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REVIEW article

CRISPR-Cas9-based functional analysis of PARP inhibitor response in BRCA-mutated breast cancer and its implications for precision pharmacogenomics

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Abstract: BRCA1 and BRCA2 are tumour suppressor genes that play an essential role in homologous recombination and repair damaged DNA, maintaining genomic stability. Mutations in these genes can lead to inaccurate DNA repair, increasing the risk of breast cancer, and can be effectively targeted by PARP inhibitors. This review summarises the role of CRISPR-Cas9-based genome-wide screening to identify genetic determinants of PARP inhibitor response and resistance in BRCA-mutated breast cancer. These PARP inhibitors block the activity of the PARP protein, causing the single-strand DNA break to double-strand DNA break during replication due to replication fork collapse. Thus, the cancer cell DNA impairs DNA repair through defective homologous recombination. However, BRCA-mutated cells may endure resistance to PARP inhibitors due to BRCA reversion mutations, restoration of homologous recombination, 53BP1 pathway alterations, replication fork protection, drug efflux mechanisms, and the tumour microenvironment. CRISPR-Cas9 technology is an emerging genome-editing tool designed to identify the gene causing drug resistance and response. It systematically identifies determinants, synthetic lethal partners, and DNA damage response regulators using sgRNA libraries and next-generation sequencing. These findings support the development of combination therapy, PARP inhibitors with ATR or other DNA-damage response inhibitors, involved in biomarker discovery, personalised treatment, and improving precision medicine in breast cancer.

Introduction

Breast cancer (BC) is one of the most common types of cancer worldwide, primarily affecting women. About 25.0% of cancers in women are BC, which is estimated to be up to 1.5 million [1-4]. The risk factors for BC are gender, age, family history, gene mutation, lifestyle, and hormonal factors [5]. BRCA1 and BRCA2 are the tumour suppressor genes involved in repairing damaged DNA through homologous recombination. 5.0% - 10.0% of BA cases are due to hereditary or family history, and above 20.0% is caused by a mutation in the BRCA1 and BRCA2 genes [6]. Usually, the PARP protein activates the base excision repair pathway and repairs the single-strand DNA break. When PARP inhibitors are administered, they block PARP activity and inhibit the DNA repair in the BRCA-mutated cancer cells, which have defective homologous recombination [7]. Although PARP inhibitors are effective in the treatment of BC, there is a chance of developing resistance due to reverse mutation, restoration of replication fork and homologous recombination, and drug efflux. This is reducing the effectiveness of PARP inhibitors [8]. CRISPR Cas9 technology is a genetic editing tool to edit the genomic sequence through sequence-specific guide DNA and identify the gene causing drug resistance

and sensitivity. The two main components of the technology are single guide RNA (sgRNA) and Cas9 nuclease; the sgRNA guides Cas9 to the target site to introduce a double-strand break (DSB) [9]. This review understands the role of BRCA and its mutations in DNA repair through homologous recombination, the mechanism of PARP inhibitors and their resistance, the pathway in BRCA-mutated BC, and the role of the CRISPR-Cas9 gene-editing tool in identifying the gene responsible for drug resistance, drug sensitivity, and its implications in precision pharmacogenomics.

Molecular basis of BRCA-mediated DNA repair: The genome is challenged by environmental agents such as radiation and reactive oxygen species, causing the breakage of both strands of DNA, leading to DNA double-strand breaks [10]. Due to this, BRCA1, a tumour suppressor, is rapidly recruited to the damaged site, and the damaged ends of the DNA will be rapidly processed to produce 3′ single-stranded DNA (ssDNA) overhangs by DNA end resection. These ssDNA will initially be coated with Replication protein A (RPA) to prevent degradation of ssDNA for the stabilisation of DNA. Then BRCA2 is recruited, which is involved in the displacement of RPA and promotes the loading of RAD51 recombinase onto ssDNA, forming a RAD51 nucleoprotein filament. This RAD51 filament is involved in searching for a homologous sequence in the sister chromatid. The damaged ssDNA will match with the homologous sequence of the sister chromatid and copy its genetic information. DNA polymerase enzyme uses a homologous sequence as a template and adds nucleotides to restore the missing genetic information. Therefore, the damaged DNA is accurately repaired, and genomic stability is maintained as explained in **Figure 1** [11, 12].

