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Original Articles

Hypoxia but not normoxia promotes Smoothened transcription through upregulation of RBPJ and Mastermind-like 3 in pancreatic cancer

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ABSTRACT

We previously demonstrated that Hedgehog (Hh) signaling is activated under hypoxia through upregulation of transcription of Smoothened (SMO) gene. However, the mechanism of hypoxia-induced activation of SMO transcription remains unclear. In the analysis of altered expressions of genes related to Hh signaling between under normoxia and hypoxia by DNA microarray analysis, we picked up 2 genes, a transcriptional regulator, recombination signal binding protein for immunoglobulin-kappa-J region (RBPJ) and a transcriptional co-activator, Mastermind-like 3 (MAML3). Expressions of SMO, MAML3 and RBPJ were increased under hypoxia in pancreatic ductal adenocarcinoma cells (PDAC). RBPJ and MAML3 inhibition under hypoxia led to decreased SMO and GLI1 expressions, whereas SMO expression in MAML3-inhibited and RBPJ-inhibited cells under normoxia showed no change. However, overexpression of RBPJ under normoxia led to increased SMO expression. Additionally, cells knocked down for MAML3 and RBPJ inhibition under hypoxia showed decreased invasiveness through matrix metalloproteinase-2 suppression and decreased proliferation. Xenograft mouse models showed that MAML3 and RBPJ knockdown inhibited tumorigenicity and tumor volume. Our results suggest that hypoxia promotes SMO transcription through upregulation of MAML3 and RBPJ to induce proliferation, invasiveness and tumorigenesis in pancreatic cancer.

Introduction

In vivo environment, especially tumor local site locates under hypoxic condition [1,2]. The oxygen tension in tumor local sites is <1.3% O_2, compared with 5.3% in mixed venous blood and 3.3–7.9% in well-vascularized organs [2,3]. Accordingly, much research has been focused on elucidating the mechanisms and pathways underlying hypoxic conditions. We previously demonstrated that Hedgehog (Hh) signaling is activated under hypoxic condition through upregulation of transcription of Smoothened (SMO), a key protein that drives Hh signaling [4]. Furthermore, we showed that Hh signaling activation by hypoxia leads to the induction of malignant phenotypes, such as proliferation and invasiveness, in pancreatic cancer [4,5]. Consistent with our results, Lei et al. recently revealed that hypoxia induces epithelial to mesenchymal transition through hypoxia-related SMO upregulation, Hh signaling activation [6]. Moreover, Spivak-Kroizman et al. have shown that hypoxia triggers Hh-mediated tumor–stromal interactions in pancreatic cancer [7]. However, the mechanism of hypoxia-induced activation of SMO transcription remains unclear.

Currently, many Hh signaling inhibitors are under clinical study [8–10] and the US Food and Drug Administration recently approved a SMO inhibitor, vismodegib, for the treatment of metastatic or unresectable basal cell carcinomas of the skin [11]. However, promising results in pancreatic cancer treatment have not yet been reported. To develop a more effective therapeutic strategy against pancreatic cancer using Hh signaling inhibitors, understanding the mechanism underlying hypoxia-induced upregulation of SMO transcription is critical.

In the present study, we aimed to clarify the mechanism underlying altered transcription of SMO under hypoxic conditions to help contribute to the development of new effective therapeutic strategies for refractory pancreatic cancer by improving the effects of Hh signaling inhibitors.

Materials and methods

Cell culture and reagents

Two human pancreatic ductal adenocarcinoma cells (PDAC) lines (ASPC-1 and SUIT-2) were maintained in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan)

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supplemented with 10% fetal calf serum (FCS; Life Technologies, Grand Island, NY) and antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin). For normoxic conditions, cells were cultured in 5% CO_2 and 95% air. For hypoxic conditions, cells were cultured in 1% O_2, 5% CO_2, and 94% N_2 using a multigas incubator (Sanyo, Tokyo, Japan). PDAC cultured under normoxia or hypoxia for 24 h were used for experimental analyses as described below.

Cell proliferation assay

PDAC (1×10^5 cells/well) were plated to 6-well plates and were cultured under hypoxia for 3 days. Cell numbers were counted by light microscopy every day. The experiments were performed in triplicate wells.

