Oncolytic Virus Affects the RAS Pathway in Cancer: RNA Sequence Analysis

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Abstract

Background: Approximately 45% of individuals diagnosed with Colorectal Cancer (CRC) also possess KRAS mutations. One developing therapeutic method for this disease is reovirus treatment. It is theorized that reovirus treatment on patients with KRAS mutated CRC cells would be successful due to the virus' innate oncolytic properties [1]. Reovirus, a stable form of nonenveloped double-stranded RNA, causes minor infections in humans under normal circumstances. However, when the virus encounters KRAS mutated cells, it has the potential to lyse them [2]. While this method of treatment to CRC has shown signs of success, we are still some ways from universal administration of reovirus as a treatment. This review seeks to utilize various studies, as well as our original research data, to investigate reovirus as an efficient method of treatment, with a focus on select growth, apoptotic and RAS-related genes, and their effectiveness of mitigating KRAS mutated CRC post reovirus treatment. Furthermore, the review highlights transcriptome analysis as an effective tool to examine these genes and their activity. It has been shown that reovirus treatment induces apoptosis and mitigates growth related gene activity.

Conclusions: This review confirms the novelty of our findings on the efficacy of reovirus in CRC treatment. The study that this review article discusses concluded that 10 apoptotic and lymphocyte-related genes were found to be upregulated and 6 angiogenesis and Ras-related genes were found to be downregulated post reovirus treatment. These findings enforce the notion that reovirus could be used as a novel treatment for KRAS mutated CRC.

Keywords: Oncolytic Virus; Transcriptome; Reovirus; CRC; KRAS

Introduction

Viruses are double edged obligate parasites; while some cause cancer, such as HPV and EBV, ongoing clinical trials have shown oncolytic viruses, including reovirus, to be immensely beneficial in fighting cancer [3]. Oncolytic viruses have the ability to replicate, kill cancer cells, and spread within a given tumor, all the while not harming normal tissue [4]. In addition to direct oncolytic activity, oncolytic viruses can also effectively induce select immune responses to themselves and to the infected tumor cells. Oncolytic viruses encompass a broad diversity of DNA and RNA viruses that are naturally cancer selective or that can be genetically engineered [4].

Reovirus is a double-stranded RNA virus that has shown preferential replication in cancer cells harboring oncogenic mutations [3]. In the pre-clinical setting, reovirus administration has displayed anticancer activity across many malignancies. Given the virus' potential to reproduce well in cancer cells, interest in therapeutic reovirus treatment has been proposed since the 1970's [2]. Reovirus is thus perceived as an attractive agent for combination therapy for cancer due to its relative non-pathogenicity [3]. Reovirus has been shown to replicate remarkably well in cells that have a mutated KRAS gene. After infection within KRAS-mutated tumor cells, viral replication occurs resulting in cell death, which

leads to viral progeny infecting nearby tumor cells. Since upstream activation of the KRAS proteins may play a role in more than two thirds of human metastatic cancers, reovirus shows unique potential as an alternative treatment method for metastatic colorectal cancer [1]. Because reovirus induces several biological reactions, transcriptome analysis has been shown to be the best tool to get a comprehensive overview of the effects of reovirus administration. Other studies have accumulated important information regarding alterations in gene expression due to host virus interaction. To illustrate, it has been shown that Influenza virus produces a differential effect of Interleukin-17A upon infection in human cells [5]. Additionally, it has been shown that EBV infection results in viral lytic genes that are coexpressed with cellular cancer-associated pathways [6]. Previous studies performed by our group, which also utilized transcriptome analyses to analyze reovirus treatment on CRC, have shown that post reovirus treatment, KRAS mutated cells induced autophagic machinery activity [7] and adaptive and innate immune responses, specifically lymphocyte and NK cell activity [8]. These studies reinforced transcriptome analysis as an effective tool for determining the biological responses, including the oncolytic effects, of reovirus treatment.