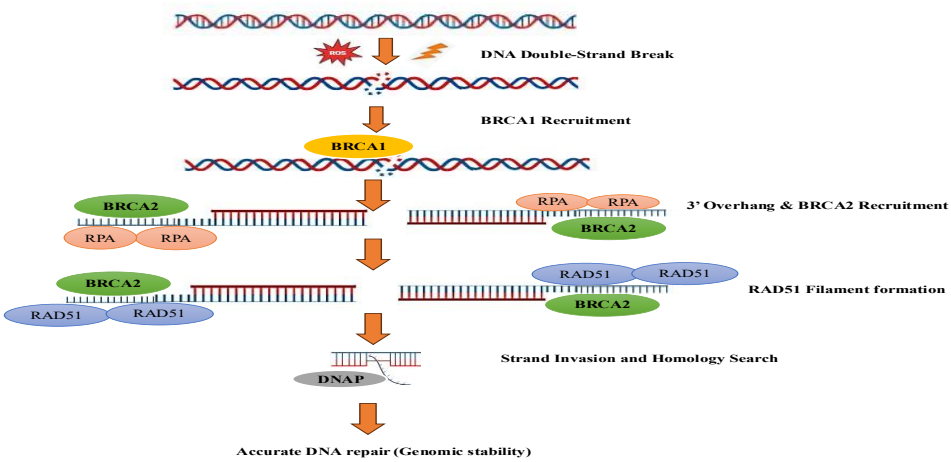

Figure 1: Molecular basis of BRCA-mediated DNA repair

Non-homologous end joining (NHEJ) is the alternate pathway for damaged DNA repair, which acts directly by ligating the broken DNA ends without extensive end processing, and it is active throughout the cell cycle [13]. In this pathway, DSBs are rapidly recognised by the Ku70/Ku80 heterodimer. Ku prevents further degradation by binding to cohesive ends, blunt ends, or damaged ends of DNA. Ku-bound DNA ends will recruit DNA-dependent protein kinase catalytic subunit (DNA-PKcs), forming DNA-Protein kinase holoenzyme, which undergoes autophosphorylation and causes conformational changes to facilitate DNA end alignment and further processing [14]. Various enzymes like Artemis, specialised Polymerases (Pol μ and Pol λ), and Nucleases will trim damaged termini and fill in missing nucleotides, generating ligatable ends for subsequent sealing by the XRCC4-DNA ligase IV complex. Then the complex will dissociate, chromatin structure will be restored after ligation, and the cell cycle will continue [15]. Several studies have demonstrated that BRCA1 and BRCA2 are ubiquitously expressed DNA repair genes and that mutations in these genes mainly contribute to breast and ovarian cancer development. Loss of BRCA1 and BRCA2 genes disrupts double-strand break repair, leading to chromosomal aberrations, copy number alterations, and large-scale genomic rearrangements. Repeated cycles of defective repair will generate characteristic genomic scars, including loss of heterozygosity, telomeric allelic imbalance, and large-scale state transitions [16].

PARP inhibitors in BRCA-mutated breast cancer: PARP inhibitors are targeted anticancer therapies used to block the activity of PARP (poly (ADP-ribose) polymerase) involved in the repair of single-strand DNA breaks. These drugs are discovered and developed to treat BRCA-mutated BC, but are used to treat ovarian and prostate cancer [17]. The DNA repair mechanism is less effective in cancer cells due to mutations in the BRCA1 and BRCA2 genes [18]. PARP inhibitors block PARP activity and prevent cells with homologous recombination deficiency from repairing, causing apoptosis [19].

Mechanism of action of PARP inhibitors: **Figure 2** explains the mechanism of action of PARP inhibitors in normal versus BRCA-mutated cells. In normal cells, single-strand DNA breaks will be rapidly recognised by the PARP1 and then bind to the damaged end of DNA, activating the PARylation process, which recruits DNA repair proteins at the damaged site. The damaged DNA will be efficiently repaired, genomic stability will be maintained, and the cell will survive [20].

