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Research Article

Application of Bioinformatics Tools for the Prediction of Helper MicroRNAs for Improvement of Oncolytic Virus Efficacy

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Purpose. Oncolytic Reoviruses, as a self-limiting virus, can be used in cancer treatment, because they have the ability to replicate in tumor cells selectively and destroy them. Studies show that some immune response proteins may interfere with the virus life cycle. So, the main aim of this bioinformatic study is to check which microRNA is able to target some reovirus inhibitory proteins. *Experimental Design*. By use of online bioinformatics software, the microRNAs that could target inhibitory genes were selected. Then, other features like content ++ score and cell type were checked and finally the eligible microRNAs were determined. *Results*. After choosing 15 inhibitory proteins, analysis was performed and finally 37 microRNAs which could target inhibitory proteins in colorectal cell lines were selected. In the end, by investigation of web-based tools, just two microRNAs were finalized. *Conclusions and Clinical Relevance*. This bioinformatic study shows that microRNA-140 and microRNA-92a have the potential to target some inhibitory proteins which interfere with oncolytic Reovirus replication and it may help in the optimal use of this virus as a cancer treatment. Because selective reproduction of Reovirus in tumor cells, as a nonchemical therapy, can be a good way to overcome this disease with broad advantages.

1. Introduction

Colorectal cancer (CRC) is one of the most frequent malignancies in the world and is the third leading cause of cancer-related mortality in both men and women. Studies estimate that there are 1.36 million new cases yearly with 694,000 CRC-related deaths. Of course, the mortality rate in recent years is decreased due to early diagnosis and improved treatment methods. By the way, it has been estimated that about 30-50% of cured patients eventually face disease recurrence [1, 2]. Many studies have shown that ageing, lifestyle ,and eating habits play a key role in CRC development. For instance, smoking, alcohol abuse, or excessive use of meat and fats are the most important factors in getting CRC. In addition, it is said that age over 50 years, family history of CRC, inflammatory bowel disease, etc. are other risk factors [3].

Oncolytic viruses (OVs) are a group of genetically engineered or intact viruses which can selectively replicate in cancer cells and destroy them by direct lytic effect or induction of immune responses [4]. Different clinical studies agree with their safety even as monotherapy use or combination therapy [5]. Naturally occurring reoviruses are a sort of oncolytic virus that has a direct oncolytic effect [6]. These nonenveloped, double-shelled viruses contain 10 segments of dsRNA and so, replicate in the cytoplasm. The infection of these viruses is mild with minor respiratory or enteric symptoms. Studies show that, in different societies, reovirus infection is common and about 50-100% of adults are seropositive. As

an oncolytic virus, they selectively replicate in transformed cells and could be a safe choice in cancer therapy [7–10] .

The microRNAs (miRNAs), which are known as 18-22 nucleotides sequences, are sort of noncoding RNAs that participate in gene expression regulation. Actually, they bind to 3′UTR of some specific mRNAs and either degrade the mRNAs or inhibit their translation. Different studies have shown that miRs play an important role in central biological processes such as proliferation, cell development, and apoptosis. Moreover, improper expression of miRs in many cancers is associated with initiation, progression, and also metastasis [11–13]. They also can regulate genes functions which are involved in cancers; therefore, they can be nominated as new biomarkers for cancers [14].

Nowadays, different studies are focusing on increasing the oncolytic reovirus capacity for tumor lysis and using them in combination therapies [15, 16]. Because, although many clinical trials show the safety of reovirus in patients with cancers, it has been revealed that reovirus therapeutic ability is limited [17]. For instance, after reovirus infection, antiviral mechanisms like cytokines production and cell death response, try to inhibit virus diffusion [18]. Activation of protein kinase R (PKR) results in phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) which is necessary for viral transcription [16]. Also, reovirus infection can activate the PI3K/Akt pathway as a regulator of virus replication and blockage of this pathway, which impairs the induction of INF-stimulated genes (ISGs) and IFN-stimulated response elements (ISRE), enhances the virus infectivity [9, 19].

