Targeting Super-Enhancer associated oncogenes in esophageal squamous cell carcinoma

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SUPPLEMENTARY MATERIALS AND METHODS

Construction and Infection of CDK7 shRNA-Expressing Lentivirus

The pLKO.1-CDK7-shRNA was generated by inserting double-stranded oligonucleotides into pLKO.1-puro lentiviral vector, and was confirmed by DNA sequencing. Recombinant lentiviral vectors and packaging vectors (pCMV-dR8.91 and pMD2.G-VSVG) were co-transfected into 293T cells using Lipofectamine 2000 according to the manufacturer's instruction. Supernatants containing lentivirus expressing shRNA were harvested 48h after transfection, TE7 and KYSE510 cells were infected with the lentiviruses and supplemented with 8 mg/ml Polybrene (Sigma-Aldrich).

Cell Proliferation Assay

To measure proliferation, cells were seeded onto 96 well plates (2,000-5,000 cells per well), and exposed to either vehicle or different concentrations of inhibitors at indicated time points. Cell viability was assessed using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) staining method.

Cell-Cycle Analysis

Cells were harvested after 24 and 48 hr treatment with either vehicle or the inhibitor, and fixed (75% ethanol) overnight at - 20℃. Cell pellets were obtained by spinning at 15,000 RPM for 5 min, washed with cold PBS, and finally resuspended in PBS, with 0.1% triton, RNAse A and propidium iodide (PI). After 30 min of incubation, samples were analyzed by LSR II Flow Cytometer System (BD Biosciences, San Jose, CA).

Cell Apoptosis Analysis

Cells were harvested after exposure to either vehicle or inhibitor, washed twice with PBS, double-labeled with Annexin V-fluorescein isothiocyanate (FITC) and Propidium iodide (PI) using the Rh Annexin V/FITC kit (Bender Medsystem, San Bruno, CA) and measured by

LSR II Flow Cytometer System.

Colony Formation Assay

Colony formation assay was performed by plating cells in 6-well plates. After 2 weeks, cells were fixed with methanol and stained with crystal violet. The number of colonies was counted by ImageJ software. Data were presented as mean ± SD from 3 independent experiments in triplicate wells.

Antibodies and Chemicals

Antibodies: CDK7 (Cell Signaling Technology, 2916), CDK2 (Cell Signaling Technology, 2546), CDK4 (Cell Signaling Technology, 12790), CDK6 (Cell Signaling Technology, 13331), CDK9 (Cell Signaling Technology, 2316), RNAPII CTD S2 (Bethyl, A300-654A), RNAPII CTD S5 (Bethyl, A300-655A); RNAPII CTD S7 (Cell Signaling Technology, 13780); RNAPII (Santa Cruz, sc-899); DNAJB1 (Cell Signaling Technology, 4871S); YAP1 (Novus Biologicals, NB110-58358); SREBP2 (Abcam, ab30682); RUNX1 (Abcam, ab35962); Phospho-RUNX1 (Cell Signaling Technology, 4327); PAK4 (Cell Signaling Technology, 3242); Phospho-PAK4 (Santa Cruz, sc-135774); GAPDH (Abcam, ab46540); Anti-rabbit IgG (Cell Signaling Technology, 7074); Anti-mouse IgG (Cell Signaling Technology, 7076); H3K27ac (Abcam, ab4729); Rabbit anti-IgG (Abcam, ab46540).

Chemicals: THZ1 (ApexBio, A8882); AT7519 (Selleck Chem, S1524); SNS-032 (Selleck Chem, S1145); LEE011 (Selleck Chem, S7440); Flavopiridol (Selleck Chem, S1230); Roscovitine (Selleck Chem, S1153); JNJ-7706621 (Selleck Chem, S1249). KPT9274 (Karyopharm Therapeutics)

RNA-Seq Analysis

Total RNA from each sample was extracted according to the manufacturer's instruction of RNeasy Mini kit (QIAGEN). RNA library was prepared using TruSeq Library Prep Kit (Illumina) according to the manufacturer's instructions and subjected to massive parallel

sequencing using Hiseq (Illumina) at Beijing Genomics Institute. To analyze the RNA-Seq results, we first aligned 100 bp paired-end sequencing reads to human reference genome (build GRCh37/hg19) using STAR aligner with ensemble gtf (v75) provided as junctions file[1]. Cufflinks were used to measure the expression in terms of FPKM (Fragments Per Kilobase of transcript per Million mapped reads) against Ensemble transcripts (v75)[2]. Those transcripts with mean expression of FPKM >1 were considered as actively transcribed and were used for subsequent analysis. Heatmaps were drawn with R software using log2 fold change values. Wiggle tracks for RNA-seq data were generated using rseqc (bam2wig.py command) and normalized for rpm[3].

Chromatin Immunoprecipitation

Cells were first crosslinked with 1% formaldehyde solution and incubated for 10 min at room temperature. Cells were neutralized by 1 mL 1.25 M glycine for 5 min, washed three times in ice-cold PBS, centrifuged at 2,000 rpm for 5 min to remove PBS and resuspended in nuclear extraction buffer A (10mM HEPES, 10mM KCL, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5 Mm PMSF) by gentle pipetting or vortex, on ice for 15 min. 25ul of ice cold 10% NP-40 was added per 400 ul of Buffer A and vortexed for 10 sec, kept on ice for 1min and vortex again for 10 sec, centrifuged for 1-5 min at 16,000 ×g. 200 ul SDS lysis buffer was added to the pellet (0.5% SDS, 50 mM Tris, pH 8, 10 mM EDTA, 1 complete protease inhibitor (Roche). Chromatin was sheared in a Bioruptor Sonicator (Diagenode) with 26 cycle, 30 sec. ON, 30 sec. OFF, on high mode at 4℃. Sonication resulted in most small fragments being 200-250 bp in length. The sonicated lysates were collected and centrifuged for 10 min at 4 °C. Supernatants were collected and four parts of dilution buffer (1.25% Triton X-100, 12.5 mM Tris, pH 8, 187.5 mM NaCl, 1 complete protease inhibitor) were added. Sonicated lysates were cleared and incubated overnight at 4℃ with magnetic beads bound with antibody to enrich for DNA fragments bound by the indicated factor. Precipitated

immunocomplexes were washed, for 5 min each, as follows: once with low-salt buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris, pH 8, 2 mM EDTA, 150 mM NaCl and 1 Complete protease inhibitor), once with high-salt buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris, pH 8, 2mM EDTA, 500 mM NaCl and 1 Complete protease inhibitor), twice with LiCl buffer (0.7% sodium deoxycholate, 1% NP-40, 20 mM Tris, pH 8, 1 mM EDTA, 500 mM LiCl and 1 complete protease inhibitor) and once with Tris-EDTA buffer (with protease inhibitor). DNA was eluted with elution buffer (50 mM TrisHCl pH 8.0,10 mM EDTA, 1% SDS). Cross-links were reversed overnight. RNA and protein were digested using RNase A and Proteinase K, respectively and DNA was purified with QIAquick PCR spin kit (QIAGEN).

Gene Ontology Analysis

Gene Ontology analysis was performed using Goseq Bioconductor package[4]. All actively transcribed genes were considered as assayed genes (background) and genes with log2 fold change less than one (50nM THZ1 treatment at 6 hours) were considered as “THZ1-sensitive transcripts”. Similar analysis was performed for SE-associated genes.

Reference

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