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Possibility to use attenuated Hepatitis A virus vaccine as anticancer agent against liver cancer

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Abstract

Attenuated viral vaccines that originally developed for infectious disease prevention, have established potential in cancer therapy by selectively infecting and lysing tumor cells while stimulating anti-tumor immune responses. Current work aims to try the In Vitro Immuno-Independent experiments for discover the anti-tumor activity of live attenuated Hepatitis A virus vaccine (LAHAVV) against rat hepatocellular Carcinoma (HC) Cancer cell line as well as against Rat Embryonic fibroblast (REF) Cell line as normal cells. Six concentrations of LAHAVV [35, 30, 25, 20, 15 and 10 viral particle per microliter (V/ µl)] were tested against both cancer (HC) and normal (REF) cell lines. Using the Crystal Violate Cytotoxicity Assay. Cells killing percentages were assessed for each of the six concentrations of vaccine, thus vaccine Ic50 were calculated. Cytopathological effects such apoptotic cells were evaluated under an inverted microscope. With significant statistical differences at a probability level lower than 0.01. The results found a toxic effect of LAHAVV on all HC cancer cell line, while for the normal REF cell line, this toxic effect was correlated only with the highest concentrations of vaccine. Moreover, the study explored that the multiplicity of infection (MOI) of LAHAVV against cancer cell was greater than its MOI against normal cell (2.5 vs 1). Cytological changes were observed in cancer cells that have been treated by LAHAVV with insignificant effect on non-cancer (normal) cells.

Keywords: Hepatitis A vaccine, Cancer immunotherapy, Vaccine repurposing, Antitumor activity, Apoptosis.

Introduction

Hepatocellular carcinoma (HCC) is the most prevalent primary liver malignancy, accounting for approximately 77–85% of all liver cancer cases globally. It is closely linked to chronic liver diseases, particularly persistent infections with Hepatitis B virus

(HBV) and Hepatitis C virus (HCV), as well as liver cirrhosis and various metabolic disorders (1).

Although diagnostic modalities and treatments for HCC have improved greatly over the past several decades, hepatocellular carcinoma remains a leading cause of cancer-related death worldwide due to its highly aggressive nature., poor prognosis, often late presentation, and lack of treatment options (2). The current treatment methods such as resection, liver transplantation, Systemic treatments (Tyrosine Kinase Inhibitors (TkI), immune checkpoint inhibitors) offer limited extension of survival, especially for those diagnosed at late stages. Oncolytic virotherapy has recently gained attention as an anticancer strategy over the past few years (3). Re-purposing attenuated viral vaccines, initially developed for preventing contagious illnesses, have demonstrated the ability to target and kill tumor cells and potentiate anti-tumor immunity (4).

Several attenuated viruses such as PIV3, measles virus, and Mycobacterium bovis Bacillus Calmette-Guérin have been studied in pre-clinical and clinical trials for oncolytic and immunomodulatory capabilities. Microbes can be used as platforms to potentiate tumor-specific immunity and may allow for reversing HCC associated immune suppression (4). While attenuated viral vaccines offer a unique method for HCC treatment, there are many difficulties to be addressed such as viral targeting, off-target effects, and patient safety (1). Ongoing research and clinical trials are crucial to evaluating their efficacy and establishing their role in combination therapies. The integration of viral-based immunotherapy with existing treatment modalities may provide a new avenue for improving patient outcomes in HCC (5). Existing work aims to attempt In Vitro immuno-independent experiments to discover the anticancer activity of live attenuated Hepatitis A virus vaccine (LAHAVV) against rat hepatocellular Carcinoma (HC) cancer cell line and against Rat Embryonic Fibroblast (REF) cell line as normal cells.

Materials and Methods

Seeding of Cell Lines

Two different cell lines rat Hepatocellular Carcinoma (HC) The transformed Rat Embryonic Fibroblast (REF) cell line was obtained from the Cell Bank Unit at the Iraqi Center for Cancer and Medical Genetics Research, Al-Mustansiriyah University. Cells were maintained in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin.

