on human lung cancer cell line in comparison to a control group and the virus cytotoxic effect was increased according to virus load increment, in parallel with time. Figure. 3 explained that there was significant difference in inhibitory effect of measles virus on human lung A549 compared with control group after 24, 48, 72, and 96 hr post exposure ($P < 0.001$).

The percentage of growth inhibition was correlated positively and significantly with the viral load in cancer cell line if considered together which means that a higher viral load will lead to high percentage of growth inhibition, because a high titer of virus destroy a large number of cancer cells, while a lower viral load would destroy lower percentage of cell number.

The result consistent with previous studies, documented that measles virus has antitumor activity. Blechacz et al.[20] reported that MV-Edmonton, successfully infected human hepatocellular carcinoma cell, resulted in transgene expression and syncytium formation in dependent on expression of CD46 receptor, and tumor cell killing was assessed by crystal violet assay. Also, McDonald et al.[21] who found that MV has potent antitumor activity against breast cancer lines and xenografts, resulting in significant cytopathic effects consisting of extensive syncytia formation and massive cell death after 72–96 hr from infection.

Induction of cancer cell apoptosis is the key to its treatment. The Annexin-V affinity assay was a widely used as method for apoptosis analysis, as well as for the discrimination between apoptotic and viable cell. This test is based on the changes in the cell membrane caused by apoptotic processes, Loss of plasma membrane asymmetry is an early step in apoptosis. The rate of apoptotic cell is increased significantly with the duration intervals and the dose of measles virus, associating with a decrease in expression of Ki-67 protein.

The present results consistent with other previous studies that documented that oncolytic virus induces apoptosis. Liu et al.[22] revealed that the measles vaccine virus-induced apoptosis and leading to cytotoxicity against glioma cell line *in vitro*. On other hand, Li et al.[23] found that adenovirus could inhibit Ki-67 expression and cellular proliferation and induce apoptosis of prostate carcinoma *in vivo*. The present study revealed that measles virus can replicate and destroy cancer cells, and yield high load of new progeny viruses, which spread, infect and kill more cancer cells around it. Also, a high load of measles virus increased inhibition of cancerous cells as compared with low-virus titer.

Conclusion

Live attenuated measles virus strain induced cytotoxic effect against human lung cancer cell line (A549) by induction of apoptosis as an important mechanism of anti-tumor activity, in addition, it indicated a correlation between the quantity of MV genomes and percentage of growth inhibition. This relation has proved that measles virus had anticancer effect.

Acknowledgment

We would like to thank the staff of Cancer Research Laboratory, in Pharmacology Department, College of Medicine, Babylon University for their assistance of this research work.

