Cancer Letters 385 (2017) 137–143

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Article

CRISPR-mediated targeting of *HER2* inhibits cell proliferation through a dominant negative mutation

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ARTICLE INFO

Article history:
Received 19 August 2016
Received in revised form
20 October 2016
Accepted 22 October 2016

Keywords:
Cas9
ERBB2
Breast cancer
PARP inhibitor
Trastuzumab
Therapeutic

ABSTRACT

With the discovery of the CRISPR/Cas9 technology, genome editing could be performed in a rapid, precise and effective manner. Its potential applications in functional interrogation of cancer-causing genes and cancer therapy have been extensively explored. In this study, we demonstrated the use of the CRISPR/Cas9 system to directly target the oncogene *HER2*. Directing Cas9 to exons of the *HER2* gene inhibited cell growth in breast cancer cell lines that harbor amplification of the *HER2* locus. The inhibitory effect was potentiated with the addition of PARP inhibitors. Unexpectedly, CRISPR-induced mutations did not significantly affect the level of HER2 protein expression. Instead, CRISPR targeting appeared to exert its effect through a dominant negative mutation. This HER2 mutant interfered with the MAPK/ERK axis of HER2 downstream signaling. Our work provides a novel mechanism underlying the anti-cancer effects of HER2-targeting by CRISPR/Cas9, which is distinct from the clinical drug Herceptin. In addition, it opens up the possibility that incomplete CRISPR targeting of certain oncogenes could still have therapeutic value by generation of dominant negative mutants.

Introduction

Cancer is a disease that stems from genetic alterations that includes point mutations, gene amplifications and translocations [1]. Most therapeutic approaches aim to target the phenotype of cancer, such as rapid cell division, a dysregulated signaling pathway, or an activated kinase that fuels growth. Unfortunately, most of these characteristics are shared by normal cells, thus resulting in undesirable side effects of chemotherapy. A therapeutic approach that directly targets the genomic changes could be valuable since it should, in principle, have no effect on wild type cells. Recent development of genome editing tools such as the clustered regularly interspaced short palindromic repeats (CRISPR) system could provide such an opportunity.

Type II CRISPR/CRISPR-associated protein nuclease (Cas9) system derived from *Streptococcus pyogenes* has been successfully employed for genome engineering in mammalian cells and animals [2–7]. In its most widely used form, CRISPR/Cas9 is composed of two components: the DNA endonuclease Cas9 and a chimeric single guide RNA (gRNA). The chimeric gRNA binds and recruits Cas9 to a specific genomic target sequence [8,9]. Specificity is conferred by the 20 nucleotides at the 5′ end of the gRNA that is complementary to the desired DNA sequence. In addition, a protospacer adjacent motif (PAM) located immediately downstream of the target sequence is essential for Cas9-mediated DNA cleavage at the target site. Cas9-induced double-stranded breaks (DSBs) can lead to error-prone repair by nonhomologous end-joining (NHEJ) [10], thereby enabling targeted disruption of specific genes.

In this study, we employed the CRISPR/Cas9 technology to target the *HER2* (*ERBB2*) gene in HER2-amplified breast cancer cells. *HER2* is a well-known oncogene and the therapeutic target for the monoclonal antibody Herceptin (trastuzumab). We showed that CRISPR/Cas9-mediated targeting of *HER2* inhibited cell proliferation and tumorigenicity. Furthermore, we demonstrated that the effect of CRISPR/Cas9 was markedly enhanced by the treatment of poly-ADP ribose polymerase (PARP) inhibitors. Surprisingly, CRISPR targeting of HER2 did not significantly reduce its protein expression level, though the downstream MAPK/ERK and PI3K/AKT signaling cascades were abrogated. These effects could partly be mediated by a dominant negative truncated form of HER2 generated through a frame-shift insertion in a *HER2* exon.

