Molecular Therapy
Oncolytics
Original Article

NEBL and AKT1 maybe new targets to eliminate the colorectal cancer cells resistance to oncolytic effect of vesicular stomatitis virus M-protein

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This study compares the oncolytic effect of vesicular stomatitis virus (VSV) wild type and M51R M-protein on the colorectal tumors of different invasive intensity on SW480 and HCT116 cell lines and 114 fresh colorectal cancer primary cell cultures. Fresh tumor samples were divided into two groups of lower stages (I/II) and higher stages (III/IV) regarding the medical records. The presence of two mutations in the PIK3CA gene and the expression of NEBL and AKT1 genes were evaluated. The cells were transfected with a plasmid encoding VSV wild-type and M51R mutant M-protein. Results showed either wild type or M51R mutant can kill SW480 and stage I/II primary cultures while mutant M-protein had no apoptotic effects on HCT116 cells and stage III/IV primary cultures. NEBL and AKT1 expression were significantly higher in resistant cells. Elevated caspase-9 activity confirmed that the intrinsic apoptosis pathway is the reason for cell death in lower-stage cells. Different tumors from the same cancer exhibit different treatment sensitivity due to genetic difference. NEBL and AKT1 gene expression may be responsible for this difference, which may be the target of future investigations. Therefore, tumor staging should be considered in oncolytic viral treatment as an interfering factor.

INTRODUCTION

Most people are not aware that colorectal cancer (CRC) is going to be the most common cancer worldwide.[1–3] Common CRC treatments such as surgery, chemotherapy, and radiotherapy have unavoidable disadvantages. Oncolytic virus (OV) therapy has recently been recognized as a promising therapeutic approach for cancer treatment.[4,5] Viruses preferentially infect, propagate, and kill tumor cells without causing damage to normal cells.[6] Over the past decade, enormous research has been reported on potential OVs and significant preclinical success.

Some OVs are genetically modified to reduce pathogenicity, improve tumor cell selection, and code for therapeutic genes. vesicular stomatitis virus (VSV) is a prototype member of the family rhabdoviruses.[7–9] VSV replicates in the cytoplasm. The VSV genome codes for five proteins: nucleocapsid, phosphoprotein, matrix (M) protein, glycoprotein, and large viral polymerase.[9,10] The M-protein is a small molecule consisting of 229 residues (26.6 kDa). This protein is a multifunctional protein and its roles are not restricted to virus assembly and budding,[11,12] but also induce cytopathic effects and apoptosis.[12] M-protein restrains host gene expression at three different stages: (1) host transcription inhibition by blocking all three RNA polymerase subclasses, (2) interference with host nucleocytoplasmic RNA transfer, and (3) host translation machinery alteration by the eIF4F complex.[13,14]

It has been proposed that modification of M-protein by substitution of an arginine for a methionine residue at position 51 (M51R) eliminates the virulence of VSV in healthy cells but not in tumor cells.[15] Therefore M-protein mutant viruses can be efficient oncolytic agents because they can specifically kill tumor cells *in vivo* without causing disease.[16] Previous studies on VSV have shown preclinical success against various types of malignancies such as prostate cancer, breast cancer, melanoma, hepatocellular carcinoma, and glioblastoma.[17] Evidence supports each cancer cell line differing in their susceptibility to OVs, even if these cancer cells derive from the same tissue type.[18] There is controversy about the effect of the M-protein mutant on the induction of apoptosis in different cancer cells. In the present study, the oncolytic potentials of wild-type VSV (WT) and M51R-mutant M-proteins were compared in human CRC cell lines

Received 13 August 2021; accepted 18 November 2021;
https://doi.org/10.1016/j.omto.2021.11.013.

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Table 1. Statistical results for two PIK3CA gene polymorphisms, and the fold change of expression of the AKT1 and NEBL genes in 114 cancer and 114 adjacent normal tissues

		PIK3CA, E545D/K				PIK3CA, H1047R					
Total		Wild		Mutant		Wild		Mutant		NEBL cancer versus normal expression	AKT1 cancer versus normal expression
Cancer	Normal	Cancer	Normal	Cancer	Normal	Cancer	Normal	Cancer	Normal		
Stage I/II											
38	42	20	29	18	13	24	38	14	4	+0.2 fold	+0.3 fold
p value = 0.6		p value = 0.07		p value = 0.06		p value = 0.06		p value = 0.01			
Stage III/IV											
76	72	8	42	68	30	12	41	64	31	+3.7 fold	+2.2 fold
p value = 0.8		p value = 0.0001		p value = 0.0001		p value = 0.0002		p value = 0.0001		p value = 0.0018	p value = 0.0001

HCT116 and sw480 using several cytological and proteomics methods.

RESULTS

Fresh tumor analysis

The youngest and oldest patients were 36 and 84 years old, respectively, with an average of 55.6 years, consisting of 58 male and 56 female patients. Most of the samples were resected from the descending colon (72 cases) compared with the transverse colon (29 cases) and ascending colon (13 cases) (Table 1). Only 38 of 114 primary cell cultures were stable after three passages in which 23 were successfully transfected by the constructed plasmid. Figure 1 shows the results of the PIK3CA gene polymorphisms and the expression of Alpha-Serine/Threonine Kinase 1 (AKT1) and Nebulette (NEBL) in 114 tumor and adjacent normal tissues.

