Impact of oHSV-2 on the Carcinoembryonic antigen (CEA) Expression in Colorectal cancer (CRC)

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Abstract

Colorectal cancer (CRC) is the third most prevalent form of cancer and the second leading cause of cancer-related deaths worldwide. oncolytic virotherapy shows promise as a targeted therapy for various cancer types, including CRC. In this study, we constructed a novel oncolytic herpes simplex virus type 2 (oHSV-2) are derived from the wild type HSV-2, that isolated from semen samples of suspected infertile men. We investigated the effects of oHSV2 in vitro on expression the Carcinoembryonic antigen (CEA) in LS174T colorectal adenocarcinoma cell line model compare with a normal vero cell line. Tumor markers expression monitoring by the enzyme-linked immunosorbent assay (ELISA), of untreated cells and that treated with wHSV-2, oHSV-2 and after treatment with the traditional chemotherapeutic agent 5 flouro uracil 5FU. The results demonstrated a downregulation and decline in the concentration of Carcinoembryonic antigen (CEA), these results were statistically non-significant (p=0.302) when compared with control (untreated cells) level of CEA, (p=0.302). The drug 5FU revealed CEA down regulation with positive correlation coefficient with dose dependency (r^2=0.9797) and IC50 of 47.29µg/ml. we concluded that our virus is a safe and effective therapeutic agent for down regulation and decline in the concentration of Carcinoembryonic antigen (CEA).

Keywords: Colorectal cancer (CRC), oHSV-2, Carcinoembryonic antigen (CEA), 5 flouro uracil 5FU, ELISA.

DOI: 10.48047/ecb/2023.12.8.485

Introduction

Colorectal cancer (CRC) is the third most prevalent form of cancer and the second leading cause of cancer-related deaths worldwide (Ohishi *et al*.,2023). While there are varying treatment recommendations for different stages of CRC, the standard approach remains surgical removal of part of the colon. However, this procedure carries the risk of side effects, Additional treatment options, including chemotherapy, radiotherapy, immunotherapy, and combination therapies, are commonly used but are not universally effective against all cancer types. Consequently, there is a need to develop an alternative therapy for CRC patients that minimizes side effects. One such therapy is oncolytic virotherapy, which involves using viruses to selectively infect and destroy cancer cells while sparing normal tissues (Ma and Yu, 2006; Urruticoechea *et al*., 2010). Oncolytic virotherapy, a new and promising cancer treatment approach, utilizes oncolytic viruses (OVs) to target

various types of cancer. OVs, whether native or engineered, selectively multiply in cancer cells, inducing a cytolytic effect. This method differs from traditional cancer therapies in its ability to effectively harness and manipulate the host's cellular processes, leading to a high expression of both virus and therapeutic genes (Hong and Yun, 2018). The use of live viruses for cancer treatment dates back to the 19th century, and with recent advancements in genetic engineering, the viral genome can now be easily altered for increased specificity and lower potential side effects (Lin *et al*., 2018). Clinical trials have tested various oncolytic viruses (OVs), including *herpesviruses*, *adenoviruses*, *retroviruses*, *poliovirus*, and *vaccinia virus*, which vary in size, genome, and replication efficiency (Kaufman *et al*., 2015; Lawler *et al*., 2017). Studies have been conducted on OVs against colorectal cancer, both in preclinical and clinical settings, and many have shown promising results. (Kana and Essani, 2021). Oncolytic herpes simplex virus (oHSV) has emerged as a highly promising therapy for various solid tumors (Zhao *et al*., 2014). The process of infection, replication, and transmission of oHSV within solid tumors plays a crucial role in achieving efficient destruction of infected cancer cells, leading to tumor cell death and the release of tumor antigens that can stimulate anti-tumor responses (Hong *et al*., 2022). Oncolytic herpes simplex virus type 2 (oHSV2) is a conditionally replication-competent HSV-2 engineered through the deletion of both the ICP34.5 and ICP47 genes, along with the insertion of granulocyte-macrophage colony-stimulating factor (GM-CSF). Deletion of the ICP34.5 gene enables selective oncolytic activity and reduces pathogenicity. Deletion of the ICP47 gene promotes antigen presentation, oncolytic selectivity, improved antitumor immunity, and enhanced tumor killing (Kaufman *et al*., 2010). Carcinoembryonic antigen (CEA) is a tumor-associated antigen, which was first extracted from colon cancer and embryonic tissues by Gold and Freedman in 1965. It is related to the progression of various solid tumors (Krupey *et al*.,1968). Serum CEA is an important biomarker for diagnosis, prognosis, recurrence, metastasis monitoring, and the evaluation of the effect of chemotherapy in CRC (Liu *et al*.,2018) .5-Fluorouracil (5-FU) is an essential component of systemic chemotherapy for colorectal cancer and to treat different malignant tumors, including breast, pancreatic, skin, stomach, esophageal, and head and neck cancers (Vodenkova *et al*.,2020). This research aims to assessment the effect of the recombinant HSV-2 and the chemotherapy 5-FU on the expression of Carcinoembryonic antigen (CEA) in LS174T colorectal adenocarcinoma and normal vero cell lines.

