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**shRNA and Control shRNA Cell Lines**

MSH6 knockdown HT1080 and MGG152 cells were generated with lentiviral transduction as described previously (20). For knockdown of MSH6, 293T cells were transfected with lentivirus vector packaging plasmid DNA containing 6 μg of human MSH6 shRNA (#1, TRCN0000286578, Sigma-Aldrich), 4 μg of pCMV-dr8.2-dvpr, and 2 μg of pCMV-VSVG with ScreenFect (Wako Chemicals). GIPZ lentiviral human MSH6 shRNA (MSH6 #2, V3LHS\_318784) was purchased from GE Dharmacon. MGG152 or HT1080 cells were infected with lentivirus with polybrene (8 μg/mL) for 8 h. After 1 week, the cells were selected with puromycin (0.2–0.5 μg/mL) for 7 days and used in experiments. pLKO Non-silencing shRNA Control (SHC002, Sigma-Aldrich) was used as a non-silencing control.

Sequences were as follows:

pLKO Non-silencing shRNA (Sigma-Aldrich, SHC002): ATCTCGCTTGGGCGAGAGTAAG

Plasmid: pLKO. MSH6 shRNA (MSH6 #1, Sigma-Aldrich, TRCN0000286578): GCCAGAAGAATACGAGTTGAA

Plasmid: GIPZ lentiviral MSH6 shRNA (MSH6 #2, Dharmacon/Horizontal Discovery, V3LHS\_318784): TACACATTACTTTGAATCC

**Molecular Analyses**

Genomic and bisulfite-modified DNA was extracted using AllPrep DNA/RNA/miRNA Universal Kit and EpiTect Bisulfite kits (Qiagen) per the manufacturer’s protocol. For cell line fingerprinting, Sanger Sequencing, Pyrosequencing, FISH, MLPA, multiplex PCR technology (SNaPshot), and microsatellite instability analysis were used. To detect single nucleotide variants (SNVs), insertion/deletion (indels), and copy number in genomic DNA, the multiplex polymerase chain reaction (PCR) technology Anchored Multiplex PCR (AMP(3), SNaPshot) was used on the ArcherDx platform in conjunction with Illumina NextSeq next-generation sequencing (NGS). After enzymatic shearing, the genomic DNA was end-repaired, adenylated, and ligated with a half-functional adapter. Two hemi-nested PCR reactions were performed to generate a sequencing library targeting hotspots and full exons. Then, NovoAlign was used to align the Illumina NextSeq 2 × 150-base paired-end sequencing results to the hg19 human genome reference. An ensemble variant calling approach, using MuTect1 (4), LoFreq (5), GATK (6-8), and a laboratory developed hotspot caller, was applied for SNV and indel variant detection. A laboratory developed copy number caller using a coverage distribution from a panel of normal controls was applied for copy gain and loss detection. Variants are reported according to Human Genome Variation Society (HGVS) protein and DNA nomenclature rules and are followed by the referenced Ensembl transcript ID. This validated procedure detects SNV and indel variants at ≥ 5% allelic frequency in target regions with sufficient read coverage. The assay shows the following limitations for copy number calling: (1) aneuploidy cannot be distinguished from gene-specific copy gains or copy losses, (2) it has limited sensitivity for borderline/weak copy gains or copy losses, (3) it has limited sensitivity in specimens with low tumor cellularity, (4) it has limited performance in poor-quality specimens showing suboptimal coverage, and (5) absolute copy number status cannot be determined.