Western blot analysis

The expression of WT and M51R VSV M-protein was validated by western blotting. Transfection of the cells with pCDNA-WT and pCDNA-M51R M-protein plasmids resulted in detectable expression of the M-protein using an anti-M monoclonal antibody, after 48 and 72 h of incubation. However, the protein expression bands were clearer at 72 h compared with 48 h post-transfection. The bands were hardly detectable after 24 h post-transfection. A distinct band was seen about 26 kDa in transfected cells, but not in the non-transfected ones (Figure 2).

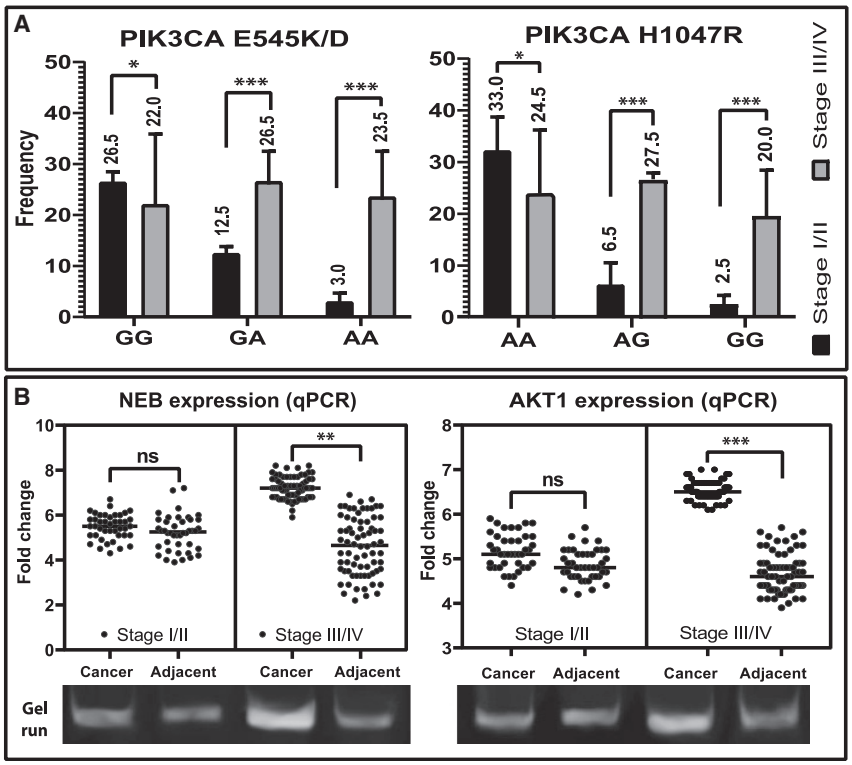

Figure 1. (A) Comparison of stages "I/II" versus "III/IV" for two PIK3CA gene polymorphisms (RFLP-PCR) (average of three times repeat), (B) The expression of AKT1 and NEBL genes (RT-qPCR) in 114 cancer and 114 adjacent normal tissues.

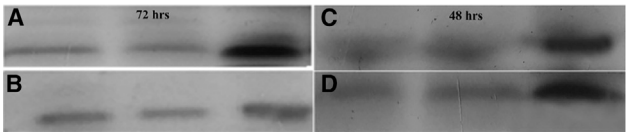

Figure 2. M-protein Expression aproval

Western blotting M-protein expression of WT (left) and M51R (middle) in transfected HCT116 (A and C) and SW480 (B and D) cells, which was carried out at 48 and 72 h post-transfection. Commercial M-protein was used as the positive control (A–D, right column).

Cytotoxicity test-MTT assay

As shown in Figure 3, the viability of Hct116, SW480, and all of 23 primary cultured cells was reduced by at least 67% when were transfected with 200 ng pCDNA-WT plasmid compared with the untransfected cells. The viability of the mutant M-protein expressing SW480 cells and 12 stages "I/II" primary cultured cells was observed to be decreased as well. However, no considerably disrupted cells were observed in 11 stages "III/IV" primary cultured and Hct116 cells expressing M51R mutant protein. Experiments were repeated three times and the same results were observed.

Caspase-3, -8, and -9 activity assays

A 3-fold increase in caspase-9 and a 2-fold increase in caspase-8 concentration were observed in two cultured cell lines (SW480, HCT116) that express WT M-protein compared with control cells. As showed in Figure 4, in HCT116 cells like four of stage C/D primary cultured CRC cells, the level of caspase-3 was about 3-fold more than cells transfected with empty pCDNA3.1 after 48 h and it slightly increased after 72 h. The SW480 cells transfected with M51R plasmid resulted in a caspase-8 activity depletion from 48 h to 72 h post-transfection, its level is almost the same as cells transfected with empty plasmid. The same results of caspase-8 activity depletion were observed in four primary cultured CRC cells of stage A that were expressing M51R M-protein. Furthermore, the activity of caspase-9 was almost equal to cells transfected with empty plasmid after 48 h and was dramatically elevated about 2-fold for 72 h either in SW480 or the four A stage CRC cells. Interestingly, no considerable changes were seen in the concentration of either caspase-8 or -9. Caspase-3 was decreased slightly in HCT116 cells from 48 to 72 h post-transfection with M51R M-protein plasmids. These changes are interpreted more in the discussion section (Figure 4).