In the early 2000s RNA research made an incredible breakthrough with the development of next generation RNA-Seq [9]. Researchers are particularly interested in RNA studies because of the dynamic role that these molecules play in biological systems. A comprehensive understanding of their sequences can provide a researcher with a plethora of knowledge of specific biological pathways, including genetic coding/decoding, gene regulation and gene expressions of specific genes. An understanding of the functionality of RNA molecules can allow for manipulation of the expression and regulation of certain genes in beneficial pharmaceutical and treatment methods (our original research data). The two primary methods by which researchers use RNA-Seq to assemble transcriptomes are the De novo and Genome guided methods. In the Genome guided method, the RNA sequences are aligned within a specific, known, genome, like that in the DNA alignment process [10]. This method differs from De novo in that a genome is already provided.

The primary purpose of this review is to affirm the antitumorigenic effects of reovirus on various signaling and growth-factor related genetic pathways. As our published paper has shown, and as this review highlights, reovirus-mediated immune modulation results in antitumor activity that is realized via innate and adaptive immune responses [11]. These responses are influenced by the altered expression of genes within vital biological pathways. The Ras-related biological pathways of miR in Lymphocytes, Angiogenesis, Apoptosis, RAS, MAPK, EGFR, PI3K-Akt are the focus of this review, as they are all involved in growth-related processes that are integral to cancer proliferation.

Discussion

Increased lymphocytic activity results from the expression of CHORDC1 and RTN4 post-reovirus

MicroRNAs (miRNA) within the human biological system represent a family of non-coding RNAs that are integral to post-transcriptional gene regulation and play important roles in lymphocytic development and maturation [12]. miRNAs are crucial in tumorigenesis, modulating target gene expression through translation repression or mRNA degradation [13]. CHORDC1 serves as a key regulator of centrosome duplication. This regulation results from its inhibition of the kinase activity of ROCKII [14]. The downregulation of CHORDC1 has been shown to promote the interaction between ROCKII and NPM to result in an increase in ROCKII kinase activity, which leads to centrosome amplification [14]. CHORDC1 also ensures the localization of the tyrosine kinase receptor EGFR to the plasma membrane, which in turn regulates EGFR activity and EGF-induced actin cytoskeleton remodeling [15]. Additionally, there exists a novel ADP-dependent HSP90 interaction with the CHORDC1 [16]. This interaction requires the presence of a linker region between the CHORD domains in CHORDC1 [16]. CHORDC1, as other HSP90 co-chaperones, suppresses the aggregation of denatured proteins. HSP90 and its co-chaperones mediate protein conformation shifts during the cell cycle [17]. It has been shown that reduction of CHORDC1 leads to defective chromosome condensation and spindle formation, as well as tumorigenesis, as a result of altered HSP90 activity [17]. RTN4 belongs to the family of reticulon encoding genes which are associated with the endoplasmic reticulum. Specifically, RTN4 has been shown to be required to induce the formation and stabilization of endoplasmic reticulum tubules [18]. Depending on their tissue expression specificities, isoforms of this gene regulate membrane morphogenesis in the endoplasmic reticulum by promoting tubular ER production, influence nuclear envelope expansion, induce nuclear pore complex formation, and ensure proper localization of inner nuclear membrane [19,20]. An important isoform of this gene, RTN4B, is required to promote various macrophage-related functions such as the cytokine/chemokine gene expression involved in tissue repair [21]. Additionally, it mediates ICAM1 induced migration of leukocytes and acute inflammation and is necessary for immune responses triggered by nucleic acid sensing TLRs. This gene also reduces the anti-apoptotic activity of Bcl-xl and Bcl-2 [21]. RTN4C, another isoform of this gene, is essential in hepatocellular carcinoma regulation. Specifically, it inhibits cell growth and promotes apoptosis [22]. Our original research data showed that post-reovirus, RTN4 and CHORDC1 expression are significantly upregulated at multiple time points indicating that lymphocytic activity is increased, while tumorigenesis is effectively impeded [11].

