Acta Scientific Gastrointestinal Disorders (ISSN: 2582-1091)

Volume 5 Issue 3 March 2022

Research Article

ATR Mediated Activity of Molecular Editors on Oncological Cells

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Received: December 14, 2021

Published: February 25, 2022

Abstract

CRISPR Cas 9, a biologist editing guide has conquered molecular genetics with its supreme ability to edit the human genome thus eradicating the complexity involved in proteomics and recombinant DNA technology to cure many genetic diseases. In this study, the CRISPR Cas 9 machinery has been applied to demonstrate anti tumoral activity with the aid of molecular sensors working in the DNA replication like ATR that, at times of normal replication allows the stalled replication forks to move further that will eliminate circumstances leading to apoptosis. This biomolecule is used as anti- cancer tool to prevent detrimental effects of malignancies associated with the cancer. During the progression S-phase, most of the times, the cell accurately completes the replication but when cell senses the mutation or any physical damage in its way of replicating DNA, the replication machinery gets halted thus accumulating many ssDNA molecules and this stimulates ATR molecule to bind and pave the way for completion of replication. This review juxtaposed the anticancer effects in mammalian and mouse cells and indicated ATR to be important molecule in diagnosis of cancer.

Keywords: CRISPR Cas 9; Proteomics; Molecular Sensors; ATR; Apoptosis; Malignancies

Introduction

CRISPR Cas 9 has been used to treat many kinds of genetic diseases like cystic fibrosis, severe combined deficiency syndrome, sickle cell anaemia, X- linked chromosomal disorders etc. The main reason behind the supremacy of this bio- weapon is to eradicate the need for any such enzymes or endonucleases that act at a particular site and thus curing the disease by just correcting or editing the human genome with significant amount of accuracy [1]. This palindromically enriched system was first deciphered in model organism *Escherichia coli* and then in around 20 varying prokaryotic organisms. But the functional utility was decoded only when the CRISPR gene was transfected with bacteriophages containing a foreign gene where it demonstrated its real significance. Clustered Regularly Interspaced Short Palindromic Repeats sequence extracted from Streptococcus pyogenes when undergoes transfection was suppressed by the glycoprotein enriched peptide sequences G8Ps inherited in the bacteriophage genome and were rendered as only anti-CRISPR mediators [2]. These CRISPR molecules exhibiting excellent molecular memory when come in contact with any foreign gene reminisces the genomic framework of that DNA. This versatile molecule can disrupt any inheritable disorders or infection with specific action because when this modified biomolecule undergoes the process of transcription thus yielding a short RNA sequence that along with spacer sequence also contain a region that is homologous to the gene sequence previously found upon exposure with CRISPR molecule that will instantly interact upon contact with diseased site [3]. This site-killing feature of CRISPR Cas 9 can also be used as tool while in case of killing tumours that develop several malignancies. Based on the Cas 9, many attempts were made by the researchers to classify the CRISPR molecule but the most popularly accepted was the one in which the Cas 9 endonuclease was utilized by CRISPR molecule. Notably, anti- CRISPR

RNA does not functional individually to drive Cas9 but with the aid of surrounding RNA molecules like trans RNA. This complex along with guide RNA naturally incorporated with the CRISPR genome can be used against several diseases like cancer by just manipulating the guide RNA. ATR molecule with kinase activity intiates a cascade of events is stimulated only when the replication forks are halted at times of replication. This activity of ATR is utilized as a tool to study oncogenes. In a study, oncogenes such as MYC and CYCE were taken for expression without the presence of any agents that can eliminate the tumour cells and ATR activity was observed in such mouse cells (Murga., *et al.* 2011; Schoppy., *et al.* 2012). In this case, we have cultured mouse embryo cells that has Dox- associated Cas 9 gene and tetracycline – associated Cas 9 gene that will enhance working efficiency of CRISPR gene containing libraries. The Cas 9 with small guide RNA sequence releases CDC25A that in case of cancer cells conjugately works with ATR molecule while in the normal cells, upon secretion inhibits ATR [4]. This phosphatase will certainly drive the ATR in correct orientation when suppressing replication in cancer cells.

