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APPLICATIONS OF CRISPR/CAS9 IN IMMUNOTHERAPY

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ABSTRACT

Cancer is among the deadliest diseases worldwide, though years of studies and research has made the understanding of the tumor cells really conventional helping to inculcate various treatment options, immunotherapy is one of the advanced options for reversing the tumor activity, and implementing CRISPR/cas9 in the gene editing with best possible results is taking onco-immunology to the next extent. Various genes are associated with the negative regulation of the CAR cells which are essential in T-cell therapy. These genes like, B2M, PD-1, TRAC, LAG-3 can be knocked down very precisely by easy to use and immensely prompting gene edited technique of CRISPR/cas9. Various studies have been conducted incorporating CRISPR/cas9 in enhancing the scope of immunotherapy. This review is subjected to study the genes and their downregulation by CRISPR/cas9 and challenges this technique is still holding along with the future implementations to well orient oncology along with immunology helping in the eradication of cancer.

KEYWORDS: CRISPR/cas9, immunotherapy, PD-1, LAG-3, lung cancer.

INTRODUCTION

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a bacterial acquired immune system[1] [2] that can alter the human DNA sequence like a very precise and easy to use pair of scissors.[3] It was first used in the form of genome editing tool in 2013 in the mammalian cells[4] [5] [6], the toolkit of CRISPR/cas9 with two key component a chimeric single guide (sgRNA) and the DNA endonuclease Cas9[7] [8] [9] has been constantly expanded,

allowing not only the modification of the genomic sequence of cells and organisms but also the introduction of epigenetic and transcriptional modifications.[10] CRISPR/cas9 is widely being used in various fields like drug discovery, drug resistance, creating disease models for testing the drug efficiency, tumor therapies, immunoregulation of tumor, studying particular oncogenes or tumor suppressor genes, gene diagnosis.[11] [12] [13] There are basically three types of CRISPR/cas system each further sub categorised.[14] The most prevalently used is typeII CRISPR/cas9, which is composed of three components. The first component is an endonuclease (cas9), then a CRISPR RNA (crRNA), and a transactivating crRNA (tracrRNA).[14] [15] The crRNA along with tracrRNA forms guide RNA (gRNA) a duplex structure which is reinstated by sgRNA making the application of CRISPR/cas9 in various aspects more reliable and easier.[16] The sgRNA is composed of a unique set of 20 base pair sequence which are similar to the target DNA site in accordance with the short DNA sequence called as PAM (protospacer adjacent motif) necessary for the compatibility with cas9 protein.[17][18] This gene editing tool takes in account the cas9 and sgRNA to create a high efficiency, double or single stranded cleavage at specific location of the genome where complementary sequences of genomic DNA and sgRNA are present.[8] The sgRNA has unique 20 base pair sequence that are complementary to the target sites and to the protospacer adjacent motif (PAM), the compatibility of the PAM with cas9 is extremely important[19] PAM along with the cas9 forms a ribonucleotide (RNP) complex that in turn is directed to the target DNA site by the sgRNA, here the RNP complex binds to the target DNA site by Watson Crick base pairing and the RNA guided cas9 enzyme cleaves the DNA at the target site producing double stranded break DSBs[20] [Figure 1]