Materials and methods

1- Cells and virus

Two types of cell lines that used in this study African green monkey kidney cell line (Vero) and LS-174T- Colorectal Adenocarcinoma was grown in RPMI medium supplied with 10% fetal calf serum (FCS). The pH of both media ranged from 6.8 to 7.1. To prevent bacterial contamination, antibacterial agents, namely streptomycin at a concentration of 100 µg/ml and penicillin G at a concentration of 100 IU/ml, were added to various media and solutions. Additionally, Nystatin, an antifungal agent, was added to achieve a final concentration of 25 IU/ml. the cells incubated at 37 C in a humidified atmosphere of 5% CO2.

The wild type HSV-2 isolated from semen samples after detection the virus by Real-Time- qPCR , the samples pretreated with antibiotics, then propagated via cell culture technique using Vero cell line. The serial dilution of propagated WT HSV-2 stock sample prepared by added 400 µl from WT HSV-2 to 3600 µl from

maintenance media, then mix with 100 µl from antibiotic , after this the tube incubate for 30 min. the 10 fold serial dilution prepare by add 1800 µl from maintenance media to each tube then transfer 200 µl from the stock tube to another respectively.

2- Recombinant virus generation

The constructed of recombinant HSV-2 by singular or dual deletion of ɤ 34.5 and ICP47 genes described previously in other paper (under publishing).

3-CEA Measurement

The cell culture supernatant samples were collected and stored at a temperature of -80°C until they were ready for use. Prior to conducting the assay, the samples underwent centrifugation at 1000 x g for a duration of 20 minutes. This process resulted in the separation of the supernatants, which were then collected and immediately subjected to the assay.

Tumor marker expression monitoring

Tumor markers expression monitoring was done by using Human CEA, ELISA kits**:** These kits are sandwich enzyme immunoassay for invitro quantitative measurement of Human CEA, in human and cell culture biological fluids. The micro-plate supplied has been pre-covered with a specific antibody to tumor markers CEA,. Standards or specimens are then added to the proper micro-plate wells with a biotin-conjugated antibody specific to tumor marker. After that, a conjugation of Avidin conjugated and Horseradish Peroxidase (HRP) is introduced to each micro-plate well and incubated. When TMB substrate solution is applied, only the wells that have positivity to tumor marker, biotin-conjugated antibody and enzyme-conjugated Avidin will show a color difference. The intensity of color is proportional with the concentration of the tumor marker. The enzyme-Substrate reaction is ended by the applying of sulphuric acid solution and the color difference is detected by spectrophotometer at a wavelength of 450 nm. The concentration of tumor marker in the specimens is then detected by comparing the data of O.D. for the specimens to the standard curve of that biomarker accordingly**.** Human CEA, concentrations were measured for untreated cells and that treated with different titer (1, 0.1 and 0.01)pfu/cell treatments of wHSV-2, oHSV-2 of singular and dual ɣ34.5 and ICP 47 gene deletion, as compared with the concentration of tumor marker after treatment with chemotherapeutic drug; 5FU of 1, 10 and 100 µg/ml by final absorbance reading at 450nm wavelength ELISA reader. CEA standard curve concentrations include 1250pg/ml, 625pg/ml, 312.5 pg/ml, 156.3pg/ml, 78.2pg/ml, 39.1pg/ml and 19.6pg/ml.