Morphological changes in cells

The apoptotic shrunk cells can be observed detached from the surface and appear to be floating in the medium. Most of the cells remained attached to the surface with a cone shape after 24 h post-transfection, which represents an alive and healthy condition. After 48 h, some round shaped floating cells were observed (Figure 5).

Flow cytometric assays for Annexin V-fluorescein isothiocyanate/propidium-iodide

Flowcytometric Annexin V-fluorescein isothiocyanate (FITC)/propidium-iodide (PI) assays were performed 72 h post-transfection. Cells double-stained for Annexin V and PI to discriminate the cells in the earlier stages of apoptosis (annexin V-FITC positive, PI negative) from those in later stages of apoptosis or that were already dead (annexin V-FITC positive, PI positive).

Most of the cells, 92.3%, and 97.9% were viable in non-transfected SW480 and HCT116 cells, respectively. The count of the apoptotic cells increased in both cell lines that were transfected with pCDNA-WT M-protein; 7.3%, 73.5%, and 19.1% of viable, early apoptosis, and late apoptosis were detected in SW480 cells expressing WT M-protein, respectively (Figure 6). Meanwhile, these same rates were 15.3%, 56.6%, and 28.1% for the HCT116 cells expressing WT M-protein (Figure 6, middle-down).

In the M51R M-protein expressing cell lines, 20.5% of the cells were viable for SW480, and 79.1% for HCT116 cells, whereas the cells that underwent early apoptosis were 5.3% and 8.9% for SW480 and HCT116 cell lines, respectively (Figure 6).

DISCUSSION

VSV is a potent OV candidate.[19] VSV-M-protein plays an essential role in virus assembly as well as the cytopathic effect on host cells by disruption of the host cytoskeleton. Moreover, M-protein is responsible for apoptosis in the host cell through inhibition of transcription through host RNA polymerase dumping as well as interruption of nucleocytoplasmic transport of RNAs.[20–22] Researchers have identified wild M-protein as the key harmful molecule for infected cells.[23]

However, there is controversy about the effectiveness of the M-protein mutant. Several researchers reported no significant oncolytic effects for M-protein apart from viral particles. These researchers believe a fundamental role of other viral components in addition to M-protein for induction of apoptosis,[24,25] whereas others have noted apoptosis in cells with extra expression of recombinant M-protein.[14,23,26–28] Gaddy and Lyles[14] described that M-protein itself can interfere with the nucleus transcription.[29] Likewise, our previous work illustrated the effects of recombinant VSV M-protein on apoptosis in CRC SW480 cells.[23] Douzandegan et al have also reported both VSV WT and M51R M-protein provoke apoptotic cell death in KYSE-30 esophagus cancer cells.[36]

Similar controversy has been proved on prostate cancer cells. Researchers have reported that LNCaP prostate cancer cells are sensitive to VSV, while PC3 prostate cancer cells are resistant to this virus.[29,30] Other reports have demonstrated the diverse effects of WT and mutant M-proteins in human CRC cell lines. ARKO cells showed high sensitivity, LoVo cells were resistant, whereas HCT116 has limited susceptibility to VSV.[19] This proposes the idea that different cancer cells exhibit different susceptibility to OVs and therefore the need to investigate the mechanism.

In the present study, we analyzed the oncolytic effect of WT and M51R VSV M-proteins on two human CRC cell lines of different

