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RESEARCH ARTICLE

In silico–guided Design and Endonuclease-Based Functional Validation of sgRNAs Targeting *ERBB2* Transmembrane and Kinase Domains

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Abstract

BACKGROUND: Erythroblastic oncogene B2 (ERBB2) overexpression promotes breast cancer progression through activation of phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and mitogen-activated protein kinase (MAPK) signaling pathways. Although CRISPR–Cas9 enables precise gene disruption, sgRNA efficiency is strongly influenced by target sequence and structural features, necessitating careful *in silico* design and validation. This study aimed to design and evaluate sgRNAs targeting the transmembrane- and tyrosine kinase–encoding regions of *ERBB2*.

METHODS: Candidate sgRNAs were selected using CHOPCHOP, CRISPOR, and CRISPR RGEN, followed by secondary structure analysis with RNAfold to assess folding stability and scaffold integrity. sgRNAs were synthesized via *in vitro* transcription and assembled with Cas9 protein to form ribonucleoprotein (RNP) complexes. *In vitro* endonuclease assays were performed using PCR-amplified *ERBB2* fragments derived from SK-OV3 genomic DNA, corresponding to exon 22 (523 bp) and exon 25 (933 bp). sgRNAs targeting *eIF4E1* served as positive controls.

RESULTS: Two sgRNAs meeting all *in silico* criteria were selected. Secondary structure prediction confirmed that both sgRNAs possessed essential structural elements required for Cas9 interaction including the repeat–anti-repeat region and three stem–loop structures. *In vitro* endonuclease assays demonstrated that the sgRNA targeting exon 22 of *ERBB2* successfully cleaved its target DNA, producing fragments of 266 bp and 257 bp. Similar cleavage activity was observed in the two control sgRNAs, which generated fragments of 384 bp and 303 bp, and 486 bp and 201 bp, respectively. In contrast, the sgRNA targeting exon 25 of *ERBB2* exhibited no detectable cleavage activity.

CONCLUSION: The sgRNA targeting exon 22 of *ERBB2* demonstrated effective DNA cleavage activity *in vitro*, whereas the sgRNA targeting exon 25 showed no endonuclease activity.

KEYWORDS: CRISPR–Cas9, endonuclease, ERBB2, *in vitro*, breast cancer, RNP, sgRNA

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Introduction

Breast cancer is the most commonly diagnosed malignancy in women worldwide and the second leading cause of cancer-related death. Its development is influenced by multiple factors, encouraging extensive research to identify effective therapeutic strategies.(1-4) Human epidermal growth factor receptor 2 (HER2)-positive breast cancer is caused by dysregulated growth signaling due to overexpression of the HER2 or also known as erythroblastic oncogene B2 (ERBB2) protein.(5) ERBB2 contains a transmembrane domain and an intracellular tyrosine kinase domain, both of which are essential for maintaining oncogenic activation through receptor dimer stabilization and continuous kinase activity.(6)

Structurally, the HER2 receptor consists of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. In certain conditions, a truncated isoform known as p95HER2 lacks the extracellular domain but retains the ability to form stable homodimers, leading to constitutive signaling activity. The transmembrane domain plays an essential role in receptor dimerization and stabilization of the active receptor conformation. This domain contains two conserved dimerization motifs, SmxxxSm (GxxxG), located at the N- and C-terminal regions, which regulate helix orientation and influence the positioning of the juxtamembrane and kinase domains, thereby acting as a structural switch between inactive and active receptor states.(7,8) Exon 22 encodes the transmembrane domain, making it a critical structural region involved in HER2 receptor dimerization and activation.

The intracellular tyrosine kinase domain is responsible for catalytic activity and the initiation of downstream signaling pathways that regulate cellular proliferation, growth, survival, and angiogenesis. Activation of this domain triggers several major oncogenic signaling cascades, including the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), and phospholipase Cγ/protein kinase C (PLCγ/PKC) pathways.(5,9) Exon 25 encodes a portion of this kinase domain and contributes directly to HER2 catalytic activity and signal transduction. Therefore, targeting exons encoding these key functional domains may disrupt receptor activation and downstream oncogenic signaling. Based on these structural and functional considerations, exon 22 and exon 25 were selected in this research as target sites for CRISPR–Cas9-mediated gene disruption of *ERBB2*.

