METTL14 promotes prostate tumorigenesis by inhibiting THBS1 via an m6A-YTHDF2-dependent mechanism

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N6-methyladenine (m6A) is the most predominant RNA modification, which has been shown to be related to many types of cancers. However, understanding of its role in prostate cancer (PCA) is largely unknown. Here, we report an upregulation of METTL14 that was correlated with poor prognosis in PCA patients. Functionally, knocking down METTL14 inhibited tumor proliferation both in vitro and in vivo. Mechanically, RNA-seq and MeRIP-seq analyses identified THBS1 as the downstream target of METTL14 in PCA. METTL14 downregulated THBS1 expression in an m6A-dependent manner, which resulted in the recruitment of YTHDF2 to recognize and degrade Thrombospondin 1 (THBS1) mRNA. Thus, our findings revealed that METTL14 acted as an oncogene by inhibiting THBS1 expression via an m6A-YTHDF2-dependent manner. METTL14 could be a potential prognosis marker and a therapeutic target.

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INTRODUCTION

Prostate cancer (PCA) is the most common male cancer and represents the sixth cause of cancer death in men worldwide [1, 2]. Nowadays, for patients with advanced PCA, androgen deprivation therapy (ADT) has been the primary therapy for decades of years. Despite the initial response to androgen deprivation therapy (ADT), the majority of patients relapse with a poor prognosis stage of castration-resistant prostate cancer (CRPC). Patients with CRPC cannot be cured currently and the mortality remains high [3, 4]. Thus, understanding of the mechanisms underlying PCA progression is essential for molecular diagnosis and targeted therapy.

Traditionally, epigenetic regulations consist of diverse modifications on DNA and histone, which are a series of reversible biological processes regulating gene expression without changing the genome sequences [5]. Previous studies have found that many epigenetic regulators on DNA and histone methylation have important effects on PCA progression [6]. These studies have provided a novel epigenetic view for exploring promising targeted therapies for PCA. Apart from these regulators on DNA or histone, in recent years, accumulating studies have focused on RNA modifications, especially methyladenine (m6A) modification, which is the most prevalent post-transcriptional alteration [7, 8]. As a dynamic and reversible process, m6A modification is installed to RNA by m6A methyltransferase (writers), including methyltransferase-like 3 (METTL3) [9], methyltransferase-like14 (METTL14) [10], and Wilms tumor 1 associated protein (WTAP) [11], and removed by alkylation repair homolog protein(ALKBH5) and Fat mass and obesity-associated protein (FTO), which both act as m6A demethylases (erasers) [12]. Besides, there are some proteins, called m6A readers, exerting their functions in recruiting and binding to m6A sites, like YTH domain-containing family protein 1/2/3 (YTHDF1/2/3) and insulin-like growth factor 2 mRNA binding proteins 1/2/3 (IGF2BP1/2/3) [12]. The alterations of these m6A effectors have been implicated in many types of cancers, such as lung cancer [13], hepatocarcinoma [14], and colorectal cancer [15]. It has been previously shown that METTL3 drives migratory and invasive capacities of PCA cells via mediating m6A modification of USP4 mRNA in a YTHDF2-dependent manner [16]. METTL14, as the indispensable allosteric activator of METTL3, has been shown to play an important role in tumor progression in many types of cancers [17–19]. However, the biological significance of METTL14 in PCA has not been elucidated.

In this study, we report that METTL14 promoted the progression of PCA and identified Thrombospondin 1 (THBS1), an endogenous inhibitor of angiogenesis [20], as its downstream target. Moreover, a member of m6A readers, YTHDF2, was recruited for THBS1 mRNA decay. Collectively, our investigation proposes that METTL14 may be a novel prognosis marker and a potential therapeutic target for PCA.

