**Genome Editing and Engineering**
**Prof. Utpal Bora**
**Department of Bioscience and Bioengineering**
**Indian Institute of Technology, Guwahati**

**Module - 11**
**Personalized Therapy**
**Lecture - 46**
**Genome editing and personalized therapy**

Welcome to my course on Genome Editing and Engineering. We are discussing about Genome editing and personalized therapy. This is lecture 2 of this module number 11.

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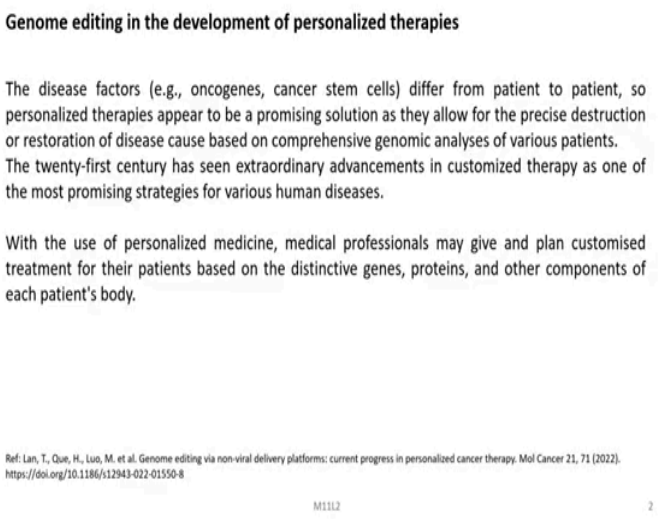


Genome editing in the development of personalized therapies, the disease factors like oncogenes, cancer stem cells, differ from patient to patient. So, personalized therapies appear to be a promising solution as they allow for the precise destruction or restoration of disease cause, based on comprehensive genomic analysis of various patients.

The 21st century has been has seen extraordinary advancements in customized therapy as one of the most promising strategies for various human diseases, with the use of personalized medicine. Medical professionals may give and plan, customized treatment for their patients based on the distinctive genes, protein markers and other components of each patient's body.

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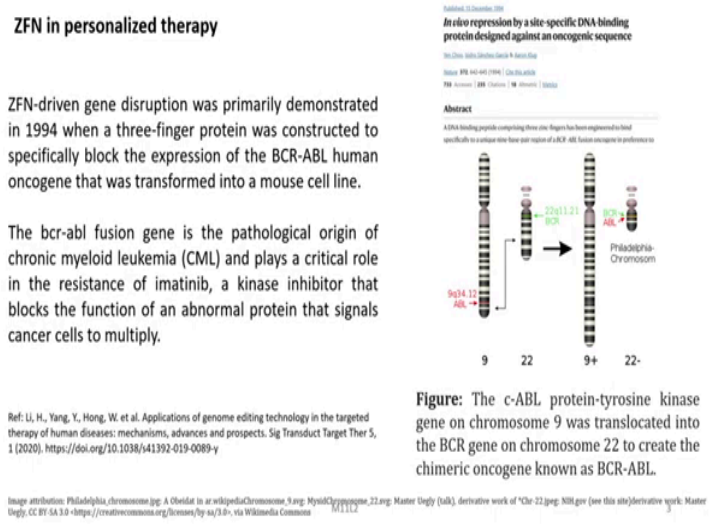


The use of ZFN in personalized therapy. ZFN-driven gene disruption was primarily demonstrated in 1994, when a three-finger protein was constructed to specifically block the expression of the BCR-ABL human oncogene that was transformed into a mouse cell line. The BCR-ABL fusion gene is the pathological reason of chronic myeloid leukemia and plays a critical role in the resistance of imatinib, a kinase inhibitor that blocks the function of an abnormal protein that signals cancer cells to multiply.

So, in this publication by Choo et al., the in vivo preparation by a site-specific DNA binding protein design against an oncogene sequence was published, in the journal Nature in 1994 and you can see here the c-ABL protein tyrosine kinase gene on chromosome 9, was translocated into the BCR gene on chromosome 22 to create a chimeric oncogene known as BCR-ABL.

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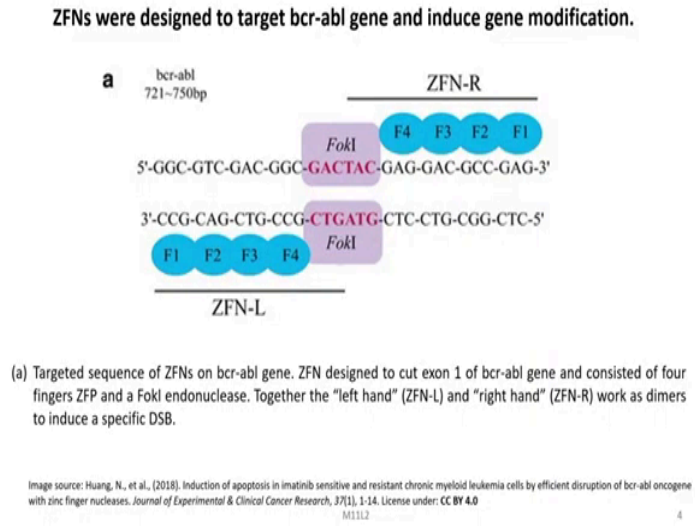


(a) Targeted sequence of ZFNs on bcr-abl gene. ZFN designed to cut exon 1 of bcr-abl gene and consisted of four fingers ZFP and a FokI endonuclease. Together the "left hand" (ZFN-L) and "right hand" (ZFN-R) work as dimers to induce a specific DSB.



The ZFNs were designed to target BCR-ABL gene and induce gene modification. So, here in this figure a, you can see targeted sequence of ZFN on the BCR-ABL gene. ZFN was designed to cut exon 1 of BCR-ABL gene and it consisted of four zinc fingers and a Fok endonuclease. Together the "left hand" and the "right hand" work as dimers to induce the specific DSB. The mechanism of which has been discussed in length in under the lectures of ZFN technologies.

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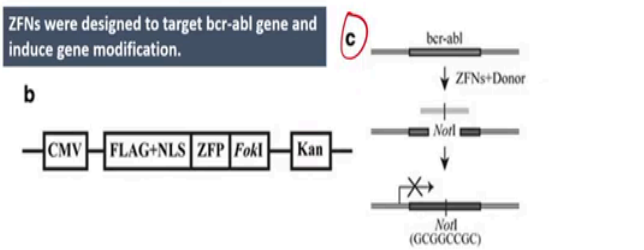


(b) The structure of pAd-Track-ZFN vector. ZFP fused to FokI endonuclease, a nuclear localization signal (NLS) and FLAG tag. The expression of Kanomycin resistance gene (Kan) was regulated by CMV promoter.

(c) Sketch of the donor construct and HDR detection scheme. Cleavage of bcr-abl gene created a substrate for HDR, which may use the donor DNA fragment containing a NotI site as a repair template. The introduction of NotI site, which involved 8-bp, may result in termination of translation.

In these figure b, you can see the structure of pAd-Track-ZFN vector, ZFP was fused to Fok1 nuclease, a nuclear localization signal and the flag tag the expression of kanomycin a resistance gene was regulated by the CMV promoter.

And in figure c, you can see the sketch of the donor construct and HDR detection scheme cleavage of BCR-ABL gene created a substrate for HDR, which may use the donor DNA fragment containing a Not1 side, as a repair template. The introduction of Not1 site, which involve 8 base pair may result in termination of translation.

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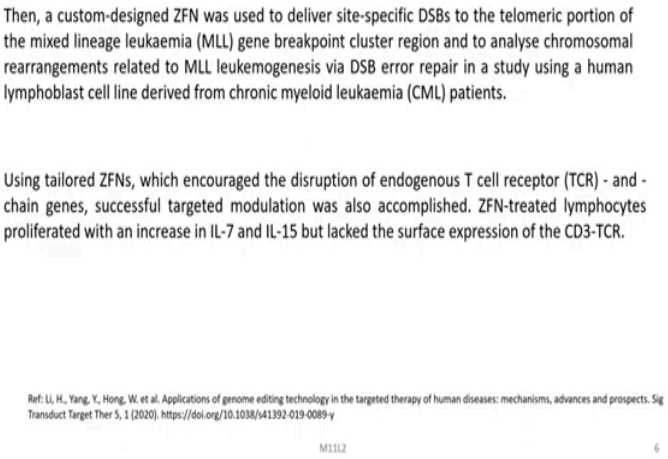


A custom-designed ZFN was used to deliver site specific double strand bricks to the telomeric portion of the mixed lineage leukaemia gene breakpoint cluster region and to analyse chromosomal rearrangements related to MLL leukemogenesis via DSB error repair in a study using a human lymphoblast cell line derived from chronic myeloid leukaemia patients.