Although surgery, chemotherapy, hormonal therapy, and radiotherapy are widely used, their effectiveness remains limited by therapy resistance and metastatic recurrence.(10) Genome editing technologies provide new opportunities for biological research and therapeutic development, with human genome editing offering significant scientific and clinical potential.(11) CRISPR–Cas9 enables precise and efficient genome modification without random transgene integration, making it a promising strategy for breast cancer gene therapy.(12-15) However, cleavage efficiency depends strongly on single-guide RNA (sgRNA) design, and *in silico* predictions alone are insufficient, requiring experimental validation.(16-18) Previous studies show that only a small fraction of designed sgRNAs exhibit high cleavage activity. For example, only approximately 5% of 1,841 sgRNAs demonstrated high efficiency.(19) Similarly, it was also reported that only 129 out of 218 designed sgRNAs exhibited satisfactory efficiency.(14,20)

A previous study targeted exons 5, 10, and 12 of *ERBB2*, which encode the extracellular domain of HER2, using a plasmid-based CRISPR–Cas9 system. It was reported that only the sgRNA targeting exon 12 showed effective cleavage, while the others exhibited no significant activity. Importantly, this approach did not target the intracellular tyrosine kinase domain, which is directly responsible for HER2 autophosphorylation and remains active in truncated variants.(21)

In contrast, the present study proposes a novel strategy by targeting exon 25, which encodes the transmembrane and kinase domains of *ERBB2*, and by using a ribonucleoprotein (RNP)-based CRISPR–Cas9 system. This system allows faster and more precise genome editing with potentially fewer off-target effects.(22) In addition, the combination of *in silico* sgRNA design and *in vitro* endonuclease validation provides a more comprehensive approach for selecting and optimizing sgRNAs specific to the *ERBB2* kinase domain.

Based on this rationale, this study designed sgRNAs targeting the transmembrane (exon 22) and tyrosine kinase domains (exon 25) of the *ERBB2* gene and evaluated their ability in guiding Cas9 through *in vitro* endonuclease activity. These findings represent an important step toward developing CRISPR–Cas9–based gene therapy strategies for HER2-positive breast cancer, with the specific aim of generating highly specific sgRNAs and validating their cleavage efficiency *in vitro*.

Methods

DNA Target Preparation

Genomic DNA Extraction

Genomic DNA was extracted from SKOV3 cells using the Wizard® Genomic DNA Purification Kit (Cat. No. A1120; Promega, Madison, WI, USA) following the manufacturer's protocol. DNA integrity was assessed by 1% agarose gel electrophoresis, while DNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Amplification and Cloning of ERBB2 Exon 22 and 25

Genomic fragments encoding the transmembrane domain (exon 22) and kinase domain (exon 25) of the *ERBB2* gene were amplified using GoTaq® Green Master Mix (Cat. No. M7122; Promega, Madison, WI, USA) and specific primer pairs (Supplementary 1). PCR products were verified by Sanger sequencing (Genetika Science, Jakarta, Indonesia) to confirm that the sgRNA target regions within exon 22 and exon 25 matched the *ERBB2* reference sequence and did not contain mutations or polymorphisms that could affect sgRNA binding or Cas9 cleavage efficiency. The amplified fragments were purified using the GenepHlow™ Gel/PCR Kit (Cat. No. DFH100; Geneaid, New Taipei City, Taiwan).

Cloning of ERBB2 Target Fragments

Purified PCR products were ligated into the pGEM®-T Easy Vector (Cat. No. A1380; Promega, Madison, WI, USA) and transformed into competent *Escherichia coli* DH5α cells by heat shock at 42°C for 45 s. Positive clones were selected by blue–white screening, and recombinant plasmids were isolated and confirmed by Sanger sequencing to ensure that the cloned *ERBB2* fragments retained sequences identical to the reference gene. Sequences identical to the *Homo sapiens* ERBB2 reference were used as templates for sgRNA design and as targets for *in vitro* Cas9 endonuclease assays.

The sgRNA Preparation

sgRNA Design

Candidate sgRNA targeting *ERBB2* exon 22 and exon 25 were designed by using the CHOPCHOP (23), CRISPOR (24), dan CRISPR RGEN (25) web-based tools. These tools were used to evaluate sgRNA specificity by predicting potential off-target sites across the human genome and ranking candidate sgRNAs based on specificity and efficiency scores. Secondary structures of the sgRNAs were predicted in RNAfold program.(26)

Amplification of sgRNA Cassettes

The sgRNA cassette, consisting of an EcoRI restriction site, T7 promoter, spacer sequence, sgRNA scaffold, and rrnB T1 terminator (Supplementary 2), was synthesized as double-stranded DNA (dsDNA) along with specific forward and reverse primers for PCR amplification (Supplementary 1) by Integrated DNA Technologies (IDT, Coralville, IA, USA). The sgRNA cassette was amplified by PCR using MyTaq™ DNA Polymerase (Cat. No. BIO-21105; Bioline, London, UK) for 25 cycles. PCR products were purified using the GenepHlow™ Gel/PCR Kit (Cat. No. DFH100; Geneaid, New Taipei City, Taiwan) and verified in a 2% agarose gel electrophoresis.