RESULTS

Upregulation of METTL14 is correlated with poor prognosis of PCA patients

To quantify METTL14 expression in PCA patients, we performed IHC staining on a tissue microarray containing prostate tumor specimens (n = 49) and adjacent normal prostate tissue specimens (n = 11). The staining results indicated that METTL14 was highly expressed in PCA tissues compared to the normal ones.
Similarly, the higher expression levels of METTL14 mRNA (Fig. 1C) and protein (Fig. 1D) were observed in human PCa cell lines compared with the normal prostate cell line RWPE1, a prostatic epithelial cell line. Besides, the relative m6A quantification of RWPE1 and PCa tumor cell lines. Two independent shRNA sequences targeting METTL14 (shMETTL14-1 and shMETTL14-2) were separately transfected into DU145 cells. The relative m6A quantification of control and METTL14 knockdown in DU145 cells. The overall survival of PCa patients with high and low METTL14 mRNA level using Kaplan–Meier survival curve analysis methods based on the TCGA dataset. Overall survival of PCa patients with high and low m6A expression classified by consensus clustering. The data in B is presented as the mean ± SEMs. The other data are presented as the mean ± SDs. *p < 0.05; **p < 0.01; ***p < 0.001 (Student’s t-test).

Fig. 1 Upregulation of METTL14 is correlated with poor prognosis of PCa patients. A Representative images of IHC staining of PCa tumor tissues (n = 49) and normal ones (n = 11) with the anti-METTL14 antibody. up: scale bar: 400 μm, down: scale bar: 100 μm. B The calculation of percentage of positive area of METTL14 in PCa tumor tissues (n = 49) and normal ones (n = 11) for TMA. C, D The relative mRNA (C) and protein (D) levels of METTL14 in RWPE1 and PCa cell lines detected by qPCR and western. E The relative m6A quantification of RWPE1 and PCa tumor cell lines. F Two independent shRNA sequences targeting METTL14 (shMETTL14-1 and shMETTL14-2) were separately transfected into DU145 cells. The relative m6A quantification of control and METTL14 knockdown in DU145 cells. G Overall survival of PCa patients with high and low METTL14 mRNA level using Kaplan–Meier survival curve analysis methods based on the TCGA dataset. H Overall survival of PCa patients with high and low m6A expression classified by consensus clustering. The data in B is presented as the mean ± SEMs. The other data are presented as the mean ± SDs. *p < 0.05; **p < 0.01; ***p < 0.001 (Student’s t-test).

(METTL14) promotes PCa cell proliferation in vitro and in vivo
To explore the role of METTL14 in PCa, we established the stable METTL14 knockdown and overexpression cell lines based on CRPC cell lines DU145 (Fig. 2A, L, Supplementary Fig. 1B) and PC3 (Fig. 2D, Supplementary Fig. 1C). We found that depleting METTL14 reduced the cell proliferation and the colony formation efficiency remarkably in DU145 (Fig. 2B, C) and PC3 (Fig. 2E, F) cells. On the contrary, overexpression of METTL14 amplified the proliferation and colony numbers of tumor cells (Fig. 2M, N). Furthermore, cell
Cycle assays were carried out to further confirm the function of METTL14 for cell proliferation. The results showed that the proportion of cells in the G2 phase was increased and the proportion of cells in the S phase was decreased in METTL14 knockdown cell lines (Fig. 2G-I), whereas, elevated expression of METTL14 effectively decreased the proportion of the G2 phase and increased the proportion of cells in S phase (Fig. 2O).

In addition, to validate the function of METTL14 for tumor growth in vivo, we subcutaneously injected stably transfected METTL14 knockdown DU145 cell lines in nude mice. Then after...
6 weeks, the data showed that the depletion of METTL14 significantly restricted the tumor growth. Compared to the control group, the shMETTL14 group exhibited smaller tumor volumes and weights (Fig. 2J, K).

These data together suggest that METTL14 results in accelerated cell proliferation in PCa.

Identification of METTL14 targets via RNA-seq and MeRIP-seq
To further investigate the mechanism underlying how METTL14 promotes tumor progression and identify its downstream targets in PCa, we conducted RNA-sequencing (RNA-seq) and Methylated RNA immunoprecipitation sequencing (MeRIP-seq) using shNC and shMETTL14 DU145 cells. RNA-seq analysis showed that compared to the control group, 204 genes (log2 FC < -0.5) were downregulated, and 172 genes (log2 FC > 0.5) were upregulated when METTL14 was knocked down in tumor cells (Fig. 3A, B, Supplementary Table 3). Differential genes were found to be linked with regulation of mRNA stability, cell cycle G2/M phase transition, positive regulation of binding, and RNA transport using Gene Ontology (GO) analysis (Fig. 3C, Supplementary Table 4).