DNA microarray

The cRNA was amplified, labeled, and hybridized according to the recommended protocol from Agilent. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software. We calculated Z-scores and ratios from the normalized signal intensities of each probe for comparison between ASPC-1 cells cultured under hypoxia for 2 days and ASPC-1 cells continuously cultured under normoxia. We selected genes that were related to Hh signaling that showed a change in expression of ≥2.0-fold.

Real-time PCR

Total RNA was extracted using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) and quantified by spectrophotometry (Ultrospec 2100 Pro; Amersham Pharmacia Biotech, Cambridge, United Kingdom). For real-time RT-PCR, 1 μg of RNA was treated with DNase and reverse transcribed to cDNA with the Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Reactions were run with iQ™ SYBR Green Supermix (Bio-Rad) on a DNA Engine Option 2 System (MJ Research, Waltham, MA). All primer sets amplified fragments less than 200 bp long. The primer sequences used were as follows: *SMO*, forward, CAGGTGGATGGGGACTCTGTGAGT, reverse, GAGTCATGACTCCTCGGATGAGG; *MAML3*, forward, 5′-AAGCCCAGGGACCGAGGCAA-3′, reverse, 5′-GCAGCCTTGGAGGGGCTTGG-3′; *GLI1*, forward, 5′-GGTTCAAGAGCCTGGGCTGTGT-3′, reverse, 5′-GGCAGCATTCTC AGTGATGCTG-3′; *RBPJ*, forward, 5′-CGCA TTATTGGATGCAGATG-3′, reverse, 5′-CAGGAAGCGCCATCATTTAT-3′; *matrix metalloproteinase (MMP-9)*, forward, 5′-TGGGCTACGTGACCTATGACAT-3′, reverse, 5′-GCCCAGCCCACCTCCACTCCTC-3′; *MMP-2*, forward, 5′-TGATCTTGACCAGAATACCA TCGA-3′, reverse, 5′-GGCTTGCGA GGGAAGAAGTT-3′; and *β-actin*, forward, 5′-TTGCCGACAGGATGCAGAAGGA-3′, and reverse, 5′-AGGTGGACAGCGAGGCCAGGAT-3′. The amount of each target gene in a given sample was normalized to the level of *β-actin*.

Matrigel invasion assay

Cell invasion assays were performed using Matrigel-coated transwell inserts as described previously [12]. In brief, the upper surface of a filter (pore size, 8.0 μm; BD Biosciences, Heidelberg, Germany) was coated with Matrigel basement membrane (BD Biosciences). Cells were suspended in RPMI-1640 with 10% FCS. Next we added 0.8×10^5 cells to the upper chamber and incubated the cells for 16 h under hypoxia. After incubation, the filter was fixed and stained with Diff-Quick reagent (International Reagents, Kobe, Japan). All cells that had migrated from the upper to the lower side of the filter were counted under a light microscope (BX50; Olympus, Tokyo, Japan) at a magnification of ×100. Tumor cell invasiveness was performed in triplicate wells.

RNA interference

Small interfering RNA (siRNA) for *MAML3* (ON-TARGET *plus* SMART pool, L-013813), siRNA for *HIF-1α* (ON-TARGET *plus* SMART pool, L-004018), siRNA for *RBPJ* (ON-TARGET *plus* SMART pool, L-007772) and negative control siRNA (ON-TARGET *plus* si CONTROL non-targeting pool, D-001810) were purchased from Dharmacon RNA Technologies (Chicago, IL). Cells (0.2×10^6 cells/well) seeded in 6-well plates were transfected with 100 nM siRNA under normoxia using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. Cells were used for experiments at 2 days after transfection. Then, cells were cultured additionally under normoxia and hypoxia in each experiment.

Plasmids

Plasmids pFN21A HaloTag CMV Flexi-RBPJ vector and pFN21AB5901 control empty vector were purchased from Promega (Madison, WI, USA). Cells (0.2×10^6 cells/well) seeded in 6-well plates were transfected with 2.5 μg plasmids under normoxia using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Cells were used for experiments at 1 day after transfection.

