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REVIEW ARTICLE

Gene Editing in Clinical Practice: Where are We?

Rama Devi Mittal[1]

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Abstract Multitude of gene-altering capabilities in combination with ease of design and low cost have all led to the adoption of the sophisticated and yet simple gene editing system that are clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR). The CRISPR/Cas9 system holds promise for the correction of deleterious mutations by taking advantage of the homology directed repair pathway and by supplying a correction template to the affected patient's cells. CRISPR is a tool that allows researchers to edit genes very precisely, easily and quickly. It does this by harnessing a mechanism that already existed in bacteria. Basically, there's a protein that acts like a scissors and cuts the DNA, and there's an RNA molecule that directs the scissors to any point on the genome one wants which results basically a word processor for genes. An entire gene can be taken out, put one in, or even edit just a single letter within a gene. Several platforms for molecular scissors that enable targeted genome engineering have been developed, including zinc-finger nucleases, transcription activator-like effector nucleases and, most recently, CRISPR/CRISPR-associated-9 (Cas9). The CRISPR/Cas9 system's simplicity, facile engineering and amenability to multiplexing make it the system of choice for many applications. CRISPR/Cas9 has been used to generate disease models to study genetic diseases. Improvements are urgently needed for various aspects of the CRISPR/Cas9 system, including the system's precision, delivery and control over the outcome of the repair process. However, there are still some glitches to be mended like how to regulate gene drives and its safeguards. The creation of gene knockouts is one of the first and most widely used applications of the CRISPR–Cas9 system. Nuclease-active Cas9 creates a double-strand break at the single guide RNA-targeted locus. These breaks can be repaired by homologous recombination, which can be used to introduce new mutations. When the double-strand break is repaired by the error-prone nonhomologous end joining process, indels are introduced which can produce frame shifts and stop codons, leading to functional knockout of the gene. Precedence modification have to be done on mechanism of CRISPR/Cas9, including its biochemical and structural implications incorporating the latest improvements in the CRISPR/Cas9 system, especially Cas9 protein modifications for customization. Current applications, where the versatile CRISPR/Cas9 system is to be used to edit the genome, epigenome, or RNA of various organisms is debated. Although CRISPR/Cas9 allows convenient genome editing accompanied by many benefits, one should not ignore the significant ethical and biosafety concerns that it raises. Conclusively lot of prospective applications and challenges of several promising techniques adapted from CRISPR/Cas9. Is discussed. Although many mechanistic questions remain to be answered and several challenges to be addressed yet, the use of CRISPR–Cas9-based genome technologies will increase our knowledge of disease process and their treatment in near future. Undoubtedly this field is revolutionizing in current era and may open new vistas in the treatment of fatal genetic disease.

Keywords Genome editing · CASPR Cas9 systems · Molecular scissors · Palindromic repeats · Nucleases · Gene targeting · Gene therapy embryo · Ethics · Potential pitfalls

✉ Rama Devi Mittal
ramamittal@gmail.com

[1] Department of Urology and Renal Transplantation, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raebareli Road, Lucknow, Uttar Pradesh 226014, India

Introduction

Over the past several decades the drive behind those in the field of genetic engineering has been to improve our understanding of normal and disease processes and therewith enable the design of new diagnostic and treatment procedures. These techniques might eventually be applicable to gene therapy. The progression of the field from transgenesis to gene targeting, to embryonic stem cells (ESCs), to induced pluripotent stem cells (iPSC), and recently to gene editing has contributed immensely to fulfill some of the dreams.

The emergent technology of next-generation sequencing, which has greatly powered genome-wide association studies, has successfully identified numerous common genetic defects associated with important human diseases [1, 2] Amongst some genetic tools, the most talked about gene editing tool is CASPR–Cas9 which has made the headlines all over the world because of its potential to change the human race. CRISPR gene-editing technology has been taking the medical world by storm, showing potential for treating diseases ranging from cancer to type 2 diabetes. The technology has been moving full-steam ahead, with a trial in humans already started, even as the repercussions of gene editing remain largely unknown.