The miRs target databases show that either one miR can regulate many genes or one gene can be regulated by different miRs. But generally, a one-to-one relationship between miR and its target is much more common [20]. Because some immune system proteins have an antireoviral effect, in this study, by using online software, we tried to predict an operator miR which may target some reovirus inhibitory proteins in colorectal cancer cell lines. We think that by targeting these inhibitory genes by miRs, we can improve the virus multiplicity as much as possible and use this optimization process for early manipulating of tumor response in near future for the improvement of therapeutic cancer vaccines.

2. Materials and Methods

2.1. Investigation of Cellular Target Genes which Inhibit Oncolytic Reovirus Replication. In order to find inhibitory genes which interfere with reovirus replication, we referred to different articles. It was observed that various proteins can block or alter the expression of genes involved in reovirus replication. After that, by checking different signaling pathways for each cell protein and their functions some imperative genes were selected.

2.2. miR Prediction Using Bioinformatics Tools. To predict miRs which could target the selected genes, the TargetScan database version 7.2 (https://www.targetscan.org/) was examined. Prediction of this bioinformatics tool is based on complementary sites in gene sequences which bind to the seed region of miRs and gives each miR a P_{CT} (Probability of Conserved Targeting) or Context ++ score. By choosing humans as species and entering every gene name, probable miRs sequences which can target the gene were displayed.

2.3. The microRNAs Target Sites Frequency Comparison. In the extracted miR populations, the number of replicates of each miR were counted separately. These numbers showed how many times a specific miR may target the genes and alters their expression.

2.4. Gene Expression Pattern in CRC Model Cell Lines. In this study, we decided to evaluate the enhancement of the oncolytic reovirus effect on the CRC cells model by applying suitable miRs, so we referred to the human protein atlas database version 19.0 (http://www.proteinatlas.org/humanproteom/cell) and checked the selected genes expression profile. The function of this web-based tool is drawing human proteins in different body organs by use of omics technology and deep sequencing. So, for every gene, a panel of different cells with the amount of the gene expression was shown.

2.5. Checking miR's Performance. To avoid any unwanted side effects of miRs, we referred to two web-based tools, miRpath version 3.0 and miRNApath version 3.15. This step was performed to investigate which signaling pathways would be affected by the combinatory *effect* of microRNAs in *pathways* if a specific miR was selected and what would be the final consequence.

2.6. GEO Analysis. By referring to the NCBI site and GEO (Gene Expression Omnibus) database, the quantity of miRs expression in desired cell lines was examined. In this web-based tool, the genes were compared based on different performed arrays. It has two main parts, GEO profiles which contain processed data and GEO datasets which belong to raw or semiprocessed data. By referring to GEO datasets, data were analyzed according to the needs of the study.

3. Results

3.1. Inhibitory Genes in Reovirus Replication Cycle. The aim of this study was the identification of inhibitory proteins which interfere with the reovirus infectious life cycle. Therefore, 40 proteins were initially selected. Then we checked KEGG software and based on their degree of hub, we excluded some proteins. Because this software introduces proteins that are more important in signaling processes and so those that were less important in inhibiting the virus, were excluded from the study. Finally, 15 proteins which seemed to play a more important role, were nominated and selected for further study. These proteins were PKR, STAT1, IFITMs (1, 2, and 3), MAVS, IRF3, RIG-I, Viperin, TLR3, MDA5, OAS3, and ISG15, 54, and 56 (Table 1).

3.2. TargetScan Predicted Hundreds of Targeting miRs. As mentioned before, this bioinformatics tool gives a context ++ score to each gene. So, miRs with context scores over

Table 1: The probable immune system proteins which interfere with reovirus replication.