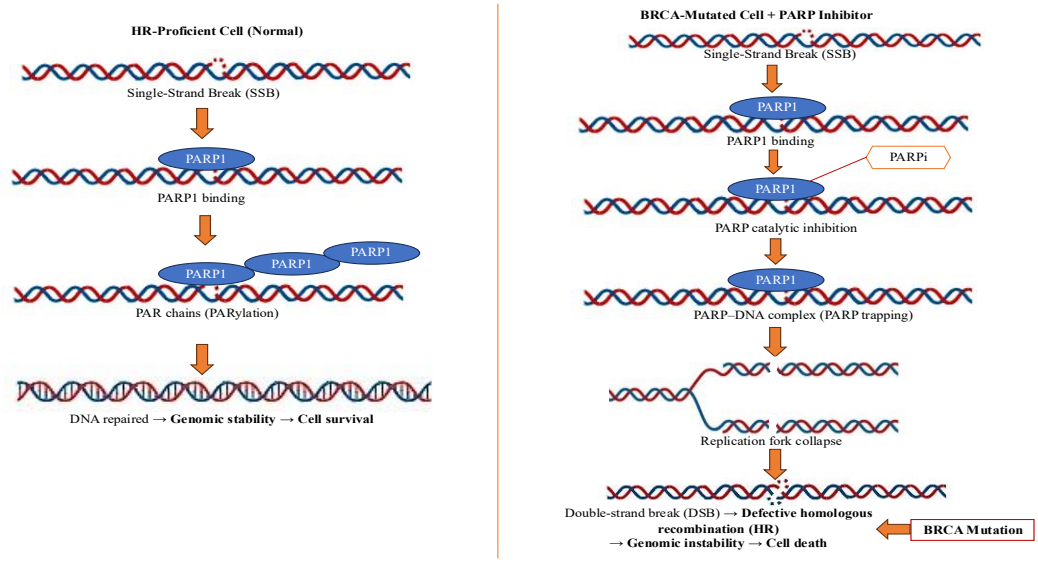

Figure 2: Mechanism of action of PARP inhibitors in BRCA-mutated cells

In BRCA-mutated cells, the homologous recombination pathway is defective. When PARP inhibitors are administered, they will block the activity of PARP, and single-strand DNA breaks will not be repaired. These SSBs will be converted into DSBs due to the replication fork during replication. The DSBs cannot be repaired due to defective homologous recombination in BRCA-mutated cells, leading to genomic instability and finally apoptosis. Thus, synthetic lethality is produced due to impairment in the homologous recombination pathway and PARP-mediated SSB repair, selectively targeting BRCA-mutated cells while sparing normal cells [21].

Table 1: Regulatory status, clinical significance, and adverse effects of PARP inhibitors in breast cancer

Drug	Regulatory status	Clinical significance	Adverse effects
Olaparib [22]	FDA-approved (metastatic and adjuvant, gBRCA-mutated HER2-negative BC)	Improved Progression-Free Survival and Invasive Disease-Free Survival; first approved PARPi in breast cancer	Nausea, fatigue, and anaemia
Talazoparib [23]	FDA-approved (metastatic gBRCA-mutated HER2-negative BC)	High response rate; strong PARP-trapping activity	Anaemia, thrombocytopenia
Niraparib [24]	Investigational in breast cancer (approved in ovarian cancer)	Studied in HER2-negative BRCA-mutated BC; broader Homologous Recombination Deficiency evaluation	Thrombocytopenia, hypertension
Rucaparib [25]	Limited data in breast cancer (approved in ovarian cancer)	Evaluated in BRCA-mutated solid tumours, including BC	Nausea, anaemia, ↑ liver enzymes
Veliparib [26]	Investigation of breast cancer	Studied with carboplatin/paclitaxel in BRCA-mutated metastatic BC	Neutropenia, nausea
Pamiparib [27]	Investigation of breast cancer	Evaluated in BRCA-mutated and HRD-positive tumours	Hematologic toxicity

Mechanisms of resistance to PARP inhibitors: Though PARP inhibitors such as Olaparib and Talazoparib are widely used in BRCA-mutated BC, resistance will remain one of the barriers to therapeutic response. Resistance may occur when the cancer cell restores its DNA repair ability or reduces the drug-induced damage [28]. CRISPR-Cas9 technology helps identify and study genes responsible for resistance and sensitivity in BRCA-mutated BC cells, enabling personalised, precise treatment (**Figure 3**, [29]).