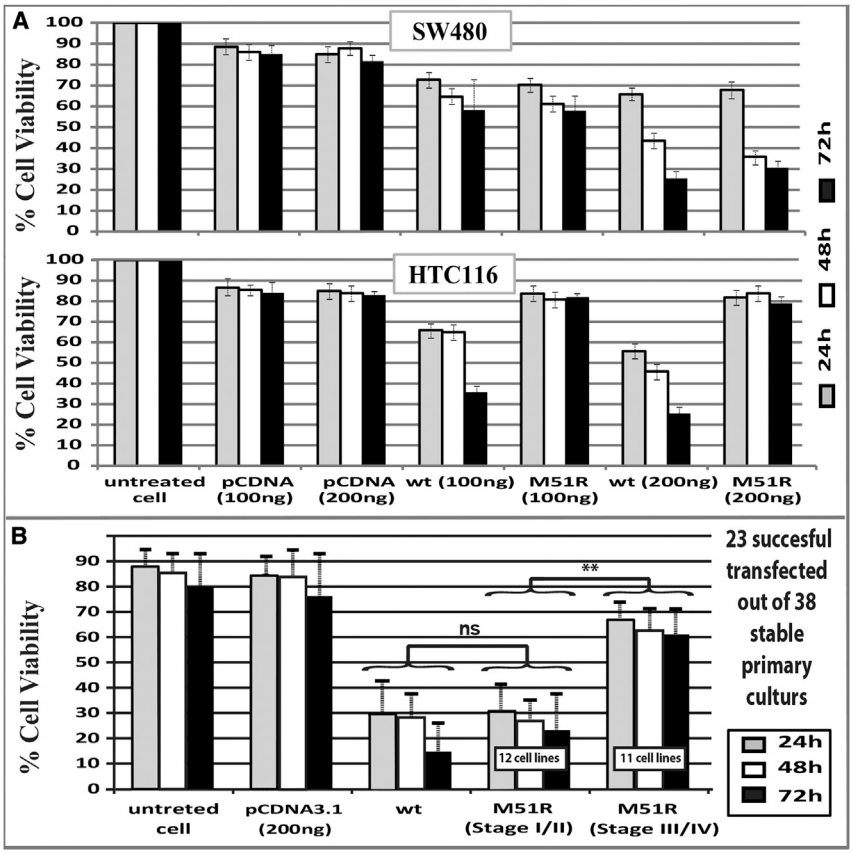

Figure 3. Effects of M-protein on the cell viability

(A) MTT assay (average of three times repeat), cell viability of SW480 and HCT116 cells transfected with 100 ng and 200 ng (24, 48, and 72 h post-transfection) was determined by MTT assay. Cells transfected with empty plasmids were considered as control cells. (B) MTT assay to comparison of viability of primary CRC cell cultures on untreated, treated with empty and wt M-protein plasmids, with cells transfected by mutant M-protein plasmids (including different stages) after 24, 48, and 72 h post-transfection.

cancer stages. Western blot analysis was performed to confirm the expression of WT and M51R M-proteins in transfected cells at 24, 48, and 72 h post-transfection. As mentioned in the results, a very thin band was visible at the 26 KDa position at 24 h, which represents a very low concentration of expressed M-protein. These specific bands were more visible and widened from 48 to 72 h, which may describe the increase of the M-protein expression (Figure 2). An increasing cell death rate is expected with this increasing M-protein concentration. The MTT Assay can validate this hypothesis.

As determined by the MTT assay, SW480 cells expressing WT and M51R proteins show a significant increase in cell death compared with control cells 24, 48, or 72 h post-transfection. HCT116 cells treated with WT M-protein showed a 45% decrease, while no considerable cytotoxic effect was observed for cells expressing M51R protein. These results suggested that the expression of mutant M-protein in HCT116 cells may not affect cell viability.

As seen in Figure 3, the amount of plasmid used for transfection of cells was optimized with 100 and 200 ng of plasmids. The amount of applied plasmid does not affect cell viability in the HTC116 cell line, while in the SW480 cells, the more applied plasmid (200 ng) coefficient with less viable cells after 24, 48, or 72 post-transfections. This different sensitivity to the number of transfection plasmids suggests a significant difference between the physiopathology of the two case study cell lines.

To investigate this physiopathology difference, the Caspase-3, -8, and -9 levels were evaluated. Although caspase-3 has been recognized as one the of crucial components in the apoptosis cascade but this protease also has been known to participate in a variety of non-apoptotic phenomena, such as tumor aggressiveness, invasiveness, and proliferation of cancer cells. Also, caspase-3 is involved in another programmed mode of cell death called pyroptosis. So, the activation of caspase-3 does not mean necessarily that cells are going to undergo apoptosis. Therefore, we did complementary investigations to confirm apoptosis cell death such as measurement of caspase-9 and -8. Also, we performed flow cytometry (annexin V/PI) and morphological observation to validate our result.

It is well known that caspase-8 involves in the extrinsic pathway and caspase-9 activates via the intrinsic apoptosis pathway. Caspase-3 is an execution enzyme that activates in response to caspase-8 or -9 activations and is considered a hallmark of apoptosis.[31,32]

Our experiment showed that the caspase response to WT M-protein expression was similar in either the SW480 or HCT116 cell line, including a dramatic elevation of caspase-9 and -3 and slightly in caspase-8 (Figure 4). However, mutant M-protein results in no considerable increase of caspase-3, -8, or -9 in HCT116 cells. Meanwhile, SW480 cells expressing M51R M-proteins showed higher activity of caspase-3 and -9. Similarly, in several studies, caspase-9 has been reported as the dominant enhancing component of apoptosis in the presence of M-protein.[7,31,33] Other researchers have also reported this prominent role for caspase-9 in flaring up the apoptosis cascade due to WT M-protein activity in HeLa and BHK cells.[24,25,32]

Gaddy and Lyles have two different hypotheses for the mechanism of apoptosis in response to M51R M protein expression. They report caspase-9 and intrinsic pathway as the main mechanism