Inhibitory pathways	Protein content	Effects
JAK-STAT	STAT, IRF9, BCL2, P21, mTOR, AKT, PI3K, Ras, Raf, GRB, VIPERIN, and Tyk [5, 6, 9, 17, 19, 21]	Antiviral effects, development of immune system, hematopoiesis, activation or suppression of genes transcription, and regulation of apoptosis following Reovirus infection
IFNI	RIGI, Mda5, IPS-1, TBK1, IKKe, IRF3, IRF7, TRIF, NAP1, FADD, PKR, RIP, TRAF6, IRAK, TAK1, P50, P65, NF-κB, ISRE, TLR3, ISG15, IFITM, and MAVS [6, 9, 17–19, 22]	The first line of antiviral defense, activator of JAK-STAT pathway, and early mediators of innate immune response
Intrinsic apoptosis pathway	Noxa, bid, ISG54, and ISG56 [5, 9, 17]	Antireoviral effect, a regulator for physiological growth control, regulation of tissue homeostasis, and regulation of tumor formation
RNase L	OAS3 [22]	An immune response to RNA viruses and activator of IFN signaling pathway

70 were selected contractually. This step was performed for every gene separately and finally, a document with about 5000 miRs was obtained for further evaluation.

3.3. Some miRs Could Target Genes 18 Times. As mentioned above, miRs with contexts score over 70 were selected. Then, by searching every miRs separately, the number of their repeats was counted. These numbers showed that different miRs could target different genes between 1-18 times, hence the miRs with 10-18 times repeats were selected contractually.

3.4. Gene Expression Pattern in CRC Cell Lines. The human protein atlas site, showed a page containing the full name of the gene, its function, cell dispersion pattern, etc. for each gene. Then, according to the main goal of this study, we found that some genes were not expressed in the CRC cell lines. Therefore, the study was continued with RIGI, IRF3, PKR, MAVS, Mda5, STAT1, OAS3, ISG15, and IFITM3 genes.

3.5. Checking miR's Performance. To prevent any unwanted function after the miRs expression, miRNApath and miRpath databases were checked. So, no undesirable interaction was seen and it means if we overexpress the selected microRNAs, they do not interfere with other cellular functions. It is important because we do not want to make any other changes in the normal cellular processes.

3.6. GEO Analysis. In this database by defining two groups as control (normal cells) and test (CRC cell lines), the expression of nominated miRs was compared. For this purpose, all P values were checked and values that had no significant differences between test and control groups were accepted. This final step revealed that there are several miRs that meet our desired condition, so we checked their context ++ score in TargetScan and miRs that had a better overall score were selected and result of this selection were miR-140 and miR-92a (Table 2).

4. Discussion

Oncolytic viruses are considered an emerging therapeutic agent that has relatively effective anticancer effect. OVs selectively infect and destroy the different cancer cell models in vitro and in vivo. The major complication using of OVs is their limited potential for production of high titer virus with infinite effect and their delivery to tumor microenvironments [6, 23, 24]. Mesenchymal stem cells (MSCs) are used as carriers for OVs and can act as biological factories for viral genome replication and increase the final viral titer [6].

Bioinformatics is used as a tool for the evaluation of the structure and functions of genes and proteins. Although, it is a broad scientific research field that is moving forward using a variety of powerful tools and its goal is to analyze genes and their transcripts [25, 26]. It has enabled the collection and analysis of the genome, transcriptome, and proteome of different organisms for predictions of their effect on the regulation of gene expression. Using bioinformatics for miR prediction is the basis of many molecular studies. Actually, it provides a complete and in-depth view of the content [27]. Based on this description and according to previous similar studies, we decided to design a study in which by using web-based tools, introduce a miR which is able to target reovirus replication inhibitory proteins and so helps the virus replication.

Previous studies show that miRs, by regulating mRNA expression, commonly in a negative way, decrease the protein expression [28]. This bioinformatics study revealed that miR-140 could bind to 3′UTR of EIF2AK2, MAVS and STAT1 genes and then hinders their performance. Moreover, miR-92a has the potential to target EIF2AK2, MAVS, IRF3, and RIG-I.

miR-140, which is produced by the miR-140 gene, is expressed in chondrocytes and is involved in cell differentiation [29]. It was predicted by bioinformatics tools, that miR-140 can target three important inhibitory genes which are part of the innate immune system and so, it may help the reovirus replication cycle as its oncolytic activity. PKR, MAVS, and STAT1 proteins as said before are three reovirus

Table 2: Two microRNAs expressed in colorectal cancer cell line compared with control colorectal cell according to data from the GEO database.