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http://dx.doi.org/10.1016/j.canlet.2016.10.033
0304-3835/

Materials and methods

Molecular cloning of CRISPR/Cas9, HER2 and guide RNA design

To inactivate all isoforms of *HER2*, exons 5, 10 and 12 were chosen for targeting by single guide RNAs (gRNAs). Three pairs of gRNAs were designed using DNA2.0 gRNA Design Tool (https://www.dna20.com/eCommerce/cas9/input). The pCas9_GFP plasmid expressing *S. pyogenes* Cas9 was obtained from Addgene. Individual gRNAs were cloned into gRNA_Cloning vector (Addgene) according to the methodology online (http://www.addgene.org/41824/). *HER2*-specific gRNA sequences are as follow: exon5 (GTGCCAGTCCCGAGACCCAC), exon10 (GAGGGCCGGTATACATTCGG) and exon12 (GGGCATGGAGCACTTGCGAG). HER2 or gRNAs were cloned into pMX retroviral vector (Addgene), utilizing BamH1 and Not1 restriction sites via Gibson assembly. Wildtype HER2 cDNA clone (NM_004448.2) was purchased (Genecopoeia; EX-Z2866-M61) and single "C" insertion in *HER2* exon12 was generated using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies).

Cell culture and drug treatments

Human breast cancer cell lines BT-474, SKBR-3 and MCF-7 were purchased from American Type Culture Collection (ATCC). BT-474, SKBR-3 and MCF-7 were maintained in RPMI (Gibco), McCoy's 5A (Modified) (Gibco) and 4500 mg/L glucose DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin antibiotics (Gibco) at 37 °C with 5% CO_2, respectively. Human Embryonic Kidney (HEK) 293FT cells were cultured in DMEM under similar conditions. Cells were treated with Herceptin (10 μg/ml; a gift from Motoichi Kurisawa), SCR7 (10 μM; MedKoo), NU7441 (1 μM; Cayman Chemical), ME0328 (2 μM; Tocris Bioscience) and Veliparib (2 μM; Selleck Chemicals) for the indicated periods prior to functional assays.

Retrovirus production and transduction

pMX retroviral transfer vectors were co-transfected with VSV-G (Addgene) envelop plasmid into HEK293GP2 cells using FuGENE HD (Promega) or calcium phosphate (Clontech). Supernatants containing viral particles were harvested 24 h and 48 h post-transfection and were concentrated by Retro-Concentin according to the manufacturer's protocol (System Biosciences). For transduction, cells were incubated with virus-containing supernatants in the presence of 6 μg/ml polybrene (Sigma–Aldrich) for 8 h before replenishment with normal culture medium.

Transfection and cell proliferation assay

Breast cancer cell lines were transfected with Cas9 and gRNAs expression vectors using X-tremeGENE DNA transfection reagent according to the manufacturer's protocol (Roche). Briefly, cells were transfected or infected at 2×10^5 cells/well in 12-well plates for 3 days. They were then seeded in 96-well plates at 1×10^4 cells/well (BT-474 and SKBR-3) or 4000 cells/well (MCF7) for AlamarBlue cell proliferation assay. Fluorescence was measured at 530 nm and 590 nm wavelengths by Infinite M200 PRO plate reader (TECAN).

Soft agar colony formation assay

Transiently transfected cells were suspended in 0.4% agarose and culture media supplemented with 10% FBS and seeded over a basal layer of 0.6% agarose. The experiments were set up in 6-well plates at 1×10^5 cells/well in triplicates. Images from 6 different fields were captured for each biological replicate. The number of colonies ≥50 μm in size was scored 1–3 weeks after culture at 37 °C.