Using tailored ZFNs which encouraged the disruption of endogenous T cell receptors and chain genes, successful targeted modulation was also accomplished. ZFN treated lymphocytes proliferated with an increase in interleukin-7 and interleukin-15, but like the surface of surface expression of the CD3-TCR.

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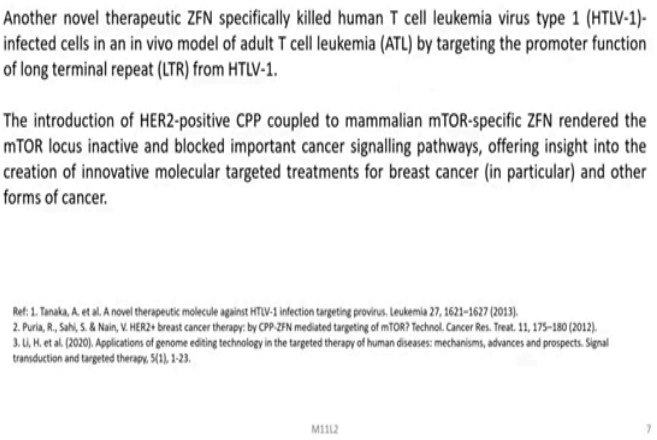


Another noble therapeutic ZFN specifically killed human T cell leukaemia virus type 1, infected cells in an in vivo model of adult T cell leukemia by targeting the promoter function of long terminal repeat from HTLV-1.

The introduction of HER2-positive CPP coupled to mammalian mTOR-specific ZFN rendered the mTOR locus inactive and blocked important cancer signaling pathways, offering insight into the creation of innovative molecular targeted treatments for breast cancer in particular and other forms of cancer.

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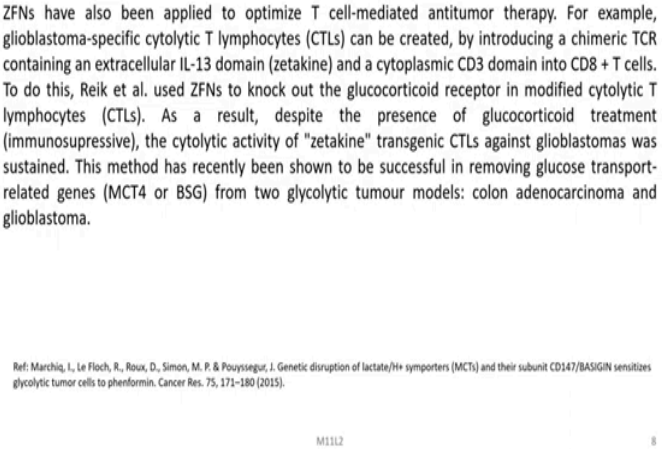

ZFNs have also been applied to optimize T cell mediated antitumor therapy. For example, glioblastoma specific cytoytic T lymphocytes can be created, by introducing a chimeric TCR containing an extracellular IL-13 domain in a cytoplasmic CD3 domain into CD8 plus T cells.

To do this, Reik et al. used ZFNs to knock out the glucocorticoid receptor in modified cytolytic T lymphocytes. As a result, despite the presence of glucocorticoid treatment immunosuppressive the cytolytic activity of "zetakine" transgenic CTLs against glioblastomas was sustained. This method has recently been shown to be successful in removing glucose transport related genes from two glycolytic tumor models: colon adenocarcinoma and glioblastoma.

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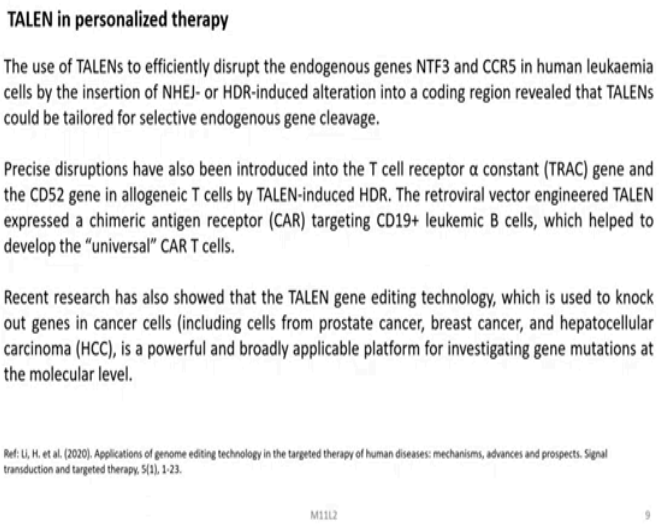


The application of TALEN in personalized medicine. The use of TALENs to efficiently disrupt the endogenous genes NTF3 and CCR5 in human leukaemia cells by the insertion of NHEJ or HDR-induced alteration into a coding region revealed that TALENs could be tailored for selective endogenous gene cleavage.

Precise disruptions have also been introduced into the T cell receptor alpha constant TRAC gene and CD52 gene in allogeneic T cells by TALEN induced HDR. The retroviral vector engineered TALEN expressed a chimeric antigen receptor targeting CD19 plus leukemic B cells, which helped to develop the "universal" CAR T cells.

Recent research has also showed that the TALEN gene editing technology, which is used to knock out genes in cancer cells including cells from prostate cancer, breast cancer, and hepatocellular carcinoma, is a powerful and broadly applicable platform for investigating gene mutations at the molecular level.

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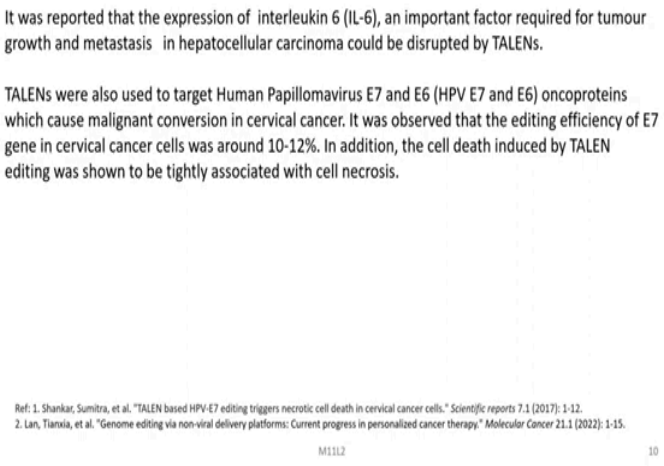


It has been reported that the expression of IL-6 an important factor required for tumor growth and metastasis in hepatocellular carcinoma could be disrupted by TALENs. TALENs were also used to target human papillomavirus E7 and E6 oncoproteins which cause malignant conversion in cervical cancer. It was observed that the editing efficiency of E7 gene in cervical cancer cells was around 10 to 12 percent.

In addition, the cell death induced by TALEN editing was shown to be tightly associated with cell necrosis.

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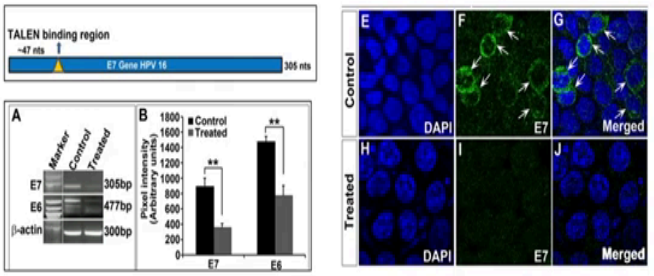


**Figure:** (A) RT-PCR analysis for the transcripts corresponding to *E7* and *E6* showed a significant reduction in treated (TALEN-transfected) group when compared to controls. (**B**) Graph represents expression levels of *E7* and *E6* in control and treated groups.

Immunocytochemical analysis also indicated that E7 protein levels were significantly decreased in TALEN-treated SiHa cells (**H–J**) when compared to controls (**E–G**).