Cloning and sgRNA Production by in vitro Transcription (IVT)

Purified sgRNA cassettes were ligated into the pGEM®-T Easy Vector (Cat. No. A1380; Promega, Madison, WI, USA) and transformed into competent *E. coli* DH5α (CCMB80) cells. sgRNAs were synthesized by *in vitro* transcription using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit (Cat. No. E2050S/L; New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions.

RNP Complex Formation and Endonuclease Activity Assay

The *in vitro* CRISPR–Cas9 RNP cleavage assay was performed as an initial screening step to evaluate sgRNA-guided Cas9 activity prior to potential cellular genome editing experiments. Cas9–sgRNA RNP complexes were assembled by mixing 5 ng of *Streptococcus pyogenes* Cas9 (Cat. No. 1081058; Integrated DNA Technologies, Coralville, IA, USA), 349 ng of sgRNA, and Cas9 dilution buffer (30 mM HEPES, 150 mM KCl, pH 7.5 in nuclease-free water) to a final volume of 10 µL, followed by incubation at 25°C for 10 min. RNP complex formation was confirmed by electrophoretic mobility shift assay (EMSA) in a 2% agarose gel.

For the *in vitro* endonuclease assay, 2 µL of Cas9–sgRNA RNP, 72 ng of DNA template, 2 µL of 10× Cas9 nuclease reaction buffer (0.2 M HEPES, 0.1 M $MgCl_2$, 5 mM DTT, 1.5 M KCl in nuclease-free water), and nuclease-free water were combined to a final volume of 20 µL. Reactions were incubated at 37°C for 30, 60, 90, and 120 min. Endonuclease activity was visualized in a 2% agarose gel electrophoresis. Two sgRNAs targeting the eIF4E1 gene, as previously reported (27), were used as positive controls.

Results

Genomic DNA Isolation, Amplification, and Cloning of the *ERBB2* Target Fragment

Genomic DNA extracted from SKOV3 cells exhibited an A260/A280 ratio of 1.91, and agarose gel electrophoresis showed intact genomic DNA without smearing (Supplementary 3A). PCR amplification using exon-specific primers produced single bands of 523 bp for exon 22 and 933 bp for exon 25 (Supplementary 3B and 3C), consistent with the expected sizes and confirming specific amplification. Exon 22 (523 bp) and exon 25 (933 bp) of *ERBB2* were successfully cloned into pGEM-T Easy vector as indicated by single amplicons corresponding to the expected target sizes (Supplementary 4). Sanger sequencing analysis (Supplementary 5) confirmed that the exon 22 and exon 25 sequences were identical to the *Homo sapiens* ERBB2 reference sequence (NG_007503), indicating that the sgRNA target regions did not contain mutations that could interfere with Cas9 recognition or cleavage. After sequence verification, the exon 22 and exon 25 fragments were re-amplified from the confirmed constructs and used as substrates for the *in vitro* nuclease assay.

Computational Design and Evaluation of sgRNA Candidates Targeting *ERBB2* Exons 22 and 25

The sgRNA targeting *ERBB2* exon 22 was selected based on sequence consistency identified by both the CHOPCHOP and CRISPOR platforms. In contrast, no identical spacer sequence was found across all three design tools for exon 25; therefore, the candidate generated by CHOPCHOP was selected based on optimal GC content and a high score specific for knockout applications. The selected spacer candidates for exon 22 and exon 25 exhibited GC contents of 50% and 60%, respectively, with a self-complementarity value of 0 and predicted cleavage efficiencies of 66.69 and 69.79 (Table 1).

CHOPCHOP off-target analysis (0–3 mismatches) indicated that the exon 22 spacer possessed only one 0-mismatch site corresponding to the intended target, whereas the exon 25 spacer showed one 0-mismatch site and two sites with three mismatches. CRISPOR output further revealed that the *ERBB2* exon 22 spacer displayed a high MIT specificity score (≥92), moderate-to-high efficiency scores according to the Doench'16, Moreno–Mateos, and Doench RuleSet 3 models, and favorable predictions for out-of-frame indels and Lindel scores, supporting its potential to induce a functional knockout with a low off-target profile (0–4 mismatches) (Table 2).