LAHAVV serial dilution preparation

Six concentration of LAHAVV were prepared depending on the MOI of Hepatitis A virus, 35, 30, 25, 20, 15 and 10 viral particle per microlitter (V/ µl) through addition of 35, 30, 25, 20, 15 and 10 µl of the vaccine stock solution into six prepared test tubes contain 500 µl of free serum-RPMI solution medium to make the six below concentration.

Sustaining Transplanted Cells

Cancer and transformed for cytotoxicity assessment, cells were seeded into 96-well microplates at a density of 1×10^4 cells per well and exposed to six designated preparations of LAHAVV as described in the vaccine formulation protocol. Serum free

medium was used as a negative control in each experiment for assay validation. All plates were incubated at 37 °C for 48 hours in a humidified incubator with 5% CO2.

Table. 1: LAHAVV serial dilution preparation

Number of vaccine diluents	Concentration of vaccine (V/ µl)	Number of viral particle	Number of seeded cells in single well
1	35	35000	10000
2	30	30000	10000
3	25	25000	10000
4	20	20000	10000
5	15	15000	10000
6	10	10000	10000

Cytotoxicity test of the LAHAVV

Cell viability/cytotoxic effect of LAHAVV on various cell lines was determined using crystal violet staining method. Cells were treated with crystal violet solution (200 µg/mL) for ~20 min at 37 °C with mild shaking. Following the recommended washing and drying steps, the OD of each well was determined by spectrophotometry.

Determination of Killing Percentage

For the absorbance readings, the results were obtained using a microplate reader with a wavelength of 485 nm. To calculate the percentage of growth inhibition or cytotoxicity, the following formula was used:
Killing Percentage = [(A - B) / A] × 100,
where A is the optical density of the control wells (COD) and B is the optical density of the treated wells (TOD).

Growth Inhibition Rate and IC_{50} Determination

The growth inhibition (GI) rates and the half-maximal inhibitory concentration (IC50) values, which are the concentrations required to inhibit cell viability by 50%, for both cancer and normal cell lines, were computed using the GraphPad Prism software (version 7.0, 2016).

Morphological Study of Treated Cells

The cytopathological changes were observed using an inverted phase contrast microscope coupled to a digital imaging system. The morphological parameters observed included apoptotic induction (AI), cell membrane damage (DCM), cell shrinkage (CS), disruption of normal cell architecture, and apoptotic bodies (AB).

Statistical Analysis

All experiments were performed in triplicate, and data are expressed as mean ± standard deviation (SD). For comparing different groups statistically, one-way analysis of variance followed by multiple comparison tests was used. Data normalization and nonlinear

regression analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was considered at $P < 0.05$. The coefficient of determination (R^2) and P-values were calculated to assess model fitting and statistical reliability.

Results

Evaluation of Antitumor Activity

The antitumor efficacy of LAHAVV was investigated by assessing its inhibitory effect on the proliferation of HC and REF cell lines. Cells were exposed to six different concentrations of LAHAVV (35, 30, 25, 20, 15, and 10 V/µL). Growth inhibition percentages and IC_{50} values were calculated relative to untreated control cells. The inhibitory effect demonstrated a clear dose-dependent pattern, with increasing concentrations of LAHAVV resulting in progressively higher growth suppression. As presented in Table 1, the maximum cytotoxic activity was observed in the HC cell line, with a killing percentage of 80.4%.at vaccine concentration (35 Virus/ µl) followed by REF cell line (66.9%) at vaccine concentration (35 Virus/ µl). Moreover, IC50% of the vaccine on the HC cancer cell line was (22.9 Virus/ µl) while regarding REF cell line IC50% was (27.5 Virus/ µl).

Table. 2: Cytotoxic effect of LAHAVV on HC and Ref at different concentrations

Cell Line	Vaccine concentration Virus/ µl	The highest killing%	IC 50% (Virus/ µl)	S.D.	P-value
HC	35	80.4	22.2 %	0.23	<0.001
	30	74.2		0.56	
	25	61.3		0.11	
	20	60.1		0.93	
	15	59.5		0.15	
	10	46.9		0.57	
REF	35	80.4	27.5 %	0.49	
	30	70.4		0.23	
	25	69.3		0.41	
	20	68.8		0.84	
	15	56.4		0.57	
	10	50.1		0.89	

Furthermore, inhibition of HC cancer cell line growth by the LAHAVV vaccine appeared to be dose dependent, with R2 > 0.97. Normal RF cell lines showed minimal growth inhibition, while cancer cell lines showed significant growth suppression ($p < 0.005$) (Figure.1).