In this figure in (A), we can see the RT-PCR analysis for the transcripts corresponding to E7 and E6 showed a significant reduction in treated group when compared to the control group. In figure (B), we can see the graphs which represents expression levels of E7 and E6 in control and in the treated groups.

Immunocytochemical analysis also indicated that E7 protein levels are significantly decreased in TALEN treated SiHa cells H I J when compared to the control from (E) to (G).

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The development of CRISPR-Cas based approaches opens up a new opportunity for customised genetic element screening, including the identification of genes and enhancers that affect many aspects of cancer growth and the subsequent development of treatments.

The ultimate objective of CRISPR/Cas9 cancer treatment is to eliminate malignant mutations and replace them with normal DNA sequences.

Ref: Das, S., Bano, S., Kapse, P. *et al.* CRISPR based therapeutics: a new paradigm in cancer precision medicine. *Mol Cancer* **21**, 85 (2022). https://doi.org/10.1186/s12943-022-01552-6

The development of CRISPR-Cas based approaches opens up a new opportunity for customized genetic elements screening, including the identification of gene and enhancers that affect many aspects of cancer growth and the subsequent development of treatments. The ultimate objective of CRISPR-Cas 9 cancer treatment is to eliminate malignant mutations and replace them with normal DNA sequences.

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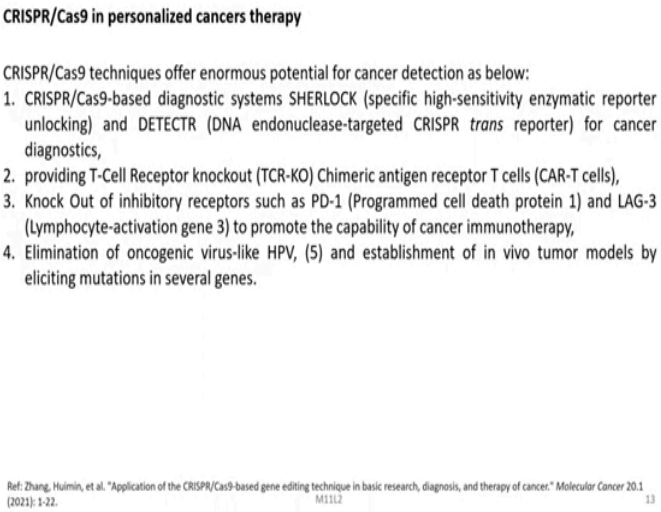


Let us discuss about the CRISPR-Cas9 applications in personalized cancer therapy and its potential. The CRISPR-Cas9 techniques offer enormous potential for cancer detection as below: it CRISPR-Cas9 based diagnostic systems SHERLOCK specific high sensitivity enzymatic reporter unlocking and DETECTR, DNA endonuclease targeted CRISPR trans reporter for cancer diagnostics.

Providing T cell receptor knockout TCR-KO chimeric antigen receptor T cells CAR T cells about which we have discussed at length in the earlier lecture also. Knockout of inhibitory receptors such as PD-1 programmed cell death protein 1 and LAG-3 lymphocyte-activation gene 3 to promote the capability of cancer immunotherapy. Elimination of oncogenic virus-like HPV and establishment of in vivo tumor models by eliciting mutations in several genes.

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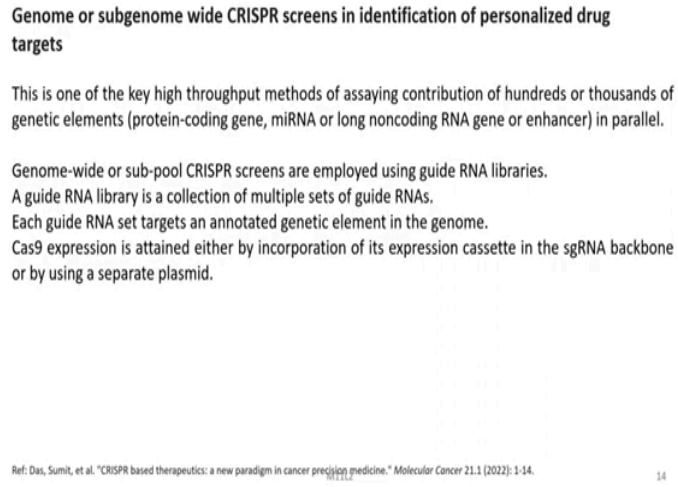


Genome or subgenome wide CRISPR screens in identification of personalized drug targets this is one of the key high throughput methods of assaying contributions of hundreds or thousands of genetic elements in parallel.

Genome wide or sub-pool CRISPR screens are employed using guide RNA libraries, a guide RNA library is a collection of multiple sets of guide RNAs, each guide RNA sets targets and annotated genetic element in the genome. Cas9 expression is attained either by incorporation of its expression cassette in the sgRNA backbone or by using a separate plasmid.

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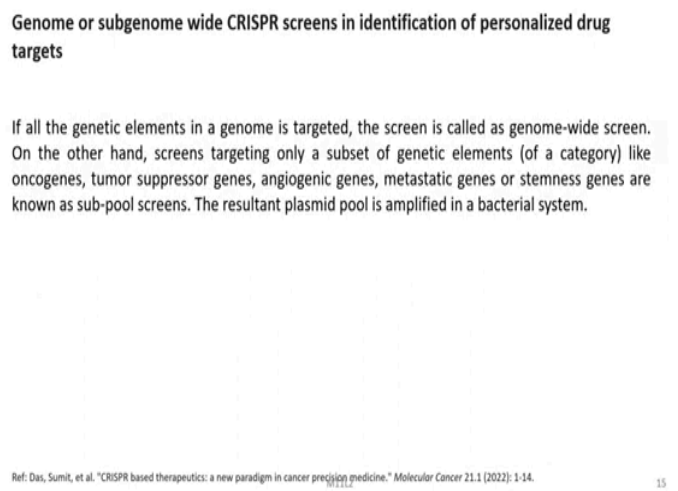

If all the genetic elements in a genome is targeted, the screen is called as genome wide screen. On the other hand screens targeting only a subset of genetic elements like oncogenes, tumor suppressor genes, angiogenic genes, metastatic genes or stemness genes are known as sub pool screens. The resultants plasmid pool is amplified in a bacterial system.

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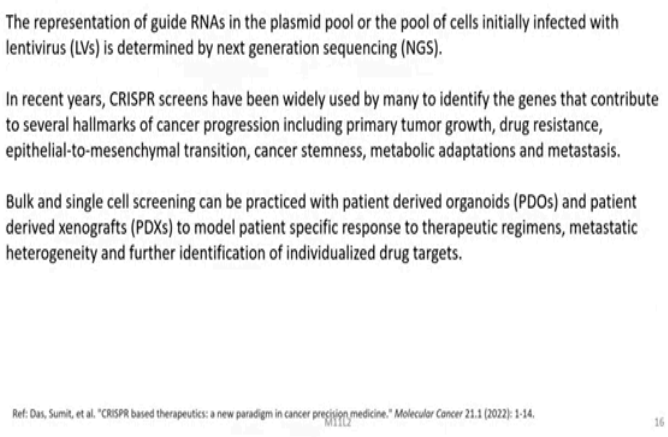


The representation of guide RNAs in the plasmid pool or the pool of cells initially infected with lentivirus is determined by next generation sequencing. In recent years, CRISPR screens have been widely used by many to identify the genes that contribute to several hallmarks of cancer progression including primary tumor growth, drug resistance, epithelial-to-mesenchymal transition, cancer stemness, metabolic adaptation and metastasis.

Bulk and single cell screening can be practiced with patient derived organoids and patient derived xenografts to model patent specific responses to therapeutic regimens, metastatic heterogeneity and further identification of individualized drug targets.

