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Abnormal RNA splicing and genomic instability after induction of *DNMT3A* mutations by CRISPR/Cas9 gene editing

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ABSTRACT

DNA methyltransferase 3A (DNMT3A) mediates *de novo* DNA methylation. Mutations in *DNMT3A* are associated with hematological malignancies, most frequently acute myeloid leukemia. *DNMT3A* mutations are hypothesized to establish a pre-leukemic state, rendering cells vulnerable to secondary oncogenic mutations and malignant transformation. However, the mechanisms by which *DNMT3A* mutations contribute to leukemogenesis are not well-defined. Here, we successfully created four *DNMT3A*-mutated K562 cell lines with frameshift mutations resulting in truncated DNMT3A proteins. *DNMT3A*-mutated cell lines exhibited significantly impaired growth and increased apoptotic activity compared to wild-type (WT) cells. Consistent with previous studies, *DNMT3A*-mutated cells displayed impaired differentiation capacity. RNA-seq was used to compare transcriptomes of *DNMT3A*-mutated and WT cells; *DNMT3A* ablation resulted in downregulation of genes involved in spliceosome function, causing dysfunction of RNA splicing. Unexpectedly, we observed *DNMT3A*-mutated cells to exhibit marked genomic instability and an impaired DNA damage response compared to WT. CRISPR/Cas9-mediated *DNMT3A*-mutated K562 cells may be used to model effects of *DNMT3A* mutations in human cells. Our findings implicate aberrant splicing and induction of genomic instability as potential mechanisms by which *DNMT3A* mutations might predispose to malignancy.

1. Introduction

DNA methyltransferase 3A (DNMT3A) is the member of the DNA methyltransferase family primarily involved in *de novo* gene methylation [24]. DNA methylation is an epigenetic modification involving the addition of a methyl group to cytosine residues to form 5-methylcytosine (5-mC), usually in the context of a cytosine-guanine (CpG) dinucleotide pair [11]. DNA methylation of CpGs in gene regulatory regions influences gene expression, with high levels of DNA methylation generally associated with gene silencing [47].

Aberrant DNA methylation has been broadly implicated in the pathogenesis of cancer [8]. In particular, mutations in the *DNMT3A* gene are associated with a wide range of hematological malignancies. *DNMT3A* mutations are found in 20–40% of acute myeloid leukemia (AML) patients [18,29] and are also reported in myelodysplastic syndrome (MDS), myeloproliferative neoplasms, and T-cell acute lymphoblastic leukemia [43]. *Dnmt3a* loss in mouse hematopoietic stem cells (HSCs) predisposes to malignant transformation, further supporting a role of DNMT3A in preventing malignancy [22]. Clinically, many studies have demonstrated that the presence of somatic *DNMT3A* mutations is associated with poor patient prognosis in myeloid neoplasia [28,40].

DNMT3A mutations may act as driver mutations, producing a pre-leukemic state by rendering cells vulnerable to secondary oncogenic mutations and malignant transformation. *DNMT3A* mutations are typically present at higher variant-allele frequencies in patients with hematological malignancies, suggesting they occur early, perhaps arising months or years before the development of disease [41]. In AML patients, mutations in *DNMT3A* often coexist with secondary lesions in leukemia-related genes such as *ASXL1, FLT3, IDH1/2,* and *TET2*, supporting the hypothesis that *DNMT3A* mutations predispose to secondary oncogenic lesions [18]. Furthermore, AML patients harbor phenotypically normal HSCs with *DNMT3A* mutations but without coincident *NPM1* mutations present in peripheral blasts, and these HSCs retain the ability to differentiate into multiple lineages, suggesting that *DNMT3A* mutations confer a pre-leukemic state [34]. Similarly, clonal

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hematopoiesis driven by leukemia-associated genes, with *DNMT3A* being the most frequent driver mutation, is common in humans and increases with age. Healthy individuals with such clonal hematopoiesis are at increased risk of developing leukemia and all-cause mortality [10,15]. We have also recently described a large cohort of aplastic anemia (AA) patients, in whom the presence of adverse somatic mutations, including *DNMT3A*, was associated with inferior overall survival and more frequent progression to MDS or AML [46].

The mechanisms by which *DNMT3A* mutations contribute to malignant transformation and ultimately to poor patient outcomes are not well-defined. In mice, *Dnmt3a*-mutated HSCs preferentially self-renew rather than differentiate, leading to accumulation of mutated clones in the bone marrow [2]. Further, *Dnmt3a* loss drives hypomethylation and subsequent activation of leukemia-related genes [20,44]. However, these findings have not been recapitulated using human tissue.

The goals of our study were to determine the effects of *DNMT3A* mutations which contribute to malignant transformation in human cells. To this end, we created *DNMT3A* mutated (MT) human cell lines using the gene-editing technology CRISPR/Cas9. Compared to conventional gene editing techniques such as RNA interference, CRISPR/Cas9 leads to permanent and complete loss of gene function by altering the genetic code, analogous to mutations that occur *in vivo* during the development of hematologic malignancy. Our generation of *DNMT3A*-mutated K562 clones establishes this cell line as a model that can be used to study human *DNMT3A*-driven leukemogenesis. In addition, we provide potential mechanisms by which *DNMT3A* mutations predispose to malignancy, including the novel association of *DNMT3A* loss with spliceosomal dysregulation and genomic instability.