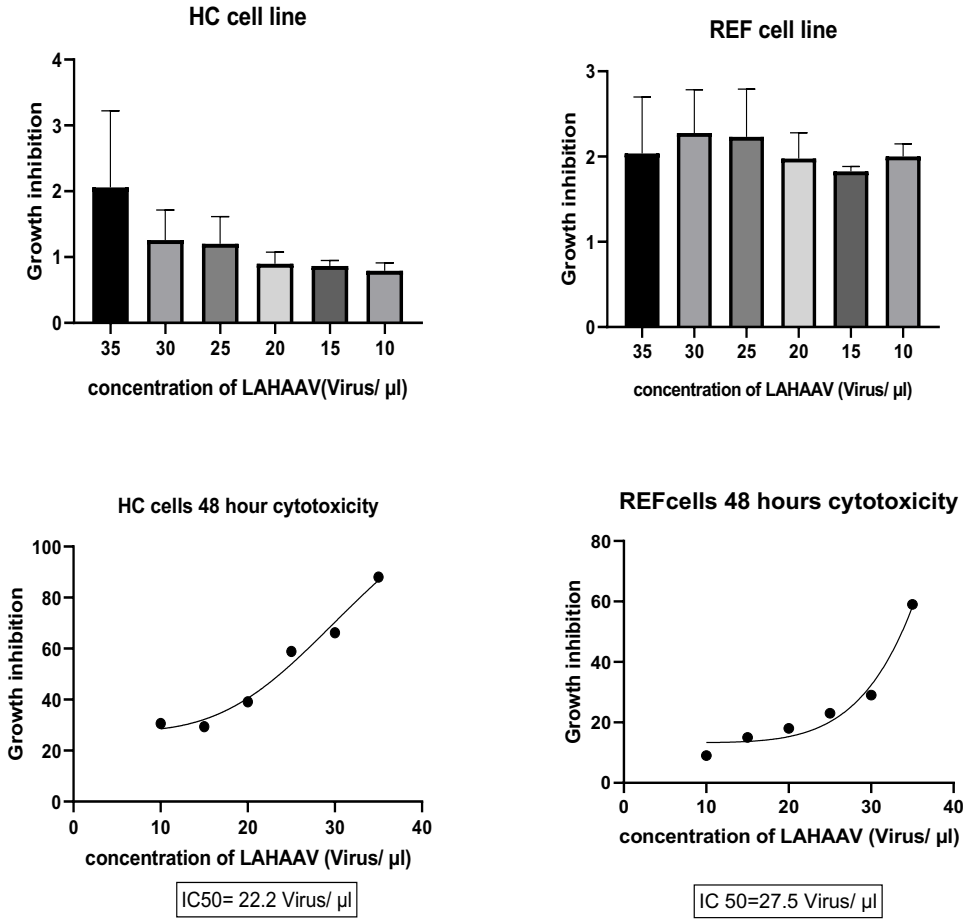

Figure. 1: IC50% of the LAHAV vaccine in HC and REF cell lines. Values are mean and SD for three replicates from three experiments.

Morphological Assessment by Crystal Violet Staining

Morphological alterations in treated and untreated cells were evaluated using crystal violet staining and documented via inverted microscopy (Figure 2). Untreated cells maintained morphological characteristics consistent with the original cell line phenotype. In contrast, cells treated with LAHAVV showed pronounced structural changes such as membrane disruption and cellular shrinkage. These changes were accompanied by a lack of normal cellular architecture and the presence of spherical apoptotic bodies.