No.	ID	miRNA-ID	logFC	*P* Value	Adj. *P* Value
1	MIMAT0004508	Hsa-miR-92a-2	-1.001	0.02703	0.961
2	MIMAT0004597	Hsa-miR-140-3p	0.628	0.08317	0.961
3	20500765	Hsa-miR-125a	0.757	0.013698	0.451
4	20519435	Hsa-miR-4652	1.05	0.017581	0.473
5	1274	Hsa-miR-4763	0.627	0.6767	0.979
6	1386	Hsa-miR-509-3	0.708	0.070621	0.979
7	1281	Hsa-miR-4768	-0.466	0.094282	0.979
8	236	Hsa-miR-16	-0.637	0.103716	0.979
9	251	Hsa-miR-183	-0.338	0.132184	0.979
10	Hsa-miR-7-1	Hsa-miR-7-1	2.1851	2.82E-08	1.27E-06
11	Hsa-miR-582	Hsa-miR-582	2.9043	2.15E-04	4.07E-03
12	Hsa-miR-222	Hsa-miR-222	2.2535	2.04E-02	1.84E-01
13	Hsa-miR-153	Hsa-miR-153	0.6972	5.55E-02	3.74E-01
14	MIMAT0019798	Hsa-miR-4701	-0.824	0.01507	0.961
15	MIMAT0019781	Hsa-miR-4691	1.169	0.01826	0.961
16	MIMAT0019899	Hsa-miR-4756	-0.443	0.18841	0.961
17	MIMAT0019912	Hsa-miR-4763	0.693	0.19499.	0.961
18	MIMAT0002823	Hsa-miR-512	0.441	0.26914	0.961
19	MIMAT0005872	Hsa-miR-1207	-0.562	0.25695	0.961
20	MIMAT0004568	Hsa-miR-221	-0.644	0.29228	0.961
21	BM10792	Hsa-miR-495	1.048	1.70E-01	4.06E-01
22	BM10629	Hsa-miR-19b	-0.929	2.27E-01	4.92E-01

replication inhibitors and based on this analysis, we think that miR-140 by downregulating these genes as well as having a negative effect on their downstream proteins, allows the reovirus replication cycle to be done and thereby, tumor cell destruction.

MAVS is an adaptor protein, with a length of 540 aa, is composed of three domains: CARD, a proline-rich domain, and a transmembrane domain. Its main function is triggering innate antiviral immunity responses against RNA viruses [30]. Activation of MAVS protein by affecting NF-κB, IRF1, and IRF3, leads to an increase in inflammatory cytokines [31]. MAVS also, in response to reovirus infection, inducing rapid apoptosis may block the virus spread in somatic cells [32]. In this study, it is expected that by interfering with MAVS function, apoptosis is inhibited and viruses spread easily (Figure 1).

PKR as a serine/threonine kinase is a 551 aa length protein and is coded by the EIF2AK2 gene. It has an important role in significant cellular interactions such as mRNA translation, transcription control, and apoptosis regulation. One of the activators of PKR is dsRNA which induces an immune response [33]. PKR, as a PRR (Pattern Recognition Receptor), is a part of the innate immunity that triggers the production of cytokines and other defense factors. PKR, as a translation regulator, has an undeniable role in modulating reovirus replication; therefore reducing its oncolytic ability [17] (Figure 2).

STAT1 is a transcription factor which has an important role in immune response and triggers different cell processes like cell death. It is a transcription mediator of IFNs. STAT1 is activated by JAKs and is an important inhibitory protein in the reovirus replication cycle [21, 34, 35].

