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The Current Role and Future Potential of CRISPR-Cas9 in Breast Cancer Research

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Abstract. Breast cancer is one of the most prevalent and deadly cancers affecting women globally. It is characterized by the uncontrolled proliferation of epithelial cells originating in the ducts of breast tissue. It is one of the leading causes of death in women. Despite advances and continued development in conventional treatment options, like chemotherapy and endocrine therapy, drug resistance and recurrence remain longstanding obstacles. Therefore, CRISPR-Cas9 emerges as a promising treatment due to its high precision and effectiveness. This study reviews the mechanism by which CRISPR- Cas9 functions to correct and remove parts of the human genome. It also examines how CRISPR-Cas9 combats drug resistance: the major challenge in breast cancer treatment. The review highlights challenges in the applications of CRISPR-Cas9, focusing on off-target effects and ethical concerns. The review also addresses the future research areas for the further development of CRISPR-Cas9 including combating problems with off-target effects, delivery systems, and pre-existing immunity. Ultimately, while CRISPR-Cas9 has great potential in breast cancer treatment, there are still future steps needed to be taken to ensure a better prognosis for patients.

Keywords: breast cancer, CRISPR-Cas9, personalized therapy, gene

1. Introduction

According to the World Health Organization (WHO), in 2022, 2.3 million women globally were diagnosed with breast cancer with 670000 reported deaths [1]. Such high statistics remind readers that breast cancer remains an immense health burden on global health. Furthermore, according to predictions, breast cancer cases and deaths are projected to rise in the following years. Scientists have predicted that the number of breast cancer cases will increase by 40%, possibly reaching 3 million cases by 2040, with breast cancer deaths increasing by over 50% and hitting 1 million in 2040 [2]. These concerning statistics emphasize the need for new strategies to prevent, detect, and cure breast cancer. Breast cancer is the most frequently diagnosed cancer in women. where 99% of breast cancer cases occurring in females. Breast cancer risks increase by age, and is also influenced by family history and reproductive history. However, gene mutations are the primary reason for a higher breast cancer risk. Specifically, patients with mutations in the BRCA1, BRCA2, and PALB2 genes are much more susceptible to breast cancer [1]. Breast cancer being a disease where abnormal

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cells grow to become tumors that metastasize throughout the body can become extremely fatal if not treated and caught early.

Complicating treatments further, breast cancer is an extremely heterogenous disease. With multiple stages and multiple subtypes that require differentiating treatments and yield different results, personalized therapy has become urgent due to the relatively high rates of drug resistance in standard treatments such as immunotherapy [3]. Therefore, despite the continuous progress in conventional treatment and detection efforts, breast cancer cases are still projected to grow in the future. This highlights a pressing need for personalized therapy that can provide patients with personalized treatment to overcome drug resistance challenges. This is where the development and usage of CRISPR-Cas9 as a treatment has become extremely promising. CRISPR-Cas9 provides an highly personalized treatment option, enabling scientists to target the necessary genes to insert, delete, or correct the defective genes. Given its precision and versatility, CRISPR-Cas9 has the potential to overcome one of the largest barriers in breast cancer treatment, such as drug resistance [4]. However, CRISPR-Cas9 is not perfect, as its clinical applications still face challenges both technologically and ethically. It still holds possible risks, such as off-target effects and ethical concerns [4]. Nevertheless, the future prospects of CRISPR-Cas9 applied in breast cancer treatment are still bright and promising, This review will explore current applications of CRISPR-Cas9, evaluate how it is able to overcome drug resistance, underline challenges, and discuss the future prospects of this technology in breast cancer research.