Fig. 3 Identification of METTL14 targets via RNA-seq and MeRIP-seq. A Volcano plot of DEGs identified by RNA-seq in shNC and shMETTL14 DU145 cells. Gray, unchanged genes (|log2(fold change)| < 0.5 or p > 0.05) when comparing shNC and shMETTL14 group. Red, upregulated genes (log2(fold change) > 0.5 and p < 0.05) in shMETTL14 group. Blue, downregulated genes (log2(fold change) < -0.5 and p < 0.05). B Heatmap of upregulated and downregulated DEGs. C GO analysis of DEGs. D The consensus motif of DU145 cells identified by MeRIP-seq. p = 1e-79. E The number of m6A peaks in shNC (n = 5461) and shMETTL14 (n = 2861) group. F The m6A signals were largely enriched in 3'UTR. G Distribution of peaks in DU145 cells H Determination of shared genes of RNA-seq and MeRIP-seq. 11 genes were measured. THBS1 was identified as the potential target of METTL14.
For MeRIP-seq, consistent with previous studies [22], GGAC was the most frequent m6A motif enriched in our detected peaks (Fig. 3D). In total, MeRIP-seq analysis identified 5461 and 2861 peaks in shNC and shMETTL14 cells, respectively (Fig. 3E). The expression and location of METTL14 and THBS1 were measured by Immunofluorescence (IF) assays in DU145 or PC3 shNC and shMETTL14 cells. Scale bar: 20 μm.

To assess whether the changed gene expression was induced by m6A modification, we then overlapped 282 reduced m6A peaks of MeRIP-seq (Supplementary Table 5) and 377 altered genes of RNA-seq, and found that 11 genes were both detected (Fig. 3H). Among these genes, we found that THBS1, an endogenous inhibitor of angiogenesis [20], presented the most consistent decreased m6A level, and its mRNA level became increased in shMETTL14 PCa cells compared to control cells (Supplementary Fig. 1E, F), we therefore chose THBS1 as a potential targeted gene after METTL14 m6A modification for further studies.

**Fig. 4** THBS1 is regulated by METTL14-mediated m6A modification and acts as a potential tumor suppressor in PCa cells. A The relative m6A enrichment of THBS1 determined by MeRIP-qPCR. B The mRNA level of THBS1 when knocking down (left) or overexpressing (right) METTL14 in DU145 (B) and PC3 (C) cells. D The protein levels of THBS1 after METTL14 deletion in DU145 and PC3 cells. E The expression and location of METTL14 and THBS1 measured by Immunofluorescence (IF) assays in DU145 or PC3 shNC and shMETTL14 cells. F The decay rate of THBS1 mRNA determined at 0, 3, 6 h after treating with actinomycin D (5μg/ml) in METTL14 knocking down and METTL14 overexpression DU145 cells. G, H The siRNA sequences targeting THBS1 (siTHBS1) were transfected into DU145 or PC3 cells. Cell proliferation assays (G) and colony formation assays (H) were conducted. All data are presented as the mean ± SDs. *p < 0.05; **p < 0.01; ***p < 0.001 (Student’s t-test).

**THBS1 is regulated by METTL14-mediated m6A modification**

To validate whether THBS1 is a target of METTL14-mediated m6A modification, as identified in MeRIP-seq, we performed MeRIP-qPCR in PCa cells. The m6A enrichment of THBS1 was remarkably decreased when METTL14 was knocked down (Fig. 4A). Furthermore, THBS1 mRNA level was increased in PCa METTL14 knockdown cells and decreased in PCa overexpression cells (Fig. 4B, C). Moreover, the protein level of THBS1 was also increased when METTL14 was knocked down (Fig. 4D). We also found that the METTL14 protein was located in the nucleus while THBS1 protein was located in the cytoplasm by Immunofluorescence (IF) assays. METTL14 expression exhibited a rise in PCa cells after METTL14 was knocked down (Fig. 4E). In addition, the half-life of THBS1 RNA was extended in METTL14 knockdown cells after the cultures were treated with actinomycin D, which is a transcriptional inhibitor.
Taken together, our results reveal that METTL14 mediates mRNA degradation via an m6A-dependent manner to inhibit THBS1 expression. THBS1 is a potential tumor suppressor in PCa To investigate the role of THBS1 in PCa progression, cell proliferation assays were conducted and the data showed that after knocking down THBS1 using targeted siRNA (Supplementary Fig. 1D), the growth of PCa cells was increased (Fig. 4G). The same trend was also observed in colony formation assays, we found that knocking down THBS1 significantly facilitated tumor cells colony formation ability (Fig. 4H). Collectively, these results point out that THBS1 may act as a potential tumor suppressor to suppress PCa proliferation.