There have been reports about immunosuppression by targeting genes by various miRs. Zhang et al., for example, used bioinformatics tools to show that miR-1343-5p intensifies feline panleukopenia virus (FPV) replication by targeting the IFN-I signaling pathway [36] or Lagos et al. claimed that miR-132 regulates innate immunity in Kaposi's sarcoma-associated herpesvirus (KSHV) infection by inhibiting p300 expression [37]. So far, various studies have been conducted on targeting immune components by miR-140. For example, Li et al. showed that miR-140 inhibits the IL6 and IL8 secretion by targeting TLR4 and blocking proliferation [38]. Or Fang et al., in a study, claimed that miR-140 prevents proliferation, migration, and invasion of tumor cells by targeting YES1 (YES Proto-oncogene 1) [39]. Like these studies, we also expect that miR-140 inhibitory function improves reovirus replication in colorectal cancer cells. Especially since miR-140 itself is a tumor suppressor in CRC, bile duct cancer, etc. Actually, it targets Smad2 and results in lower cell invasion and also increases cell cycle arrest [39, 40]. So, we hope that these two features of miR-140 make it a good candidate for cancer treatment.

This bioinformatics study also showed that miR-92a, in addition to EIF2AK2 and MAVS, can also target RIG-I and IRF3. miR-92a is a member of the miR-92a family that are highly conserved microRNAs and are involved in organogenesis and also blood vessel formation [41].