Post-reovirus, the expression of VEGFB and FAM96A results in a reduction of angiogenesis

The angiogenesis pathway represents a family of genes that control blood vessel growth from the body's existing vasculature [23]. Tumor angiogenesis is an essential factor in the basis for tumor growth, progression, and metastasis [23]. A key angiogenesis related gene, VEGFB, is a member of the VEGF family (a subfamily of growth factors) which stimulates the growth of blood vessels. VEGF genes promote the growth of VEGF proteins, which play significant roles in vascular genesis and angiogenesis [24]. VEGF is produced by many cell types including tumor cells, macrophages, platelets, keratinocytes, and renal mesangial cells. However, the activities of VEGF also regulate normal physiological functions such as bone formation, hematopoiesis, wound healing, and development [25]. With levels of high expression, VEGFB acts as a potent survival factor for different types of cells by inhibiting apoptosis via suppressing the expression of BH3-only protein and other apoptotic genes [26]. VEGFB produced by tumor cells remodels tumor microvasculature to form leaky vascular networks that allow for tumor cell invasion [27]. FAM96A, which plays a role in chromosome segregation through the establishment of sister chromatid cohesion, is another gene that makes up the angiogenesis pathway. FAM96A is an evolutionarily highly conserved protein, suggesting that it performs important functions in cell proliferation and angiogenesis [28]. FAM96A is a component of the cytosolic iron-sulfur protein assembly (CIA) complex. In collaboration with CIAO1, FAM96A matures ACO1 and stabilizes IREB2, thus connecting cytosolic iron-sulfur protein maturation with cellular iron regulation [29]. It has been shown that FAM96A increases cytotoxic T lymphocyte responses, interferon-γ release, and T lymphocyte infiltration [30]. Studies have shown that FAM96A is a novel pro-apoptotic tumor suppressor that is lost during tumorigenesis. FAM96A has also been found to enhance the induction of apoptosis by binding to the apoptotic peptidase activating factor APAF1 [31]. The oligomerization of the APAF1 protein at the onset of apoptosis is triggered by the binding of cytosolic cytochrome c, which prevents the autoinhibition of the APAF1 gene [31]. As indicated by our original research data, reovirus administration effectively promotes the expression of FAM96A and decrease the expression of VEGFB, thus inhibiting cell proliferation and angiogenesis [11].

Reovirus-induced apoptosis is enacted through the actions of CASP8 and CASP9

Apoptosis is an important aspect of several routine cellular processes such as normal cell turnover and proper cellular development [32]. This programmed cell death mechanism intimately regulates the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death.

The inappropriate execution and regulation of apoptosis has severe implications, resulting in neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer [32]. As such, the modulation of apoptosis is well-researched and has immense therapeutic potential. The CASP8 gene encodes cysteine-aspartic acid protease (caspase) family and plays a significantly important role in apoptosis. This protein is involved in the apoptotic pathway by Fas and various apoptotic stimuli when it binds to the adapter molecule FADD [33]. In order to mediate this response, CASP8 cleaves and activates effector caspases CASP3, CASP4, CASP6, CASP7, CASP9 and CASP10 [34]. It has been shown that in addition to regulating apoptosis, CASP8 stimulates cell proliferation, malignant transformation and tumor progression when it is dysfunctional or has significant low gene expression [35]. The kinase activity of RIPK1 limits the TNF-induced apoptosis, necroptosis and inflammatory response. By cleaving RIPK1 at 'Asp-324', CASP8 acts as a negative regulator of necroptosis by inhibiting RIPK1 kinase activity. As such, CASP8 promotes extrinsic apoptosis over necroptosis [36]. In addition, CASP8 initiates pyroptosis by mediating the cleavage and activation of GSDMD. GSDMD cleavage triggers pyroptosis by promoting the release of the N-terminal moiety that binds to membranes and forms pores [37]. In a similar fashion, CASP9 is involved in the activation cascade of caspases responsible for apoptosis execution. Specifically, CASP9 binds to pro-apoptotic genes such as APAF11 to activate the protease which cleaves and activates CASP3 [38]. In response to DNA damage, CASP9 induces apoptosis in an ABL1-dependent manner by proteolytically cleaving Poly-ADP-ribose Polymerase [39]. TNFα-promoted apoptosis of cancer cells can also be induced through CASP9 upregulation through NF-κB and its target miR-1276 [40]. Our original research data showcased the fact that reovirus treatment was able to significantly increase apoptotic activity in KRAS mutated CRC through the heightened expression of CASP8 and CASP9 [11].