Biological interaction between ATR and cells

ATR which is known as ataxia telangiectasia-mutated and Rad 3- associated protein kinase belongs to the family of phosphoinositide-3-kinase family of proteins. This ATR is naturally complexed with another protein named ATRIP which is called ATR-recruitment protein. When the cells suffers through unfavourable replication conditions, it leads to accumulation of large amount of single stranded DNA [5]. Then RPA protein on the single stranded DNA present at the replication fork signals ATR- ATRIP complex to bind the fork but it cannot activate the complex. Inactive ATR can be transformed into active ATR with the help of other mediators like Topoisomerase II binding protein 1 (TOPBP1) and Ewing tumour-associated antigen 1 (ETAA1), while TOPBP1 stabilizes ATR-ATRIP complex but ETAA1 gets bound to the RPA protein on ssDNA molecule [6,7]. This TOPB1 cannot bind on its own but with upon signalling induced by 9-1-1 complex which is already present at junction created by interaction of single stranded DNA and part of double stranded DNA present at the replication fork [8]. Now, this ATR activator needs to be loaded on DNA polymerase for activation of ATR molecule. This can be achieved with the aid of Rad 17 associated replication factor C which another molecule that loads ATR associated activator and becomes activated [9]. After the ATR has been activated by phosphorylation, although the mechanism of phosphorylation of ATR in active state is still unclear. It gets conjugated with another biomolecule named CHK that restricts DNA replication in early S- phase that will not allow the formation pre-initiation complex at start of replication that can conserve the energy needed for replicating DNA under unfavourable conditions [10]. This CHK further phosphorylates renders the cell cycle in inactive form by disrupting the activity of CDC25 family of proteins that whose concentration when decreases in cell will lower affinity for binding of ATR molecule [11]. This property can be beneficial in case of cancer cells where replication forks need to be stalled in order to prevent the multiplication of tumorigenic cells. Activity of CDC25 proteins has already been observed *invitro* in stem cells where the increment in proliferation leads molecule to sensitized state. In case of eukaryotic organisms, at times of replication, initiation complex needs numerous mediators like helicase to break to hydrogen bonds and the major component is MCM 2-7 complex. The genomic origin requires the presence of another molecule named CDC45 which is induced by this MCM 2-7 complex [12]. Hence, when the ATR comes initially, it tries to block many such mediators like CDC45 that may act like a backbone to recruit the replication of parental DNA. It can be hypothesized that human cancer cells can prevented from proliferation and differentiation by causing induced mutations in CDC45 molecule that will ultimately the formation of origin which is base for replication phenomenon. A study conducted on mouse cells in blastocyst stage of cell cycle noticed that all the ATR activity fluctuated in response to varying conditions (Brown and Baltimore, 2000). Oncogenic Ras and Myc genes were observed to eliminate the activity of ATR molecule to some extent when they undergo expression rather than normal levels that will ultimately restrict activation of ATR cascade (Gilad., *et al.* 2010; Murga., *et al.* 2011; Schoppy., *et al.* 2012). A study conducted on the colorectal cancer cell lines to determine the action ATRi (ATR inhibition) demonstrated significant amount of replication stress on the oncological cells when the ssDNA was formed in presence of ATRi inhibitor (Kinzler and Vogelstein, 1996).

Materials and Methods

Materials

We have evaluated MCF10A cell line used for culturing of breast cancer cells in Dulbecco Modified Eagle medium/ F12 Ham's media(Sigma Aldrich) with supplements such as 5% equine serum, 20ng/ml epidermal factors and hydrocortisone, 10ug/ml of insulin, 100 ng/mL toxicant extracted from virus causing cholera and antibiotics like penicillin as well as streptomycin at a concentration of 100 units/mL and 100 μg/mL respectively.

Transfection of cells with lentiviral libraries

Lentivirus libraries (Traver Hart's Laboratories) consisting of around 70,948 guide RNA along with 18,053 genes obtained from proteome of organism [13]. After 24 hours of incubation, the lentivirus was subjected to transfection with the MCF10A cells along with packaging vector, enveloped vector as well as Transfecting agent (Roche)at concentration of around 8 ug, 4ug, 60ug respectively. Then, Opti-MEM (Life Technologies) medium was added at a concentration of 2ml in the medium. After a interval of 48-72 hours, post-transfected viral cells were subjected to centrifugation at around 1500 rpm for 5-6minutes.

- The MCF10A cells were transfected with the lentiviral library and then screened for the presence of small guide RNA. The cells were observed for every 24 hours and after analysing morphology, they were transferred to a puromycin- containing media at concentration of 1.5ug/ml.
- After an interval of 3-6 days, the genome of the MCF10A cells were isolated under aseptic condition suspended in a mixture of ethanol and sodium chloride with the aid of (QIAamp Blood Maxi Kit (Qiagen) and subjected to PCR amplification followed separation and visualization by gel electrophoresis.
- For such screens, we chose concentration around 20% that will prevent the growth of the cells that drive guide RNAs in sequence specific manner and will make effective action of drug on the cell line taken into consideration. The effective screening of CRISPR library was analysed with comparison amongst the crucial and non-crucial genes that were further statistically tested with correlation coefficient>0.9 as obtained by Bayesian Analysis of Gene Essentiality algorithm which confirmed the reduction of crucial genes containing guide RNA thus making the cells sensitized to inhibition by ATR.

Identification of cells capable of ATR inhibition

- After the cells with minimal guide RNA has been confirmed, there is a need to determine those cells that act as mediators of inhibition of ATR activity in the tumoral environment. Henceforth, 10 sets of those gene reacting to DNA damage within cell microenvironment are chosen out of which two gene sets are sensitized to inhibit the activity of ATR. Those genes that play keen role in ATR-CHK pathway utilized in this screening indicated that when any one of the genes of this pathway are mutated, this can also lead to the loss of inhibition.
- Many such gene sets were tested for ATRi but we only gained interest in RNASEH2 gene [4]. RNASEH2 sequencing revealed the MCF10A cells when treated with ATRi has low levels of guide RNA expression. Thus, it is hypothesized that RNASEH2 targeted small guide RNA can reduce the ATR inhibition.