Secondary structure prediction of the sgRNAs using RNAfold revealed minimum free energy (MFE) values of −21.29 kcal/mol for exon 22 sgRNA and −22.30 kcal/mol for exon 25 sgRNA (Figure 1). Both sgRNAs formed the characteristic scaffold structure comprising the repeat–anti-repeat region and three stem–loop structures, with the spacer located at the 5′ end, supporting efficient sgRNA–Cas9 complex formation.

Successful PCR Amplification of sgRNA Transcription Cassettes Targeting *ERBB2* Exons 22 and 25

The sgRNA transcription cassettes targeting exon 22 (sgRNA exon 22) and exon 25 (sgRNA exon 25) were successfully amplified by PCR, yielding single amplicons of ~200 bp (Supplementary 6), consistent with the designed fragment size. Colony PCR analysis confirmed that three white colonies contained the expected insert, as indicated by the presence of a single ~200 bp band (Supplementary 7). Colonies that produced a single band of the expected target size in PCR analysis were selected, and plasmids isolated from these colonies were subsequently used for the IVT step, based on the consideration that a single band represents the most likely correct and specific plasmid construct candidate.

Successful *in vitro* Transcription of ERBB2-Targeting sgRNAs

Successful *in vitro* transcription yielded *ERBB2*-targeting sgRNAs for exon 22 and exon 25. Visualization showed intact single bands of approximately ~200 bp with no indication of RNA degradation (Supplementary 8). The sgRNAs generated by *in vitro* transcription were employed for RNP complex formation in Cas9 endonuclease activity assays targeting exon 22 and exon 25 of the *ERBB2* gene (Figure 2).

Successful Cas9 Endonuclease Activity of *ERBB2*

The endonuclease assay showed that the Cas9–RNP complex containing the exon 22–targeting sgRNA efficiently cleaved the target DNA, producing the expected 266 bp and 257 bp fragments (Figure 2A). Previously validated sgRNA196 and sgRNA300 served as functional controls to confirm Cas9 catalytic activity under identical conditions and generated the anticipated fragments (486 bp/201 bp and 384 bp/303 bp, respectively), although sgRNA196 displayed partial cleavage efficiency. In contrast, the exon 25–targeting Cas9–RNP complex failed to produce the predicted 750 bp and 183 bp fragments (Figure 2), despite confirmed RNP formation by EMSA (Supplementary 9), and alterations in incubation time or temperature did not restore cleavage activity (Supplementary 10). These results suggest that differential cleavage efficiency is attributable to intrinsic sgRNA properties rather than experimental conditions.

Table 1. sgRNA spacer design using CHOPCHOP.

Target	Target Sequence	Genomic Location	Strand	GC Content (%)	Self-compl.	MM				Efficiency
						0	1	2	3	
Exon 22	TTGGGATCCTCATCAAGCGA ***CGG***	Seq: 249	+	50%	0	1	0	0	0	66.69
Exon 25	GACCATGTCCGGGAAAACCG ***CGG***	Seq: 115	+	60%	0	1	0	0	2	69.79

Seq: sequence; Self-compl.: self-complementarity.

Table 2. sgRNA spacer design using CRISPOR.

Target	Position/ Strand	*Guide Sequence* + PAM	MIT Spec. Score	CFD Spec. Score	Predicted Efficiency			Outcome		Off-Target for 0-1-2-3-4 MM + Next to PAM
					Doench' 16	Mor-Mateos	Doench RuleSet3	Out of frame	Lindel	
Exon 22	269/fw	TTGGGATCCTCATCAAGCGA ***CGG***	96	96	67	64	95	41	78	0-0-0-0-46

Spec. Score: specificity score.

Discussion

This study aimed to design sgRNAs targeting the transmembrane and tyrosine kinase domains of the *ERBB2* gene and to evaluate their ability to guide Cas9 for specific DNA cleavage. Exons 22 and 25, which encode the transmembrane and tyrosine kinase domains respectively, were selected because of their essential roles in HER2-driven oncogenic signaling. As these exons represent the initial coding regions of their respective domains, their disruption is expected to impair proper domain formation and prevent the production of a fully functional HER2 protein. CRISPR–Cas9–induced frameshift mutations in these exons are therefore anticipated to inactivate oncogenic HER2/ERBB2 activity, suppress cancer cell proliferation, and provide a precise molecular strategy to control HER2-driven tumorigenesis.

In the present study, sgRNA activity was evaluated using an *in vitro* CRISPR–Cas9 RNP cleavage assay with PCR-amplified target DNA. This approach provides a rapid and efficient preliminary screening strategy to confirm sgRNA-guided Cas9 cleavage before performing genome editing experiments in living cells. Such *in vitro* validation is commonly used as an initial step to assess sgRNA functionality prior to cellular genome editing and functional assays.