YTHDF2 facilitates THBS1 mRNA decay in an m6A-dependent manner

The m6A methylation added by m6A writers needs to be recognized by m6A readers for increasing or decreasing gene expression [23, 24]. Among them YTHDF2 is known to be the regulator of promoting mRNA degradation [25], which may be recruited by METTL14 to mediate THBS1 mRNA decay. To test this conjecture, we firstly conducted RNA immunoprecipitation (RIP) assays to assess whether YTHDF2 can directly bind to THBS1 mRNA. The RIP analyses showed that the groups with antibodies against YTHDF2 bound much more THBS1 mRNA than those against IgG in DU145 and PC3 cells (Fig. 5A). Besides, the binding between YTHDF2 and THBS1 was weakened when METTL14 was inhibited (Fig. 5B). To further examine the effect of YTHDF2 binding to THBS1 mRNA on their expression levels, we knocked down YTHDF2 with two targeted siRNA and found that both mRNA and protein expression of THBS1 was elevated (Fig. 5C-F). Furthermore, the RNA stability assays revealed that after treatment with siRNA targeted YTHDF2, the decay rate of THBS1 exhibited a significantly slower trend (Fig. 5G). These data demonstrate that YTHDF2 recognizes METTL14-methylated THBS1 mRNA and accelerates THBS1 mRNA decay.

DISCUSSION

Prostate cancer (PCa) has the highest incidence among all cancers in men and the treatment options for CRPC are limited [1, 2]. Nowadays, looking for new mechanisms underlying PCa progression and finding novel therapeutic targets are urgently needed. Recent research has demonstrated that the abnormal gene expression of m6A regulators plays a significant role in PCa...
exploring the pathogenesis of PCa. METTL14 could be a potential all these studies provide a novel epigenetic dimension for dynamic methylation and demethylation, m6A modi
of proteins, called writer, eraser, and reader, respectively. Via its performed to explore how METTL14 affects the metastasis ability degradation of the tumor suppressors [29]. However, functioning as the oncogene in the pathological processes of PCa and metastasis of PCa [16]. It is also found that one of the m6A readers, YTHDF2 functions as promoting mRNA decay by binding to m6A sites in the cytoplasm [34]. Moreover, the sequencing results show that METTL14 binds to THBS1 3’ UTR region, so the specific binding site needs to be further investigated by luciferase reporter assay and single-nucleotide m6A detection methods. Additionally, THBS1, as a tumor suppressor inhibiting PCa proliferation, can be considered as the potential therapeutic target in the future. Taken together, our study reveals an oncogenic role of METTL14 in PCa progression. Mechanistically, we identified a “METTL14- YTHDF2-THBS1” axis in PCa cells, which provides a novel epigenetic dimension for exploring the pathogenesis of PCa. Thus, METTL14 could be a potential prognosis marker and a therapeutic target for PCa.

MATERIALS AND METHODS
Cell culture and reagents
All cell lines were obtained from the American Type Culture Collection (ATCC). Human prostate cancer cell lines DU145 were cultured in DMEM medium (Gibco, Grand Island, NY), while PC3 was cultured in RPMI-1640 medium (Gibco, Grand Island, NY). Both mediums were supplemented with 10% fetal bovine serum (FBS). Cells were grown at 37 °C in a cell culture incubator containing 5% CO2.

TCGA dataset analysis
RNA-sequencing (RNA-seq) data of TCGA-PRAD cohort were achieved through the Cancer Genome Atlas portal (https://portal.gdc.cancer.gov/). Fragments Per Kilobase of transcript per Million mapped reads (FPKM) was transformed into Transcripts Per Million (TPM) values for subsequent analysis. The survival data of TCGA-PRAD cohort was obtained from the TCGA Pan-Cancer Clinical Data Resource (TCGA-CDR) [35]. We divided samples into METTL14 high and low groups by median TPM of METTL14. Consensus Clustering was performed based on gene expressions of m6A and identified m6A low and high groups.