2. Materials and methods

2.1. Cell culture and cytogenetic analysis

The K562 cell line and HAP1 GeneArt engineered *DNMT3A* KO cell line was purchased from the American Type Culture Collection (ATCC) and Thermo Fisher Scientific, respectively. All cell lines were cultured in IMDM medium supplemented with 10% fetal bovine serum and antibiotics and were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells in the logarithmic growth stage were cytospun on slides using the Shandon Cytospin 4 and subjected to staining with the StainRITE® Wright-Giemsa Stain Solution (Polysciences, Warrington, PA) to examine their morphologies. Standard G-band karyotype analysis was performed using passage-matched parental cells within 7 days of thawing (Karyologic, Inc., Durham, NC, USA).

2.2. Genome editing

Two pU6-based plasmids were purchased from Santa Cruz Biotechnologies (sc-400323 and sc-418922): a plasmid containing a *DNMT3A*-targeting guide RNA (gRNA) and Cas9-GFP for generation of *DNMT3A* MT cell lines; and a non-targeting 20-nucleotide scramble gRNA and Cas9-GFP for creating transfected K562 *DNMT3A* WT cell lines. Each plasmid (2 μg) was transfected into K562 cells using the Amaxa Cell Line Nucleofector kit according to the manufacturer's instructions. After transfection and electroporation, cells were seeded onto 12-well plates, and GFP-expressing cells were sorted singly into 96-well plates by fluorescence-activated cell sorting (FACS). Individual single-cell clones were subsequently expanded and genotyped *via* Sanger sequencing.

2.3. Validation of DNMT3A mutations

Sanger sequencing was utilized to validate *DNMT3A* gene ablation and to determine the mutation induced by the CRISPR/Cas9 system. DNA extracted using the Qiagen DNeasy Blood and Tissue kit was amplified by PCR with primers targeting genomic regions surrounding the CRISPR/Cas9 gRNA target sites using the TaKaRa LA Taq polymerase kit. Purified PCR amplicons were then subjected to TA cloning *via* the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA).The TempliPhi 100 Amplification Kit (GE Healthcare Life Sciences, Pittsburgh, PA) was used to prepare the DNA templates for sequencing, and the amplified product was subsequently sequenced with adequate primers using the BigDye Terminator v3.1 Cycle Sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA).

Whole-exome sequencing of extracted DNA was performed to confirm *DNMT3A* mutations. Library construction, exon capture, and sequencing was performed by Otogenetics (Atlanta, GA, USA). In brief, paired-end libraries were generated using the Illumina TruSeq DNA sample preparation kit. Exons were enriched using the Agilent Human All Exon V5 (51 Mb) capture system. Illumina HiSeq2500 was used for sequencing with a paired-end sequencing length of 100–125 bp and approximately 70 million reads per sample (data deposited in the National Center for Biotechnology Information [NCBI] Gene Expression Omnibus [GEO] database [GSE96635, subseries GSE96625]).

2.4. Protein extraction and immunoblotting

Cells (1×10^7) were directly lysed using 300 μL of the protein gel loading buffer, boiled at 95 °C for 10 min, and subjected to gel electrophoresis by loading 45 μL (1.5×10^6 cells/well) of cell lysate onto 8% SDS gels (12-well Novex WedgeWell gels). After transferring onto PVDF membranes, immunoblotting was performed. Briefly, the membranes were incubated with the following primary antibodies (Abs): anti-DNMT3A (C-12; Santa Cruz; Dallas, TX, USA) and anti-β-tubulin (9F3; Cell Signaling; Danvers, MA, USA).Subsequently, membranes were subjected to incubation with anti-mouse IgG (Santa Cruz) or anti-rabbit IgG (Cell Signaling) conjugated with horseradish peroxidase. Signals were detected with a 1:1 mixture of the SuperSignal West Dura Extended Duration Substrate and the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher, Waltham, MA) using the Image Quant LAS 4000 system (GE Healthcare Life Sciences).

2.5. Functional assays

To assess cell growth, four replicates of each K562 cell line were seeded into a 75 mm^2 flask at 0.2×10^6 cells/mL and incubated for 8 days without changing the media. Cell numbers were measured daily by the Beckman-Coulter Vi-Cell XR Cell Viability Analyzer. All experiments were conducted using passage-matched parental cells within 7 days of thawing.

To assess apoptosis and the DNA-damage response, exponentially-growing K562 cells were seeded at a density of 5.0×10^5 cells/mL in a 12-well plate and treated with 5-fluorouracil (5-FU) at concentrations of 50 μM, 100 μM, and 250 μM. After 48 h of culture, apoptotic activity was measured using the FITC Annexin V Apoptosis Detection kit with Propidium Iodide (BioLegend) according to the manufacturer's instructions. For DNA damage, cells were washed twice with PBS, fixed, and permeabilized. Cells were then stained with anti-pH2AX-FITC (BD Biosciences, San Jose, CA) for 30 min at room temperature followed by flow cytometry analysis.

To assess megakaryocytic differentiation, K562 cells in the exponential growth phase were seeded at a density of 5.0×10^5 cells/mL in a 12-well plate and treated with 10 ng/mL phorbal 12-myristate 13-acetate (PMA, Sigma) for 16 and 24 h. Cells were harvested, stained with anti-CD61-PE (BD Biosciences) for 30 min at room temperature, and subjected to flow cytometry analysis. To assess erythroid differentiation, cells in the exponential growth phase were seeded at a density of 4.0×10^5 cells/mL in 3.5 mL/well in a 6-well plate and treated with 40 μM Hemin (Sigma Aldrich, Saint Louis, MO) for 48 and 72 h. Cells were harvested, stained with anti-CD71-APC and Glycophorin A-PE (BD Biosciences) for 20 min at room temperature and then subjected to flow cytometry analysis. Samples stained with isotype controls (BD