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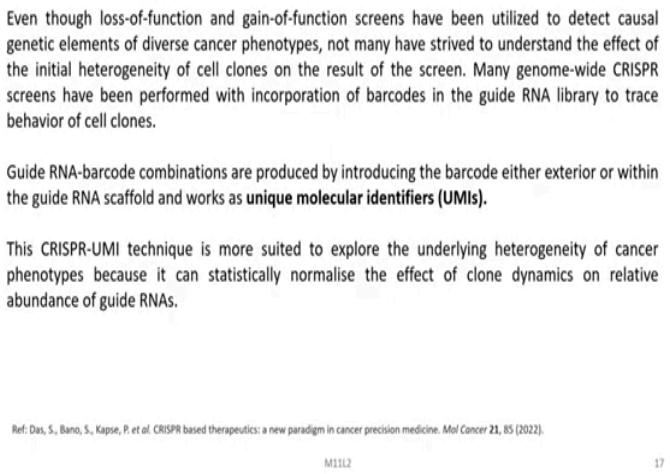


Even though loss-of-function and gain-of-function screens have been utilized to detect causal genetic elements of diverse cancer phenotypes, not many have strived to understand the effect of that initial heterogeneity of cell clones on the result of the screen. Many genome wide CRISPR screens have been performed with incorporation of barcodes in the guide RNA library to trace behavior of cell clones.

Guide RNA-barcode combinations are produced by introducing the barcode either exterior or within the guide RNA scaffold and works as unique molecular identifiers. The CRISPR-UMI technique is more suited to explore and the underlying heterogeneity of cancer phenotypes because it can statistically normalise the effect of clone dynamics on relative abundance of guide RNAs.

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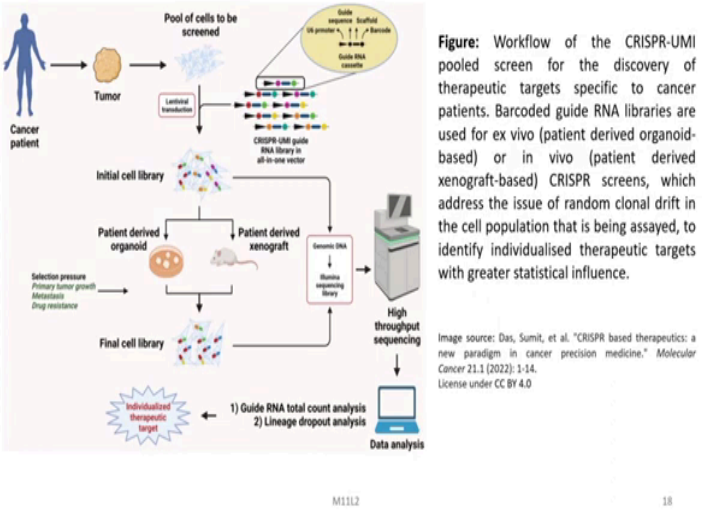


**Figure:** Workflow of the CRISPR-UMI pooled screen for the discovery of therapeutic targets specific to cancer patients. Barcoded guide RNA libraries are used for ex vivo (patient derived organoid-based) or in vivo (patient derived xenograft-based) CRISPR screens, which address the issue of random clonal drift in the cell population that is being assayed, to identify individualised therapeutic targets with greater statistical influence.

Image source: Das, Sumit, et al. "CRISPR based therapeutics: a new paradigm in cancer precision medicine." *Molecular Cancer* 21.1 (2022): 1-14.
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This figure shows the workflow of the CRISPR-UMI pool screen for the discovery of therapeutic targets specific to cancer patients. Barcoded guide RNA libraries are used for ex vivo patient-derived organoid based or in vivo patient-derived xenograft based CRISPR screens, which address the issue of random clonal drift in the cell population that is being assayed, to identify individualised therapeutic targets with greater statistical influence.

So, we have a cancer patients from which we obtain a tumor and have a pool of cells to be screened and there is the lentiviral transduction where we have the U6 promoter, guide sequence, scaffold and barcode and this is the guide RNA cassette or CRISPR-UMI guide RNA library in all in one vector. And due to this lentiviral transduction we get a initial cell library, which may go for genomic DNA sequencing or STS and finally, there will be data analysis

And on the other hand these initial cell library can be used for developing of patient derived organoid studies or the patient derived xenograft studies. And there is a selection pressure due to primary tumor growth metastasis drug resistance and we obtain the final cell library as a result of this. And then these data along with the original initial cell library data we go for sequencing and then the data analysis.

So, this will give us information on the guide RNA total count analysis in the lineage dropout analysis from which we try to develop the individualized therapeutic target.

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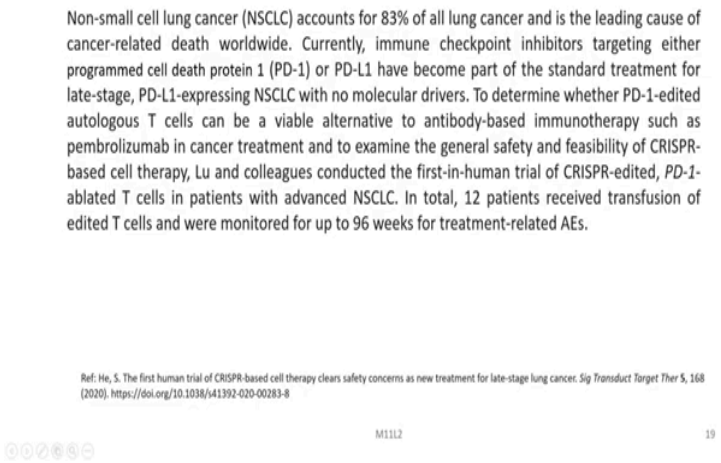


Non-small cell lung cancer (NSCLC) accounts for 80 percent of all lung cancer and is the leading cause of cancer-related deaths worldwide. Currently, immune checkpoint in inhibitors targeting either program cell that protein 1 or PD-L1 have become part of the standard treatment for late stage PD-L1 expressing NSCLC with no molecular drivers.

To determine whether PD-1 edited autologous T cells can be a viable alternative to antibody-based immunotherapy such as pembrolizumab in cancer treatment and to examine the general safety and feasibility of CRISPR based cell therapy, Lu and colleagues conducted the first-in-human trial of CRISPR edited PD-1 ablated T cells in patients with advanced NSCLC.

In total, 12 patients receive in transfusion of edited T cells and were monitored for up to 96 weeks for treatment related AEs.

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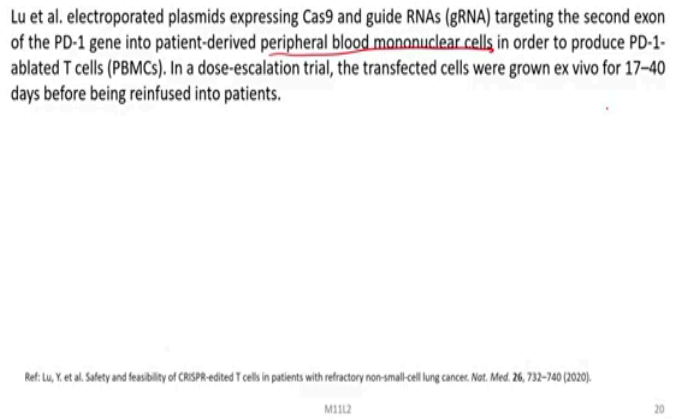


They electroporated plasmids expressing Cas9 and guide RNAs targeting the second exon of the PD-1 gene into patient derived peripheral blood mononuclear cells in order to produce PD-1 ablated T cells. In a dose escalation trial, the transfected cells were grown ex vivo for 17 to 40 days before being reinfused into the patients.

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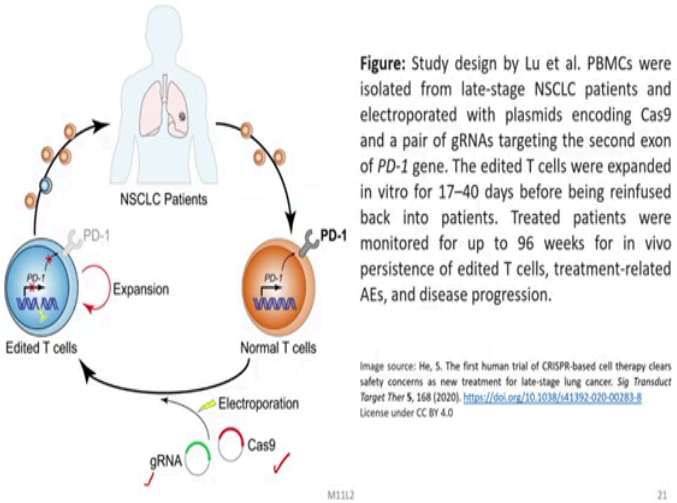


So, these figure shows the study design by Lu et al, where PBMCs were isolated from late-stage NSCLC patients and then electroporated with plasmid encoding the Cas9 and a pair of gRNAs targeting the second exon of PD-1 gene. The editing T cells were expanded in

vitro, there is a expansion of these edited T cells for 17 to 40 days as already mentioned, before being reinfused back into the patient.