2. A comprehensive overview of CRISPR-Cas9

CRISPR-Cas9 originally discovered as an adaptive form of immunity in prokaryotes has now become a transformative and effective tool of genome editing in living organisms that has a comprehensive range of applications including medicine, agriculture, and biotechnology [5]. This tool comprises two key components: a Guide RNA (gRNA) and a CRISPR associated Cas9 protein. CRISPR, clustered regularly interspaced short palindromic repeat, and Cas9, its associated protein, work together to recognize, cleave, and revise genes [6]. The CRISPR-Cas9 system originated from the adaptive immunity against bacteriophages and viruses in prokaryotes. CRISPR was first discovered by Ishino and his team in 1987 in Escherichia coli, where unusual repetitive palindromic DNA sequences interrupted by spacers were found. In 1990, Mojica noticed similar sequences in other prokaryotes, and thus named it CRISPR. Not until 2007 did researchers confirm the essential role of CRISPR in bacterial adaptive immunity, enabling the bacteria to recognize and defend themselves form future infections by the same virus. Finally, in 2012, Doudna and Charpentier discovered the use of CRISPR-Cas9 as a gene editing tool. Ever since, it has been one of the most effective, efficient, and accurate gene editing tools for scientists [6].

The CRISPR-Cas9 mechanism consists of three mains steps: recognition, cleavage, and repair [6]. The first step of recognition is critical for a precise cleavage. This step begins with the formation of a complex between the Cas9 protein and the sgRNA (single-guide RNA)/ Then the Cas9 nuclease is able to successfully recognize and bind the target site only when it detects the protospacer adjacent motif (PAM), ensuring accurate and site-specific gene targeting [5]. Once Cas9-sgRNA complex successfully binds to target DNA, the cleavage by CRISPR-Cas9 is initiated.

After recognition, the DNA duplex near the target site is locally unwound, enabling the sgRNA to base-pair with the complementary DNA strand, forming a DNA-RNA heteroduplex [5]. This formation triggers the activation of two nuclease domains in the Cas9 protein: HNH and the RuvC domain. The HNH domain cleaves the DNA strand complementary to the sgRNA, while the RuvC

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domains cleaves the non-complementary end. Together, HNH and the RuvC domain create a site-specific double-stranded break (DSB) [6] .

For repair, there are two main repair machineries: non-homologous end joining pathway (NHEJ) and homology-directed repair pathway. Only in the presence of DSBs can these two repair machineries be activated. NHEJ is a repair mechanism where the two broken ends of a DNA double-stranded break (DSB) are directly rejoined to restore the DNA strand. However, this method may lead to random insertions or deletions at the target site. The second method: homology-directed repair requires either a single or double-stranded homologous DNA template. This method allows for a more precise correction for inserting point mutations and inserting new DNA sequences [5]. Overall, CRISPR-Cas9 is a powerful and precise gene editing tool with broad applications across the science and medical field. In particular, CRISPR-Cas9 has become especially prominent in the field of cancer research and treatment.

3. CRISPR-Cas9 against drug resistance in breast cancer

The importance of overcoming along with evidence that it is a significant issue in cancer, cannot be overstated. Although there are many therapeutic options available for breast cancer treatment, the concern of drug resistance has been a persisting problem for many of these therapeutics. For instance, drug resistance is a significant problem for the drug tamoxifen, a selective ER modulator, one of the most successful treatment options for ER+ breast cancer. Although this drug is able to reduce risk of recurrence by 41% and mortality rates by 34%, more than 40% of ER+ patients with advanced disease are treated with tamoxifen the drug become unresponsive due to the adaptive resistance [7]. This acquired resistance is not only a demanding problem in this one drug, overall 90% of unsuccessful treatments for breast cancer are a result of drug resistance [7]. Therefore, that these high statistics present a rising and persisting concern to overcome drug resistance in breast cancer. Throughout the progression of breast cancer, the risks of cellular genes mutating to become oncogenes are extremely. These oncogenes with ER or HER2 do pose significant potential for treatment. ER mutations are generally helpful to helping scientists understand resistance to endocrine therapy. Studies have shown that endocrine therapy resistance is largely caused by either ER mutations or translocations. For instance, abnormal expressions of ER truncated isoforms are related to decrease effectiveness of a breast cancer medication: tamoxifen [7]. The HER2 mutation is also responsible for endocrine resistance, due to its crosstalk with ER mutations, this means that even if ER pathways are targeted by therapy, HER2 pathways can still send signals that promote cancer cell growth and survival [7].

