

## Basic Medical Research

### TMEPAI genome editing in triple negative breast cancer cells

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#### ABSTRAK

**Latar belakang:** Clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) merupakan teknik untuk mengedit genom dengan efektif. Teknik ini membutuhkan RNA-guided DNA endonuclease Cas9 dan single guide RNA (gRNA). Ekspresi keduanya secara bersamaan dapat memotong gen target dengan baik sehingga terjadi pembentukan double-strand break (DSB). Melalui perbaikan NHEJ (non-homologous end joining) atau HDR (homology-directed repair) pada DSB akan terjadi perubahan pada DNA. Penelitian ini bertujuan membuat galur sel kanker payudara tipe triple negative yang tidak mengekspresikan transmembrane prostate androgen-induced protein (TMEPAI) menggunakan teknik CRISPR-Cas9.

**Metode:** gRNA yang dirancang bekerja di gen target disintesis, disambungkan dan diklon ke dalam vektor yang dapat digunakan untuk mengekspresikan pada galur sel kanker payudara. Vektor gRNA tersebut, bersama dengan vektor RNA-guided DNA endonuclease Cas9-GFP dan vektor resistensi puromisin ditransfeksikan secara bersamaan ke dalam galur sel kanker payudara dengan polyethylenimine (PEI). Setelah 24 jam, sel diseleksi menggunakan puromisin selama 3 hari. Klon sel yang bertahan setelah seleksi di sub-kultur dan kemudian digunakan untuk analisis genom dan protein untuk membuktikan bahwa sel tersebut tidak mengekspresikan TMEPAI.

**Hasil:** Teknik CRISPR-Cas9 dalam penelitian ini telah berhasil membuat galur sel kanker payudara tipe triple negative, Hs578T, yang tidak mengekspresikan TMEPAI (Hs578T KO). Hal ini dikonfirmasi melalui pemeriksaan genomik dengan sekuensing dan proteomik melalui pemeriksaan western blot. Hasil sekuensing menunjukkan adanya mutase frameshift. Mendukung hasil tersebut, pemeriksaan western blot menunjukkan tidak adanya pita TMEPAI pada sel Hs578T-KO.

**Kesimpulan:** Galur sel kanker payudara tipe triple negative yang tidak mengekspresikan TMEPAI (knock-out/Hs578T KO) berhasil dibuat menggunakan teknik CRISPR-Cas9. Hal ini dikonfirmasi dengan pemeriksaan genomik dan proteomik.

#### ABSTRACT

**Background:** Clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) is a powerful genome editing technique. It consists of RNA-guided DNA endonuclease Cas9 and single guide RNA (gRNA). By combining their expressions, high efficiency cleavage of the target gene can be achieved, leading to the formation of DNA double-strand break (DSB) at the genomic locus of interest which will be repaired via NHEJ (non-homologous end joining) or HDR (homology-directed repair) and mediate DNA alteration. We aimed to apply the CRISPR/Cas9 technique to knock-out the transmembrane prostate androgen-induced protein (TMEPAI) gene in the triple negative breast cancer cell line.

**Methods:** Designed gRNA which targets the TMEPAI gene was synthesized, annealed, and cloned into gRNA expression vector. It was co-transfected into the TNBC cell line using polyethylenimine (PEI) together with Cas9-GFP and puromycin resistant gene vector. At 24-hours post-transfection, cells were selected by puromycin for 3 days before they were cloned. Selected knock-out clones were subsequently checked on their protein levels by western blotting.

**Results:** CRISPR/Cas9, a genome engineering technique successfully knocked-out TMEPAI in the Hs578T TNBC cell line. Sequencing shows a frameshift mutation in TMEPAI. Western blot shows the absence of TMEPAI band on Hs578T KO cells.

**Conclusion:** TMEPAI gene was deleted in the TNBC cell line using the genomic editing technique CRISPR/Cas9. The deletion was confirmed by genome and protein analysis.