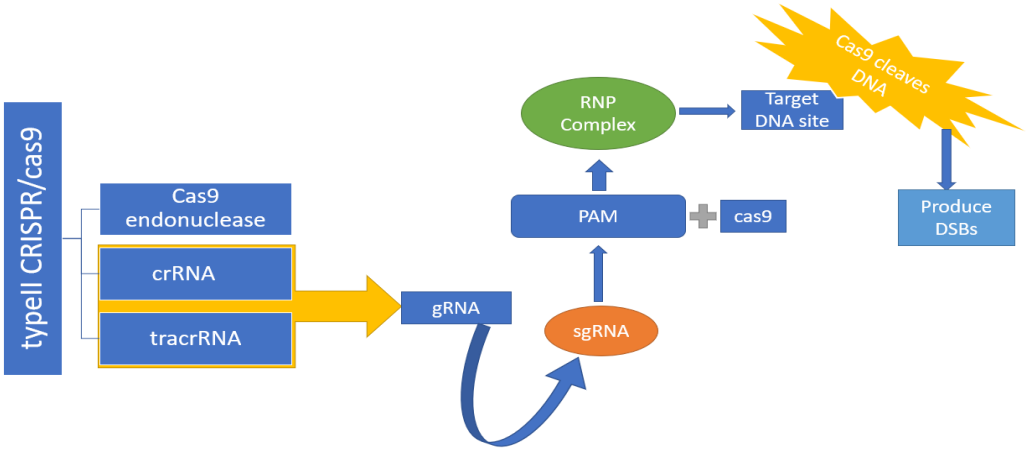

Figure 1: - Depicting the components and functioning of typeII CRISPR/cas9 in cleaving the DNA at target sites and producing the DSBs.

CANCER AND CRISPR/cas9

Cancer is a disease that has been around for centuries, but it never had such an impact on public health as it has now. After cardiovascular diseases, cancer is the crucial and second most cause of death globally.[21] Nonetheless, major discoveries and progress has been made in the field of oncology, enhanced perception of underlying tumour biology being one of them. Various treatment options like surgery, chemotherapy, hormone therapy, radiotherapy have made notable progress in the treatment of cancer.[22] However, all the mentioned treatment options have their own side effects, short term side effects being fatigue, nausea and vomiting, high risk of thrombosis[23] while long term side effects are multiple organ failure, cognitive dysfunctional, leukaemia etc.[24][25] Therefore, new treatment options are being introduced, and out of many treatment options, immunotherapy is one of them.

Immunotherapy is the artificial stimulation of the immune system to fight cancer, either by antibodies, adoptive T-cell transfer, cytokines or vaccines.[26] There are various types of immunotherapies like oncolytic virus therapy, cancer vaccines, T-cell therapy and monoclonal antibodies.[27] The gene edits of the immune cells can be done by CRISPR/cas9 along with T-cell therapy being the most advanced implementation of CRISPR/cas9 in the field of onco-immunology.[28] Enhancement of T-cell production and function is extremely crucial for the immunotherapy[29], various genes are involved that negatively regulates the T-cell and downregulation of those genes by CRISPR/cas9 is proving to be highly efficient in the T-cell therapy for immunoregulation.[30] These genes are B2M which is primarily associated with the MHC class I[31], LAG-3 associated with activated T-cell surface[32], TRAC gene involved with CAR cells[33] and PD-L1 is the ligand that downregulates the activity of the T-cell which in turn is activated by programmed death-1 (PD-1) receptor being attaching on activated T cells.[30][34] CRISPR/cas9 is deliberately playing an important role in knocking out the mentioned genes resulting in drastic decrease in the proliferation and differentiation of the tumour cells.

APPLICATIONS OF THE CRISPR/CAS9 IN IMMUNOTHERAPY

Chimeric antigen receptors (CAR) are the artificial receptors consisting of an extracellular domain that binds target with respective antibody, a hinge region, a transmembrane domain and a signaling moiety that is intracellular and can activate T cells.[35] T cells are extremely crucial for therapies against tumor due to their tendency of immunomodulation, clonal expansion, cytotoxicity, migration and long immunological memory.[36] Numerous studies