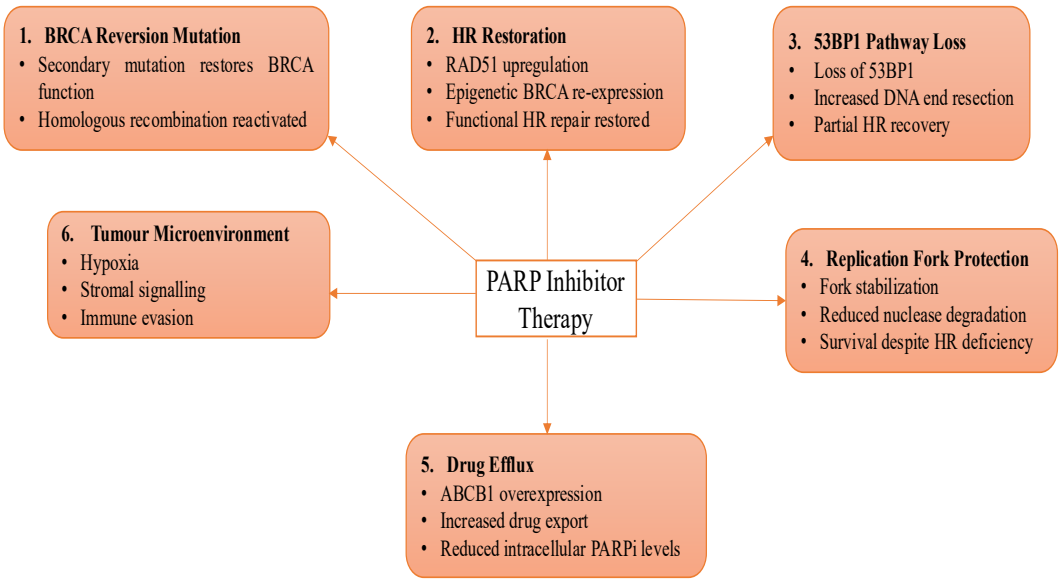

Figure 3: Molecular mechanisms of resistance to PARP inhibitors in BRCA-mutated breast cancer

One mechanism for resistance is BRCA Reversion Mutations, where the BRCA gene will undergo secondary or reversion mutations, restoring the BRCA functions, thereby reactivating the homologous recombination pathway [30]. Even though the BRCA is mutated, the cell may increase RAD51 and related proteins that help in DNA repair, or the BRCA gene may be activated due to epigenetic changes restoring HR repair [31]. 53BP1 proteins are involved in preventing the cells from undergoing DNA repair. If this protein is lost, then cancer cells may partially restart HR repair, leading to a reduction in treatment efficacy and causing drug resistance [32]. Tumour cells may develop replication fork protection and become resistant by reducing the nuclease enzyme that may destroy the fork or by binding certain proteins like RAD51 to these forks and preventing DNA damage [33]. One of the mechanisms through which the drug efficacy can be reduced is the increase in the efflux of the drug and a reduction in the intracellular concentration [34]. Several pieces of evidence suggest that the tumour microenvironment, including hypoxia and stromal signalling, can prevent DNA damage-induced apoptosis [35]. **Figure 3** explains the mechanism of resistance to PARP inhibitors, where the cancer cells will restore the ability to repair damaged DNA and thereby reduce treatment efficacy. To identify genes causing PARP resistance or synthetic lethality, researchers use the CRISPR-Cas9 tool that allows them to turn off or turn on specific genes in cancer cells [36].