Previous studies have demonstrated the application of CRISPR–Cas9-mediated gene knockout followed by functional assays to investigate cancer-related genes. For example, it was reported that the knockout of the *EGFL6* gene in SKOV3 ovarian cancer cells using CRISPR–Cas9. The sgRNA activity was initially validated using a T7E1 cleavage assay on genomic DNA extracted from transfected cells, showing cleavage efficiencies of approximately 82–83%. Subsequent functional assays, including Cell Counting Kit-8 (CCK-8) proliferation assays, wound-healing migration assays, transwell invasion assays, and human umbilical vein endothelial cell (HUVEC) tube formation assays, demonstrated significant reductions in tumor cell proliferation, invasion, and angiogenesis.(28)

CRISPR–Cas9–mediated gene knockout is achieved by inducing double-strand breaks (DSBs) that are repaired through the error-prone non-homologous end joining (NHEJ) pathway, generating insertion–deletion (indel) mutations. These mutations can cause frameshifts or premature stop

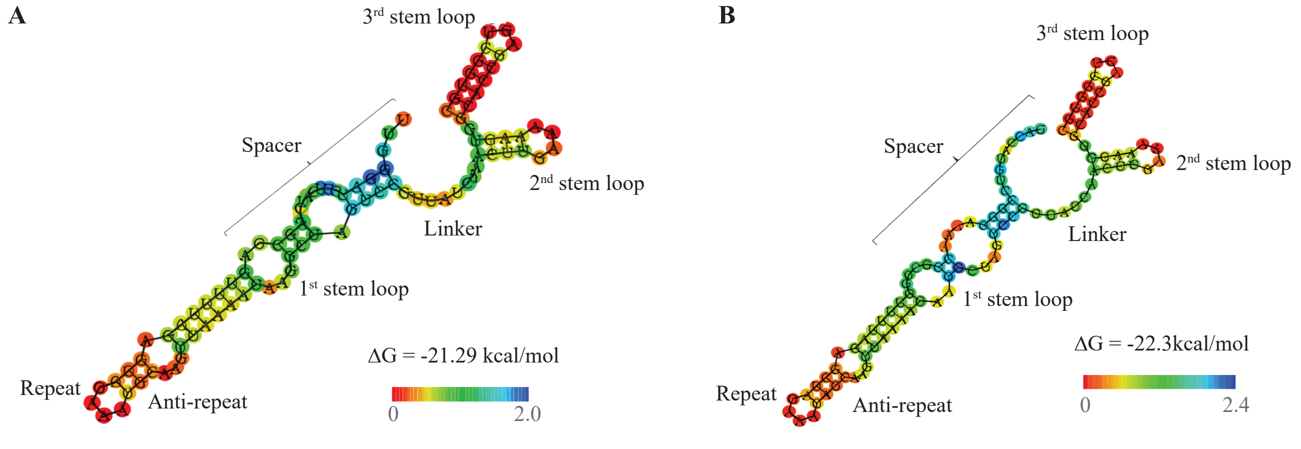

Figure 1. Predicted secondary structures of sgRNAs generated using RNAfold for exon 22 (A) and exon 25 (B) target of *ERBB2* gene. The structures illustrate the arrangement of the repeat–anti repeat region, spacer, linker, and the first to third loops together with the thermodynamic stability distribution along each molecule. The Gibbs free energies of the respective structures are ΔG=-21.29 kcal/mol for the exon 22 sgRNA and ΔG=-22.3 kcal/mol for the exon 25 sgRNA, indicating stable secondary conformations that are compatible with efficient RNP Cas9 complex formation.