have been explaining that the CAR T-cell therapy has the tendency to induce complete remissions (CRs) which are durable in patients with several types of hematologic and solid cancers, majorly in multiple myeloma and acute lymphoblastic leukaemia (ALL) with impressive retaliation rate of 80-100%(9). Currently, there are four CAR-T cell products introduced namely, Kymriah, Yescarta, Tecartus, Breyanzi.[37] Kymriah, scientifically called *Tisagenlecleucel* as first in CAR-T cell product was accepted in August 2017 by, The United States Food and Drug Administration (FDA).[37][38] Kymriah is actively been used in the treatment of Acute Lymphoblastic Leukemia (ALL) and the Diffuse Large B-cell Lymphoma (DLBCL).[39] Yescarta, scientifically called as *Axicabtagene Ciloleucel* was the second in cell therapy product to be approved and receive the marketing agreement in Europe (August 2018) and in US (October 2018).[40][41] Yescarta is being actively tested for incorporation in the treatment of, chronic lymphocytic leukaemia (CLL), multiple myeloma (MM), second-line DLBCL and follicular lymphoma (FL)[42] Tecartus i.e., *brexucabtagene autoleucel* is another cell product which was accepted by The United States Food and Drug Administration (FDA) in July 2020 whereas by EC in December 2020.[37][43][44] Tecartus is extensively being tested for the administration in the treatment of Multiple myeloma (MM) and autologous stem cell transplantation (ASCT) and primarily for Mantle cell lymphoma (MCL)[45] The most recently approved CAR-T cell product is breyanzi, scientifically called as *lisocabtagene maraleucel* particularly involved in the treatment of high-level B-cell lymphoma, it was approved by the FDA in February 2021[37](17). To be a potential CAR-T cell the two main hurdles to qualify are, allogenic T-cells infused in the recipient being rejected i.e., graft-versus-host disease (GVHD) and enhanced biosafety or less cell toxicity profile for more advanced disease-targeted activity(18). CTLA-4, TIM-3, LAG-3 and PD-1 are the naturally occurring signalling molecules or receptors that inhibits the T cell response.[47] The blockage of LAG-3 and PD-1 downregulates the T cell enervation and synergistically upgrades the therapeutic activity in various models of cancers and chronic infection.[48][49][50][51] The homeostasis, activation and multiplication of the T-cells is attuned by the lymphocyte activation gene-3 (LAG-3) which is a negative co-stimulatory receptor.[52][53] The CD-4 independent T-cell remains unaffected by LAG-3 whereas CD-4 dependent T-cells function is inhibited by its cytoplasmic domain.[54] Knockout of the LAG-3 gene was carried out using the highly efficient gene editing tool i.e., CRISPR/cas9. CAR-T cells devoid of LAG-3 showed extreme antigen-specific antitumor activity in murine xenograft model and cell culture with respect to the standard CAR-T cells.[32]

CAR-T cell therapy, majorly specific to CD19 has been an exceptional novel gene therapy method in the treatment of antitumor activity of non-Hodgkin's lymphoma and B-cell acute lymphoblastic leukaemia (ALL). Nevertheless, this method also signifies in vivo neurotoxicity and eventually cause cytokine release syndrome.[55][56] CRISPR/cas9 incorporated in specifying the CD19 specific CAR to the T-cell receptor alpha constant (TRAC) ensued consistent CAR expression in human peripheral blood T-cells, magnified T-cell activity, significantly transcending the regular CAR T-cells of acute lymphoblastic leukaemia in a mouse model.[57] [58] [33] The individualistic gene knockout study of two genes (TRAC and B2M) and three genes (TRAC, PD-1 and B2M) of CAR-T cells was carried out using CRISPR/cas9 which showed a significant loss in the tumor activity.[31] Directing CAR to TRAC site prevents the stimulatory CAR signalling as well as exhibiting constructive incorporation and re-articulation of CAR backing single or multiple antigen revelation, detaining the depletion and differentiation of effector T-cell (31). The ability of the CAR-T cells to exterminate the tumor activity is immensely dependent on the programmed cell death protein 1 (PD-1) and its interconnection with activated T cells and its ligand present on a target tumor. Therefore, blockage or knockout of the PD-1 can consequently intensify the function of CAR T cells.[59] CRISPR/cas9 was first clinically evaluated in mediating PD-1 gene knockout in T-cells of lung cancer patients.[60][59][61] The 4 loci of CTL-4 and PD-1 were successfully knocked out in a synchronized manner creating the allogenic universal T-donor cells.[62] The EvCAR-T cells were incorporated with sgRNA/cas9 expression vectors that were exclusively designed to disturb the PD-1 target region downregulating its expression, which had an inhibitory impact on EGFRvIII- expressing glioblastoma GBM cells, without changing the T-cell phenotype and not interfering with the expression of the other checkpoint receptors.[63] Another crucial, application of CRISPR/cas9 in knocking out the PD-1 gene was with GPC3-CAR T cells, the local PD-L1 exhibiting hepatocellular carcinoma (HCC), since the extent of expression anti-apoptotic protein BCL-XL and stage of Akt phosphorylation in GPC3-CAR T cells devoid of PD-1 were extremely high as compared to wild-type GPC3-CAR T cells following simultaneously culturing with PLC/PRF/5 (38). CRISPR/cas9 being an electroporation mediated intrinsically disrupting PD-1 gene non-viral transfection method does not hinder the proliferation of primary T-cells while it upregulates cytotoxicity and cellular immune response of cancer cell lines.[64] The PD-1 gene was knocked out in primary T-cells to study the increase in the cytotoxicity depicting that in spite of adding any clinical agent for blocking the PD-1 receptor signaling, the T-cells altered by knocking out the PD-1 receptor is still serving as dynamic cell therapy(40).