Treated patients were monitored for up to 96 weeks, for in vivo persistence of edited T cells treatment related AEs, and disease progression.

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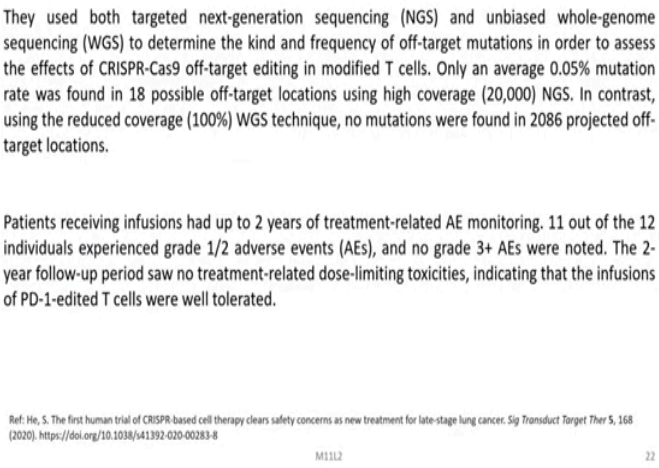


They used both targeted next generation sequencing and unbiased whole genome sequencing to determine the kind and frequency of off-target mutations in order to assess the effects of CRISPR-Cas9 off-target editing in modified T cells. Only on average 0.05 percent mutation rate was found in 18 possible off target locations using high coverage NGS.

In contrast, using the reduced coverage WGS technique no mutations have found in 2086 projected off target locations. Patients receiving infusions had up to 2 years of treatment related AE monitoring, 11 out of the 12 individuals experience grade 1 by 2 adverse events and no grade 3 plus AEs were noted. The 2 year follow up period saw no treatment related dose limiting toxicities indicating that the infusions of PD-1 edited T cells were all well tolerated.

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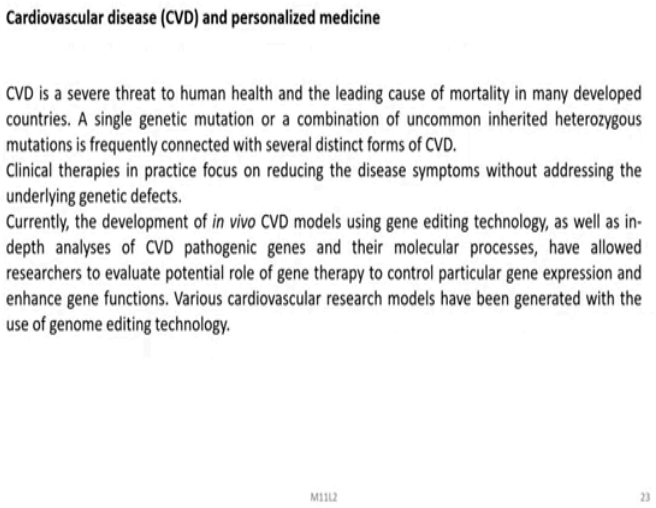


Let us now discussed about another disease in context of the personalized medicine cardiovascular diseases. Cardiovascular disease is a severe trait to human health and the leading cause of mortality in many developed countries. A single genetic mutation or a combination of uncommon inherited heterozygous mutations is frequently connected with several distinct forms of cardiovascular disease.

Clinical therapies in practice focus on reducing the disease symptoms without addressing the underlying genetic defects. Currently the development of in vivo CVD models using gene editing technology, as well as in depth analysis of CVD pathogenic genes and their molecular processes have allowed researchers to evaluate potential role of gene therapy to control particular gene expression and enhance gene functions.

Various cardiovascular research models have been generated with the use of genome editing technologies.

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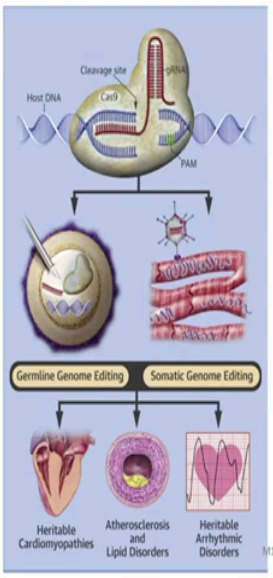


**Figure:** Germline and somatic genome editing approaches and treatable cardiovascular conditions



These figures show germline and somatic genome editing approaches and treatable cardiovascular conditions, like heritable cardiomyopathies atherosclerosis and lipid disorders and heritable arrhythmic disorders.

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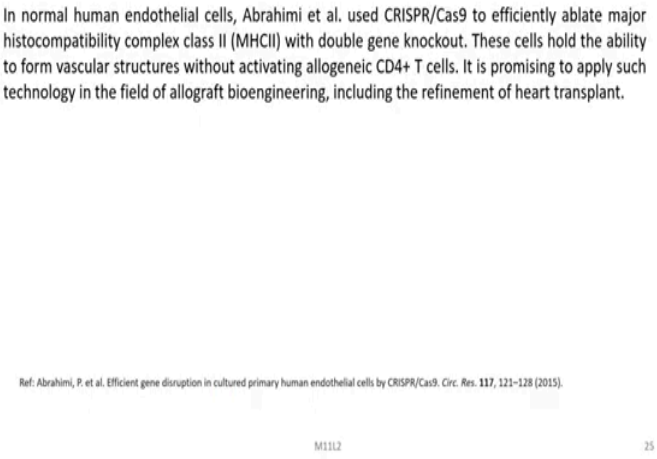


In normal human endothelial cells, Abrahimi et al. used CRISPR-Cas9 to efficiently ablate major histocompatibility complex class II with double gene knockout. These cells hold the ability to form vascular structures without activating allogeneic CD4 plus T cells. It is

promising to apply such technology in the field of allograft bioengineering including the refinement of heart transplant.

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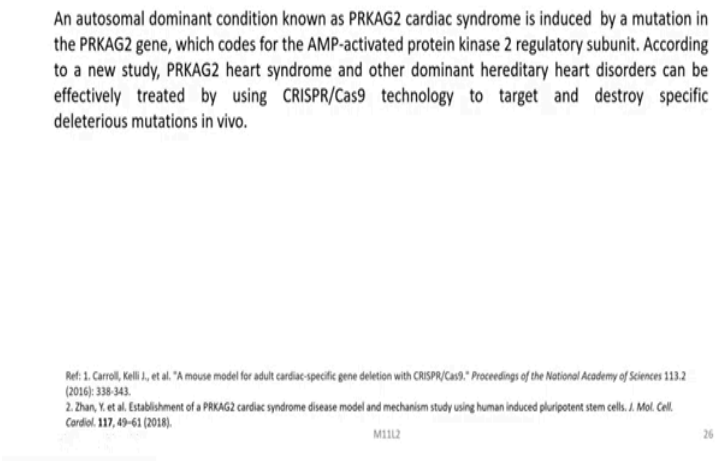


An autosomal dominant condition known as PRKAG2 cardiac syndrome is induced by a mutation in the gene, which codes for the AMP-activated protein kinase 2 regulatory subunit. According to a new study PRKAG2 heart syndrome and other dominant hereditary heart disorders can be effectively treated using CRISPR-Cas9 technology to target and destroy specific deleterious mutations in vivo.

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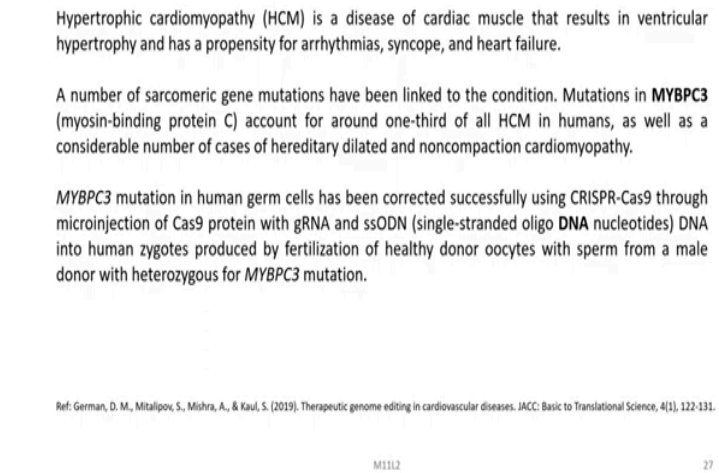

Hypertrophic cardiomyopathy is a disease of cardiac muscle that results in ventricular hypertrophy and has a propensity for arrhythmias, syncope, and heart failure. A number of sarcomeric gene mutations have been linked to the condition. Mutations in MYBPC3 myosin binding protein C, account for around one-third of all HCM in humans, as well as a considerable number of cases of hereditary dilated and noncompaction cardiomyopathy.