Altered expression of signaling genes NOS3 and ANGPT1 post reovirus inhibits cancer progression

The RAS Signaling pathway controls a plethora of genes that regulate cell growth in all eukaryotic cells [41]. This pathway has been identified as the central modulator in signal transduction pathways that respond to diverse extracellular stimuli, including peptide growth factors, cytokines, and hormones. The GDP/GTP cycle functions as a regulator to the biological activity of the RAS signaling pathway [41]. The MAPK, PI3K-Akt, and EGFR pathways are the main cellular pathways in which the RAS pathway operates. These various pathways are vital in normal cells in controlling several functions, such as cell growth and survival [42]. Within these three pathways, dimerization and autophosphorylation result from the binding of various growth factors on receptor tyrosine kinases. This process eventually produces signal cascades that allow cellular growth and evasion of

apoptosis [42]. NOS3 encodes for a nitric oxide synthetase protein that synthesizes nitric oxide from L-arginine. Nitric oxide is a free radical that serves as a mediator in several biologic processes, such as neurotransmission [43]. Production of nitric oxide due to NOS3 activity is regulated using effector proteins such as Ca^{2+} calmodulin and through posttranslational modifications such as phosphorylation via protein kinase B [43]. Specifically, the nitric oxide produced by NOS3 controls vascular smooth muscle relaxation through a cGMP-mediated signal transduction pathway. Nitric oxide also mediates VEGF-induced angiogenesis in coronary vessels and promotes blood clotting through the activation of platelets [44]. It has been shown that tumor-derived NOS3 promotes tumor growth and metastasis by stimulating tumor cell migration, invasiveness and angiogenesis [45]. NOS3 has been implicated as an essential mediator in many signaling pathways in CRC, such as the Wnt/β-catenin and extracellular-signal-regulated kinase (ERK) pathways, which are closely associated with cancer initiation, metastasis, and inflammation. In CRC, endogenous levels of NO promote colon neoplasms and lead to cytotoxic functions [46]. ANGPT1 plays an important role in the regulation of angiogenesis, endothelial cell survival, proliferation, migration, adhesion and cell spreading, reorganization of the actin cytoskeleton, and maintenance of vascular quiescence [47]. ANGPT1 oligomers recruit TEK, which leads to preferential activation of phosphatidylinositol 3-kinase and the AKT1 signaling cascades. ANGT1 activates PTK2/FAK and downstream kinases MAPK1/ERK2 and MAPK3/ERK1, and ultimately stimulates angiogenesis [48]. Increased expression of ANGPT1 has been shown to promote cancer progression by by promoting the intraperitoneal growth of cancer cells [49]. In metastatic CRC, this gene is generally upregulated, pointing the cellular proliferation of cancer cells. Our original research data showed that ANGPT1 and NOS3 expression were successfully inhibited post reovirus treatment, lending more evidence to the reduction of cell signaling activity and cancer progression [11].

Taken altogether, our transcriptome assay has shown that oncolytic virus treatments, including reovirus treatment, can cause meaningful alterations in gene expression, producing responses which have been proven, based on the referenced studies in this review, to lead to the regression of tumor mediators and the promotion of antitumor immune responses, as shown in Figure 1 and Table 1. Meaningful alteration of these genes post-oncolytic reovirus treatment can lead to favorable antitumor responses in CRC.

| Gene Analyzed	Biological Pathway Correlation	Pathway Reference	Expression Post-reovirus Treatment
RTN4	miR in Lymphocytes	Sklar S, Hanspers, miR-targeted Genes in Lymphocytes (Homo Sapiens). https://www.wikipathways.org/index.php/Pathway:WP2004	Upregulated
CHORDC1	miR in Lymphocytes	Sklar S, Hanspers, miR-targeted Genes in Lymphocytes (Homo Sapiens). https://www.wikipathways.org/index.php/Pathway:WP2004	Upregulated
VEGFB	Angiogenesis	Helix, Willighagen E, et al: Angiogenesis Overview (Homo Sapiens). https://www.wikipathways.org/index.php/Pathway:WP1993	Downregulated
FAM96A	Angiogenesis	Helix, Willighagen E, et al: Angiogenesis Overview (Homo Sapiens). https://www.wikipathways.org/index.php/Pathway:WP1993	Upregulated
CASP8	Apoptosis	Zambon A, Pico A, et al: Apoptosis Overview (Homo Sapiens). https://www.wikipathways.org/index.php/Pathway:WP254	Upregulated
CASP9	Apoptosis	Zambon A, Pico A, et al: Apoptosis Overview (Homo Sapiens). https://www.wikipathways.org/index.php/Pathway:WP254	Upregulated
NOS3	RAS Signaling	Hanspers K, Slenter D, et al: Ras Signaling (Homo Sapiens). https://www.wikipathways.org/index.php/Pathway:WP4223	Downregulated
ANGPT1	RAS Signaling	Hanspers K, Slenter D, et al: Ras Signaling (Homo Sapiens). https://www.wikipathways.org/index.php/Pathway:WP4223	Downregulated
The list of 8 genes that were analyzed and the pathway it significantly alters is shown, along with the respective level of gene expression post-reovirus treatment in KRAS mutated CRC. Though, as indicated by the original paper, these genes possess interactions with several other pathways, their affiliation was designated in this review by virtue of their high significance in the pathways indicated above.			