Another crucial gene involved in the negative regulation of CAR is B2M, it ciphers the accessory chain and forms the heterodimer with major histocompatibility complex (MHC) class I molecules and is essential for surface expression of them.[66][67][68] Elimination of B2M is an inveterate method for eroding the surface expression of MHC class I resulting into the production of hypoimmunogenic cells exclusive for transplantation and adoptive immunotherapy.[69] CRISPR/cas9 is playing an advanced role by targeting the two clinically pertinent genes, CCR5 and B2M in the primary human CD34+ and CD4+ T cells hematopoietic stem and progenitor cells (HSPCs).[70][69] The B2M gene knockout in induced pluripotent stem cells (iPSCs) leads to the human leukocyte antigens (HLA) class I depletion was generated using CRISPR/cas9.[71] The downregulation of B2M in iPSCs showed a decline of HLA I expression on the surface of cells, these cells must have decreased the immunogenicity to allogenic CD8+ T cells. The knockout of the B2M in the iPS cell lines was mediated by CRISPR/cas9 using the transfection of pSpCas9(BB)2AGFP plasmid having cas9 along with guide RNA followed by GFP based cell sorting.[72] A B2M homozygous gene knockout was carried out in the somatic cell nuclear transfer induced embryonic stem cell (SCNT-ESC) line by incorporating the gene targeting technique CRISPR/cas9.[73]

C-C chemokine receptor type 5 (CCR5) being an HIV-1 co-receptor, the off-target cleavage using CRISPR/cas9 was generated consequently limiting HIV-1 infection.[74][75] Similarly, the downregulation of CXCR4 or CXCR7 was done using the CRISPR/cas9 which resulted in the decreased cell proliferation, differentiation, migration and invasion of the tumor cells.[76]

CRISPR/CAS9 IN LUNG CANCER

Cancer is the continuous proliferation and multiplication of the normal cells until they form tumour and when the development of this tumour occurs in the lung cells it is called as primary lung cancer. But when the cancer begins in any other body part and outspreads to the lungs then it is said to be lung metastasis. The treatment of primary lung cancer is done in an alternate manner as compared to the lung metastasis. Based on the histology, lung cancer is subjected to be of two types, non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), pertaining to which type of cell the cancer begins in.[77]

NSCLC is further categorised into various classes; it is called adenocarcinoma if the non-small lung cancer begins in the glandular cells which are present on the outer part of the lung,

squamous cell carcinoma if the non-small lung cancer starts in the squamous cell lining of the bronchi, and large cell carcinoma though this category is very rare. Apart from these, sarcomatoid and sarcoma are also rare forms of non-small cell lung cancer.[78]

When the cells which line the bronchi in the centre of the lungs are afflicted then it is said to be small-cell lung cancer (SCLC). Small-cell lung cancer is further classified into small cell carcinoma and an assorted tumour with glandular or squamous cells called combined small cell carcinoma.[79]

Of all the diagnosis that are run for the cancer, 14% are turned out to be the lung cancer, out of which 80-85% are the non-small cell lung cancer (NSCLC) making it the presiding kind. Small-cell lung cancer (SCLC) is the more threatening type of lung cancer and accounts for 10-15% of all the lung cancer cases.[80]

The predominance of lung cancer is increasing globally and so in India 57,795 cases were announced in 2010 which were predicted to touch the mark of 67,000 new cases by the year 2020.[81]

Therefore, it is extremely crucial to inculcate more sustainable treatment options, even though traditional methods of RNA interference (RNAi) mediated knockdown and overexpression of cDNA have already been incorporated to alter the known function of the tumor suppressor genes and oncogenes in the studies done via cell culture, xenograft, allograft and transgenic mouse models.[77]