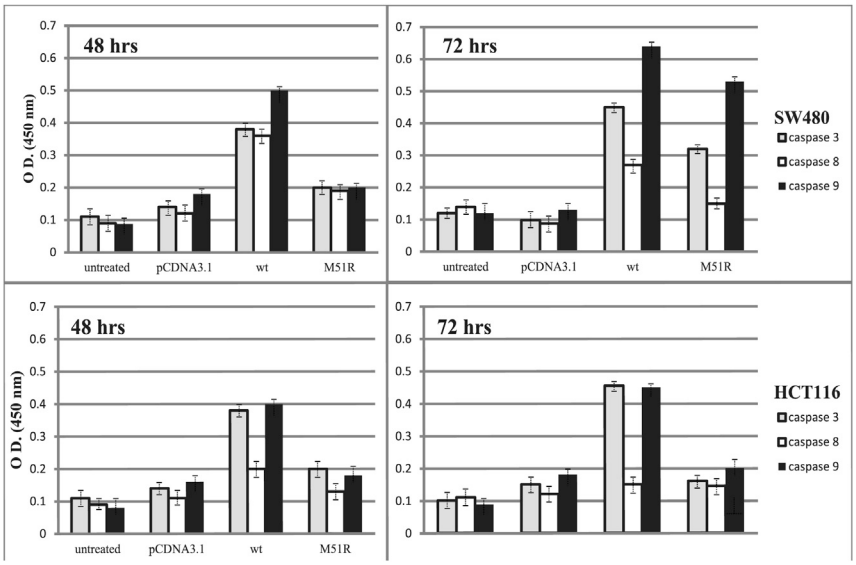

Figure 4. Activity of caspase-3, caspase-8, and caspase-9 (average of three times repeat)
Transfected SW480 cells with WT and M51R M-protein showed an increase of caspase-3 and -9 representing the intrinsic apoptosis pathway compared with untransfected cells (up). Transfected HCT116 cells with WT showed an increase in caspase-3, -8, and -9, while cells expressing M51R M-protein did not show any significant increase of caspase-3, -8, or -9.

in their primary studies while introducing caspase-8 as the most important apoptotic pathway following M51R M-protein expression in the presence of viral particles.[14,34] Our observations also verified the effect of caspase-9 and intrinsic pathways on apoptosis due to M51R-M expression in the lower-stage but not in higher-stage cancer cells.

Previous studies have shown that VSV-ΔM51 M-protein can initiate apoptosis in most PDAC cell lines except for three, Hs766T, HPAC, and HPAF-II, which were resistant to apoptosis and showed no or low amount of caspase-8 and caspase-9. We explore the difference between these cell lines and found that these three cell lines also originated from the higher cancer stages.[34]

Kopecky et al., as well as Gaddy and Lyles, suggested that apoptosis induced by mutant M-protein is due to the expression of some new genes in the host cell that are responsible for mitochondrial cell switching death, whereas apoptosis caused by WT M-protein is due to global inhibition of host gene expression.[32,35] However, in one study examining the effect of different VSV M-proteins on host gene expression in human embryonic kidney BHK21 cells, it was reported that the substitution of Methionine 51 with Arginine weakened the apoptotic potential of M-protein to the minimum level and led to the halter caspase-3 induction.[36]

Furthermore, flow cytometry with Annexin V/PI staining confirmed that the cytotoxicity of the expressed M-protein correlates with the induction of apoptosis. Based on flow cytometric results, WT VSV M-protein induces apoptosis in both SW480 and HCT116 cells while M51R only has an apoptotic effect on SW480 cells.

According to this different apoptotic susceptibility, Mouradov et al reported a clear distinction between CRC cell lines. They have extensively studied 70 CRC cell lines for mutations and molecular differences.[46] They categorized cell lines into eminent hypermutated and non-hypermutated groups in terms of mutations ranging from 6.6 to 260 per million base pairs. Based on their results, 86.3% of hypermutated cell lines such as HCT116 exhibited high microsatellite instability (MSI), a higher number of insertion/deletion (41.1-fold; $p < 0.001$), and single nucleotide polymorphisms (9.7-fold).; $p < 0.001$) compared with non-hypermutant cell lines such as SW480.[37]

The mutations and molecular differences of cancer cells have a significant impact on the stage of cancer, and therefore, treatment and prognosis. The Dukes' staging for CRC has been mentioned in several reports that classified SW480 as stage B and HCT116 cell line as stage D. Dukes' stage D represents a metastatic and more invasive cancer. Dukes' B stage restricts the muscle layer and is therefore a less invasive tumor.[38]

Regarding the cellular and molecular differences of tumor cells at various stages, two promising hypotheses need to be investigated in future works. Berg et al. in 2017 reported a severe upregulation of AKT pathway genes in SW480 but not in HCT116 cells.[47] Dunn and Connor in 2011 showed that viral M-protein can inactivate AKT.[48] The first hypothesis is that the apoptotic response of SW480 cells to mutated M-protein may be explained by inhibition of AKT1 via M-protein and consequently increased apoptosis. As the second hypothesis, SW480 cells have several mutations in the TP53 gene while HCT116 harbored WT TP53.[38,41] Moreover, HCT116 showed lower BCL-2 family activity compared with SW480 cells.[42] Furthermore, previous research has reported the M-protein effects on BCL suppression.[20] This difference in BCL and TP53 may be another reason for the responsiveness of HCT116 cells to WT M-protein and not to the mutant one, so it may be referred to as the BCL/TP53 hypothesis. The SW480 may be the better target for the M51R M-protein due to having more BCL activity. The present work reconfirmed the second hypothesis in which there is apoptosis of SW480 cells in the presence of the mutant M-protein by flow cytometry method (Figure 6, upper right, right). NEBL and Akt1 are the biological markers of the cancer staging.[38,43,44] A higher level of NEBL expression was detected in HCT116 and all of the higher-stage cells of primary tumor cell cultures