CRISPR, or clustered regularly interspaced short palindromic repeat, is at the most basic level a very precise way of tinkering with genes. Whereas gene editing was once a very imprecise and expensive process, scientists can now go into your DNA and essentially cut and paste it at specified places. The technology can be traced back to bacteria, which protect themselves by cutting out invading viruses' DNA and inserting it into their own, then replicating the new sequences. In 2012, researchers refined the system and revealed that any DNA (not just bacteria) has this ability—and the process works in humans. In 2012, scientists turned CRISPR from a bacterial shield into a gene-editing tool. They replaced the bacterial CRISPR RNA system with a modified guide RNA. This RNA acts as a kind of 'wanted poster'—it tells a bounty hunter enzyme called CAS9 where to look. The enzyme scans the cell's genome to find a DNA match then slices for the DNA in the cell's enzymes. To repair damage at that point, scientists can change or add DNA within the cell. By feeding CAS9 the right sequence or guide RNA, scientists can cut and paste parts of the DNA sequence, up to 20 bases long, into the genome at any point.

Genome editing was selected by Nature Methods as the 2011 Method of the Year. CRISPR was first used from Osaka University researcher Yoshizumi Ishino in 1987, who accidentally cloned part of a CRISPR together with the iap gene, the target of interest [3.] CRISPR technology i.e. CRISPR–Cas9 consists of two key molecules that introduce a change (mutation) into the DNA…. This acts as a pair of 'molecular scissors' that can cut the two strands of DNA at a specific location in the genome so that bits of DNA can then be added or removed. a piece of RNA called guide RNA (gRNAThe protein Cas9 (or "CRISPR-associated") is an enzyme that acts like a pair of molecular scissors, capable of cutting strands of DNA [4, 5]. CRISPR–Cas9 is a unique technology that enables geneticists and medical researchers to edit parts of the genome by removing, adding or altering sections of the DNA sequence. It is currently the simplest, most versatile and precise method of genetic manipulation and is therefore causing a buzz in the medical field. The protein Cas9 (or "CRISPR-associated") is an enzyme that acts like a pair of molecular scissors, capable of cutting strands of DNA [4]. One would wonder how CRISPR works. CRISPR "spacer" sequences are transcribed into short RNA sequences ("CRISPR RNAs" or "crRNAs") capable of guiding the system to matching sequences of DNA. When the target DNA is found, Cas9—one of the enzymes produced by the CRISPR system—binds to the DNA and cuts it, shutting the targeted gene off. Now one would wonder about working of CRISPR for which a desired gene to be manipulated is first selected. Where it has to be expressed is decided by the choice of expression system. The target sequence is thereupon selected and gRNA then designed for further action. Here Cas9 (CRISPR associated protein 9) which is an RNA-guided DNA endonuclease enzyme associated with the clustered regularly interspersed short palindromic repeats (CRISPR) is adaptive immunity system in Streptococcus pyogenes, among other bacteria. Since CRISPR (clustered regularly interspaced palindromic repeats) is a defense mechanism, present in bacteria and archaea, which confers immunity against phages…. The CRISPR system protects prokaryotic cells by destroying viral DNA after it has entered the cell. The CRISPR–Cas9 components can be delivered as DNA, RNA, or protein, as indicated, and introduced into the cell or embryo through injection, transfection, electroporation.

Several researchers made key contributions to the field of CRISPR genome editing, including molecular biologists Charpentier and Doudna [6–9]. But it wasn't until Zhang's team demonstrated the use of engineered CRISPR–Cas9 to edit the genomes of living mouse and human cells in 2013 that its full potential became evident. In everyone's view, Zhang's article in Science is a landmark that transformed molecular biology. Today, thousands of researchers use this molecular scalpel to edit DNA for research and potential therapeutic purposes. In 2013, Feng Zhang through his continuous efforts opened the window through which genome editing became a therapeutic possibility [10] when he engineered a novel version of CRISPR–Cas9

to edit human genomes [10]. The speed and efficiency of CRISPR–Cas9 is a remarkable leap in research. This feature can enable it to increase the identification of genes that are associated with human diseases and facilitate the development of therapies to correct the mutated gene [8]. Due to its unparalleled genetic specificity, scientists are using CRISPR–Cas9 genomic editing technology to facilitate discoveries in cancer biology. Cancer models have been developed using CRISPR–Cas9. The models better reflect the disease in humans [6]. Feng Zhang and Nobel laureate Phillip Sharp [11] successfully engineered a mice model using CRISPR–Cas9 to model the deleterious effects of mutations in cancer. The ability of their system to introduce loss of function mutations in tumor suppressor genes and gain of function in proto-oncogenes facilitate screening of causal genetic mutations [6].

These applications make CRISPR/Cas9, a technology of choice to edit disease causing mutations as well as the epigenome more efficiently than ever before. Meanwhile its application in in vivo and ex vivo cells is encouraging the scientific community for more vigorous gene therapy and in clinical setups for therapeutic genome editing.