Tissue microarray (TMA) and immunohistochemical (IHC)
TMA chip was purchased from Outdo Biotech, Ltd (HProA060PG01, Shanghai, China), consisting of the prostate tumor tissue specimens (n = 49) and adjacent normal prostate tissue specimens (n = 11). IHC was executed on a TMA chip using primary antibodies against METTL14 (HPA038002, Sigma). The Image J software was used to quantify the protein levels by calculating the integrated optical density per stained area (IOD/area). The clinical information of the patients was provided in Supplementary Table 1.

Quantitative PCR (qPCR) assays
Total RNA from tumor cells was extracted with Trizol (15596-026) (Invitrogen, Carlsbad, CA) and reverse transcribed using the HiScript III 1st Strand cDNA Synthesis Kit (R123-01, Vazyme, China). The qRT-PCR assay was performed using ChamQ SYBR Master Mix (Q311-02) (Vazyme, Nanjing, China). Relative expression of mRNA was calculated by normalizing to the housekeeping gene ACTB as the endogenous control. At least three independent replicates were included for analysis. The primers used were listed as follows: METTL14: sense: 5’-TTTCTTGCAGGCTTTGGTCT-3’; antisense: 5’-GTTGGAACATGGATAGCCGC-3’; THBS1: sense: 5’-AAAGGTCTCGG-CCTTCA-3’; antisense: 5’-AGAATGCTGTCCTCGCTGTT-3’; YTHDF2: sense: 5’-AGCCCCACTTCTACCCA-GATG-3’; antisense: 5’-TGGAAATCTTATTCCCATGGC-3’.
Western blot
Cells were lysed in RIPA buffer (89901) (Invitrogen, Carlsbad, CA) including protease inhibitor (HY-K0021, MCE, NJ). The protein abundance was measured by BCA Protein Assay Kit (23227) (Invitrogen, Carlsbad, CA). The lysates were centrifuged at 12,000 × g for 10 min at 4 °C to remove cell debris. Afterwards, the supernatants were subjected to SDS-PAGE for the following subsequent experiments. The PVDF membranes with transfected protein were incubated at 4 °C overnight and then incubated with secondary antibodies (31460, Invitrogen) for 2 h. Finally, the PVDF membranes were washed using 1% TBST and detected by the Bio-Rad ChemiDoc Touch Imaging System. The antibodies used were as follows: GAPDH (30203ES05, Yeasen, China), METTL14 (HPA038002, Sigma), THBS1 (ab267388, abcam), YTHDF2 (24744-1-AP, proteintech). The original western blots were provided in Supplementary Material.

Lentivirus package and infection
Plasmids including transgenes and packaging plasmids were cotransfected into HEK 293 T cells using polyethyleneimine linear (PEI) (40815ES03, Yeasen, China). After 48 h, viruses were collected. When tumor cells grew at a density of around 50%, the collected viruses were transfected with an appropriate concentration of polybrene (ST1380-10, Beyotime, China). After 2 days, puromycin (58-58-2, Gene Operation, MA) was used to select the transfected cells.

Immunofluorescence (IF)
Cells were plated on coverslips in 12-well plates and were harvested when growing to an ~40% density. The slides were fixed with formalin for 15 min and washed twice with phosphate buffer saline (PBS). Then, 2 ml 0.2–0.5% triton X-100 was used for permeabilization for 10 min. Blocking solution was used to block the cells for 30 min and then the primary antibody was incubated at room temperature for 1 h. After combination, the slides were washed with PBS for 3 × 5 min. Next, the secondary antibody (1:1000, Invitrogen) was combined for 1 h at room temperature in the darkness and followed by three times of PBS washing. Finally, we added a drop of mounting medium on the slides for 10 min and then checked the staining results with a fluorescence microscope.

Cell proliferation and colony formation assay
For cell proliferation assay, control and transfected tumor cells were seeded into 96-well plates in a density of 1500 cells/well. For every 24 h, replicate wells were added 10% CCK8 (MA0218-3, meilunbio, China) and then the samples were left at room temperature for 30 min. After these treatments, the absorbance was measured at 450 nm for each well.