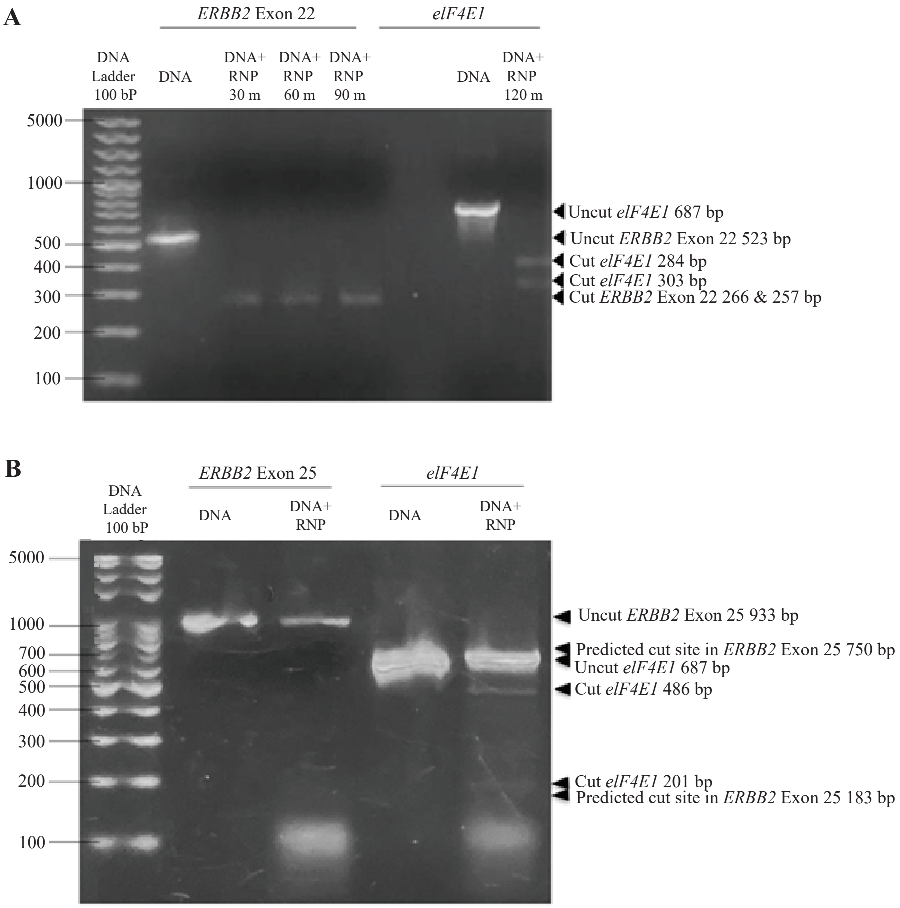

Figure 2. *In vitro* endonuclease activity assay of the Cas9–RNP complex targeting the *ERBB2* gene. A: The *ERBB2* exon 22 target exhibited cleavage activity, as indicated by the generation of DNA fragments of 266 bp and 287 bp, while the positive control (*eIF4E1*) produced cleavage fragments of 383 bp and 303 bp. B: The *ERBB2* exon 25 target showed no detectable cleavage fragments, whereas the positive control (targeting *eIF4E1* gene) generated cleavage fragments of 486 bp and 201 bp.

codons, leading to truncated proteins or mRNA degradation via nonsense-mediated decay (NMD), ultimately abolishing functional HER2 expression and disrupting oncogenic signaling pathways that promote cancer cell survival.(29)

A similar study also reported that among three sgRNAs targeting exons 5, 10, and 12 (the extracellular domain of ERBB2), only the sgRNA targeting exon 12 was effective in inducing DNA cleavage at the cellular level.(21) Although the overall mutation frequency was low, the resulting truncated HER2 protein exhibited dominant-negative activity, suppressing the MAPK/ERK signaling pathway and cell proliferation without affecting total HER2 protein levels. These findings suggest that the present study, which targets the transmembrane and tyrosine kinase domains of ERBB2, has the potential to disrupt signaling cascades and inhibit proliferation in HER2-positive breast cancer.

Efficient induction of DSBs depends on optimal sgRNA design to accurately guide Cas9 to the target sequence. In this study, sgRNAs were selected using an integrated approach combining three web-based tools: CHOPCHOP, CRISPOR, and CRISPR RGEN; and evaluated based on their structural and functional parameters. These tools also predict potential off-target sites across the genome and generate specificity scores, enabling preliminary assessment of sgRNA specificity during the design stage. This strategy addresses the limited accuracy of individual prediction platforms in estimating cleavage efficiency and off-target effects. Only approximately 65–85% of sgRNAs predicted to be efficient actually exhibit high experimental activity, with relatively low overlap among different design tools.(30) Accordingly, the use of a combination of three or more tools has been recommended to increase the probability of selecting highly effective sgRNAs.(31)

In silico analysis indicated that both spacers satisfied fundamental sgRNA design criteria; however, the exon 22 spacer demonstrated a superior overall profile. It exhibited optimal GC content (50%), was consistently ranked as the top candidate by both CHOPCHOP and CRISPOR with an identical sequence, and showed strong inter-platform agreement. Additionally, it presented a minimal off-target profile with only one 0-mismatch site, high MIT and Cutting Frequency Determination (CFD) specificity scores, robust on-target efficiency predictions, and a high probability of inducing frameshift mutations, supporting its suitability for gene knockout applications. In contrast, although the exon 25 spacer had acceptable GC content, it was prioritized by only one platform (CHOPCHOP) and lacked consistent ranking across other tools. The presence of two off-target sites with three mismatches further suggests a relatively higher theoretical risk of nonspecific cleavage. Overall, the exon 22 spacer is more favorable due to its higher specificity, stronger predicted efficiency, and consistent multi-platform validation.