References

1. Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. Nature Biotechnol. 2012;30:658–670.
2. Achard C, Boisgerault N, Delaunay T, Tangy F, Grégoire M, Fonteneau JF. Induction of immunogenic tumor cell death by attenuated oncolytic measles virus. J Clin Cell Immunol. 2015;6. http-omicsonline.
3. Singh PK, Doley J, Kumar GR, Sahoo AP, Tiwari AK. Oncolytic viruses & their specific targeting to tumour cells. Indian J Med Res. 2012;136: 571–584.
4. Dorer DE, Nettelbeck DM. Targeting cancer by transcriptional control in cancer gene therapy and viral oncolysis. Adv Drug Delivery Rev. 2009;61:554–571.
5. He S, Li P, Chen CH, Bakst RL, Chernichenko N, Yong AY, et al. Effective oncolytic vaccinia therapy for human sarcomas. J Surg Res. 2012;175: e53–e60.
6. Takano M, Takahashi, Agarikuchi T, Kurebayashi Y, Minami A, Otsubo T, et al. Histochemical fluorescent staining of Sendai virus-infected cells with a novel sialidase substrate. Virology. 2014;464:206–212.
7. Goldufsky J, Sivendran S, Harcharik S, Pan M, Bernardo S, Stern RH, et al. Oncolytic virus therapy for cancer. Oncolytic Virother. 2013;2: 31–46.
8. Zhao D, Chen P, Yang H, Wu Y, Zeng X, Zhao Y, et al. Live attenuated measles virus vaccine induces apoptosis and promotes tumor regression in lung cancer. Oncol Rep. 2013;29:199–204.
9. Knuchel MC, Marty RR, Morin TNA, Ilter O, Zuniga A, Naim HY. Relevance of a pre-existing measles immunity prior immunization with a recombinant measles virus vector. Hum Vaccin Immunother. 2013;9: 599–606.
10. Fishelson Z, Donin, N Zell S, Schultz S, Kirschfink M. Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. Mol Immunol. 2003;40:109–123.
11. Guillerme JB, Gregoire M, Tangy F, Fonteneau JF. Antitumor virotherapy by attenuated measles virus (MV). Biology. 2013;2:587–602.
12. Castro-Garza J, Barrios-García HB, Cruz-Vega D, Said-Fernández S, Carranza-Rosales P, Molina-Torres CA, et al. Use of a colorimetric assay to measure differences in cytotoxicity of *Mycobacterium tuberculosis* strains. J Med Microbiol. 2007;56:733–737.
13. Msaouel P, Iankov ID, Allen C, Morris JC, von Messling V, Cattaneo R, Galanis, E. Engineered measles virus as a novel oncolytic therapy against prostate cancer. The Prostate. 2009;69:82–91.
14. Haas C, Ertel C, Gerhards R, Schirrmacher, V. Introduction of adhesive and co-stimulatory immune functions in to tumor cells by infection with Newcastle disease virus. Int J Oncol. 1998;13:1105–1115.
15. Anne G, Samantha B, Carole S. Measles virus induces oncolysis of mesothelioma cells and allows dendritic cells to cross-prime tumor-specific CD8 response. J AACR. 2008;68:4882–4892.
16. Madlum KN, Galib RA, Ghazi FM, Ali ZH. The effects of zizyphus stem bark and termite shelter tubes on HeLa cell growth. RJPBC. 2015;6:620.
17. Hummel KB, Lowe L, Bellini WJ, Rota PA. Development of quantitative gene-specific real-time RT-PCR assays for the detection of measles virus in clinical specimens. J Virol Methods. 2006;132:166–173.
18. El Mubarak HS, De Swart RL, Osterhaus AD, Schutten M. Development of a semi-quantitative real-time RT-PCR for the detection of measles virus. J Clin Virol. 2005;32:313–317.
19. Ammour Y, Faizuloev E, Borisova T, Nikonova A, Dmitriev G, Lobodanov S, et al. Quantification of measles, mumps and rubella viruses using real-time quantitative TaqMan-based RT-PCR assay. J Virol Methods. 2013;187:57–64.
20. Blechacz B, Splinter PL, Greiner S, Myers R, Peng KW, Federspiel MJ, et al. Engineered measles virus as a novel oncolytic viral therapy system for hepatocellular carcinoma. Hepatology. 2006;44:1465–1477.

21. McDonald CJ, Erlichman C, Ingle JN, Rosales GA, Allen C, Greiner SM, et al. A measles virus vaccine strain derivative as a novel oncolytic agent against breast cancer. Breast Cancer Res Treat. 2006;99:177–184.
22. Liu C, Sarkaria JN, Petell CA, Paraskevakou G, Zollman PJ, Schroeder M, et al. Combination of measles virus virotherapy and radiation therapy has synergistic activity in the treatment of glioblastoma multiforme. Clin Cancer Res. 2007;13:7155–7165.
23. Li J, Luo J, Gu D, Jie F, Pei N, Li A, et al. Adenovirus-mediated angiotensin II type 2 receptor overexpression inhibits tumor growth of prostate cancer *in vivo*. J Cancer. 2016;7:184–191.