Dual effects of a CpG-DNAzyme targeting mutant EGFR transcripts in lung cancer cells: TLR9 activation and EGFR downregulation

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Supplementary Information

MATERIALS AND METHODS

Cell Culture and Oligonucleotide Transfection PC9/GR cells (a gefitinib-resistant lung cancer cell line harboring double mutations in EGFR; deletion of E746~A750 and a T790M point mutation) were purchased from the European Collection of Authenticated Cell Cultures (ECACC, catalogue# 90071810). Cells were cultured in RPMI 1640 medium (HyClone Laboratories, Logan, UT, USA). A549 lung cancer cells and HepG2 liver cancer cells (purchased from the American Type Culture Collection; ATCC CCL-185 and ATCC HB-8065, respectively) were cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose (HyClone Laboratories) at 37 °C in humidified air with 5% CO2. Both media contained 10% fetal bovine serum (HyClone Laboratories), 100 U/ml penicillin, and 100 μg/ml streptomycin (Welgene, Daegu, Korea). Cells were seeded onto cell culture plates and incubated at 37 °C for 12 h, and then transfected with each oligonucleotide (100 nM) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and a 6 h incubation.

RNA Cleavage with DNAzyme RNA substrates (exon 19 wild-type RNA and deletion-type

RNA, Figure 1C) for the DNAzyme assay were produced by in vitro transcription using the corresponding DNA templates and T7 RNA polymerase.31 Each RNA substrate (500 nM) was incubated with an individual DNAzyme (5 μM) in a buffer (30 mM MgCl2 and 20 mM Tris-HCl, pH 7.5), and 20 μL of the reaction mixture was incubated at 37 °C for 3 h. After incubation, 1 μL of DNase I (5 U/μL, Takara, Tokyo, Japan) was added to degrade the DNAzymes. The cleavage reaction was quenched by adding an equal volume of stop solution containing 25 mM Na2EDTA and 8 M urea, and the products were then separated by 10% (w/v) denaturing PAGE. RNA product bands were visualized with SYBR Gold (Molecular Probes, Life technologies, Eugene, USA).

Cell Proliferation Assay PC9/GR and HepG2 cells were seeded onto 24-well plates at a density of 3.0×104 cells per well and incubated at 37 °C for 12 h. The cells were transfected with 100 nM of an individual DNAzyme using lipofectamine 2000 in Opti-MEM reduced serum medium (Life Technologies, Carlsbad, CA, USA) for 6 h. The media were replaced with fresh culture media (500 μL) after washing with phosphate buffered saline (PBS) twice, and the cells were then incubated at 37 °C for 48 h. At 48 h after transfection, the cells were fixed in 500 μL of 4% paraformaldehyde for 2 h, stained with methylene blue (1% w/v in borate buffer, Sigma-Aldrich, St. Louis, MO, USA) for 10 min, and then dissolved in 0.5% HCl. The absorbance of the dissolved solution was measured at 595 nm using a VICTOR X3 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA).

Western Blot Analyses Total cellular proteins (25 μg) were separated by 8~10% gradient SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane

was immunoblotted with EGFR (2232s, 1:1,000 dilution; Cell signaling, Beverly, MA, USA), phospho-EGFR (Tyr1068) (ab32430, 1:1,000; Abcam, Cambridge, MA, USA), ERK1/2 (sc-154, 1:1,000; Santa Cruz Biotechnology), p-ERK1/2 (Tyr 204) (sc-7383, 1:1,000; Santa Cruz Biotechnology), P38 (9212s, 1:1,000; Cell signaling), phospho-P38 (Thr180/Tyr182) (9211s, 1:1,000; Cell signaling), TLR9 (ab52967, 1,1,000; Abcam), Akt (9272s, 1:1,000; Cell signaling), p-Akt (9271s, 1,1000; Cell signaling), PARP (9542s, 1,000; Cell signaling), GAPDH (MA5-15738, 1:2000; Pierce), and β-actin (sc47778, 1:2,000; Santa Cruz Biotechnology). The membrane was subsequently incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin (sc2004, 1:10,000; Santa Cruz Biotechnology) and reacted with ECL solution (ECL Western blot analysis system kit, Millipore). The signals were detected using a G:BOX with GeneSnap image program (Syngene, Cambridge, UK).