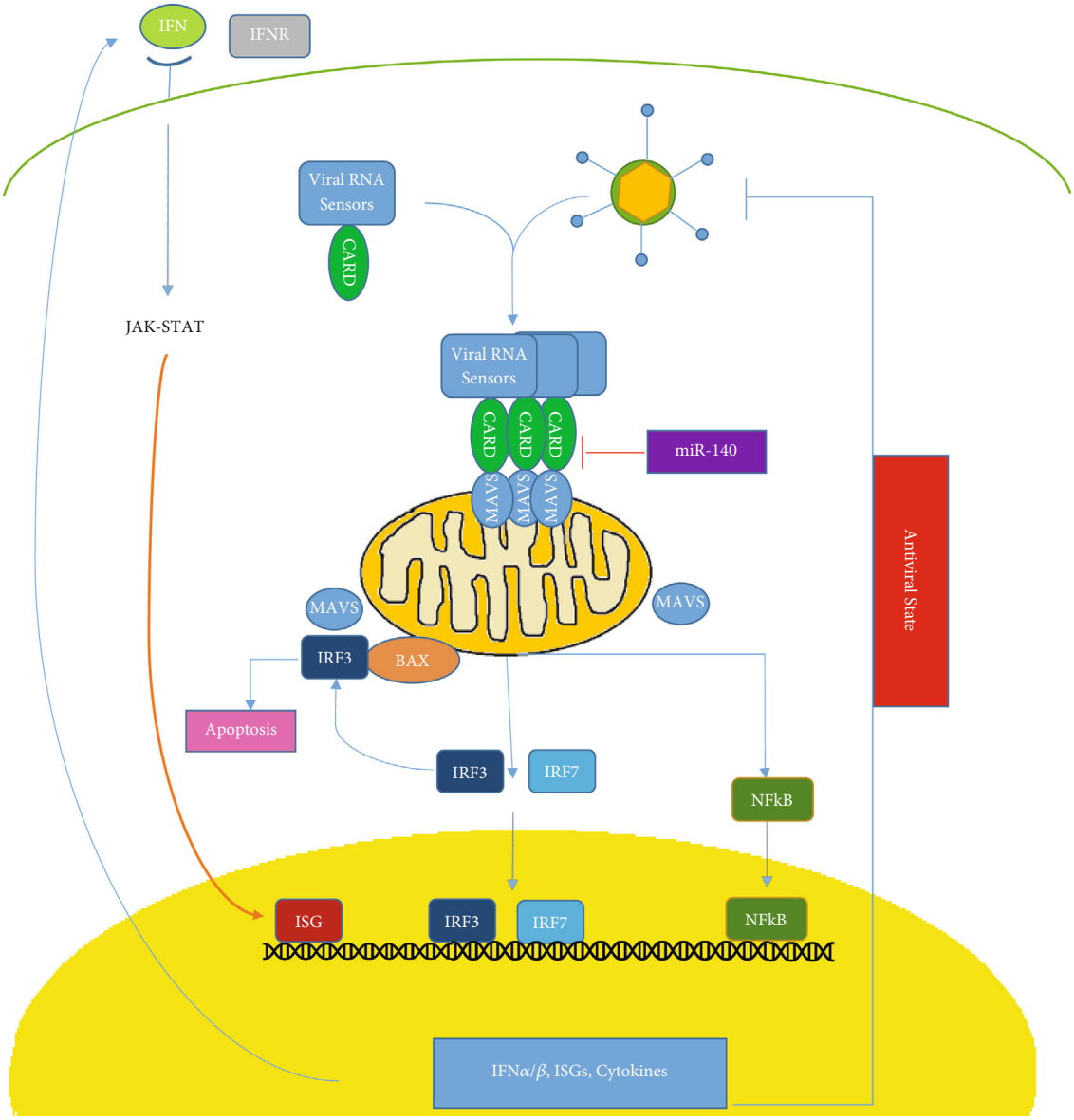

Figure 1: Overview of MAVS signaling pathway as a part of innate immune system defense. After detection of Reovirus by viral RNA sensors, they attach to MAVS protein by CARD domain and activate it. So, MAVS activation leads to secretion of different cytokines which induces the immune system and results in antiviral state. Moreover, it triggers JAK-STAT signaling pathway which intensifies virus elimination by production of different ISGs. MAVS blocking by miR-140 inhibits all these processes.

RIGI is encoded by the DDX58 gene. It is an important receptor for RNA viruses and detects both ssRNA and dsRNA viruses. It has RNA helicase activity and so is able to recognize RNAs containing 5′-triphosphate or 5′-diphosphate. Moreover, it is a necessary factor for triggering the transcription of IFN genes. It contains two CARD domains in N-terminal, a helicase domain and a C-terminal domain [42, 43].

IRF3, as an important regulator of transcription in antiviral immune responses, induces IFN-I production and after activation by MAVS, it results in the induction of many antiviral genes called interferon-stimulated genes (ISGs). It is the first transcription activator of IFN-β and IFN-α and for induction of IFN production, it must be translocated in the nucleus and binds to promoter and enhancer regions of IFN-I genes [44–46].

In a study, Zhang et al. claimed that targeting IRF3 and TAK1-mediated NF-κB signaling pathways with miR-217, can modulate the antiviral response [47]. Moreover, Sheng et al. in a recent study claimed that overexpression of miR-92a facilitates vesicular stomatitis virus (VSV) reproduction in macrophages because it targets RIGI directly and as a result, weakens immune system response [42]. Although we anticipated that miR-92a helps the virus reproduction, some studies showed that this microRNA is not beneficial for colorectal cancer treatment, because it is an early biomarker for CRC diagnosis and it is related to metastasis