**Keywords:** CRISPR/Cas9, gene editing, knock-out cell lines

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Genome editing methods have been developing recently. There are several genome editing technologies, namely zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) system. ZFNs and TALENs have been put to practical use first. Both contain tethered endonuclease catalytic domains and deoxyribose nucleic acid (DNA) binding domain for introducing targeted DNA-double stranded break (DSB) in specific locus.<sup>1</sup> Unlike them, the CRISPR/Cas9 system uses guide ribonucleic acid (gRNA) to recruit Cas9, an endonuclease, for pairing with the specific DNA region.<sup>1,2</sup> Compared to previous techniques, the CRISPR/Cas9 system is easily designed, and gives more efficient multi genes editing for variety of cells and organism.<sup>1</sup> DSB is repaired by DNA repairing pathways, non-homologous end joining (NHEJ) or homology-directed repair (HDR) and undergo genome editing.<sup>3</sup> NHEJ frequently generates insertion or deletion mutation. HDR mediates DNA editing in the presence of exogenous donor DNA.<sup>4</sup>

Triple negative breast cancer (TNBC) is a leading breast cancer causing death although it is found only in 15–25% of total breast cancer patients. It has no targeted therapy due to the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2). TNBC tends to grow more rapidly, frequently occurs in young women, more frequently metastasizes to lungs and brain, and more readily acquires drug resistance, compared to non-TNBC.<sup>5,6</sup> Therefore, understanding the molecular mechanism in pathogenesis to determine targeted therapy and drug resistance mechanism in TNBC is necessary.

TMEPAI was found highly expressed in 68.8% of TNBC patients.<sup>5</sup> Kaplan Meier (KM) plot showed that high TMEPAI expression correlates with poor prognosis. Previous research proved TMEPAI as a direct target gene and acts as a negative regulator by regulating duration and intensity of Transforming growth factor- $\beta$  (TGF- $\beta$ ) responses in TGF- $\beta$  Smad-dependent pathway.<sup>7</sup> Additionally, TMEPAI can degrade phosphatase and tensin homolog (PTEN) by recruiting E3 ubiquitin ligase through its PY motifs to activate phosphatidylinositol-3-kinase/a serine/threonine kinase (PI3K/AKT) pathway.<sup>5</sup> Furthermore, knock-down of TMEPAI

in lung cancer reduces *in vivo* the xenograft tumor forming ability and the sphere forming ability. Thus, TMEPAI is thought to be a possible oncogenic protein.<sup>8</sup> However, the molecular mechanisms of TMEPAI in TNBC is not clear yet.

We aimed to apply CRISPR/Cas9 technique to knock-out TMEPAI in the triple negative breast cancer cell line. Knocking-out cell lines will be useful for further experiments to elucidate the TMEPAI function on tumorigenesis and drug resistance.

## METHODS

### Cell culture

The TNBC cell line, Hs578T was obtained from the American type culture collection (ATCC). Hs578T were cultured in Dulbecco's modified essential medium (DMEM, Invitrogen) which is supplemented with 10% fetal bovine serum (FBS, Gibco), 10  $\mu$ g/mL insulin, and 100 units/ml of penicillin G and 0.1 mg/ml of streptomycin sulfate (Wako). Cells were maintained in 5% CO<sub>2</sub> incubator at 37°C.

### Plasmid construction

The gRNA against for TMEPAI exon2 locus was designed *in silico* using the CRISPR design online tool.<sup>9</sup> The oligonucleotide primers have a default sequence for forward TTTCTTGGCTTTATATATCT TGTGGAAAGGACGAAACACCGtgacggctcctcatcagc and reverse GACTAGCCTTATTTTAACTTGCTATTT CTAGCTCTAAAACgctgatgaaggaccgtgcac.