MYBPC3 mutation in human germ cells has been corrected successfully using CRISPR-Cas9 through microinjection of Cas9 protein with gRNA and ssODN DNA into human zygotes produced by fertilization of healthy donors oocytes with sperm from a male donor with heterozygous for MYBPC3 mutation.

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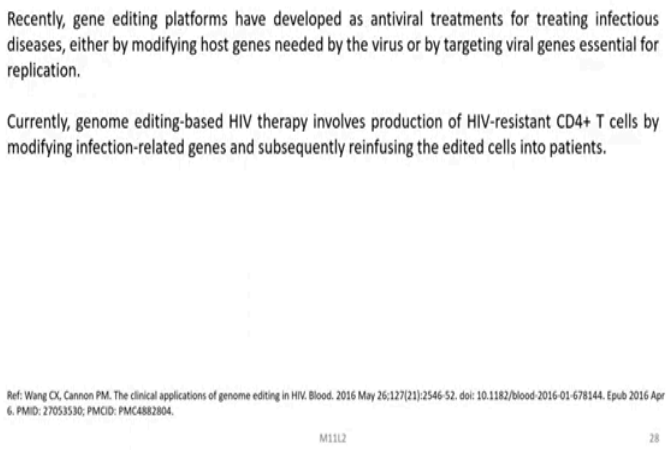


Recently gene editing platforms have been developed as antiviral treatments for treating infectious diseases, either by modifying host genes needed by the virus or by targeting viral genes essential for replication. Currently genome editing based HIV therapy involves a production of HIV resistance CD4 plus T cells by modifying infection related genes and subsequently infusing the edited cells into patients.

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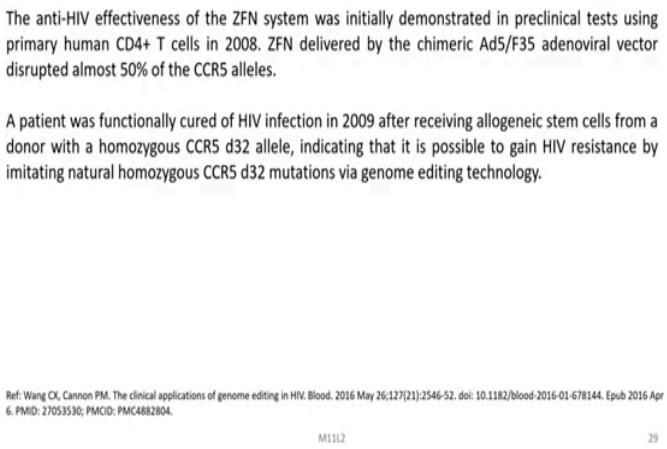


The anti-HIV effectiveness of the ZFN system was initially demonstrated in preclinical test using primary human CD4 plus T cells in 2008, ZFN delivered by the chimeric Ad5 oblique F35 adenoviral vector disrupted almost 50 percent of the CCR5 alleles.

A patient was functionally cured of HIV infection in 2009 after receiving allogeneic stem cells from a donor with homozygous CCR5 d32 allele, indicating that or delta 32 allele indicating that it is possible to gain HIV resistance by initiating imitating natural homozygous CCR5 delta 32 mutations via genome editing technology.

We have discussed about these in length earlier under the lectures on ZFN as well as TALEN and CRISPR-Cas9.

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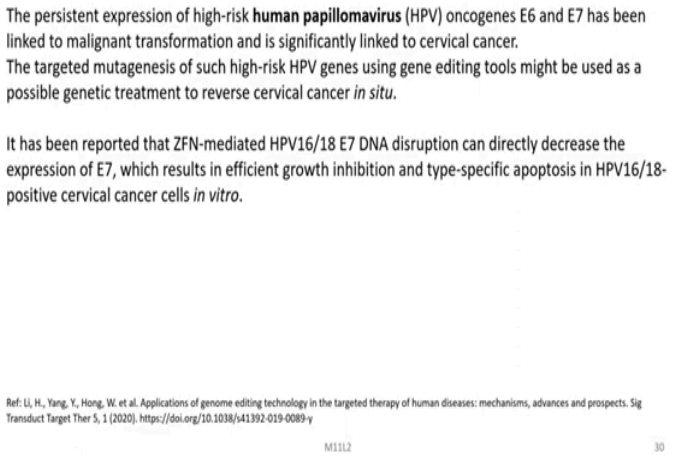


The persistent expression of high-risk human papillomavirus oncogenes E6 and E7 have been linked to malignant transformation and is significantly linked to cervical cancer.

The targeted mutagenesis of such high risk HPV genes using gene editing tools might be used as a possible genetic treatment to reverse cervical cancer in situ. It has been reported that ZFN mediated HPV 16, 18 E7 DNA disruption can directly decrease the expression of E7 which results in efficient growth inhibition and type-specific apoptosis in HPV 16 oblique 18 positive cervical cancer cells in vitro.

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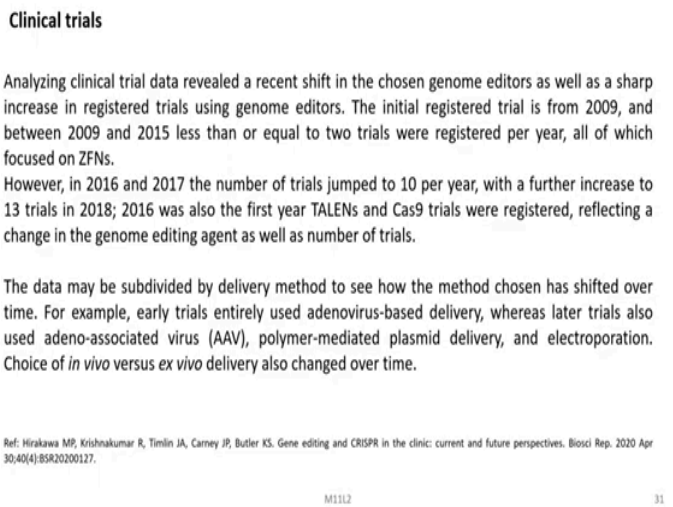

A clinical trials: analyzing clinical trial data revealed a recent shift in the chosen genome editors as well as a sharp increase in registered trials using genome editors. The initial registered trial is from 2009 and between 2009 and 2015, less than or equal to two trials were registered per year all of which focused on ZFNs.

However, in 2016 and 2017 the number of trials jumped to 10 per year with a further increase to 13 trials in 2018; 2016 was also the first year TALENs and Cas9 trials were registered, reflecting a change in the genome editing agent as well as a number of trials. The data may be subdivided by delivery method to see how the method chosen has shifted over time.

For example, early trials entirely used adenovirus based delivery whereas, later trials also used adeno associated virus polymer mediated plasmid delivery and electroporation choice of in vivo versus ex vivo delivery also has changed over time.

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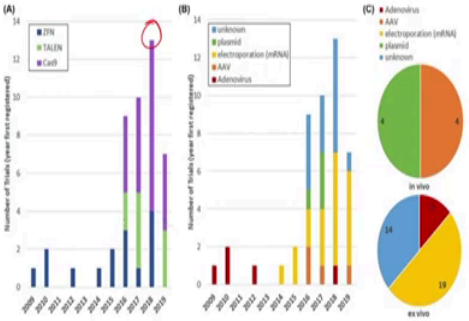


**Figure:** Genome editing trials registered in the U.S. clinical trials database by year and selected genome editor (**A**), or delivery method (**B**) and delivery method grouped by *in vivo* or *ex vivo* use (**C**). Data were accessed 1/01/2020.