Drug resistance in breast cancer, even cancer has been a longstanding problem. However, conventional methods for overcoming drug resistance have presented inconsistent outcomes. To resolve this problem scientists have utilized many methods to target onco-genes and drug-resistant genes. These methods are classified into two types: loss of function, like RNAi, and gain of function, which is cDNA base overexpression of the gene of interest. Nevertheless, these two methods do have significant drawbacks [8]. RNAi sometimes fails to fully knockdown a gene, allowing the gene to still be functional. However, a total inactivation of the gene is necessary for gene interpret a gene's role. cDNA induce supraphysiological levels of gene expression which leads to inaccurate results. Furthermore, since the development of cancer is a complex process, it has always been challenging to fully interpret the role of each mutation or multiple mutation that lead to tumorigenesis [8].

Scientists started using CRISPR-Cas9 as an alternative approach in overcoming the limitations of RNAi and cDNA. CRISPR-Cas9 has been an effective method since for overcoming drug

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resistance. It has had success in providing efficient gene disruptions and modifications for multiple cancers cells via CRISPR-Cas9 mediated NHEJ, HDR, and for targeting and disrupting cancer oncogenes. The CRISPR-Cas9 mechanism can disrupt the specific functional domains of ER or HER2, it is also predicted to target mutated exons on ER and HER2 genes. This repair mechanism has the potential to eliminate the acquired drug resistance. In addition, it also provides a form of personalized treatment for every specific patient with permanent and promised results [7]. CRISPR-Cas9 is not only an effective treatment itself, it can also be paired with other treatment methods to deliver more promising and more targeted treatment options. Specifically, CRISPR-Cas9 can reverse drug resistance for endocrine therapies which ensures efficient delivery and response of drug [7]. This could be paired with other treatment options like surgery, radiation therapy, and chemotherapy to ensure a more effective treatment.

4. Challenges in the clinical application of CRISPR-Cas9

While CRISPR-Cas9 holds great treatment promises, it does face both technical and ethical challenges when it comes to clinical applications. CRISPR-Cas9 and the idea of gene editing has always faced great controversy. These public disapprovals reach extremes especially when it comes to altering human embryos [9]. Although strict regulations do not apply to CRISPR-Cas9 when used as a treatment option for breast cancer, discussions are still constantly being made regarding its responsible use on human patients. For instance, in the past years the scientific community has hosted two summits regarding human genome editing to discuss the appropriate guidelines for the usage of CRISPR-Cas9 in both germline (inheritable) and somatic (non-inheritable) human gene editing. Overall, it has been concluded that somatic gene editing is more tolerable than ones done on germlines, but it is inevitable that in the future specific guidelines will be applicable to both germline and somatic gene editing practices [10].

In addition to ethical constraints, CRISPR-Cas9 also faces a significant technical difficulty: off-target effects. This occurs when Cas9 accidentally cuts unintended genomic sites and create cleavages leading to unwanted or harmful alterations in the genome [11]. The consequences of these potential off-target effects are significant, they may lead to genetic drift and can lead to increase of mutations and the effect of mutations in progressing generations. Off-target effects can also impact other species since it is possible to transfer these mutated genes to other species in the environment possibly through horizontal gene transfer. This may lead to certain species inheriting certain harmful genes, negatively impacting the ecosystem [10]. It is evident that although CRISPR-Cas9 is currently one of the greatest treatment options there are significant risks to it when off-target effects occur. However, scientists have found ways to monitor and possibly minimize this technical difficulty. According to research, sgRNAs (guide RNA) are usually responsible for these off-target effects, since Cas9 can compensate for 3 mismatches between the sgRNA and the DNA, allowing the Cas9 to still cut the DNA even if the sgRNA is not the most accurate [11]. Therefore, scientists have developed ways to improve CRISPR-Cas9 specificity and reduce off-target effects. Scientists have developed the use of in silico tools to predict possible off-target effects. This online software is easily accessible, however do have its own drawbacks. In silico tools mainly based on sgRNAs to make their predictions, making them especially useful for predicting sgRNA off-targets but not as accurate for other types of off-target effects. This form of computational tool is also insufficient in acknowledging the complex microenvironments in which these off-targets occur, meaning that the predictions made by this tool must be verified by other tools for accuracy [11].