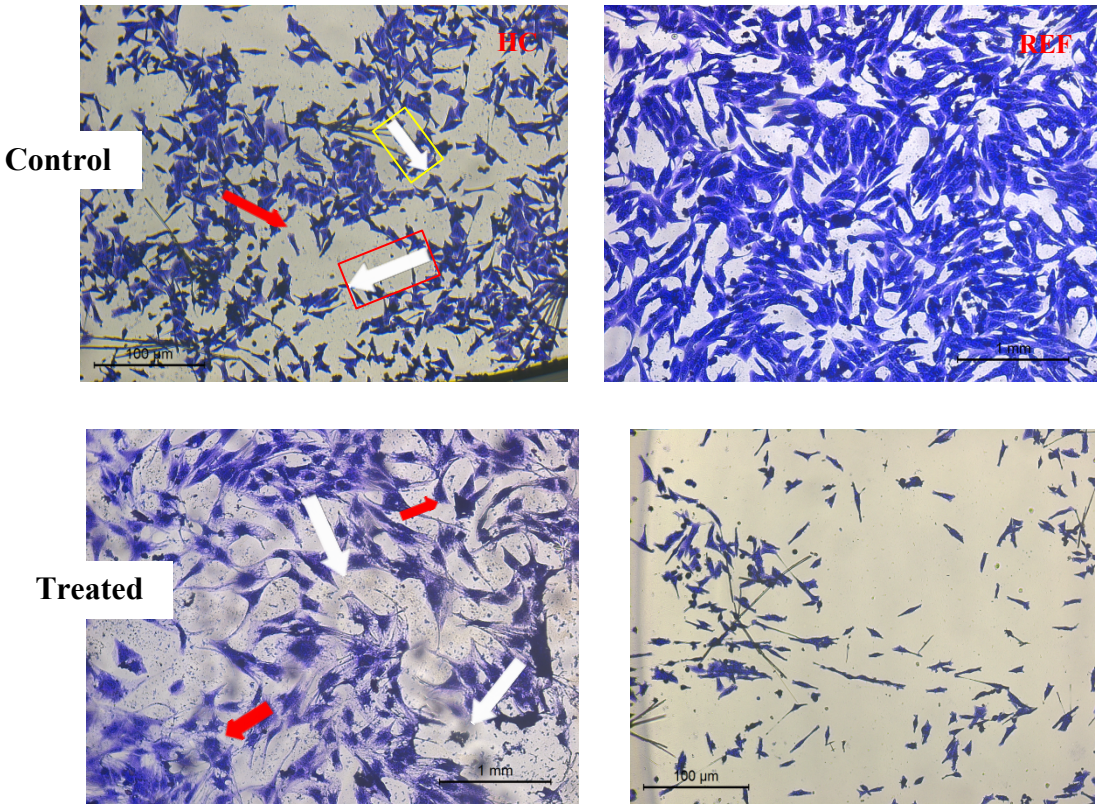

Figure. 2: A comparative cytological analysis of the HC cancer cells that had been exposed to the LAHAVV compound and the untreated cells revealed the presence of pronounced cytopathic effects after the exposure to the compound. The exposed HC cancer cells demonstrated the presence of necrotic and apoptotic cell death, as shown by the presence of rounded necrotic cells (indicated by the presence of white arrows) as well as the presence of blebbing, which indicates apoptosis (indicated by the presence of red arrows). The cells were stained using crystal violet to aid in the visualization of the changes.

Discussion

Cancer is still a major cause of morbidity and mortality at a global level. Cancer is a major public health challenge in the 21st century. In the year 2018, it is estimated that about 17 million cases of cancer were diagnosed worldwide., with an estimated 9.5 million cancer-related deaths reported during the same year (4). Among malignancies, hepatocellular carcinoma (HCC) is one of the most common and fatal primary liver cancers and is strongly associated with chronic liver disorders, particularly viral hepatitis infections (3). The rapid development of resistance to conventional chemotherapeutic agents has intensified the need for innovative therapeutic strategies and novel anticancer molecules. Despite substantial financial investments and continuous scientific efforts over recent decades, effective and durable therapeutic outcomes remain limited for many cancer types, including HCC (4). While Hepatitis B (HBV) and Hepatitis C (HcV) are well-established risk factors for HcC, the role of Hepatitis A virus (HAV) has remained mostly unexplored (6) .In the present study, we investigate the potential use of a live attenuated HaV (LAHAAV) vaccine as an anti-cancer agent against liver cancer. Although Hepatitis A virus (HAV) is not categorized as an oncolytic virus, accumulating evidence suggests