Secondary structure prediction revealed that both designed sgRNAs possessed essential structural components required to support Cas9 endonuclease activity, including the repeat–anti-repeat (RAR) region critical for Cas9 binding, stem loop 1 involved in Cas9–sgRNA–DNA complex formation, and stem loops 2 and 3, which contribute to structural stability and enhanced *in vivo* function.(32) Recent studies have emphasized that all stem loops, except stem loop 1, must maintain their secondary structures to ensure optimal genome-editing efficiency.(33)

The successful amplification and purification of the ~200 bp sgRNA fragments confirmed that the sgRNAexon22 and sgRNAexon25 transcription cassettes were obtained in sufficient quality and quantity for cloning into the pGEM-T Easy vector. The confirmation of white colonies yielding a ~200 bp band by colony PCR indicated efficient ligation and transformation, as well as correct integration of the sgRNA inserts, enabling their use as templates for sgRNA production via *in vitro* transcription. The resulting sgRNAs were subsequently employed in endonuclease activity assays to assess their efficiency in guiding Cas9 to the target DNA sequences and inducing cleavage.

In vitro CRISPR–Cas9 RNP endonuclease activity assays demonstrated that both positive control sgRNAs (sgRNA196 and sgRNA300) and the sgRNA targeting exon 22 generated DNA cleavage fragments of the expected sizes. For the 523 bp exon 22 targets, Functional *in vitro* validation of sgRNA prior to *in vivo* application is a reliable predictor of CRISPR/Cas9 activity, as reported previously that a 100% correlation between *in vitro* cleavage and *in vivo* editing efficiency in zebrafish, outperforming in silico tools such as CHOPCHOP.(34) Consistent with this principle, the present study employed a Cas9–sgRNA ribonucleoprotein (RNP)-based *in vitro* endonuclease assay using PCR-amplified ERBB2 substrates. The exon 22–targeting sgRNA produced the expected cleavage fragments (266 bp and 257 bp from a 523 bp amplicon) with stable activity at 30, 60, and 90 min (Figure 2A and 3A), whereas the exon 25 sgRNA showed no detectable cleavage despite predicted fragments of 750 bp and 183 bp and confirmed sgRNA–Cas9 complex formation by EMSA (Figure 2B and 3B). Since sgRNA–Cas9 binding is expected to activate the nuclease complex (35), these findings indicate functional differences in sgRNA performance. Control sgRNA196 and sgRNA300 verified Cas9 catalytic competence under identical conditions, supporting the reliability of the assay and reinforcing the importance of prescreening prior to therapeutic development for HER2-positive breast cancer.

The lack of detectable cleavage by the exon 25–targeting sgRNA is likely due to intrinsic structural properties. This spacer exhibited higher GC content (60% without PAM; 65.22% with PAM) compared to exon 22 (50% and 56.52%, respectively), with a notably elevated GC proportion in the PAM-proximal region (nucleotides 1–12) of 66.67% versus 50% for exon 22 (Figure 3). High GC content in this critical region has been associated with reduced CRISPR–Cas9 activity, and it was previously reported that GC content exceeding 56% in the PAM-proximal region can significantly decrease genome-editing efficiency.