Western Blotting reveals the robust nature of small guide RNA associated with RNASEH2

- After the sequential analysis, Western blotting was performed to determine the proteomic interaction RNASEH2C which is targeted by small guide RNA that revealed the reduction of MCF10A cells in which ATR activity is inhibited to some extent. Activity of RNASEH2 as potent inhibitor of ATR activity in tumoral cells *in vivo* is still unclear.
- Xenograft tumour cells obtained from mouse after the proteomic study of RNASEH2 under aseptic condition to determine the activity of potent ATR- associated inhibitor named AZD6738 for a span of 21 days which confirmed the functional importance of this inhibitory molecule and also that the RNASEH2 need to be present in the lower levels in order for the tumour cells to function appropriately.

Figure 1: This picture elucidates the microscopic examination MCF10A cell line visualized under electron microscope [15].

Figure 2: CRISPR Cas 9 inserted into the cells undergoes PCR amplification and identification and characterization by Fluorescence capillary gel electrophoresis and then KO cells are subjected to proteomic analysis [16].

Figure 3: A: Induction of CRISPR Cas 9 into two tier gene system in which in one system guide RNA is placed near Dox gene while in other system, guide RNA is placed near tetracycline operator near Col1a1locus. B: Graphical representation of two gene systems in which Dox containing gene indicated high expression of Cas mRNA. C: When gene system in ES cells were transfected with lentiviral systems expressing proteins like GFP and BFP, the Cas 9 cells that are infected with both GFP and BFP in presence of dox protein were found to be GPF negative. D: Expression of p53 was analysed in presence of tubulin as a control for guide RNA where p53 gene previously transfected with lentivirus expressed guide RNA variantly.

Results and Discussion

In this review, we have observed ATR activity in both normal and cancer cells. Oncogenes were mainly focus in this paper. This prolific technique has made job much easier for biologists to target sequence-specific oncogenes while treating several forms of cancer. The genes need to distinguished into four different areas before being screened with CRISPR Cas 9 technology. Notably, out of four genes, oncogenes are which actively dividing can be obtained from diversified tumour cell lines [14]. In the normal cells, sensitivity to ATR inhibition is needed to stall the replication forks in order for the replication forks to move forward but ATR molecule needs to restricted in case of tumorigenic cells because it will not allow the growth of cancer cells. CRISPR Cas 9 molecule has targeted those mediators successively inhibit the ATR activity in tumour cells at the time of replication [17]. The cancer cells biomarkers like ERCC1 and XRCC1 can be targeted by anti-ATR activity (ATR kinase) driven by CRISPR Cas 9 [18]. It is a proven fact that the mutated agents of ATR-CHK1 pathway is a positive factor in targeting oncogenic molecules by reducing the activity of ATR molecule [19]. The small guide RNA levels of p53 gene which is an important cancer marker if present at the low levels can be selectively killed by the ATR inhibiting molecules. Hence, it is crucial for the cancer cells to have low p53 levels in order to suppress the ATR activity [20]. However, the correlation between ATR and p53 in suppression of cancer cells is still unknown. After the analysis of many gene sets, another unique molecule RNASEH2 when reduces in absence ATR kinase induces DNA damage, apoptosis and senescence. The RNASEH2 is a very interesting molecule with a triad unit out of which only one is functional while the other two are said to be non-functional. The molecule could contribute to limiting the growth of cancer cells because of its nature of functioning opposite of ATR as while the ATR motivates the cell growth but RNASEH2 limits the cell growth by hampering cell cycle stages [21]. CRISPR technique indicated that this unique molecule not only limits the functioning of ATR but also attracts other apoptotic proteins inducing apoptosis. Thus, this molecule act as efficient biomarker for cancer cell identification and characterization and that could even lead to ATRi hypersensitivity. Another biomarker named Cdc25A is found to be over expressed in cancer cells thus can be inhibited by ATR driven inhibitors. It has been observed that a crucial relationship exists between functioning of forks as well as origins at initation site. While the replication forks is found to be slowered with increasing rate of origin firing, the ATR inhibition is found to reduce the speed of the forks [22].

Conclusion

Crispr Cas 9 technology has reduced the job of biologist to 90% in curing several genetic and infectious disorders. While in normal replication, it is preferable for ATR to allow the replication fork to move further but it can be hazardous in case of cancerous cells. In DNA replication, ATR with aid of many co-ordinating molecules removes the barriers that stall the forks that also minimizes the chances of apoptosis. ATRi (ATR inhibitors) like AZD6738 tested in Xenograft cells successively disrupted the ATR activity with RNASEH2 that functions coordinately that also inhibits potent cancerous biomarkers like CDC25A.

Conflicts of Interest

There are no conflicts of interest.

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