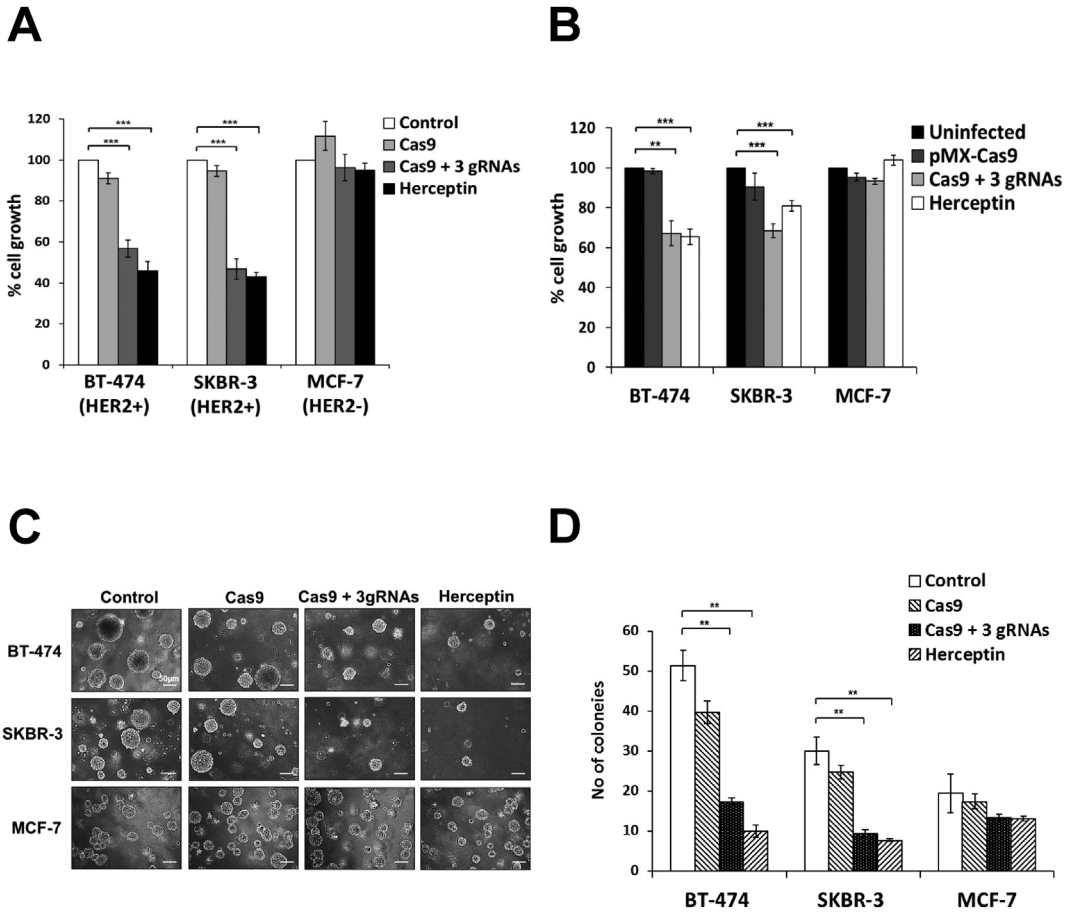

Fig. 1. Targeting of *HER2* by CRISPR/Cas9 reduced cell growth and tumorigenicity. (A) HER2-positive breast cancer cell lines BT-474 and SKBR-3 and HER2−negative MCF-7 cells were transfected with plasmids expressing Cas9 alone or together with 3 gRNAs that target exons 5, 10 and 12 of *HER2*. Cell proliferation was evaluated by AlamarBlue assay 6 days post-transfection. Cells treated with Herceptin at 10 μg/ml for 6 days served as positive control. Three independent experiments consisting of technical duplicates were performed. The values were plotted relative to the untransfected control sample for each cell line (means ± SEM, n = 6). (B) Breast cancer cells were transduced with pMX-based retroviruses encoding Cas9 alone or in combination with *HER2*-targeting 3gRNAs or subjected to Herceptin treatment (10 μg/ml) for 6 days prior to AlamarBlue assay. The data was normalized to the uninfected control sample (means ± SEM, n = 6). (C) The tumorigenic potential was assessed by soft agar colony-forming assay. Representative phase contrast images are shown. Scare bar = 50 μm. (D) The number of colonies ≥50 μm in size were scored 1–3 weeks after plating (means ± SEM, n = 3). Double and triple asterisks represent *p* value < 0.01 and < 0.001, respectively.