Results and discussion

Results of CEA expression showed down regulation and declining of CEA concentration correlated with different doses included in this study with positive correlation coefficient r^2 that equal to 0.9886, 0.8487, 0.9972 and 0.7821, and EC50 of 0.478, 0.380, 0.491 and 0.353 (pfu/cell) for ɣ34.5 ExRV, ICP47 ExRV, Dual ɣ34.5 & ICP47 ExRV and w-HSV-2 respectively. Figure (1) was revealing the effect of ɣ34.5 ExRV, ICP47 ExRV and dual ɣ34.5 & ICP47 ExRV and WT on CEA expression of LS-174T cells, presented by CEA concentration (pg/ml). Mean±SEM of absorbance at 450nm, correlation and EC50 were also shown in table (1). However these results were statistically non-significant (p=0.302) when compared with control (untreated cells) level of CEA, (p=0.302). Effect of 5 FU on CEA expression of LS-174T cells, presented by CEA concentration (pg/ml) was also shown in figure (2). The drug 5FU revealed CEA down regulation with positive correlation coefficient with dose dependency (r^2=0.9797) and IC50 of 47.29µg/ml. Dose response curves were also shown in figure (3). Since the introduction of 5-fluorouracil (5-FU) in the

1960s, 5-FU has been widely used in colorectal cancer treatment, as well as for other malignancies including gastric cancer, breast cancer, and head and neck cancer (Mayer,2000).

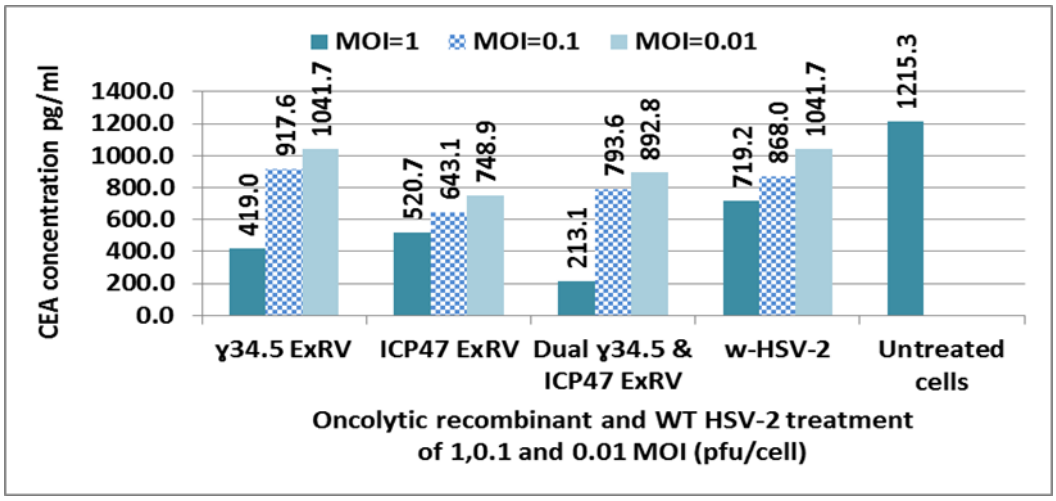

Figure (1): Effect of γ34.5 ExRV, ICP47 ExRV and dual γ34.5 & ICP47 ExRV and WT on CEA expression of LS-174T cells, presented by CEA concentration (pg/ml).

Table (1) : Effect of γ34.5 ExRV, ICP47 ExRV and dual γ34.5 & ICP47 ExRV and WT on CEA expression of LS-174T cells, presented by Mean±SEM of absorbance at 450nm, correlation and EC50.

Dose(MOI)	LS-174T cells CEA tumor marker expression				
	Mean of triplicate absorbance (OD) ± SEM				
	γ34.5 ExRV	ICP47 ExRV	Dual γ34.5 & ICP47 ExRV	w-HSV-2	P-value
1	0.499 ± 0.075	0.54 ± 0.081	0.416± 0.062	0.620 ± 0.093	0.302
0.1	0.700 ± 0.105	0.590± 0.089	0.651± 0.098	0.680 ±0. 102	
0.01	0.751 ± 0.113	0.632± 0.095	0.691±0.1035	0.751 ± 0.113	
0	0.820 ±0.123	0.820 ±0.123	0.820 ±0.123	0.820 ± 0 .123	
r^2	0.9886	0.8487	0.9972	0.7821	
EC50 (pfu/cell)	0.478	0.380	0.491	0.353	