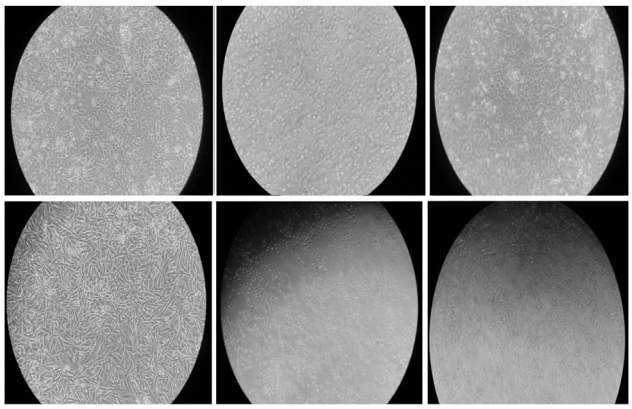

Figure 5. Morphology changes after M-protein expression

Cell morphology of HCT116 (up) and SW480 (down) cells after 72 h post-transfection, in control (left), Wt (middle), and M51R M-protein expressing cells (right), showed cell rounding, shrinking, and detachment from the surface

in the current study before induction of VSV M-protein expression. Suppressing the Akt1 expression has shown to promote apoptosis in higher stages of colorectal cancer cells.[45]

In conclusion, our results suggest that the oncolytic effects of a virus on cells even derived from the same origin may be related to the different molecular and genetic characteristics of the cells. The degree of cell differentiation may be the key factor correlating with the efficacy of M51R M-protein in SW480 cells and not in HCT116, where SW480 belongs to a better differentiated and less mutated group, and HCT116 is a metastatic tumor cell and belongs to the hypermutated cell group.[37] AKT1 and NEBL genes have been shown that are upregulated in higher stages of the cancer. These genes may be potential targets for the treatment of high-stage tumors. Tumor staging should be considered as an interfering factor in oncolytic viral cancer treatment approaches.

MATERIALS AND METHODS

This project was approved by the Institutional Research Ethics Committee of the GoUMS. Briefly, the oncolytic effect of VSV WT and M51R M-protein was compared in SW480 and HCT116 CRC cells from the different invasive intensity. Primary cell culture was performed from isolated tumor masses from 114 patients at different clinical stages, of which only 38 were stable after 3 passages. SW480 and HCT116 cell lines and 38 CRC primary cell cultures were transfected with a plasmid encoding VSV WT and M51R mutant M-protein. The expression of viral M-proteins confirmed using western blotting assay followed by cell death rate analyzing by MTT assay, observation of cell morphological changes, and evaluation of caspases-3, -8, and -9. There are too many interactions between extrinsic and intrinsic pathways in apoptosis. Caspase-9 can also be activated by extrinsic via caspase-8-mediated bid processing. Caspase-3 is also involved in pyroptosis phenomenon. Therefore, we have reconfirmed the apoptosis process by the flow cytometry method as well. Each experiment has been repeated at least three times to confirm the accuracy of the results.

We also measured the expression state of three genes that were introduced to be overexpressed or mutated in higher stages or metastatic cases of cancers, Nebulin (NEB),[38] and PIK3CA,[19] AKT1[20] in all of the 114 fresh tumor masses, to confirm the correlation of the invasiveness of the tumor with the effectiveness of the oncolytic VSV treatment. In this regard, two reported polymorphisms in the exon 9 as E545D/E545K as well as one in the exon 20 as H1047R in the PIK3CA gene were evaluated using restriction fragment length polymorphism (RFLP)-PCR. Overexpression of NEBL and AKT1 genes also were evaluated using RT-qPCR.

Fresh tumor samples

After taking the individual consent agreement, we obtained a 3-g piece of each pathologically confirmed tumor mass isolated from 120 patients with CRC of whom only 114 were in an adjacent noncancerous area, and entered the project. Tumor masses had been resected through colonoscopy by a gastroenterologist or open surgery at the university hospital. Demographic information, Dukes' staging, and histopathologic report were collected from hospital records after preparation. Primary cell culture was established from the tumor mass following the previously described protocol,[21] of which only 38 were stable after three passages.

Cell lines, plasmids, transfection

Human colon adenocarcinoma cell lines HCT116 (C570, RRID: CVCL_0291) and SW480 (C506, RRID:CVCL_0546) were selected based on their Dukes' staging, which classified SW480 as stage B (less invasive) and HCT116 cell line as stage D (most invasive).[38] Both cell lines were cultured in RPMI 1640 media (Gibco, Germany) containing 10% fetal bovine serum with 100 U/mL penicillin and 100 U/mL streptomycin, at 37°C in an atmosphere containing 5% CO_2.

The pCDNA3.1 plasmid (Invitrogen, San Diego, CA) expressing VSV WT and M51R mutant M-proteins was generated. Site-directed mutagenesis was applied to produce M51R coding plasmid with the primers that were designed using the New England Biolabs (NEB.com) web server as:

Forward: 5′-GTTGACGAGAaGGACACCTATG-3′

Reverse: 5′-TCCAAAATAGGATTTGTCAATTG-3′

Hct116 and sw480 cells as well as 38 stable primary cultured cells were seeded in six-well plates at a density of 1 million cells/well and cultured until the cells reached 80% confluency. Lipofectamine 2000 (Invitrogen) was used for transfection according to the manufacturer's protocol.