Flow Cytometry for Apoptotic Cell Detection After the cells were transfected with 100 nM oligo DNAs (scrambled DNAzyme, Ex19del DNAzyme, and oligo dT) cells were trypsinized and centrifuged. The cell pellet was washed with PBS once, resuspended in 500 μL of annexin V binding buffer, and then incubated with annexin V-fluorescein-5-isothiocyanate (FITC) and propidium iodide from an apoptosis detection kit (BD Biosciences, San Jose, CA, USA) at 37 °C for 20 min. The samples (at least 10,000 cells) were analyzed with a FACSCalibur Flow Cytometer (BD Biosciences) equipped with BD CellQuest Pro software (BD Biosciences).

Real-time Reverse Transcription PCR (RT-PCR) Total RNA was extracted from cells using TRIzol according to the manufacturer's protocol (Invitrogen) 6 h after the transfection of DNA.

Total RNA (1 μg) was reverse transcribed using a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). PCR amplification of cDNA was performed with 500 nM specific primers and a Rotor-Gene SYBR® Green PCR Kit (Qiagen, Hilden, Germany). Cycling conditions were 95 °C for 10 s and 60 °C for 45 s, and real-time amplified SYBR green signals were observed with Rotor-gene Q (Qiagen). The sequences of the specific primers were as follows: EGFR forward primer, 5'-GAT CAA AGT GCT GGG CTC C-3'; EGFR reverse primer, 5'-CTG CGT GAT GAG CTG CAC-3'; TLR9 forward primer, 5'-CAT GCC CTG CGC TTC CTA TTC A-3'; TLR9 reverse primer, 5'-TGG GCC AGC ACA AAC AGC GTC T-3'; GAPDH forward primer, 5'-ACC ACA GTC CAT GCC ATC AC-3'; and GAPDH reverse primer, 5'-TCC ACC ACC CTG TTG CTG TA-3'.

Immunocytochemistry PC9/GR cells and HepG2 cells were cultured in 12-well plates with an auto-cover glass at 37 °C. The cells were transfected with oligo DNAs for 6 h in Opti-MEM medium. To visualize the co-localization of the transfected DNAs and lysosomes, PC9/GR cells were transfected with FITC-labeled Ex19del Dz (100 nM) for 6 h and fixed in 4% paraformaldehyde. The nuclei and lysosomes were stained with TOPRO-3 (1:500 diluted, Invitrogen) and LysoTracker Red DND-99 (Molecular Probes, Eugene, OR, US), respectively. To compare TLR9 expression levels, the cells transfected with an individual oligonucleotide were fixed in 500 μL of 4% paraformaldehyde for 1 h at room temperature, and then, the cells were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. The cells were washed with PBS and incubated overnight with primary antibody (2 μg/mL; TLR9, Abcam) in PBS containing 3% BSA at 4 °C. The cells were washed with PBS three times, incubated for 2 h with goat anti-rabbit IgG (1:200; Life Technologies, Gaithersburg, MD, Molecular

Probes®/Alexa Fluor® 488), and counterstained with TOPRO-3 (1:500 diluted). The cells were washed with PBS, mounted on a cover slip with ProLong Gold antifade reagent (Invitrogen), and observed with a confocal microscope (Olympus FV-1000 spectral, Tokyo, Japan).

Enzyme-linked Immunosorbent Assay (ELISA) Cells were seeded onto cell culture plates and incubated at 37 °C for 12 h, and the cells were then transfected with each oligonucleotide at the given concentration using Lipofectamine 2000 and a 6 h incubation. Twenty-four hours after the oligonucleotide transfection, cell culture supernatants (2 mL) were collected and centrifuged for 15 min at 1000 × g to remove particulates. An ELISA kit (Cusabio Biotech, Wuhan, Hubei, China) was used to detect secreted IL-6 in the culture media following the manufacturer's instructions. After the reaction mixture was incubated for 1 h at 37 °C, the absorbance of each well was read at 450 nm using a VICTOR X3 Multilabel Plate Reader.