CRISPR-Cas9 technological screening: The CRISPR-Cas9 system is a defence mechanism found in bacteria that has been repurposed as a genome-editing tool. It allows manipulation of any genomic sequence through sequence-specific guide DNA [37]. This system consists of two major components: Cas-9 proteins and single-guide RNA (sgRNA). The sgRNA is designed to be complementary to base pairs in a genome, which guides Cas-9 nuclease to the target site. Cas9 introduces a double-strand break (DSB) at the protospacer adjacent motif (PAM) in the DNA, which is either repaired by non-homologous end joining or homology-directed repair cellular mechanisms, as explained in **Figure 4**. Mainly through non-homologous end joining, but this is prone to error, hence may cause an insertion or deletion of a gene. Whereas in homologous end-joining, the homologous DNA template is highly precise [38].

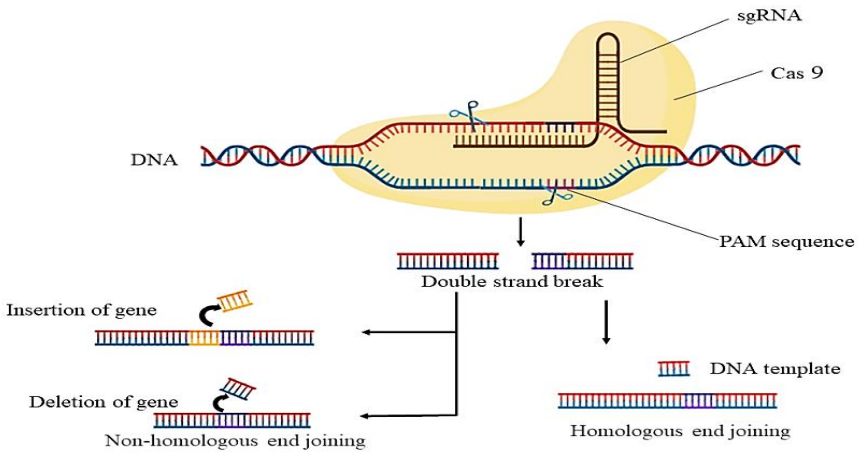

Figure 4: Mechanism of CRISPR Cas9 technology

CRISPR-Cas9 technology, through the development of sgRNA libraries, enables the targeting of thousands of genes [39]. There will be multiple sgRNAs for each gene, as it reduces false positives [40]. It is usually delivered through a helper viral vector, like a lentiviral vector, which contains the sgRNA [41]. The CRISPR-Cas9 knockout screening is done to identify genes required for the survival of the cell and genes involved in drug resistance. using lentiviral vector sgRNA libraries is introduced to the cell to express Cas-9. Each cell receives a single sgRNA for a different cell knockout of a different gene. When pressure is applied, such as a PARP inhibitor, if a cell dies due to the knockout gene, then that gene is essential for the survival of the cell, such as replication, translation, and transcription. If the cell survives, then the knockout gene is responsible for drug sensitivity [42]. There are mainly two CRISPR screens that are pooled and arrayed. In pooled library screening, thousands of different sgRNAs are mixed into a single population of cells. It is used to screen the gene responsible for survival and resistance to drugs. In an array, each sgRNA is isolated by using a different well in a multiwall plate, which is usually used for high-content screening [43, 44].

CRISPR-based identification of determinants of PARP response:

Genome-wide CRISPR screen*:* Genome-wide CRISPR screens are a powerful tool that uses sgRNA libraries to knock out genes, and selective pressure is applied, which leads to the identification of genes either enriched or deleted in the selective control population [45]. PARPi reduces the burden of tumour initially, but gradually leads to resistance in patients suffering from a mutation in BRCA1 OR BRCA2. The mechanism of PARPi resistance is identified with wide-genome CRISPR screens, which help to identify the gene responsible for resistance [46]. Genetic loss-of-function (LOF) and gain-of-function (GOF) screening is crucial in identifying genes responsible for drug resistance and new drugs for drug-resistant cancers [47].