There are various stances in which CRISPR/cas9 has already been incorporated in the deciphering of various gene manipulation treatment options. As per a study done by Choi and Meyerson, the in vitro chromosomal rearrangement of the genes EML4- ALK, KIF5B-RET, CD74-ROS1, were carried out in the target cell h HEK-293 cells using a plasmid transfection as the delivery agent against the lung adenocarcinoma.[82]

The in vivo study using the lentiviral as the delivery system against lung adenocarcinoma has been studied in mouse models as well, the loss of function of genes such as Pten, Nkx2.1 and Apc were seen to downregulate the proliferation of cancerous cells in mouse lung cells.[83] Similarly, the lentiviral and adeno-associated virus (AAV) delivery system were incorporated in the endothelial cells and neurons of the mouse and study not only the loss of function but also the site-specific mutagenesis of extremely crucial genes like p53, Kras, Lkb1 for the

downregulation of lung adenocarcinoma.[84] The loss of function of the GeCKO library in the mouse lung cells were seen against non-small cell lung cancer (NSCLC) using the CRISPR/cas9 gene editing technique.[85] Furthermore, the in vivo study against NSCLC in the mouse lung cells suing adenoviral delivery system were carried out to study the alteration of echinoderm microtubule-associated protein-like 4 (EML4) and anaplastic lymphoma kinase (ALK) gene by integrating CRISPR/cas9.[86] Similarly, another in vivo study to study the chromosomal rearrangement of EML-ALK in the mouse model was done using the lentiviral delivery system against the NSCLC.[87] A significant decrease in the lung tumor size was recorded on the knockdown of the AMPK α1 and α2 using CRISPR/cas9 in the murine lung adenocarcinoma cells.[88] There are drugs like gefitinib and erlotinib which acts as the Tyrosine Kinase Inhibitors (TKIs) in the lung adenocarcinoma caused by the epidermal growth factor receptor (EGFR) mutations, however, these drugs can become ineffective because of the ability of cancer cells to develop immunity against them, so the alteration in the EGFR T790M were carried out in the PC9 human lung cancer cell lines, here the CRISPR/cas9 gene editing tool was used in the deletion of exon 19 of EGFR gene.[89] The CRISPR/cas9 is also being incorporated in the immunotherapy pertaining to the lung cancer, for instance the first-in- human clinical trial phase I of PD-1 edited T cells using CRISPR/cas9 were done in patients with recently developed NSCLC.[90]

CHALLENGES ASSOCIATED WITH CRISPR/CAS9

Even though this gene editing technique is bringing a revolution in various fields of biological research, there still are major challenges associated with it that are being studied and looked up for further modifications in CRISPR/cas9. One of the primary challenges is to decrease the potential off target effects, since the off- target DSBs can generate small indels or extensive alteration in the genome, increased specificity of CRISPR/cas9 can reduce this effect[91] (75). Selection of appropriate nuclease platform and objectively outlining the sgRNA is still a major challenge. Additionally, the in vivo and in vitro transferring of multiple elements into the target cells needs to be depicted in a precise manner. While targeting the CXCR4 in T-cells the infrequent indels were discovered in the two anticipated off- target sites[93], so studying the robustness of the edited cells and the therapeutic approach of the edit is a challenge.

CONCLUSION

CRISPR/cas9 is unquestionably an asset of a technique when it comes to gene editing for curing various diseases especially in the field of oncology and immunology it is opening the gateways for long lost questions. Many successful gene edits have been performed and still a lot of experimental work pertaining to various diseases is going on to inculcate the more advanced version of this tool. Many positive outcomes have been accomplished in various areas, from fundamental research to the progress of potential therapies for different sorts of cancer, chronic diseases and congenital diseases. However, there are still few challenges linked with CRISPR/cas9. Upgraded strategies need to be implemented to upregulate the target efficiency and decreases the off- target effects. The CAR T- cell therapy is still not accessible to a remarkable part of the society due to its exorbitant price. Novartis' first FDA approved CAR T- cell therapy costs $475,000 per treatment, additional advancement in CRISPR/cas9 can add in the lowering of the cost.

In conclusion, this gene editing technique has a remarkable possibility for tumor treatment, gene therapy and various other diseases, the clinical trials and new improved approaches are leading the anticipation for many unanswered questions of tumor treatment and personalized targeted therapy.

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