For colony formation assay, 500 cells per well were seeded into six-well plates and cultured in the medium with 10% FBS in the incubator for 2 weeks. Then, the cells were fixed with 4% paraformaldehyde (AR-0211, Meilunbio, China) and maintained at 37 °C for 2 h. Subsequently the absorbance was measured at 450 nm for each well.

For colony formation assay, 500 cells per well were seeded into six-well plates and cultured in the medium with 10% FBS in the incubator for 2 weeks. Then, the cells were fixed with 4% paraformaldehyde (AR-0211, Meilunbio, China) and maintained at 37 °C for 2 h. Subsequently the absorbance was measured at 450 nm for each well.

Cell cycle assay
The control and transfected cells (5 × 10^4) were collected and then washed with PBS. Accordingly, 1 ml DNA Staining solution and 10 μl permeabilization solution of the cell cycle staining kit (70-CCS012, MultiSciences, China) were added into each sample, and then the samples were left at room temperature for 30 min. After these treatments, cells were measured by flow cytometry, and the data were analyzed by Cell Quest ModFit software.

Xenografts in mice
Stably transfected shMETTL14 and shNC DU145 cells (5 × 10^6 cells) suspended in a mixture of 100 μL PBS were subcutaneously injected into the right flank of male nude BALB/c mice (6–8 weeks old) to induce tumor formation. Tumor growth was measured every week using a caliper and the tumor volume was calculated by the formula (width^2 × length/2). All animal studies were consistent with the Shanghai Jiaotong University Guide for the care and use of laboratory animals.

siRNA transfection
Tumor cells were seeded on the six-well plates at a density of 2 × 10^4 cells per well. After 24 h, cells were transfected with transient knockdown of target genes by siRNA using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA). Cells were harvested 48 h for RNA abundance analysis and 72 h for protein expression analysis. YTHDF2 and THBS1 siRNA sequences and negative control sequences were designed and synthesized by Genomeditech (Shanghai, China). Sequences of siRNAs were listed in Supplementary Table 2.

m6A quantification assay
Total RNA was extracted from prostate cancer cells using Trizol. Extracted 200 ng RNA was used by EpiQuik m6A Methylation Quantification Kit (P9005-48) (Epigentek, Farmingdale, NY), and the m6A level of each sample was measured according to the manufacturer’s instructions.

RNA immunoprecipitation (RIP) assays
RIP assays were performed using Abnova RIPA Binding Protein Immunoprecipitation Kit (17–700) (Millipore, Billerica, MA) according to the manufacturer’s instructions. 2 × 10^7 DU145 or PC3 cells were collected using Lysis Buffer. Magnetic Beads Protein A/G was incubated with antibodies against YTHDF2 and IgG overnight at 4 °C. The next day after times of washing and other treatments, the purified RNA was conducted by qPCR, and data were normalized to input.

Methylated RNA immunoprecipitation sequencing (MeRIP-seq) and MeRIP-qPCR
In brief, MeRIP assays were conducted following the manufacturer’s instructions (C11051-1, Ribobio, China). Total 100ug RNA of shMETTL14 and shNC were fragmented into 100–150 bp fragments. Then, about 1/10 of the fragment RNA was divided as input. The others were coated with anti-m6A (ab190886, Abcam) or IgG for 2 h at 4 °C. After times of washing and other procedures, the methylated RNA was purified by Magen Hicup Serum/plasma miRNA Kit (R4317-03, Magen, China) and the samples were sequenced by RibBio (Guzhou, China). For MeRIP-qPCR, the purified methylated RNA from the above steps was reverse-transcribed and analyzed by qPCR.

RNA stability assays
PCA cells were plated in twelve-well plates. The following day, actinomycin D (5 μg/ml, S8964, Selleck) was added into the culture medium. Total RNA was collected at 0, 3, 6 h and analyzed by qPCR. The data were normalized by β-Actin and evaluated with a linear regression model.

Statistical analysis
Statistical analysis was carried out using the GraphPad Prism software (version 8.0). To compare two independent groups, a two-tailed Student’s t-test was used, whereas, for differences among more groups, one-way analysis of variance (ANOVA) was conducted on the data. p < 0.05 was considered statistically significant. Survival analysis was performed using Kaplan–Meier methods, and a log-rank test was used to determine the statistical significance of differences.

DATA AVAILABILITY
All data generated or used during this study are available from the corresponding author by request.

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