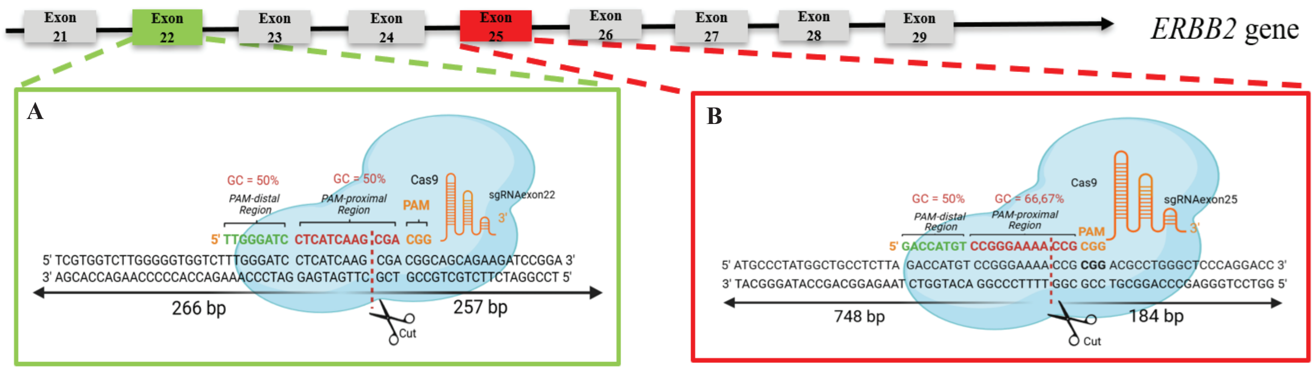

Figure 3. Predicted cleavage sites of the Cas9 RNP complex within the *ERBB2* gene. A: The Cas9 RNP complex guided by the exon 22–targeting sgRNA encoding the transmembrane domain (highlighted in green) cleaves at nucleotide position 266, generating DNA fragments of 266 bp and 257 bp. B: Whereas the exon 25–targeting sgRNA encoding the kinase domain (highlighted in red) cleaves at nucleotide position 750, producing DNA fragments of 750 bp and 183 bp. Green and red colors in the exon schematics represent the coding regions of the transmembrane and kinase domains, respectively.

Another factor that may have contributed to the reduced cleavage efficiency of exon 25 is the PAM motif composition and the position of specific nucleotides within the spacer. Cas9 activity is reduced when using a CGG-type PAM.(36) In addition, the presence of adenine at position 6 of the spacer can negatively affect cleavage efficiency.(20) Exon 25 sgRNA spacer contains an adenine at position 6 and utilizes a CGG PAM. This combination is therefore hypothesized to reduce cleavage efficiency, resulting in the absence of the expected 750 bp and 183 bp fragments, although these characteristics did not adversely affect the exon 22 sgRNA spacer. Another possible explanation is misfolding of the sgRNA secondary structure, which may hinder the formation of an active Cas9–RNP complex.(37) Collectively, these observations suggest that both sequence composition and predicted structural configuration influence sgRNA performance and underscore the value of integrating RNAfold-based secondary structure analysis with sequence-based design parameters during sgRNA selection.

In this study, the evaluation of sgRNA activity was limited to an *in vitro* CRISPR–Cas9 cleavage assay using PCR-amplified target DNA. In addition, the use of plasmid DNA containing the *ERBB2* target fragment as the substrate does not allow experimental assessment of potential off-target cleavage at other genomic loci. Although this method effectively confirms the ability of sgRNAs to guide Cas9-mediated DNA cleavage at the intended target site, it does not directly assess genome editing efficiency or the resulting biological effects in living cells. Therefore, further studies involving cellular genome editing experiments will be necessary to validate the functional impact of *ERBB2* disruption. Future investigations incorporating functional assays, such as analyses of cell proliferation, apoptosis, and migration in HER2-positive cancer models, will be important to better understand the biological consequences of *ERBB2* gene editing.

This study demonstrates that the sgRNA targeting exon 22 of *ERBB2* exhibits efficient and specific *in vitro* cleavage, whereas the exon 25 sgRNA requires further optimization. However, DNA-level editing does not necessarily correlate with RNA expression or functional outcomes, as cellular compensation mechanisms may occur. Although GSTM1 gene editing was successfully achieved, discrepancies between DNA and RNA levels and the absence of altered drug response likely reflected compensatory gene activity or cellular defense mechanisms.(38) As the present work was conducted in a cell-free system lacking chromatin context and endogenous DNA repair pathways, the observed cleavage cannot yet be interpreted as functional gene knockout, underscoring the need for validation in relevant cellular models. Overall, these findings represent an early preclinical stage in CRISPR–Cas9 RNP-based gene therapy development and emphasize the importance of quantitative analysis, sequencing-based indel profiling, and cellular validation to establish biological efficacy and therapeutic potential.

Conclusion

In summary, this study designed two sgRNAs targeting the *ERBB2* gene, specifically exon 22 within the transmembrane domain and exon 25 within the intracellular tyrosine kinase domain, which are critical for HER2 signaling. *In vitro* CRISPR–Cas9 RNP cleavage assays showed that the sgRNA targeting exon 22 successfully guided Cas9 to cleave the target DNA, whereas the sgRNA targeting exon 25 did not exhibit detectable cleavage activity under the experimental conditions used. These findings suggest that the exon 22 sgRNA may serve as a candidate for further genome editing studies targeting *ERBB2*.

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Authors Contribution

VSG ADP designed the study, performed the experiments, analyzed the data, and wrote the manuscript draft. KM contributed to study conception, scientific guidance, data interpretation, and manuscript revision. MIT supported experimental optimization, data analysis, and manuscript improvement. AC provided conceptual feedback, critical review, and academic supervision. All authors read and approved of the final manuscript.

Ethical Statement

Ethical approval and informed consent were not required for this work.

Conflict of Interest

The authors declare no conflict of interest.

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