The sgRNA libraries, which are complementary to base pairs, are selected, and sgRNA libraries targeting thousands of genes are introduced into the Cas9 nucleus, which is obtained from bacteria, delivered to the target site with the help of a lentiviral vector. Once bound to the target site, it will introduce DSB at the PAM sequence, which leads to non-homologous end joining, thus causing knockout of the gene. When treated with PRAP inhibitors like olaparib, rucaparib, niraparib, and talazoparib, this may lead to death or survival of the gene. After some time, genomic DNA is extracted, and next-generation sequencing is performed to assess the abundance of sgRNA. This leads to the identification of genes required for survival and genes that cause drug resistance. If sgRNA levels are low, Cas9 targets genes required for cell survival, and knockout genes are passed through generations in CRISPR-Cas9 technology, thereby reducing sgRNA levels due to cell death. If the sgRNA is high, the Cas9 breaks the gene that is responsible for drug resistance since the knockout gene is passed through generations in CRISPR-Cas9 technology, resulting in an increase in the amount of sgRNA due to drug sensitivity, as explained in **Figure 5** [46, 48].

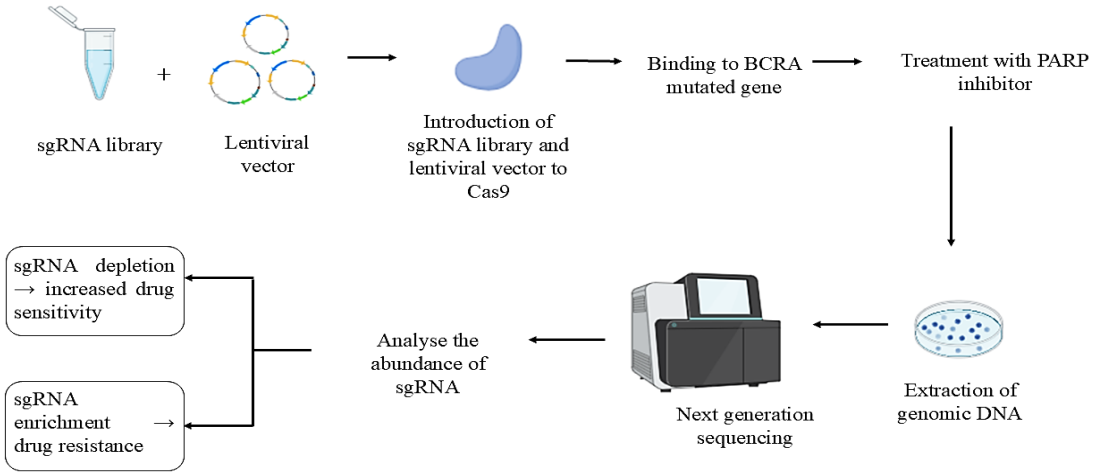

Figure 5: Genome-wide CRISPR screen for PARP inhibitor response

Some commonly used sgRNA libraries are GeCKO (Genome-scale CRISPR Knock-Out), SAM (synergistic activation mediator), and TKOv3 (Toronto Knock Out Library version 3) [46, 47]. This screening method is applied to determine the gene responsible for resistance and a synthetic legal partner of PARPi in BRCA1 and BRCA2 mutations. This screening method is performed on several cancer models, including breast, ovarian, and prostate, to identify genes that resemble homologous repair, replication fork stability, and DNA damage signalling in cancer patients with BRCA1 and BRCA2 mutations [46, 48].

Resistance gene: The loss of function of genes such as TP53BP1**,** RIF1**,** REV7, and check2 leads to resistance to PARPi. These genes can be identified using CRISPR screening. Development of resistance leads to restoration of HR in BRCA-mutated cells, hence facilitating replication even in treatment with PARPi [49]. TP53BP1, REV7 block DNA end resection, promotes NHEJ, loss of function promotes restoration of HR, DNA end resection, recruitment of RAD51 in the BRCA mutated cell when treated with PARPi, RIF1 works along with TP53BP1 to suppress HR and cause resistance [50]. CHECK2 normally regulators protein such as p53, E2F7, which suppresses BRCA2; loss of this gene increases the activity of BRCA2, improves DNA repair, as loss of this gene significantly affects DNA damage pathways such as ATM and ATR. Because restoration HR reduces DNA damage from PARPi, allowing cancer cells to survive [48].