The SNV and indel gene targets covered by this test were as follows (exons): ABL1 (4-7), AKT1 (3,6), ALK (21-23,25), APC (16), ARID1A (1-20), ATM (1-63), ATRX (1-35), AURKA (2,5-8), BRAF (11,15), BRCA1 (2-23), BRCA2 (2-27), CCNE1 (3-8,10,12), CDH1 (1-16), CDK4 (2-7), CDKN2A (1-3), CIC (1-20), CSF1R (7,22), CTNNB1 (3), DAXX (1-8), DDR2 (12-18), DDX3X (1-17), EGFR (3,7,15,18-21), ERBB2 (8,10,19-21,24), ERBB3 (2-3,7-8), ERBB4 (3-4,6-9,15,23), ESR1 (8), EZH2 (16), FBXW7 (1-11), FGFR1 (4,7-8,13,15,17), FGFR2 (7,9,12,14), FGFR3 (7-9,14-16,18), FLT3 (11,14,16,20), FOXL2 (1), GNA11 (5), GNAQ (4-5), GNAS (6-9), H3F3A (2), HNF1A (3-4), HRAS (2-3), IDH1 (3-4), IDH2 (4), JAK2 (11,13-14,16,19), JAK3 (4,13,16), KDR (6-7,11,19,21,26-27,30), KEAP1 (2-6), KIT (2,8-11,13-15,17-18), KRAS (2-5), MAP2K1 (2,3,6-7), MAP3K1 (1-20), MDM2 (2-4,6,8,10), MEN1 (2-10), MET (2,11,14,16,19,21), MLH1 (12), MPL (10), MSH6 (1-10), MSI, MYC (1-3), MYCN (3), NF1 (1-58), NF2 (1-15), NOTCH1 (25-27,34), NPM1 (11), NRAS (2-5), PDGFRA (12,14-15,18,23), PIK3CA (2,5,7-8,10,14,19,21), PIK3R1 (1-10), POLE (9-14), PTCH1 (1-23), PTEN (1-9), PTPN11 (3,13), RB1 (1-27), RET (10-11,13-16), RHOA (2-3), RNF43 (2-10), ROS1 (36-38), SDHB (1-8), SMAD2 (7), SMAD4 (2-12), SMARCA4 (3-36), SMARCB1 (2,4,5,9), SMO (3,5-6,9,11), SRC (14), STAG2 (3-34), STK11 (1-9), SUFU (1-12), TERT (1), TP53 (1-11), TP63 (1-14), TSC1 (3-23), TSC2 (2-42), TSHR (10), and VHL (1-3).

The CNV gene targets covered by this test were as follows: ABL1, AKT1, ALK, APC, ARID1A, ATM, ATRX, AURKA, BRAF, BRCA1, BRCA2, CAMTA1, CCNB1, CCND1, CCND2, CCND3, CCNE1, CDK4, CDKN2A, CDK6, CIC, CDH1, CSF1R, DAXX, DDR2, DDX3X, EGFR, ERBB2 (HER-2), ERBB3, ERBB4, FBXW7, FGF19, FGFR1, FGFR2, FGFR3, FLT3, FOXL2, GLI2, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, JAK2, JAK3, KDR, KEAP1, KIT, KRAS, MAP2K1, MAP3K1, MDM2, MDM4, MEN1, MET, MITF, MLH1, MSH6, MYC, MYCN, NF1, NF2, NKX2-1, NOTCH1, NRAS, PDGFRA, PIK3CA, PIK3R1, PLAUR, POLE, PTCH1, PTEN, PTPN11, RB1, RET, RHOA, RNF43, SDHB, SMAD2, SMAD4, SMARCA4, SMARCB1, SMO, SRC, STAG2, STK11, SUFU, TERT, TP53, TP63, TSC1, TSC2, and VHL.

ANALYSIS PIPELINE VERSION: CIDer v2.7. 2

ANNOTATION VERSION: Variant Effect Predictor v83 (8).

**2-HG Quantitation**

In each experiment, 1 × 106 cells, as indicated, were washed with cold PBS and extracted in CelLytic M Cell Lysis Reagent (Sigma-Aldrich). After a 15-min incubation at room temperature, samples were centrifuged at 13,000 rpm for 15 min and the supernatant was transferred to a new tube. Then, 40 µL of 4 M PCA was added and the tube was incubated on ice for 5 min. The sample was centrifuged at 13,000 rpm for 2 min and the supernatant was transferred to a new tube. Samples were neutralized by the addition of 2 M KOH to comprise 34% of the supernatant. After a 15-min centrifuge at 4°C, samples were transferred to a new fresh tube. After adjustment of the sample to pH 6.5–8.0 by KOH or PCA, samples were incubated with D2HG complete reaction mixture. The black-bottom plate (Falcon) was covered and incubated at 37°C for 60 min. The results were read on a multi-well fluorometer (Synergy™ HT Multi-Mode Microplate Reader, BioTek) under the following conditions: (ex = 530 nm) / (em = 590 nm).