And you can see here the data starting from 2009 to 2019 and the number of ZFN trails increasing and then decreasing and again increasing and decreasing and increasing and in 2009 not a single ZFN trial can be reported as seen by this green colored column. And you can see for the first time the appearance of in ZFN sorry TALEN and CRISPR-Cas9 technology trial in 2016.

And over the years the number of the Cas9 technologies are picking up somewhere in 2018 and almost similar in the other years. And while the number of TALEN trials also significant

and of course, there is no any reported in the year 2018. This is the genome editing trials registered in the US clinical trials database by year wise and selected genome editor as I have already shown you the various editing technologies ZFN TALEN and Cas9.

And in figure (B), you can see the chosen delivery method. So, these are unknown these are quite significantly in a way all only small in 2019 and here you can see the use of plasmids as delivery vehicles. And the majority of the times we can see that the electroportion has been used and then we have others like AAVs and adenoviruses.

And you can see the amount or number of trials based on the basis of the delivery method. And they are grouped by in vivo or ex vivo use, so this is the data for in vivo 4 and 4, 8 and this is the data for ex vivo which is much bigger when compared to in vivo.

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| Delivery vector | Nuclease | Phase | Target gene and effect | Disease | Ex vivo/in vivo | Intervention | Sponsor organization | Country | NCT number | Date posted |
|---|---|---|---|---|---|---|---|---|---|---|
| Electroporation (mRNA) | ZFN | I/II | CCR5 knockout | HIV | Ex vivo | Modified CD4+ T cells | Sangamo Biosciences | U.S.A. | NCT02225665 | 8/26/2014 |
| | ZFN | I | CCR5 knockout | HIV | Ex vivo | Modified CD4+ T cells | University of Pennsylvania | U.S.A. | NCT02388594 | 3/17/2015 |
| | ZFN | I | CCR5 knockout | HIV | ex vivo | Modified CD34+ hematopoietic stem cells | City of Hope Medical Center | U.S.A. | NCT02500849 | 7/17/2015 |
| | ZFN | I/II | Disrupt the erythroid enhancer in B-cell lymphoma/leukemia 11A (BCL11A) | β-thalassemia | Ex vivo | Modified hematopoietic stem cells | Sangamo Biosciences | U.S.A. | NCT03432364 | 2/14/2018 |
| | ZFN | I | CCR5 knockout | HIV | Ex vivo | Modified T cells with ZFN-mediated CCR5 deletion as well as the addition of CD4 CAR receptor and modified CXCR4 expression | University of Pennsylvania | U.S.A. | NCT03617198 | 8/6/2018 |

**Table: Interventional trials with ZFN**

Source: Hirakawa MP, Krishnakumar R, Timlin JA, Carney JP, Butler KS. Gene editing and CRISPR in the clinic: current and future perspectives. Biosci Rep. 2020 Apr 30;40(4):BSR20200127

Now, various delivery vectors I have already shown statistically here and this is a table, where you can see electroportion mostly ZFN is using them and particularly CCR 5 knockout for HIV is very very prominent among here. And you can see also some trial for beta thalassemia and many companies like Sangamo Biosciences.

And then you have the Universities of Pennsylvania, City of Hope Medical Center doing these kind of clinical trials with a particular number and date.

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| Delivery vector | Nuclease | Phase | Target gene and effect | Disease | Ex vivo/in vivo | Intervention | Sponsor organization | Country | NCT number | Date posted |
|---|---|---|---|---|---|---|---|---|---|---|
| Electroporation | TALEN | I | TCRα, TCRβ, CD52 knockout | Advanced lymphoid malignancy | Ex vivo | CD19-CAR modified T cells with CAR delivered by lentivirus and TALEN knockout CD52 and TCR to create universal T cells | Institut de Recherches International es Servier | U.K., U.S.A., France | NCT02746952 | 4/21/2016 |
| | TALEN | I | TCRα, TCRβ, CD52 knockout | Refractory B-ALL | Ex vivo | CD19-CAR modified T cells with CAR delivered by lentivirus and TALEN knockout CD52 and TCR to create universal T-cells | Institut de Recherches International es Servier | U.K., Belgium, France, U.S.A. | NCT02808442 | 6/21/2016 |
| | TALEN | I | Programmed cell death 1 (PD-1) and CD52 knockout | Acute myeloid leukemia | Ex vivo | CD123-CAR modified T cells with CAR delivered by lentivirus and TALEN-mediated knockouts | Cellectis S.A. | U.S.A. | NCT03190278 | 6/16/2017 |
| | TALEN | I | Programmed cell death 1 (PD-1) and CD52 knockout | Multiple myeloma | Ex vivo | CS-1-CAR modified T cells with CAR delivered by lentivirus and TALEN-mediated knockouts | Cellectis S.A. | U.S.A. | NCT04142619 | 10/29/2019 |
| | TALEN | I | Programmed cell death 1 (PD-1) and CD52 knockout | CD22+ B cell acute lymphoblastic leukemia | Ex vivo | CD22-CAR modified T cells with CAR delivered by lentivirus and TALEN-mediated knockouts | Cellectis S.A. | U.S.A. | NCT04150497 | 11/4/2019 |

**Table: Interventional trials with TALEN**

Source: Hirakawa MP, Krishnakumar R, Timlin JA, Carney JP, Butler KS. Gene editing and CRISPR in the clinic: current and future perspectives. Biosci Rep. 2020 Apr 30;40(4):BSR20200127 34

So, this table shows you mostly about the TALEN technology being used and target gene and effect like TCR alpha beta CD52 knock out and the PD-1 and CD52 knock out, we have discussed in length about this PD-1 earlier. And then most of these are falling into the ex vivo method and we can see these are being conducted by multiple countries UK, USA, France, Belgium.

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| Delivery vector | Nuclease | Phase | Target gene and effect | Disease | Ex vivo/in vivo | Intervention | Sponsor organization | Country | NCT number | Date posted |
|---|---|---|---|---|---|---|---|---|---|---|
| Electroporation/undefined | Cas9 | I/II | βTCRα, TCRβ, β-2 microglobin (B2M) knockout | B-cell leukemia | Ex vivo | CD19-CAR modified T cells with CAR delivered by lentivirus and Cas9 knockout B2M and TCR to create universal T cells | Chinese PLA General Hospital | China | NCT03166878 | 5/25/2017 |
| | Cas9 | I | TCRα, TCRβ, PD-1 knockout | Various malignancies | Ex vivo | Modified T cells with Cas9-mediated deletions and lentiviral transduction of NY-ESO-1 targeted TCR | University of Pennsylvania | U.S.A. | NCT03399448 | 1/16/2018 |
| | Cas9 | I/II | Disruption of the erythroid enhancer to BCL11A gene | β-thalassemia | Ex vivo | Ex vivo modified hematopoietic stem cells | CRISPR Therapeutics | U.K., Germany | NCT03655678 | 8/31/2018 |
| | Cas9 | I/II | Disruption of the erythroid enhancer to BCL11A gene | Sickle cell anemia | Ex vivo | Ex vivo modified hematopoietic stem cells | Vertex Pharmaceuticals Incorporated and CRISPR Therapeutics | U.S.A. | NCT03745287 | 11/19/2018 |
| | Cas9 | I/II | Creation of a CD19-directed T cell | Refractory B-cell malignancies | Ex vivo | CD19-directed T-cell immunotherapy | CRISPR Therapeutics | U.S.A., Australia | NCT04035434 | 7/29/2019 |
| | Cas9 | I | disruption of HPK1 | refractory B cell malignancies | Ex vivo | CD19-CAR modified T cells with CAR delivered by lentivirus and Cas9 knockout of HPK1 | Xijing Hospital | China | NCT04037566 | 7/30/2019 |

**Table: Interventional trials with TALEN**

Source: Hirakawa MP, Krishnakumar R, Timlin JA, Carney JP, Butler KS. Gene editing and CRISPR in the clinic: current and future perspectives. Biosci Rep. 2020 Apr 30;40(4):BSR20200127 35

And also in certain cases for example, here is the data for the Cas9, again the delivery vector is either electroportion or undefined. But here you can see then countries like Asian countries

like China has also been involved in this kind of trials including European countries like Germany and of course, Australia which is altogether in a different zone and continent.