Table 1: Genes Analyzed.
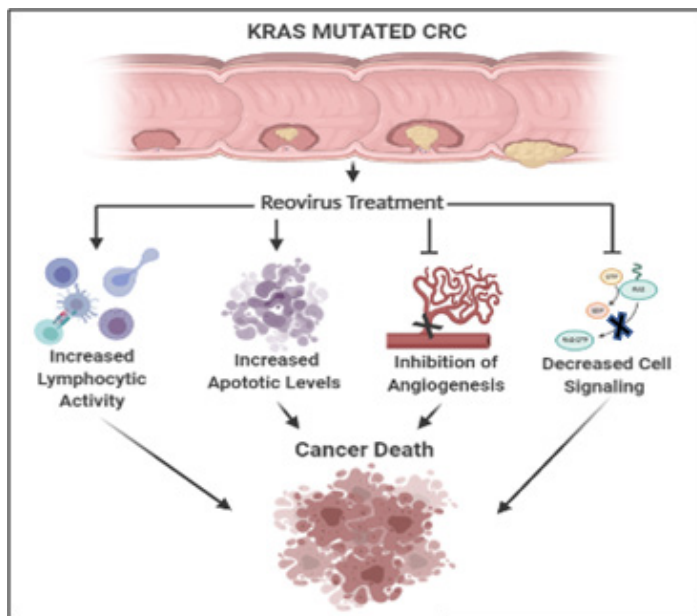
Figure 1: Immune Responses to Reovirus-treated CRC.

The immune responses that result from reovirus treatment in KRAS mutated CRC, including increased lymphocytic maturation and activity, increased levels of apoptosis, and decreased levels of angiogenesis and cell signaling.

Conclusion

As our published data has shown, reovirus promotes antitumor responses in several important biological pathways, including miR in lymphocytes, angiogenesis, apoptosis, and cell signaling [11]. Although we did not focus on other biological pathways, it is important to note that reovirus has been shown to affect the expression of genes in many other crucial pathways that affect cancer. Studies performed by our group using transcriptome analysis, including our original research data, confirms that the expression of CHORDC1, RTN4, VEGFB, FAM96A, CASP8, CASP9, NOS3 and ANGPT1 coincides with their expected outcomes, namely, immune responses such as increased lymphocytic maturation and activity, increased levels of apoptosis, a decrease in angiogenesis, and a reduction in carcinogenic cell signaling [11]. Previous research by our group has, overall, confirmed the tremendous benefits and efficacy of reovirus. First off, our research has confirmed that reovirus improves survival of patients possessing KRAS mutated CRC by replicating well within tumor cells and stimulating the immune system in beneficial ways [50]. Additionally, we have shown that reovirus induces immune stimulation with changes in cytokine profile, including significant changes in GMCSF and other vital genes [8]. Lastly, we have shown that reovirus has the ability to hijack host autophagic machinery in patients possessing KRAS mutant CRC, allowing

for the preferential oncolysis of the cancer cells [51]. All in all, this review confirms the tremendous potential of oncolytic viruses such as reovirus in producing antitumorigenic effects.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: Not applicable

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Authors' contributions:
Conception and design: S.G, R.M; Analysis and interpretation of data: A.S, E.F, R.M; Writing, re-view, and/or revision of the manuscript: A.S, E.F, S.G, R.M; Administrative, technical, or material support: S.G, R.M; Study supervision: S.G, R.M. All authors have read and agreed to the published version of the manuscript.

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