Immunoblotting

Whole cell extraction was performed with M-PER Reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Protein concentration was determined with Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), and protein samples (50 μg) were separated by electrophoresis on an SDS–polyacrylamide gel and transferred to Protran nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Blots were then incubated with anti-SMO Ab (1:200, sc-13943, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GLI1 Ab (1:100, sc-20687, Santa Cruz Biotechnology), anti-MAML3 Ab (1:100, sc-82220, Santa Cruz Biotechnology), anti-RBPJ Ab (1:100, sc-8213, Santa Cruz Biotechnology), anti-MMP2 Ab (1:100, sc-10736, Santa Cruz Biotechnology) and anti-α-tubulin Ab (1:1000, Sigma Aldrich Co., St. Louis, MO, USA) overnight at 4 °C. Blots were then incubated with the appropriate horseradish peroxidase-linked secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 1 hour. Immunocomplexes were detected with ECL plus Western Blotting Detection System (Amersham Biosciences) and visualized with a Molecular Imager FX (Bio-Rad). We used α-tubulin a protein loading control.

In vivo *xenograft tumor model*

Six-week-old female BALB/c nude mice were obtained from Charles River Laboratories Japan (Kanagawa, Japan) and acclimatized for a week. All animal procedures were approved by the Animal Care and Use Committee at Kyushu University (A25-027-1). Cultured SUIT-2 cells transfected with *MAML3*-targeting siRNA, *RBPJ*-targeting siRNA and non-targeting control siRNA were subcutaneously implanted into bilateral flank regions (5.0×10^5 cells in 50 μl of RPMI) of the BALB/c nude mice ($n = 8$). The tumor size was determined twice a week and the tumor volume was calculated with the following formula: $A \times B^2 \times 0.5$, where B is the smaller of the two perpendicular diameters. The mice were euthanized when the tumor size had reached 2 cm in diameter or if the animals had become moribund during the observation period.

Fluorescence immunohistochemistry

Slides from patients with pancreatic cancer were deparaffinized with xylene and rehydrated with alcohol; antigen retrieval was achieved by microwaving in Target Retrieval Solution (pH 6.0, DAKO, Japan) for 10 minutes. The sections were rinsed with phosphate-buffered saline (PBS) and blocked using Blocking One Histo (Nacalai Tesque) for 10 minutes at room temperature. The sections were incubated with anti-Ki67 (BD Phamingen), anti-VEGF (sc-152, Santa Cruz), anti-SMO (sc-13943, Santa Cruz), anti-RBPJ (Santa Cruz) and anti-MAML3 (Santa Cruz) antibodies overnight at 4 °C. Primary antibodies were then visualized by incubating slides with Alexa Fluor 488 conjugated donkey anti-mouse (1:1000; Invitrogen), Alexa Fluor 594 conjugated goat anti-rabbit (1:1000; Invitrogen) and Alexa Fluor 488 conjugated donkey anti-goat (1:1000; Invitrogen) for 1 hour at 37 °C, respectively. After incubation with secondary antibodies, sections were rinsed with PBS three times. Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) and then mounted by VectaShield (Vector Laboratories, Burlingame, CA, USA). The samples were examined by fluorescence microscopy (Carl Zeiss, Tokyo, Japan). Informed consent was obtained from all individuals.

Statistical analysis

The data are presented as means ± standard deviation (SD). Student's *t*-tests were used to compare continuous variables between two groups. Chi-squared test was used to analyze the tumorigenicity in mice. All statistical analyses were performed using Microsoft Excel software (Microsoft Corp., Redmond, WA, USA). *P*-values of <0.05 were considered as statistically significant.

Results

Recombination signal binding protein for immunoglobulin-kappa-J region (RBPJ) contributes to hypoxia-induced upregulation of SMO transcription

To identify genes involved in the upregulation of SMO transcription under hypoxia, we compared one of PDAC lines, ASPC-1 cells cultured under hypoxia for 2 days with ASPC-1 cells cultured under normoxia and analyzed changes in expression of genes related to Hh signaling by DNA microarray analysis. Of the genes identified by GeneMANIA as exhibiting differential expression patterns, we found four candidate genes that showed a difference of expression of ≥2.0-fold (Supplementary Table S1). RBPJ, a transcriptional regulator, was among the identified genes and thus we selected it as a candidate for further investigation.