**Western blot analysis**

Cells and tumor tissues were lysed in radioimmunoprecipitation (RIPA) buffer (Thermo Scientific) with a protease and phosphatase inhibitor cocktail (Roche). Protein (10–20 µg) was separated by 4–20% SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dry milk in TBS-T (20 mM Tris [pH, 7.5], 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and then incubated with primary antibodies at 4°C overnight. The membranes were washed using TBS-T and incubated with the appropriate peroxidase-conjugated secondary antibodies (GE Healthcare) for 1 h at room temperature. Signals were visualized using an enhanced chemiluminescence (ECL) kit (Bio-Rad).

**Immunohistochemistry**

Tumor tissue specimens for control (n=3) and TMZ (n=3) groups were fixed in neutral-buffered formalin and embedded in paraffin. Seven micrometer sections were obtained, deparaffinized, rehydrated, heated in 10 mM citrate sodium buffer for antigen retrieval, and treated with 3% H2O2 in distilled water. After being blocked using 2.5% Normal Horse Serum (Vector Laboratories), slides were incubated with Ki67 (1:200; Dako, # M7240) overnight at 4°C and then with secondary antibody (ImmPRESS HRP Horse Anti-Rabbit IgG [Peroxidase] Polymer Detection Kit; Vector Laboratories) for 30 min at room temperature. Sections were stained with liquid diaminobenzidine peroxidase chromogen substrate system (Dako) as chromogen and counterstained with hematoxylin. Images were captured with NIS Elements BR 4.6 imaging software attached to a Nikon Eclipse Ci microscope using a ×20 objective.

To exclude bias, positive cells were counted in a blinded manner by LM.

**TUNEL assay**

Tumor tissue specimens for control (n=3) and TMZ (n=3) groups were fixed in neutral-buffered formalin and embedded in paraffin. Seven micrometer sections were obtained, deparaffinized, deparaffinized, rehydrated, In situ detection of apoptotic cells was carried out using the TUNEL assay kit according to the manufacturer’s protocol (Millipore). Briefly, sections were deparaffinized and washed in PBS, followed by proteinase K pretreatment and endogenous peroxidase activity quenching. Labeling was performed by terminal deoxynucleotidyl transferase enzyme mix, and the reaction was stopped by stop buffer incubation. After PBS washes, anti-digoxigenin conjugate were incubated. Finally, the slides were visualized by liquid diaminobenzidine peroxidase chromogen substrate system (Dako) and counterstained with hematoxylin. Images were captured with NIS Elements BR 4.6 imaging software attached to a Nikon Eclipse Ci microscope using a ×20 objective.

Three slides were stained per tumor. Six pictures were captured with 20X magnification per section and used for quantitative analysis of immunopositivity. To exclude bias, positive cells were counted in a blinded manner by LM.

**In Vivo Studies**

All animal experiments were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital (Boston, MA). Non-targeting (NT) gRNA HT1080 cells and PARG gRNA2 HT1080 cells (4 × 106 each) were subcutaneously transplanted into the left and right flank of 7- to 10-week-old female athymic nude mice (weight, 21–26 g; Charles River), respectively. Mice were housed under a 12-h light: dark cycle in single cages (21–24°C with 45% humidity) with standard bedding and enrichment and ad libitum access to food and water. When the maximum tumor diameter reached 5 mm, the mice were randomized to a vehicle group (PBS, i.p., n = 5) and a temozolomide group (TMZ; 50 mg/kg, i.p., n = 5). PBS with 10% DMSO was used as vehicle control. Treatments were administered 5 times a week for 2 weeks with a 1-week off period. A digital caliper was used to measure tumor diameters 3 times a week. The volume (mm3) was calculated as length (mm) × width (mm)2 × 0.5.