Forward and reverse oligonucleotide were annealed by the standard method and followed by extension using PrimeSTAR DNA polymerase (Takara Bio). Extended 100 bps of double-strand DNA was purified using polymerase chain reaction (PCR) purification kit (Qiagen) and inserted into gRNA cloning vector (Addgene plasmid #41824), which was a gift from George Church. It amplified in *E. Coli* and purified them using a miniprep purification kit (Qiagen). It was followed by sequencing before use.

### Transfection

Hs578T cells were transfected with plasmid DNA using the polyethylenimine (PEI) transfection reagent. 1x10<sup>5</sup> cells were seeded on a 6-wells microplate one day before transfection. 1.5  $\mu$ g plasmid DNA (500 ng gRNA, 500 ng human-Cas9,

and a 500 ng puromycin resistant gene) in 4.5µL PEI, and incubate for 30 minutes. Medium was changed four to six hours after transfection.

### Western blotting

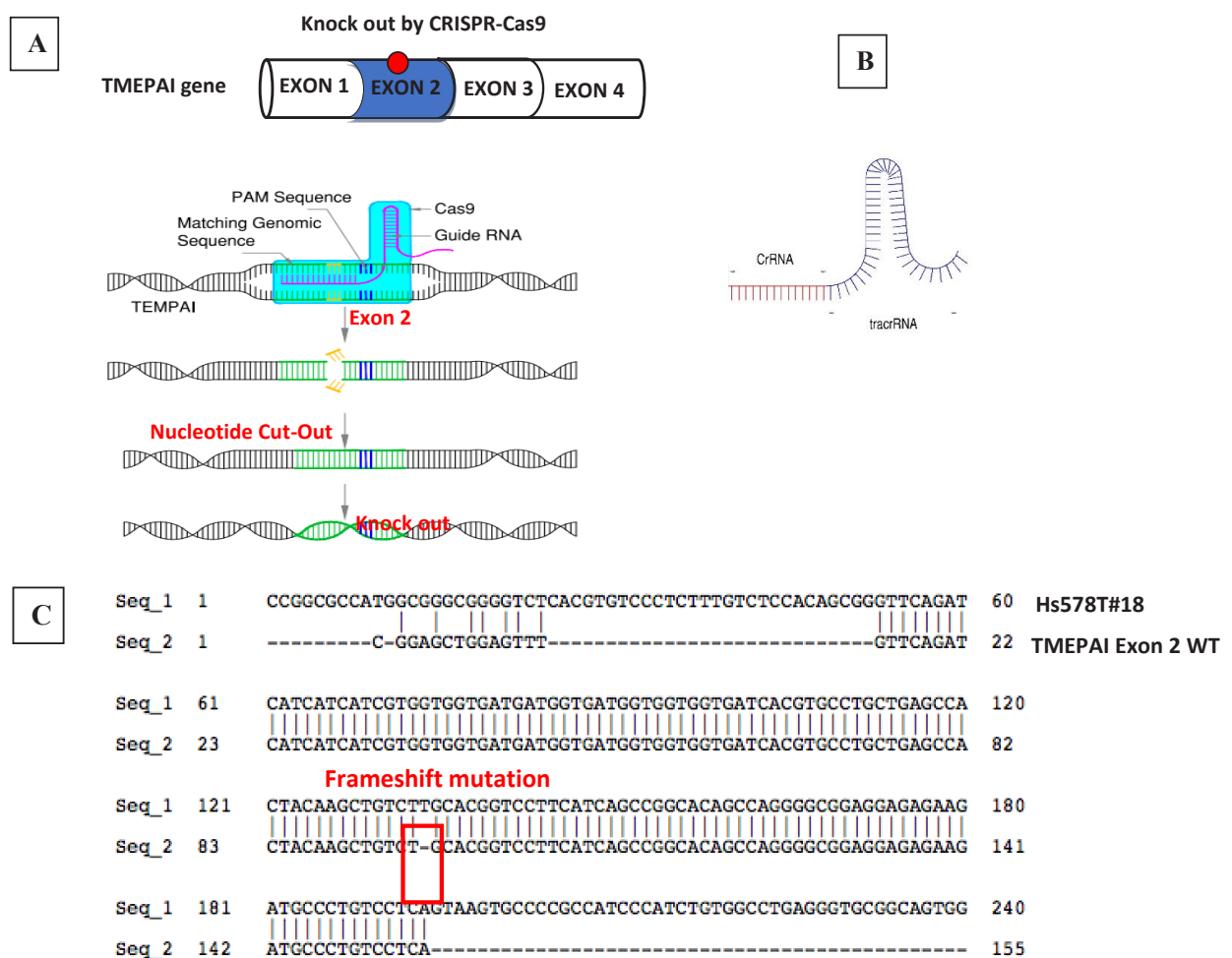
Cells were collected with Tris buffer (62.5 mM Tris-HCl pH 7.5) and lysed using sodium dodecyl sulfate (SDS)-sample buffer (4% SDS, 1.44 M β-mercaptoethanol, 20% glycerol, 125 mM Tris pH 7.4, 0.002% bromophenol blue). Cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then electro-transferred to a mix Polyvinylidene difluoride (PVDF) membrane (GE Healthcare) and subjected to immunoblot analysis. The primary antibodies used were rabbit anti-TMEPAI (homemade polyclonal antibody, 1:250)<sup>8</sup> and mouse anti-β-actin antibody (Sigma, 1:5,000). Reacted antibodies were detected with

horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare, 1:10,000), anti-rabbit IgG (GE Healthcare, 1:10,000), and Immuno-Star Zeta (Wako). EZ-capture MG (ATTO) was used for the detection of chemiluminescence.

## RESULTS

### Establishing knock-out TMEPAI in triple negative breast cancer cell line

We transfected gRNA expression vector together with human-Cas9 and puromycin resistant gene expression plasmids into Hs578T cells to induce DSB on TMEPAI exon 2 gene locus (Figure 1A). Puromycin selection was done for 72 hours since 24-post transfection. Surviving cells were seeded on a 10-cm dish to isolate single clone (two to three weeks). Then we expanded the grown



**Figure 1.** CRISPR-Cas9 system to knock-out TMEPAI. (A) CRISPR-Cas9 systems in Hs578T, the triple negative breast cancer cell line, (B) gRNA containing CrRNA which will direct Cas9 nuclease and tracrRNA which will recruit Cas9, (C) frameshift mutation due to knock-out the TMEPAI gene by CRISPR-Cas9 in Exon 2 TMEPAI.

cells for isolating protein. We analyzed TMEPAI expression by DNA sequencing (Figure 1C) and western blot (Figure 2).

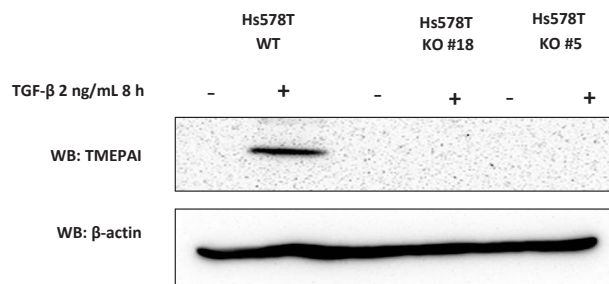
TNBC cell line, Hs578T responded to TGF- $\beta$  stimulation and expressed TMEPAI eight hours after the treatment of TGF- $\beta$  (2 ng/mL). However, KO-TMEPAI cells (clone #18 and #5) did not express TMEPAI protein (Figure 1C). The data indicate that we successfully established the KO-TMEPAI TNBC cell using the CRISPR-Cas9 system.