For target edits, targeted methods are used to investigate possible off-target edits. These methods analyze user-defined amplicons (amplified DNA fragments) allowing for close examination of less

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than 1000 base pairs. This means that any deletion that is greater than the amplicon will not be detected. Therefore, targeted methods are sufficient for detecting on-target deletions. However, studies have shown that this method has a significant drawback, due to its short read length it misses 20% of Cas9 mis-edits [12]. Scientists do have methods to obtain longer read lengths such as long-range PCR and long -read sequencing. Many scientists also check and read the area around the Cas9 cut to check for any insertions, deletions, and inversions. However, all targeted methods listed above have one limitation in common, none can detect translocations, but this can be done through unbiased detection methods. Unbiased off-target detection methods scan the whole genome to determine where the Cas9 cleaved the genome. Normally, unbiased methods work in two ways: they either scan the whole genome before and after the cut and compare, or trap DNA breakpoints then sequence those parts specifically. There are especially useful for determining small and large on and off-target edits caused by CRISPR [12]. However, it is important to note that there is not one single method that is perfect enough for checking off-target effects, each method has its own pros and cons. When it comes to analyzing DNA sequences, both methods have their own drawbacks, therefore it becomes exceptionally important to be aware and use methods to mitigate off-target cleavages. This strategy is essential to complementing detection tools and to reduce unintended edits of the genome.

Mitigating off-target effects is crucial for ensuring safety and precision in the therapeutic and clinical application of CRISPR-Cas9 genome editing. To successfully mitigate off-target effects controlling the concentration of Cas9 is essential. Overall, a higher concentration of Cas9 usually results in an increased probability of off-target editing [13]. Scientists modify the Cas9 concentration through controlling the duration of activity it has in the cell. Scientists can decrease the half-life of Cas9 by delivering it with ribonucleoprotein (RNP), mRNA, attaching Cas9 with proteasome degradation signal. Scientists can also increase the half-life by modifying the gRNA. Specific gRNA modifications like adding a hairpin loop at the end of the gRNA can increase specificity by reducing Cas9's ability to tolerate mismatches [12]. These techniques highlight a variety of approaches in solving off-target issues. In the absence of perfect detection methods, proactively engineering the CRISPR system to be more precise when executing its function is also a necessary solution to successfully prevent and minimize the high-risk of off-target effects in clinical settings.

5. Future directions for CRISPR-Cas9 in breast cancer therapy

CRISPR-Cas9 is a revolutionary tool both in the scientific community and the cancer biology field. It is effective and can efficiently target and treating multifunctional genes of cancer: suppressing oncogenes and mutations with high specificity and accuracy. However, there are major challenges that must be addressed in the future when it comes to clinical applications. One significant concern is to reduce the off-target effects of the Cas9 nuclease through controlling the behavior of the Cas9 nuclease during the gene editing process. This could be done through modifying the Cas9 protein, the concentration, the half-life, and possibly using drugs like tetracycline and doxycycline to extract the desired performance of the Cas9 nuclease. Current approaches also include using computational tools to develop more accurate sgRNAs have shown to be promising in reducing off-target effects. This again highlights that more research should be done looking into more methods to modify current Cas9 nucleases and sgRNAs to ensure a more accurate target on the cleavage site thus ensuring a more accurate and effective cleavage by the Cas9 nuclease [14].