Synthetic lethal partners: When two or more genes interact genetically, the combined loss of function of these genes causes cell death. This is known as synthetic lethality, even though the individual loss of function is still tolerable. A BC patient with BRCA mutations, a synthetic lethality therapy with treatment PARPi has been developed. CRISPR screen, along with identification of the resistance gene, is also used to identify the synthetic lethal partners of PARPi. CRISPR screen identified BRD4, HDAC1, MEPCE, and ALC1 as synthetic lethal partners of PARPi in BCCA-mutated cells. Loss of these genes increases the sensitivity of PARPi. Loss of BRD4 leads to HRD, and loss of HDAC1 causes an increase in damage to DNA, hence increasing the sensitivity of PARPi to BRCA-mutated cell death. Loss of MEPCE causes replication stress, R-loop buildup, and dysregulated RNA polymerase II (RNAPII) promoter-proximal pausing, all of which enhance transcription-replication machinery collisions and finally lead to cell death. Loss of ALC1 increases genomic instability and increases sensitivity to PARPi, which may help overcome the drug resistance [49, 50].

Shieldin complex*:* One main discovery of CRISPR screens is the role of the shieldin complex in PARPi response. Shieldin complex is four proteins consisting of SHLD1, SHLD2, SHLD3, and REV7, recruited to the site of damage by 53BP1 and RIF1**.** Normally, shielding complex blocks resection of DNA and repair of DNA by NHEJ suppresses HR in this condition. Treatment with PARPi in BCRA-mutated cells leads to the death of cancer cells. Loss of function of shieldien complex leads to recruitment of RAD51, resection of DNA, and promotes HR without BCRA. The effect of PARPi is reduced, and resistance to the drug is observed [24], [50].

DNA damage response regulators: Detection of damage to DNA, signalling, and repairing are through genes or proteins called DNA damage response (DDR) regulators, which determine whether the cell is tolerant of PARPi. DNA is blocked by PARPi. Loss of DDR regulators, such as ATM, ATR, CHK1, and FAND2, leads to an increase in sensitivity to PARPi in BRCA-mutated cells. CRISPR screening identified PARPi-sensitive DDR regulators. ATM, ATR, and CHK1 are usually responsible for detecting genomic instability, replication fork stability, and regulating the cell cycle checkpoint. Loss of function of these genes leads to defective DNA repair, increasing the damage to the DNA, leading to an increase in sensitivity to PARPi [51, 52].

Pharmacogenomic and clinical implications: CRISPR-Cas9 screens help identify the gene that causes sensitivity or resistance to PARP inhibitors, thus helping in the pharmacogenomic landscape. It was identified that loss of the HUWE1 and the histone acetyltransferase KAT5 in BRCA2-mutated cells causes resistance to PARPi, using genome-wide knockout. HUWE1 is a ubiquitin ligase; loss of this homologous recombination through RAD51 activation and loss of KAT5 leads to repair of DNA double-strand breaks by 53BP1-dependent pathways [53]. A specific mutation of RAD51 called haplotype is related to Olaparib resistance in BC, which was found by saturated CRISPR mutagenesis [54]. In BRCA wild-type MDA MB 231 cells, a triple-negative human BC line with a normal BRCA gene, CRISPR screen revealed that ARL11 is a resistance factor that activates STING-mediated innate immunity and recruits the RUVBL1/2 complex to promote homologous recombination, indicating that ARL11 or RUVBL1/2 inhibition reduces the sensitivity to PARPi [55]. Another study indicated CRISPR-mediated loss of PARP1 combined with chemotherapy in BRCA1-mutated triple-negative BC, revealing the synthetic lethal relationship between loss of BRCA1 and PARP1 inhibition [56]. The CRISPR screen identified biomarkers such as HUWE1, KAT5, RAD51 haplotypes, ARL11, and PARP1 to predict PARPi response, guide better combinations such as ATR inhibition for CHEK2 loss, and extend the benefit of PARPi beyond BRCA cancer [57]. Combining PARPi with other agents is likely to enhance therapeutic efficacy in BCRA-mutated patients [58].