## DISCUSSION

Previously, Cong et al<sup>11</sup> and Mali et al<sup>12</sup> demonstrated that Cas9 and gRNA can be used for genome editing in human cells. Instead of other techniques, it works as an advanced technique which is affordable, easy to use, and high-throughput. Nevertheless similar to other techniques, it might make off-target cuts.<sup>13</sup> The gRNA contains a targeting sequence (crRNA sequence) and a Cas9 nuclease-recruiting sequence (tracrRNA) (Figure 1B). The crRNA region (red color) is a nucleotide sequence that is homologous to a region in a TMEPAI sequence and the tracrRNA region directs Cas9 to the targeted sequence on genomic DNA. While tracrRNA interacts with Cas9, endonuclease cleaves the targeted sequence.<sup>1,2</sup> Our results showed that crispr-cas9 successfully knocked out TMEPAI in exon 2. We got the edited genomic sequence in our cell line. This edited gene is inheritable to their daughter cells. In our study, the cells which were used in genomic and proteomic analyses came from passage No. 3-5.

Our gene of interest, TMEPAI, is also known as prostate transmembrane protein androgen-induced 1 (PMEPA1) and solid tumor-associated gene1 (STAG1). The TMEPAI gene encodes four protein isoforms, a, b, c, and d. Each has a distinct sequence in exon 1.<sup>14</sup> Therefore, we could not target at exon 1. The sequence in exon 2 is commonly used for all isoforms. Hence, we targeted at exon 2 to edit all TMEPAI isoforms.

It was first found as an androgen-regulated gene which is related to a relapsed tumor in the human prostate cancer xenograft model that was strongly



**Figure 2.** Establish knock-out TMEPAI in triple negative breast cancer cell line. The expression of TMEPAI protein in WT and KO Hs578T cells was measured by western blot analysis after TGF- $\beta$  treatment.  $\beta$ -actin was used as a loading control

androgen-dependent.<sup>15</sup> Currently, TMEPAI has been proven to enhance the tumorigenic activity in ovarian, breast and lung cancer cells,<sup>8,16</sup> while in TNBC, TMEPAI promotes TGF- $\beta$  dependent growth, motility and invasion.<sup>5</sup> Taken together, TMEPAI is suspected as oncogenic protein. In contrast to above results, Fournier et al<sup>17</sup> recently reported that low of TMEPAI correlates with poor metastasis-free survival and TMEPAI knockdown increases prostate cancer metastases in a mouse model.<sup>17</sup> All the opposite results suggested that TMEPAI has an important function in cancer. However, how role of TMEPAI in cancer remained unclear. So, our cell line can be used in further experiments to elucidate the role of TMEPAI in TNBC pathogenesis to determine target therapy, drug resistance mechanism or other relevant research.

Instead of androgen, TMEPAI was induced by epidermal growth factor (EGF) in breast and ovarian cancer cell lines and in breast primary tumors,<sup>16</sup> and TGF- $\beta$  in many cancer cell lines.<sup>7,8,18</sup> Singha et al<sup>5</sup> reported that TGF- $\beta$  is a stronger inducer of TMEPAI expression compared to EGF and lysophosphatidic acid (LPA). Notwithstanding, TMEPAI is the highest inducible gene of TGF- $\beta$ , and TGF- $\beta$  has a pivotal role in cancer. Therefore, we are concerned with TGF- $\beta$  pathways to distinguish clearly between wild-type (WT) and knock-out (KO) cell lines of TMEPAI.

This study used one triple negative breast cancer cell line, Hs578T. Considering that TNBC is a diverse and heterogenous group of breast cancer type, we can not generalize this system in other triple negative breast cancer cell lines. Despite the limitation, our study clearly shows



that our designed gRNA can edited the TMEPAI genome and further disperses TMEPAI protein expression. In conclusion, the TMEPAI gene was deleted in the TNBC cell line using the genomic editing technique CRISPR/Cas9, and the deletion was confirmed by genome and protein analyses. This TMEPAI knock-out cell line can be used for further experiments to elucidate the role of TMEPAI in TNBC pathogenesis to determine target therapy, drug resistance mechanism or other relevant research.

### Conflict of interest

The authors affirm no conflict of interest in this study.

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