CRISPR/Cas9: A Cure for Cancer and Other Genetic Diseases

Alterations in genome and epigenome resulting in activation of oncogenes or inactivation of cancer suppressor genes cause cancer. Available therapies in recent years for cancers have emerged to progress prognosis in patients. Surgery, Chemotherapy, Radiotherapy have been used in combination to remission, of cancerous cells that gives benefit to patients a lifespan to a maximum of 5 years. However, harmful side effects and toxicity increases the mortality, significantly reducing the quality of life [12–14] In this aspect understanding of cancer biology is of key importance to develop novel anti-cancer therapies. The present-day advances in sequencing technology have helped to explore the cancer genome more efficiently with much lower cost.

Cancers are characterized by mutations, gene duplication and alterations in DNA and RNA including, changes in messenger RNAs. Hence integrative approach to utilize genomic and transcriptomic advances can reveal the complete picture of individual genome. This approach is also being used in clinical setting to make critical decisions regarding patient treatment [15].

Cancer exists in multiple complex forms making it difficult to prevent and/or treat. It is therefore most important to study aetiology, pathogenesis, prognosis and its phenotypes to develop new therapies. Hence Genetic engineering is now crucial in the treatment of cancer and other genetic diseases especially the formerly-niche use of clustered regularly interspaced short palindromic repeats (CRISPR) associated with Cas9. To date approximately 140 genes with delirious mutations have been reported.

Recent advances indicate CRISPR/Cas9 as a therapy of choice. There have been several important genetic mutations where CRISPRs can be repurposed to create adaptive immunity to fight carcinomas and edit genetic mutations causing it. Challenges to CRISPR technology have also been discussed with emphasis on ability of pathogens to evolve against CRISPRs in a recent good review by Khan et al. [16]. Recent developments on the function of CRISPRs with different carriers which can efficiently deliver it to target cells; furthermore, analogous technologies are also discussed along CRISPRs, including zinc-finger nuclease (ZFN) and transcription activator-like effector nucleases (TALENs All these details have been discussed in several reviews and reports [17–22] and I shall not deal with it here.

Ethical Concerns and Implications of CRISPR–Cas9 Human Germline Editing

While Genome editing of somatic cells, which is at its various clinical stages, is a promising area of therapeutic development the use of CRISPR–Cas9 embryo genome editing that could completely eradicate genetic diseases, scientists have warned that it should be treated with caution. George Daley, a stem-cell biologist at Harvard Medical School in Boston, Massachusetts, stated that even though research reported is a landmark but has a cautionary tale that the technique is not yet ready for testing to eradicate genetic diseases [23]. Since germ line modification causes genetic changes to the embryos, changes that are heritable, this technique can have unpredictable effects to the future generations. Moreover, unethical uses of the technique could emerge from gene editing of the human embryos [23]. Genome editing in human embryos using CRISPR–Cas9 could have unpredictable effects to the future generations. CRISPR–Cas9 technology could be used for non-therapeutic modifications [24].

Genome editing of the human embryo could hinder the ongoing research that involve gene editing of somatic cells that hold promise for therapeutic development. As rightly pointed out by Lanphier et al. [25], the public outcry about the ethical breach of human embryo genome editing could hinder the promising area of therapeutic development that are involved in making genetic changes in somatic cells and there should be an open discussion around the appropriate action should a compelling case arise for therapeutic benefit of germ line modification [25, 26]. The nuclease may not be as efficient. The nuclease may not necessarily cleave both copies of the target gene or the cells may start dividing before the corrections are completed, resulting in

genetic mosaic [27]. Mosaicism is the presence of the populations of somatic cells that are genetically distinct in an organism. Mosaicism is frequently masked. However, mosaicism can cause major phenotypic changes and reveal the expression of lethal genetic mutations [28]. Some of the genetic disorders that result from mosaicism include: Down syndrome, Klinefelter syndrome and Turner syndrome.