Western blotting

Western blotting was obtained 24, 48, and 72 h post-transfection to prove the expression of M-protein in transfected cells. Transfected cells with WT and mutant M-protein plasmid (SW480 and HCT116, as well as eight primary cell cultures) were lysed in lysis buffer (0.15M NaCl, 1% sodium deoxycholate, 1% Triton X-100,

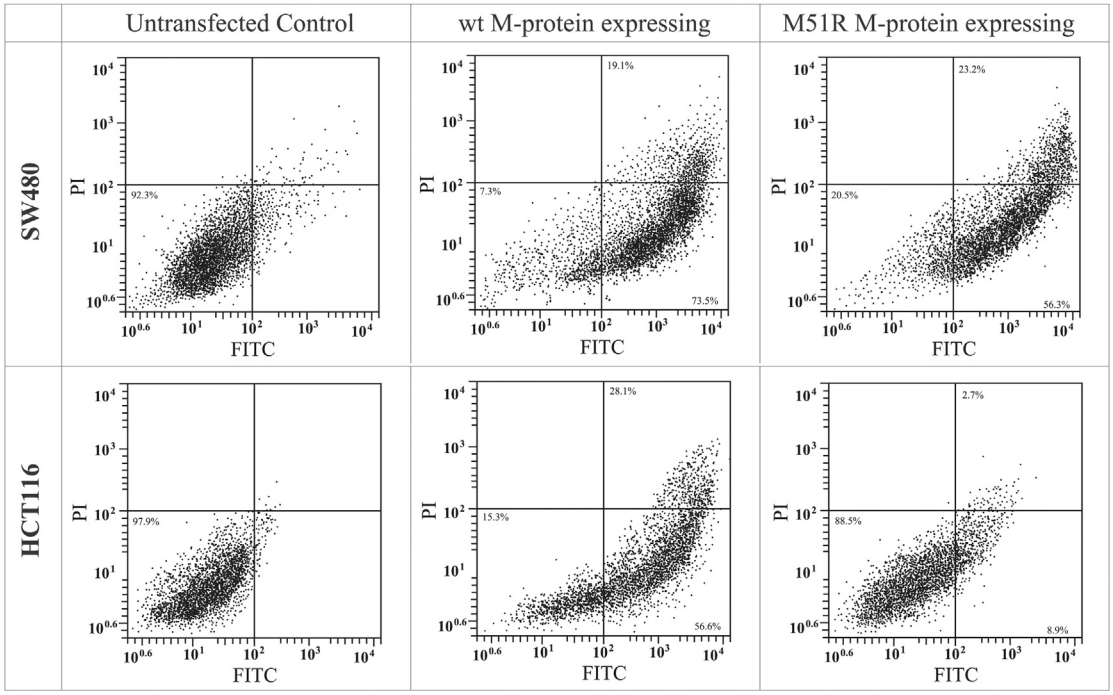

Figure 6. Apoptosis confirmation

Annexin, FITC/PI flow cytometry, shows apoptosis in either SW480 or HCT116 cells expressing WT M-protein (middle column), but only in SW480 cells expressing M51R mutant M-protein (right column). All experiments were repeated three times.

10nM Tris, pH 7.4) and protease inhibitors (protease inhibitor cocktail set 1; Calbiochem). Total cellular protein content was measured using Bradford's method to confirm the equivalent protein concentration for SDS-PAGE. Consequently, the proteins on the gel were blotted onto nitrocellulose membrane and blocked with 5% skim milk solution. VSV WT and M51R M-proteins were detected as 26 kDa with ECL reagent kit by autoradiography.

Cytotoxicity test, MTT assay

The cytotoxicity of pCDNA-WT, pCDNA-M51R plasmids on HCT116, SW480, and primary cultured cells was assessed using MTT assay. Briefly, cells were seeded 10,000 cells/well on 96-well plates. After 24 h, the cells were transfected with pCDNA-WT, pCDNA-M51R plasmids, and empty pCDNA3.1 as a control. Following 24, 48, and 72 h post-transfection incubation, the media was aspirated and 20 μL of 5 mg/mL MTT dye solution was added per well and incubated at 37°C and 5% CO_2 for 4 h. The supernatant was carefully removed and 100 μL of DMSO was added to dissolve the resulting formazan crystals. Optical absorbance at 540 nm was measured using a microplate reader. The cell viability was analyzed as described in previous studies.

Morphological observation

The cells were grown on tissue culture dishes and then transfected with WT or M51R mutant M-proteins plasmids. The morphological changes of apoptotic cells were observed using a phase-contrast inverted microscope after 24, 48, and 72 h post-transfection incubation.

Apoptosis assay (measurement of caspase-3, -8, and -9 activities)

The effect of the expression of WT and M51R mutant M-protein on caspases in HCT116, SW480, and eight primary cultured cells was determined using the commercially available caspase-3 (Abcam-colorimetric), caspase-8 (Abcam-sandwich ELISA), and caspase-9 (abnova-sandwich ELISA) ELISA kit according to the manufacturer's protocol. Due to no detectable M-protein expression observed by western blotting assay after 24 h following the transfection, apoptosis assays were performed at 48 and 72 h post-transfection.