Limitations and challenges: CRISPR Cas9 technology is a powerful genome editing tool that targets the disease-causing gene. There are many advantages of CRISPR Cas9 technology; it has its own limitations and challenges. One of the limitations of CRISPR Cas9 technology is off-target binding, although it is less compared to RNAi, but if off-targeting happens due to genomic similarity, it may lead to mutation in undesired locations. Another challenge faced by CRISPR Cas9 technology is the delivery system, as CRISPR Cas9 technology is mainly delivered with the help of a lentiviral vector, which causes an immune response as it is obtained from bacteria [59]. CRISPR Cas9 technology raises ethical concerns as it permanently alters the human genome. There are also efficiency challenges in CRISPR Cas9 technology, as it is influenced by PAM sequencing [60].

Future perspectives: Emerging evidence suggests that CRISPR-Cas9 technology will help scientists understand why BRCA-mutated BC becomes resistant to PARP inhibitors. Genome editing tools can be used to study specific mutations and test how these mutations will affect drug response. Combining CRISPR technology with patient-derived tumour models aids scientists in studying how cancer will behave in real patients. CRISPR-based research will help in the development of personalised treatment strategies, overcome drug resistance, and improve BC patient outcomes.

Conclusion: BRCA1 and BRCA2 are tumour suppressor genes involved in DNA damage repair through homologous recombination and maintain genetic stability. Mutations in the genes lead to impaired DNA repair, making the mutated cells more sensitive to PARP inhibitors. Olaparib and Talazoparib showed a significant effect in treating BRCA-mutated breast cancer. But patients will finally develop resistance due to mechanisms such as BRCA reversion mutations, restoration of DNA repair pathways, protection of replication forks, and increased drug efflux. CRISPR-Cas9 technology aids in identifying the gene responsible for resistance and synthetic lethality.

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تحليل وظيفي قائم على تقنية كريسبر-كاس9 لاستجابة مثبطات PARP في سرطان الثدي ذي الطفرة الجينية BRCA وآثاره على علم الصيدلة الجينية الدقيق

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الملخص: يُعدّ كلٌّ من BRCA1 وBRCA2 من الجينات الكابتة للأورام، والتي تلعب دورًا أساسيًا في إعادة التركيب المتماثل وإصلاح الحمض النووي التالف، مما يحافظ على استقرار الجينوم. قد تؤدي الطفرات في هذه الجينات إلى إصلاح غير دقيق للحمض النووي، مما يزيد من خطر الإصابة بسرطان الثدي، ويمكن استهدافها بفعالية باستخدام مثبطات PARP. تلخص هذه المراجعة دور الفحص الجينومي الشامل القائم على تقنية CRISPR-Cas9 لتحديد المحددات الجينية للاستجابة لمثبطات PARP ومقاومتها في سرطان الثدي ذي الطفرات في جين BRCA. تعمل مثبطات PARP هذه على تثبيط نشاط بروتين PARP، مما يؤدي إلى تحول كسر الحمض النووي أحادي السلسلة إلى كسر ثنائي السلسلة أثناء التضاعف نتيجة لانهيار شوكة التضاعف. وبالتالي، يُضعف الحمض النووي للخلايا السرطانية عملية إصلاح الحمض النووي من خلال إعادة التركيب المتماثل المعيب. مع ذلك، قد تُظهر الخلايا الحاملة لطفرة BRCA مقاومةً لمثبطات PARP نتيجةً لطفرات ارتداد BRCA، واستعادة إعادة التركيب المتماثل، وتغيرات مسار BP153، وحماية شوكة التضاعف، وآليات طرد الدواء، والبيئة الدقيقة للورم. تُعد تقنية CRISPR-Cas9 أداةً ناشئةً لتحرير الجينوم، مصممةً لتحديد الجين المُسبب لمقاومة الأدوية والاستجابة لها. وتُحدد هذه التقنية بشكلٍ منهجي العوامل المُحددة، والشركاء القاتلين التركيبيين، ومنظمات استجابة تلف الحمض النووي باستخدام مكتبات sgRNA وتقنيات التسلسل من الجيل التالي. تدعم هذه النتائج تطوير العلاج المُركب، باستخدام مثبطات PARP مع ATR أو مثبطات أخرى لاستجابة تلف الحمض النووي، مما يُسهم في اكتشاف المؤشرات الحيوية، والعلاج الشخصي، وتحسين الطب الدقيق في سرطان الثدي.