Another question that may arise regarding the embryo genome editing using CRISPR–Cas9 editing technology is the fate of the child produced by such technologies? While it is clear that people's informed consent is secured before genetically engineered somatic cells are used in clinical research, it is not clear what information would be needed from the prospective parents to adequately inform them about the risks involved in germ line modification [27]. The scientific community should engage in a dialogue to establish guidelines of research involving genetic modification of human germ cells. The discussions should involve stakeholders in different fields: the general public, scientists, bioethicists, public policy and legal experts. The discussion should make a clear distinction between genome editing in germ cells and in somatic cells. The significant progress being made in clinical development of approaches to cure deleterious diseases should not be impeded by concerns regarding the ethical implications of germline editing [27, 28]. A voluntary moratorium should be called on genetic modification. According to Harris, the side effects of germ line editing should not be used as a justification to call a moratorium on genetic modification of human germ cells. It may be ethically justifiable to make the technique available in clinics. He argues that the genetic disease may be worse than the side effects because people with genetic disease will go on reproducing [29, 30] and their progeny stand a higher chance of inheriting the defective gene responsible for a genetic disorder.

Pro and Cons of CRISPR–Cas9 Technology

In February 2017, request by the Francis Crick Institute in London to modify human embryos using the new gene editing technique CRISPR–Cas9 was approved by the Human Fertilization and Embryology Authority in the United Kingdom. The scientists here hope to emphasize on early embryo development that may eventually lead to safer and more successful fertility treatments. The embryos, provided by patients undergoing in vitro fertilization, will not be allowed to develop beyond 7 days. But ultimately in practice—CRISPR could be used to modify disease-causing genes in embryos brought to term, removing the faulty script from the genetic code of that person's future descendants as well. The supporters believe that such "human germline editing" could potentially decrease, or even eliminate, the incidence of many serious genetic diseases, reducing human suffering. However, opponents on the other hand opine that modifying human embryos is dangerous and unnatural. They also feel that modifying the embryos is like playing God. This argument rests on the evidence that natural is inherently good. But diseases are natural, and humans by the millions fall ill and die prematurely—all perfectly naturally. If we protected natural creatures and natural phenomena simply because they are natural, we would not be able to use antibiotics to kill bacteria or otherwise practice medicine, or combat drought, famine, or pestilence.

Future of Gene Editing Using CRISPR–Cas9 Technology

CRISPR–Cas9 gene editing technology though still in infancy stage has opened up a whole lot of possibilities for the treatment and prevention of human disease and genetic abnormalities. Simultaneously It's also opening a can of ethical worms regarding the limitations should be—particularly with respect to altering human embryos. There have been several stories in the news recently about human embryo gene editing: both good and bad. The development of CRISPR–Cas9 technology, one of the most recent and celebrated gene editing techniques, has resulted in a series of experiments using human embryos that have brought the ethics of gene editing to the fore.

It started in 2015 when a team of Chinese researchers used a gene editing technique called CRISPR–Cas9 to edit the genes of human embryos in an effort to 'remove' the part of the DNA that was responsible for a fatal blood disorder (β-thalassaemia) published in Protein and Cell, [31]. More recently, in August of 2017, an international team of researchers released a paper in Nature [32] in which they manipulated human embryos using CRISPR–Cas to ascertain whether or not this technique could be successfully used in the removal of a fatal genetic mutation. Research on gene editing (especially germ line editing involving human embryos) has situated researchers in the middle of an ethical quagmire. Similarly a review published in Human Genomics by Capps et al. [33–35] briefly examined the ethical issues surrounding this new technology in their paper wherein they examined the ethical complications of such research but the need to balance this with the greater good of human health and medical progress.

Whether someone is for or against genetic editing, what's clear is that researchers are eager to refine and perfect gene editing technology given the seemingly limitless potential uses that could benefit humanity in

countless ways [36]. Reliable, accurate gene editing could revolutionize the way people think about their decision to have offspring or the fact that they have tested positive for a genetic mutation linked with a specific disease or disorder. This issue therefore calls for a thorough genetic counseling Sharon Chen (MS, CGC), a Genetic Counselor at Northwell Health Division of Medical Genetics and Genomics stated that she believes this technology is here to stay and the big question is: how is this going to be used and what are the potential side effects?

What Would the Widespread Access to CRISPR–Cas9 Mean for a Genetic Counselor?

The majority of the patients Sharon counsels are expectant parents or couples that are looking to get pregnant who have been identified as at risk. Sharon considers that gene editing (especially germ line editing) could have a major impact on the options counselors would be able to provide. Currently, couples at risk are provided rather limited options: they can find out in advance if the pregnancy is impacted (in utero testing) and make an informed determination about keeping or terminating the pregnancy or they can do IVF/PGD (in vitro fertilization/pre-implantation genetic diagnosis) in which each embryo is tested and only the unaffected embryos are then implanted. If gene editing technology, such as CRISPR–Cas9, becomes more widely available then counselors have a host of new options. Furthermore, Sharon believes that "if gene editing were available, then we could then talk about a third option: 'curing' the baby. This could mean having the baby first and then curing it, or even curing it in utero."