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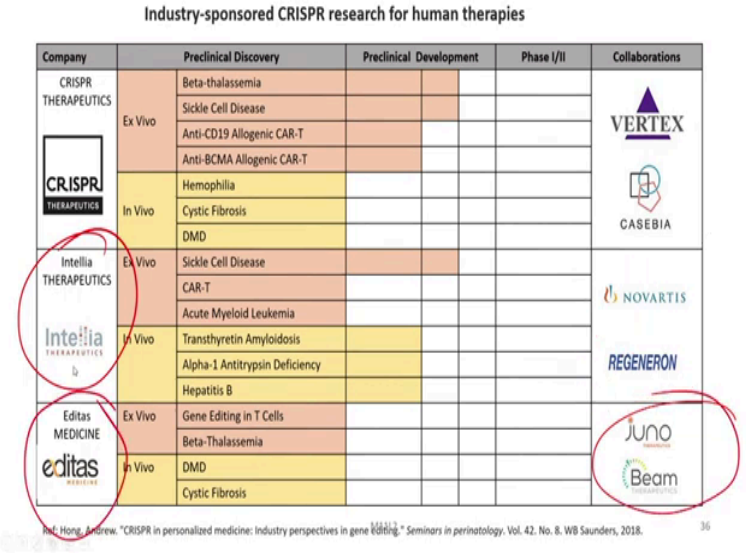


Now, this is the industry sponsored CRISPR research for human therapies, we can see companies like CRISPR Therapeutics, Intellia Therapeutics, Editas Medicine. And the pre-clinical discovery is involved in diseases like beta-thalassemia, Sickle Cell Disease, Anti CD19 Allogenic CAR-T and hemophilia, DMD. Then you have acute myeloid leukemia, then you have Hepatitis B and so on and so forth.

And also, in certain cases certain collaborators like Vertex and Casebia is involved with CRISPR Therapeutics, then you have Novartis and Regeneron is collaborating with the Intellia Therapeutics here and then Juno Therapeutics and Beam Therapeutics is our collaborative partners of Editas Medicine.

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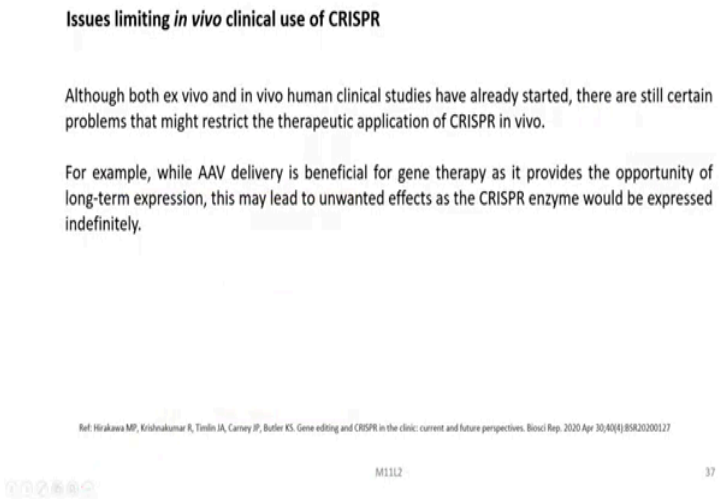


There are certain issues which limits in vivo clinical use of CRISPR. Although both ex vivo and in vivo human clinical studies have already started, and we have also discussed many examples there are still certain problems that may restrict the therapeutic applications of CRISPR in vivo.

For example, while AAV delivery is beneficial for gene therapy as it provides the opportunity of long term expression, this may lead to unwanted effects as the CRISPR enzyme would be expressed indefinitely.

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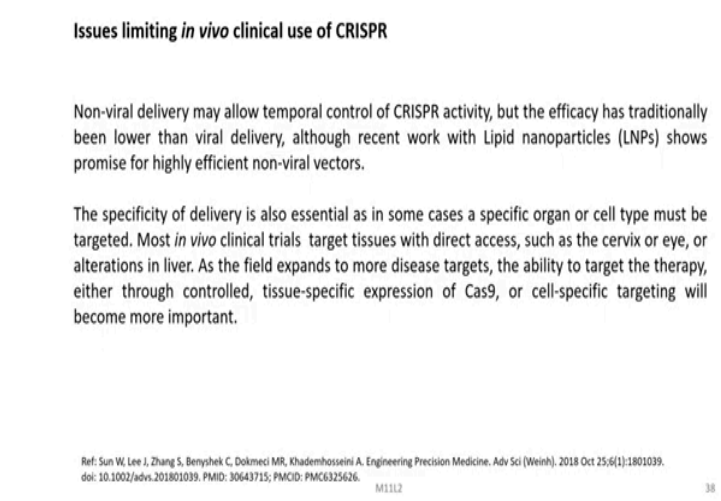

Non-viral delivery may allow temporal control of CRISPR activity, but the efficacy has traditionally been lower than viral delivery, although recent work with lipid nanoparticles show promise for highly efficient non-viral vectors. That specificity of delivery is also essential as in some cases a specific organ or cell type must be targeted.

Most in vivo clinical trials target tissues with direct assist such as the cervix or eye or alterations in liver, as a field expands to more disease targets the ability to target the therapy either to control tissue specific expression of Cas9 or cell specific targeting will become more important.

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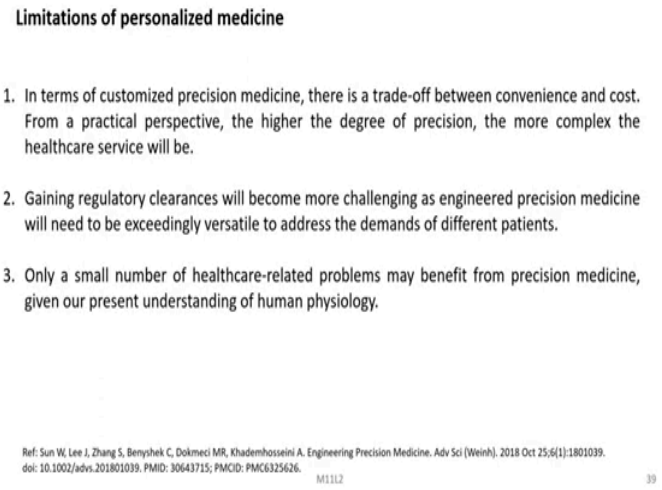


What are the limitations of personalized therapy or medicine? In terms of customized precision medicine there is a tradeoff between convenience and cost. From a practical perspective, the higher the degree of precision, the more complex the healthcare service will be.

Gaining regulatory clearances will become more challenging as engineered precision medicine will need to be exceedingly versatile to address the demands of different patients. Only a small number of healthcare related problems may benefit from a precision medicine, given our present understanding of human physiology.

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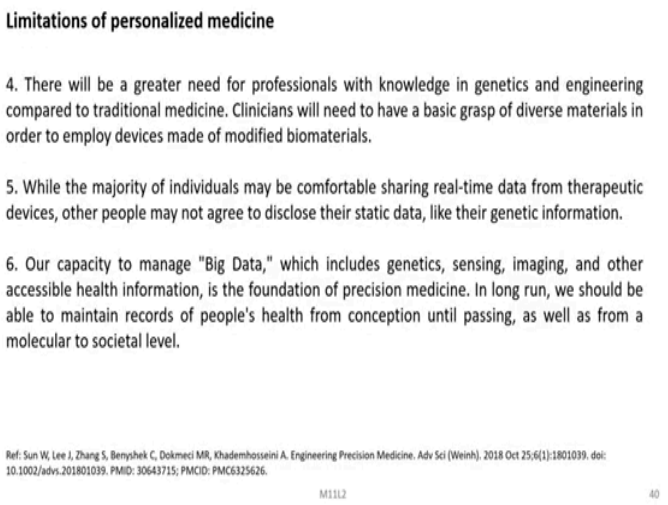


There will be a greater need of professionals with knowledge in genetics and engineering compared to traditional medicine. Clinicians will need to have a basic graphs of diverse materials in order to employ devices made of modified biomaterials. While the majority of individuals may be comfortable sharing real time data from therapeutic devices, other people may not agree to disclose their static data like their genetic information.

Our capacity to manage “Big Data” which includes genetics, sensing, imaging and other accessible health information, is the foundation of precision medicine. In long run, we should be able to maintain records of people’s health from conception until passing, as well as from a molecular to societal level.

Thank you for your patient hearing, with this we come to an end of our discussion on personalized medicine.