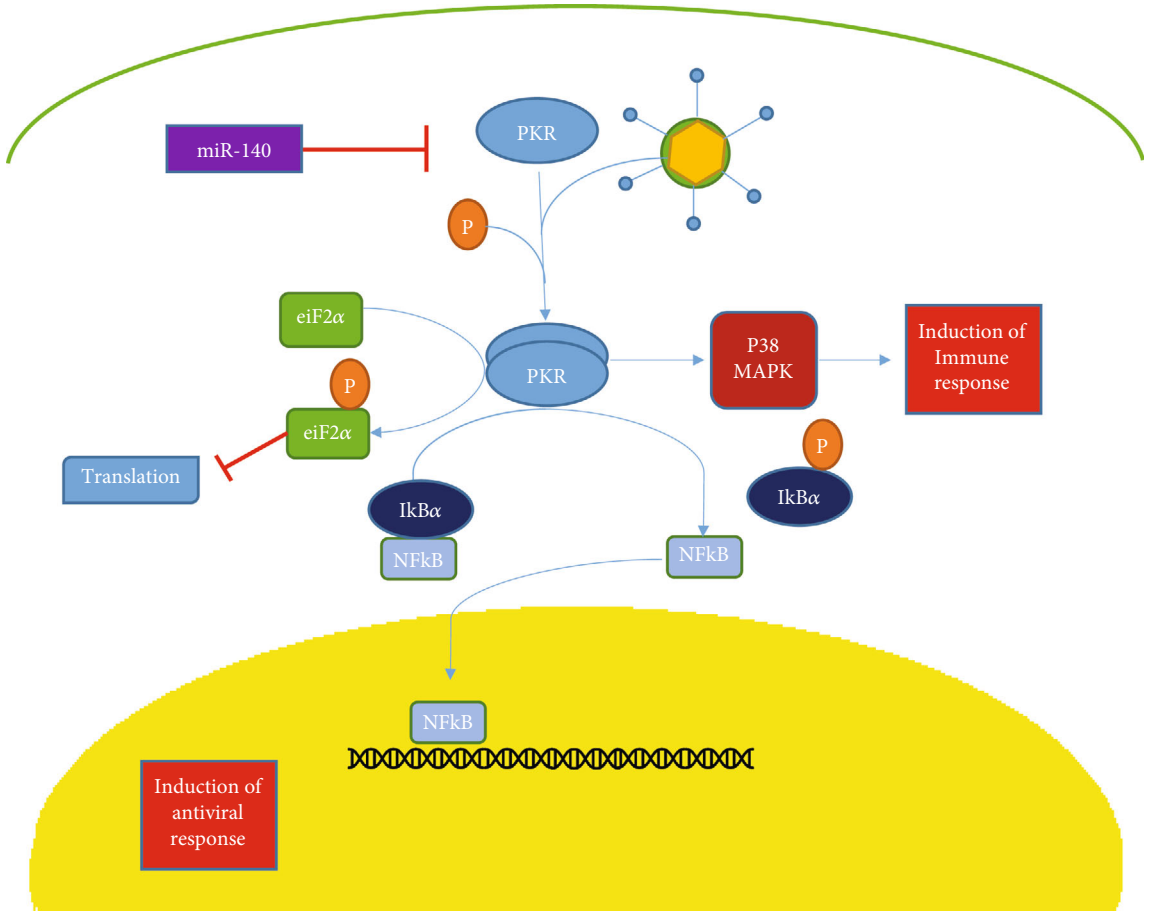

Figure 2: Overview of PKR signaling pathway. PKR is a member of innate immune system which in human is encoded by EIF2AK2 gene. After reovirus infection, PKR is phosphorylated and results in phosphorylation of eiF2α protein. eiF2α is a translation initiation factor and its inhibition leads to further cellular mRNA translation. PKR also results in nuclear placement of NF-κB which is a transcription factor and sets up an antiviral state in cell. miR-140 blocks PKR and inhibits its signaling pathway by degrading it.

and poor survival [48]. Moreover, if we target IRF3 with miR-92a, we actually helped cancer development, because IRF3 prevents tumorigenesis in CRC by inhibiting the β-actin transfer to the nucleus [49]. So, the use of miR-92a for colorectal cancer is a double-edged sword. Although it has a beneficial effect on some immune inhibitory genes and could be helpful for oncolytic reovirus, it intensifies the colorectal disease state which is not acceptable at all. So, the use of this miR in cancer treatment needs more studies and the final decision should be based on a thorough examination of its advantages and disadvantages.