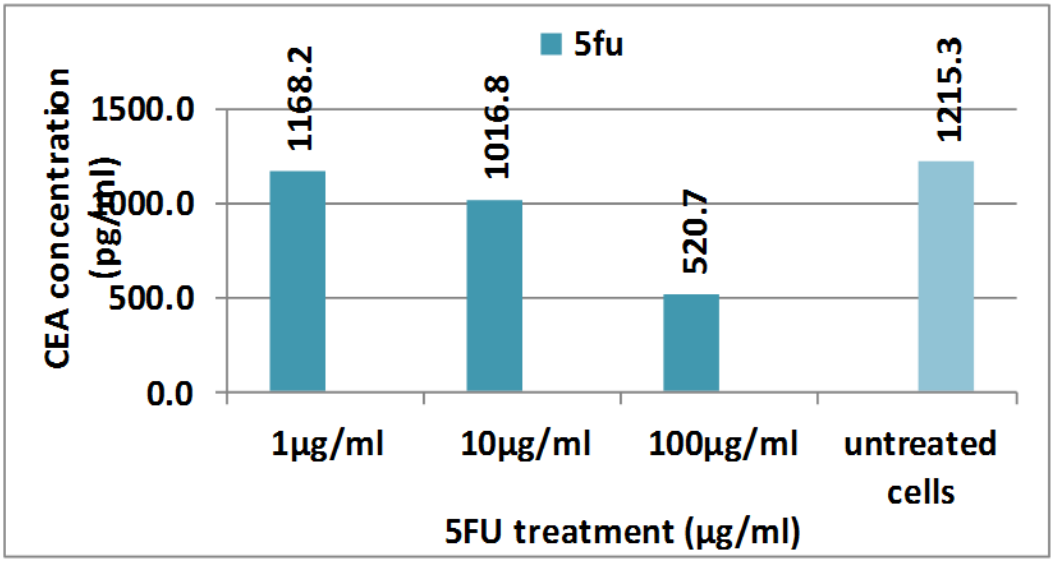

Figure (2):Effect of 5 FU on CEA expression of LS-174T cells, presented by CEA concentration (pg/ml).

Colorectal cancer is a serious disease that is characterized by rapid progression, invasiveness and high resistance to treatment. Diagnosing CRC at an early stage is not easy, as cancer is often asymptomatic. Screening requires tools and methods that are both highly sensitive and specific when diagnosing the early stages of cancer. They must be safe, cheap and widely accepted. A tumor marker can be detected in a solid tumor tissue, in a lymph node, bone marrow, peripheral blood, or other biological materials(urine, ascites, and stool). (Legolvan *et al*.,2012). Tumor markers have been sought for a number of years to detect the transformation of malignant cells at the earliest possible stage. They are usually proteins associated with a malignancy and might be clinically useful in patients with cancer. Several classical markers have been used to recognize colorectal cancer, including carcinoembryonic antigen (CEA) (Jelski and Mroczko, 2020), Carcinoembryonic antigen (CEA) is a glycoprotein oncofetal antigen that is expressed in many epithelial tumors. This relatively inexpensive blood test, first described by Gold and Freedman in 1965, was part of most recommended surveillance strategies. CEA is a glycoprotein which is formed in the cells of the large bowel. Seventy percent of patients with CRC have high CEA levels during diagnosis, which makes it a very good marker for the treatment and monitoring of the disease after resection. Although CEA is usually considered a cancer marker, its concentrations may also be elevated in a variety of benign conditions, including hepatitis, pancreatitis, obstructive pulmonary disease and inflammatory bowel disease. According to commonly accepted units of measurement values of up to 5 ng/mL are considered normal antigen level in blood. It has been observed that these values in smokers, in cases of ulcer colitis or liver cirrhosis, can be increased up to 10 ng/mL (Koness,1995). CEA serves as a robust prognostic biomarker in patients with colorectal cancer who have undergone surgical resection and received adjuvant chemotherapy (Ozawa *et al*., 2021). An elevated CEA level (>5 µg/L) at the time of new diagnosis of colorectal cancer is associated with a poor prognosis (Konishi *et al*., 2018). Fletcher conducted a review that assessed the sensitivity and specificity of CEA testing at different stages of the disease. In patients with stage I and II colorectal cancer, the sensitivity for a CEA level >2.5 ng/mL was 36%, with a specificity of 87%. The sensitivity of CEA increases with advancing disease stage, indicating that it is low in early colon cancer patients . In a study involving 358 patients diagnosed with colon cancer, only 4% of those with stage I disease exhibited an elevated CEA level (>5 ng/mL), whereas abnormal levels were observed in 25%, 44%, and 65% of patients with stage II, III, and IV disease, respectively (Wanebo *et al*., 1978). Another study involving 319 surgical patients found that CEA elevation was present in only 26% of respectable patients, but in 72% of patients with unrespectable or metastatic disease (Moertel *et al*., 1986). Other study done by Chouljenko *et al*.,2023, utilized ELISA to quantify CEA production across eight different human tumor cell lines after infected with recombinant oncolytic herpes simplex virus type 1 (VG2025 virus), including A549, BxPC3, HCC2935, LS174T, N87, SW1116, SW40, and LOVO cells . Elevated CEA was detected across multiple tumor types, with all eight tumor cell lines producing more than 5 ng/mL of CEA and 5 tumor cell lines yielding more than 100 ng/mL. CEA levels were highest in lung (HCC2935, A549), pancreatic (BxPC3), colon (LS174T), and stomach (N87) cancer cells, although the extent of CEA expression was not consistent among gastrointestinal tumors.