Annexin V-FITC/PI by flow cytometry

Since such an induced apoptotic process takes time to go through, the Annexin assay was conducted after 72 h of the transfection. HCT116 and SW480 cells were seeded (250,000 cells/well) in 24-well plates and the apoptosis was measured using the Annexin V-FITC/PI Apoptosis Detection kit (BioLegend) according to the manufacturer's protocol. Following the transfection with WT and M51R M-protein plasmids and incubation for 72 h, the cells were washed twice with ice-cold PBS; 1X binding buffer, followed by 10 μL of Annexin-V-FITC, and subsequently, 10 μL PI was added to each microtube and incubated for 30 min at 4°C in a dark chamber. The apoptosis was analyzed using flow cytometry (BD accuri c6). In the flow cytometry

analysis of Annexin V-FITC/PI double staining, the late apoptotic or necrotic cells were visible in the upper right and early apoptotic cells in the lower right quadrants, and live cells in the lower left quadrants.

PIK3CA mutation pattern

RFLP-PCR was established for the evaluation of PIK3CA exon 9 mutations, E545K and E545D, and exon 20 mutation, H1047R as described in previous research.[24] The forward primer sequence was 5′-AGAGACAATGAATTAAGGGAAAATGACA-3′, and the reverse primer was 5′-GTCACAGGTAAGTGCTAAAATGG-3′ for E545K/D mutation detection. A product of 126 base pairs (bp) resulted which using TspRI restriction enzyme could be divided into two fragments of 45 and 81 bp if there is no mutation. In case of substitution of the glutamic acid (E) at the position 545 by aspartic acid (D) or lysine (K), the enzyme recognition site will be changed and no digestion occurs, and a single 126-bp band is expected in gel electrophoresis assay. The primers for detection of the H1047R mutation are 5′-GGAGTATTTCATGAAACAAATGAATGATGCG-3′ as forward and 5′-TTGACTCTTTTACTTTCGAG-3′ as reverse, which produces a fragment of 126 bp as well. Treatment of the products with the FspI restriction enzyme will result in two fragments of 31 and 95 bp in case of the presence of a Histidine (H) to Arginine (R) substitution at position 1047. Researchers previously have reported these mutations to be significantly in correlation with higher stages of tumor.[19,24,25]

NEBL expression levels

NEBL is a cytoskeletal matrix protein expressed in several tissues. Upregulation of the NEBL gene was reported in several cancers of metastatic and higher stage.[38] We have evaluated the expression of this gene in CRC in 114 tumor masses compared with the non-cancerous marginal tissues. Total RNA was extracted from all samples using the ZymoResearch miniprep RNA extraction kit according to the manufacturer's protocol. The quantitative relative reverse transcriptase, real-time PCR (RT-qPCR) with SYBR Green was performed in a CFX96 Bio-Rad Real-Time PCR instrument. Based on previous research projects, the forward primer sequence was 5′-CACCA AATCTAAGGACCTACCG-3′ and the reverse primer was 5′-CTC AATGTAATTCGCTGGGAGC-3′, which was expected to result in a product length of 176 bp.[38] GAPDH was used as the reference gene (primers F: 5′-TCCCATCACCATCTTCCA-3′, R: 5′-CATCAC GCCACAGTTTCC-3′, product length: 376 bp). All samples were assayed in triplicate. The PCR cycling conditions were as follows: primary 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 45 s, followed by a final elongation step of 72°C for 10 min. A final step of melting curve analysis at 65°C increasing 0.5°C every 5 s up to 95°C was conducted to confirm the results. The standard compared with threshold cycle (Ct) value changes (ΔΔct) was applied to analyze the results using the $2^{-\Delta\Delta Ct}$ method.

AKT1 expression levels

AKT1[20] is also reported to overexpress in higher-stage tumors including colorectal malignancies.[20,26,27] To examine the AKT1 gene expression, the extracted total RNA from 114 tumor samples and paired normal tissues were used for the SYBR Green RT-qPCR assay. The forward primer was 5′-TCTATGGCGCTGAGATTGTG-3′ and the reverse primer was 5′-CTTAATGTGCCCGTCCTTGT-3′, which is expected to produce a product of 116 bp. GAPDH was used as the reference gene and all samples were assayed in triplicate. The PCR program was as follows: a primary denaturation of 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, followed by a final step of melting curve analysis at 65°C increasing 0.5°C every 5 s up to 95°C. The standard compared with threshold cycle (Ct) value changes (ΔΔct) was applied to analyze the results, somehow a lower ΔΔct value suggested a higher expression level.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.11.013.

ACKNOWLEDGMENTS

This work was conducted at the Department of Microbiology, Medical College of the Golestan University of Medical Sciences (GoUMS) with personal financial resources. This project was approved by the Research Ethics Committee of the GoUMS. Individual consent agreements were obtained from 120 patients with CRC for using a piece of the resected tumor mass in this project. There was no funding for this project.

AUTHOR CONTRIBUTIONS

Z.M. and M.R.K. performed experiments, analysis, and manuscript preparation; A.M. and M.R.K. were responsible for intellectual content, planning the project, supervision, and manuscript editing; A.M., M.R.K., M.M.S.D., and M.P. were scientific consultants. All data generated or analyzed during this study are included in this published article.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

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