The Risks of Gene Editing Technology in the Real World

However, as others have also noted, introducing this technology is not without its risks. "There's always the risk of unintended consequences since the technology is not yet perfect: they might edit the gene responsible for Tay–Sachs and in the process mutate the gene for tumor suppression, introducing an entirely new medical challenge." There's also the possibility, for couples that go the IVF route, that the 'fixed' embryo ends up not actually being fixed (or with a new unintended genetic mutation, which has medical consequences)—leaving them back where they started. Important ethical considerations cannot be ignored with this kind of medical intervention. Sharon notes that while this technology could be used to do so much good, there's a "slippery slope" and it's easy to imagine an emergence in wanting to make non-essential genetic edits to embryos (such as selecting for eye color, height, etc.). With no international agreement in the medical or legislative community outlining the 'rules' for the use of gene editing, it's a technology that could theoretically be used to create so-called "designer babies." Sharon points out that part of the difficulty is that researchers in China have already done things with this technology that have not been permitted in the U.S., so the lack of international guidelines for a powerful technology such as this is problematic [37].

Lastly, and possibly the most disconcerting consideration Sharon raises is the likelihood that gene editing options will only be available to those who can afford it. "As it is already, IVF is incredibly expensive (around $10,000 per treatment) and isn't covered by most insurance companies. Factor PGD into it, and you're looking at a lot more money" she noted. If gene editing were added into these costs, they could become astronomical, limiting access to only the very wealthy.

What Other Scientific Uses Might CRISPR have Beyond Genome Editing?

CRISPR genome editing allows scientists to quickly create cell and animal models, which researchers can use to accelerate research into diseases such as cancer and mental illness. In addition, CRISPR is now being developed as a rapid diagnostic [38]. To help encourage this type of research worldwide, Feng Zhang and his team have trained thousands of researchers in the use of CRISPR genome editing technology through direct education and by sharing more than 40,000 CRISPR components with academic laboratories around the world.

Conclusion

The significant contributions made all across the world by pioneer scientists in this field especially by Jennifer Doudna and Feng Zhang in 2012 on gene editing using CASPR Cas9 technology has taken tremendous leap. The CRISPR/Cas9 system's simplicity, facile engineering and amenability to multiplexing make it the system of choice for many applications. CRISPR/Cas9 has been used to generate disease models to study genetic diseases. CRISPR is the most accurate form of gene editing so far, but it isn't perfect. There are 3bn bases in the human genome so there is always a chance of a stray 20-base match and a fatal cut in the wrong place. A debate is taking place on whether to allow gene edits only outside the body (with the edited cells reinserted) or to allow editing of eggs and sperm, which changes that germline forever. Prof. Doudna however, cautions for germline editing, pointing out that mitochondrial replacement therapy, which also leads to permanent genetic alteration, is already a reality in the UK.

For now, the most exciting potential medical application is in single gene diseases, such as cystic fibrosis, sickle-cell anaemia and muscular dystrophy. This is the simplest possible task for CRISPR. Just one base has to be corrected out of the 3bn and it's not a needle in a haystack: CRISPR can find and cut and repair it. Sickle-cell anaemia is caused by a faulty haemoglobin gene, so blood can easily be withdrawn from the body, the gene edited and returned to the body. But this approach demands extreme caution.

The CRISPR/Cas9 system is poised to revolutionize functional biology, biotechnology and genomic medicine. This technology has vast applications which will significantly improve knowledge of the molecular underpinnings of key cellular processes. Additionally, it will help in generating disease models and improve the efficiency of drug discovery and development. Molecular surgery, in which nucleotides are stitched to edit causative disease sequences, will become possible. Genome editing is finally close to being able to be used at the clinical bedside, and improving the efficiency, specificity and safety of gene editing reagents will unlock myriad applications in genetic medicine. This will undoubtedly improve human life by enabling treatment of diseases that are currently beyond our control and personalized medicine for effective treatment of individuals. Germline engineering applications are troublesome, but every advancement in human civilization involves unique risks; regulations should empower research aimed at improving these tools and understanding the genetic basis of human diseases while preventing applications intended to 'improve' the species or produce 'super-humans'. Assuming that these technologies are handled and applied appropriately, CRISPR/Cas9-based genomic surgeries will undoubtedly improve human life.

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