5. Conclusion

Since there are many studies in the field of using oncolytic viruses in cancer treatment, therefore all efforts are made to optimize their use. As we mentioned before, reovirus as a mild or self-limiting infection has the potential to be used in cancer therapy, but because of inefficient reproduction, we decided to study one of the inhibitory factors which seem to play a key role in the virus lifecycle and eliminating that, make an assumption on virus improvement. In the first step, we used bioinformatics as a tool to identify and interpret data sets and data mining for investigation and selection of accurate biomarkers for targeted cancer therapy. We think that miR-140 and miR-92a are two important innate immunity regulators which have positive effect on reovirus replication for induction of apoptosis. But to prevent any unwanted side effects, it needs more studies, because miR-92a is an adjunct for virus replication and its use should be done with more caution.

Abbreviations

CRC:	Colorectal cancer
OVs:	Oncolytic viruses
miRNA or miR:	microRNA
PKR:	Protein kinase R
eIF2α:	Eukaryotic initiation factor 2α
PI3K:	Phosphatidylinositol 3-kinase
AKT:	Protein kinase B
IFN:	Interferon
ISG:	Interferon-stimulated gene
ISRE:	Interferon-stimulated response elements

P_{CT}: Probability of conserved targeting
NCBI: National center for biotechnology information
GEO: Gene expression omnibus
STAT1: Signal transducer and activator of transcription
IFITMs: Interferon-induced transmembrane proteins
MAVS: Mitochondrial antiviral-signaling protein
IRF3: Interferon regulatory factor 3
RIG-I: Retinoic acid-inducible gene
Viperin: Virus inhibitory protein endoplasmic reticulum–associated interferon-inducible
TLR3: Toll-like receptor 3
MDA5: Melanoma differentiation-associated protein 5
OAS3: 2′-5′-Oligoadenylate synthetase 3
JAK: Janus kinase
Bcl2: B-cell lymphoma 2
mTOR: Mammalian target of rapamycin
Ras: Rat sarcoma
Raf: Rapidly accelerated fibrosarcoma
GRB: Growth factor receptor-bound protein
TYK: Tyrosine-protein kinase
IPS-1: Interferon-β promoter stimulator
TBK1: TANK-binding kinase 1
IKKe: Inhibitor of kappa B kinase epsilon
TRIF: TIR-domain-containing adapter-inducing interferon-β
NAP1: Nucleosome assembly protein 1
FADD: Fas-associated death domain
RIP: Ribosome inactivating protein
TRAF: Tumor necrosis factor receptor-associated factor
IRAK: Interleukin-1 receptor-associated kinase
TAK1: Transforming growth factor β-activated kinase 1
NF-κB1: Nuclear factor κB subunit 1
NOXA: Phorbol-12-myristate-13-acetate-induced protein 1
BID: BH3interacting-domain death agonist
MSCs: Mesenchymal stem cells
EIF2AK2: Eukaryotic translation initiation factor 2-alpha kinase-2
CARD: Caspase activation and recruitment domains
PRR: Pattern recognition receptor
FPV: Feline parvovirus
KSHV: Kaposi's sarcoma-associated herpesvirus
IL: Interleukin
YES1: YES proto-oncogene 1
SMAD: Small mothers against decapentaplegic
VSV: Vesicular stomatitis virus.

Data Availability

The data used to support the findings of this study are included within the article.

Additional Points

Clinical Relevance. The role of oncolytic virus therapy and signaling pathways in anticancer combination therapy has potential clinical relevance. Signaling pathway proteins such as the mitochondrial antiviral signaling protein (MAVS), cause triggering of apoptosis and it has been considered a prominent anticancer strategy. We applied using this strategy in combination with oncolytic viruses. Based on the ineffectiveness of reovirus oncolysis, we followed the virus replication inhibitors by considering these considerations: MAVS is a proinducer protein of IFN by phosphorylation of Interferon regulatory factor 3 and resulting in the production of cytokines such as type 1 interferon that causes ineffective reproduction cycle of virus for oncolysis. Bioinformatics evidence strongly supports this target's feasibility for the application of these molecules in clinical trials in future for the treatment of human tumors.

Conflicts of Interest

The authors have declared no conflict of interest.

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