that it may influence the tumor microenvironment in a manner that suppresses tumor progression. Viral replication within hepatocytes has been associated with the induction of apoptosis and interference with pro-tumorigenic signaling pathways (7). The present study focuses on evaluating the potential anticancer properties of a live attenuated Hepatitis A virus (LAHAVV), commonly administered as a prophylactic vaccine. The live attenuated Hepatitis A vaccine (LAHAVV) was selected as a candidate therapeutic agent based on preliminary in vitro findings demonstrating cytotoxic effects against liver cancer cell lines as well as normal cell lines (8, 9). This is because they have an inherent virulence factor as a result of their capacity to invade cells and activate intracellular pathways leading to cellular destruction and death. However, attenuation greatly reduces the virulence of the virus while maintaining its immunogenicity (10). Attenuation involves altering viruses using a variety of strategies to produce live viruses with reduced virulence. These attenuated viruses play a crucial role in medicine, especially in vaccine production (11). Evidence is also emerging showing the potential antitumor effects of the LAHAV vaccine. However, its adverse effects also warrant evaluation. Consequently, it can be used either as a combination treatment or in a genetically modified form for antitumor purposes. The cytotoxic effects of the LAHAVV were also evaluated using both hepatocellular (HC) cancer cells and REF normal fibroblast cells. The results obtained indicated a concentration-dependent increase in cell death in both types of cells. Morphological changes were also observed when the cells were treated for 48 hours. The treated cells had characteristic cytopathic changes. The changes included granulation, shrinking of cells, and the formation of dense opaque structures in the treated cancer cells. Past studies have demonstrated that some viruses, such as the pertussis toxin, have the capacity to suppress antioxidant defense systems through the inhibition of catalase, peroxidase, and glutathione-containing enzyme systems. (12). Disruption of antioxidant pathways may contribute to oxidative stress–mediated cytotoxicity. Apoptosis is a tightly regulated cellular process characterized by fragmentation of nuclear DNA and subsequent clearance of cellular debris by neighboring phagocytic cells (13). Failure of apoptotic mechanisms may permit the survival and proliferation of genetically damaged cells, thereby contributing to oncogenesis. Cells that lose adhesion to adjacent cells or the extracellular matrix may undergo programmed cell death through intrinsic apoptotic signaling pathways. In malignant cells, pro-apoptotic proteins such as BH3-only proteins may accumulate; however, their activity can be counterbalanced by elevated levels of anti-apoptotic proteins, particularly Bcl-2 (14). Therapeutic agents that mimic BH3 proteins can enhance pro-apoptotic signaling, thereby shifting the balance toward activation of the apoptotic cascade and induction of cancer cell death (15). The correlation found in our study suggests the need for large-scale epidemiological studies to assess whether LAHAV vaccination reduces HCC incidence over time.

Conclusion

The current study has shown a close correlation between the use of a live attenuated LAHAV vaccine and the inhibition of hepatocellular carcinoma progression. Although the exact pathways need to be further investigated, the study shows that the vaccine has the potential to act directly on the cancer cells. These findings warrant further investigation into the use of the LAHAV vaccine as a potential new way of preventing or controlling liver cancer. If the findings of this investigation are confirmed, this would provide a cheap way of controlling liver cancer, especially in endemic areas of the world.

Author's Declarations

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Ethics statement

The authors declare that this study was conducted in accordance with the ethical standards and guidelines outlined in the journal's "Ethics Approval" section of the author guidelines. This study was carried out exclusively as an in vitro laboratory study, using commercially available Hepatitis A vaccine formulations approved by the World Health Organization available in the hospital supply system. No human subjects, specimens, or animal subjects were used in the study. Therefore, in accordance with institutional research policies, no ethical approval was required. The study was carried out in accordance with standard laboratory biosafety practices and regulations.

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Competing interest's statement
The authors declare that they have no competing interests.

Author contributions
AJQ conceptualized and designed the study, performed the experimental work, conducted data analysis, and drafted the original manuscript; **RAA** contributed to methodology development, statistical analysis, and data interpretation; **NTA** assisted in laboratory procedures, sample preparation, and data collection; **SAJ** supervised the project, critically revised the manuscript, and approved the final version for publication. All authors read and approved the final manuscript

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