Another struggle that needs to be addressed in the future is the delivery system of Cas9. As breast cancer tends to metastasize, it is important that Cas9 can be delivered to all parts of the body and

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remain functional. Currently, scientists struggle with the delivery and packaging of Cas9 due to its large size [15]. Therefore, scientists should investigate possibilities of modifying the current immunogenic AAV vectors to suit more types of delivery locations in the human body. They could modify specific packaging for each specific site of the body where Cas9 needs to be delivered. Current struggles with CRISPR-Cas9 are not only limited to delivery and off-target effects, successful binding of the Cas9 nuclease can also pose potential problems too. Research has shown that occasionally after the binding of Cas9 to the DSB (double-stranded break), Cas9 becomes stuck to the DSB, resulting in a blockage of the repair enzymes. This slows down the repair process simultaneously slowing down the entire gene-editing process. At the same time, removing the Cas9 nuclease from the DNA is also challenging. Commonly scientists utilize an RNA polymerase, however, the RNA polymerase only works in a specific direction, making the process more difficult to control [15]. Therefore, finding new ways to unbind Cas9 from DSB is required: looking into other molecular tools besides RNA polymerase that could remove bound Cas9 without the concern of the proper direction.

Current studies have also shown that CRISPR-Cas9 can lead to unwanted immune responses in the human body. The human body may potentially recognize Cas9 as a foreign bacterial protein, triggering the immune system and decreasing the effectiveness of the treatment. Researchers have identified that many patients do show strong pre-existing immune responses to the two Cas9 homologs: 79% of participants with antibodies recognizing SaCas9 and 65% recognizing SpCas9. Moreover, 46% of donors have T-cells that are able to attack SaCas9 [15]. This again confirms that a majority of breast cancer patients face a high risk of strong immune responses that can hinder the efficiency and efficacy of the treatment, greatly limiting treatment options and prognosis. Thus, in the future more research should be dedicated to utilizing and developing alternative Cas9 homologs that are less likely to be recognized by the human immune system. This could include using Cas9 variants from non-pathogenic or rare-microbial species which humans are not widely exposed to. This would minimize the pre-existing immunity of patients, allowing gene editing to be more effective with a better prognosis.

6. Conclusion

In conclusion, the development and usage of CRISPR-Cas9 has revolutionized the field of cancer biology and presents a transformative opportunity in breast cancer research and therapy. Its ability to accurately target and edit oncogenes that promote metastasizing cancer provides a promising solution in overcoming one the most urgent challenges in breast cancer: drug resistance. CRISPR-Cas9 also allows for highly personalized treatment options since it can directly address and alternate patient-specific genetic mutations. CRISPR-Cas9 despite showing great potential in clinical applications, it does face significant obstacles. The most pressing issue is the off-target effects that occur. This may lead to incorrect editing of the human genome introducing unwanted mutations into the human gene pool. These mutations can also be transferred into the environment potentially endangering the ecosystem. While current solutions include controlling Cas9 concentrations, modifying gRNAs to become more precise, and using computational tools to predict possible effects, no method can fully eliminate the possibility of off-target effects occuring. Another major challenge is the delivery of Cas9 to specific parts of the human body, some parts of the body are much harder to reach due to its larger size and sometimes the Cas9 nuclease may not be effective. Therefore, this highlights the urgent need to investigate developing new types of packaging for specific sites of delivery. Furthermore, studies have also highlighted strong pre-existing immune responses in many breast cancer patients. Patients have been found to have immunity to two

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common Cas9 homologs: SaCas9 and SpCas9. As a result, future directions should include developing other Cas9 variants to address these pre-existing immune responses. Moving forward, scientists should explore possible modifications and other current or emerging technologies that could be combined with CRISPR-Cas9 to enhance its effectiveness and safety. They should also focus on innovative and multimodal treatment approaches to provide more comprehensive and more targeted treatment options for breast cancer patients. These further enhancements can significantly improve the prognosis and quality of life for breast cancer patients.

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