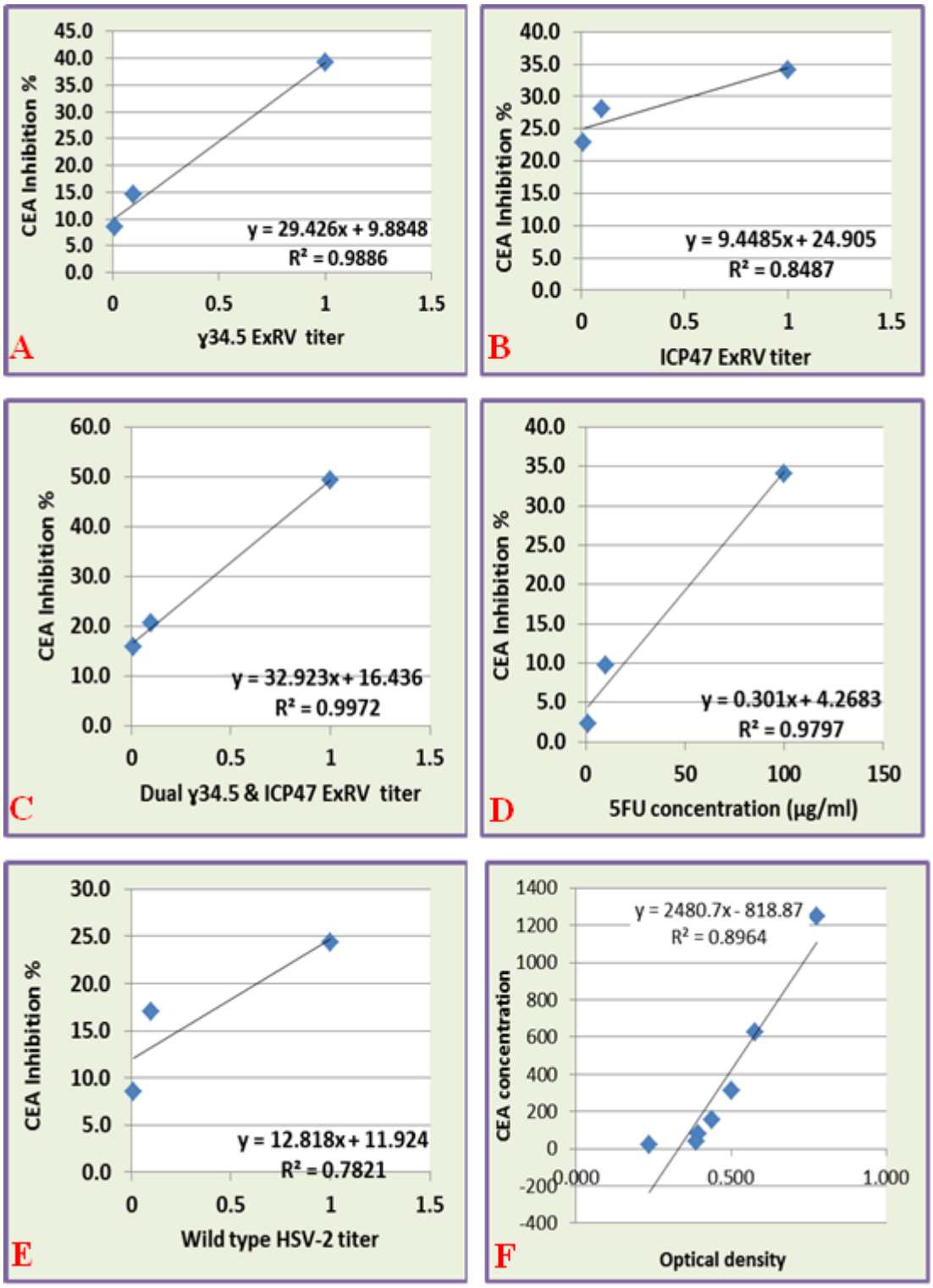

Figure (3):Carcinoembryonic antigen concentration inhibition curve after treatment on CRC LS174T cell line with γ34.5 ExRV, ICP47 ExRV and dual γ34.5 (A, B and C respectively), as compared with CEA concentration after treatment with 5FU (D)), WT HSV-2 (E) and Standard curve (F) respectively.

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