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Research Article

Abrogation of Gli3 expression suppresses the growth of colon cancer cells via activation of p53

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ABSTRACT

p53, the major human tumor suppressor, appears to be related to sonic hedgehog (Shh)–Gli-mediated tumorigenesis. However, the role of p53 in tumor progression by the Shh–Gli signaling pathway is poorly understood. Herein we investigated the critical regulation of Gli3–p53 in tumorigenesis of colon cancer cells and the molecular mechanisms underlying these effects. RT-PCR analysis indicated that the mRNA level of *Shh* and *Gli3* in colon tumor tissues was significantly higher than corresponding normal tissues ($P<0.001$). The inhibition of *Gli3* by treatment with *Gli3* siRNA resulted in a clear decrease in cell proliferation and enhanced the level of expression of p53 proteins compared to treatment with control siRNA. The half-life of p53 was dramatically increased by treatment with *Gli3* siRNA. In addition, treatment with MG132 blocked MDM2-mediated p53 ubiquitination and degradation, and led to accumulation of p53 in *Gli3* siRNA-overexpressing cells. Importantly, ectopic expression of *p53* siRNA reduced the ability of *Gli3* siRNA to suppress proliferation of those cells compared with the cells treated with *Gli3* siRNA alone. Moreover, *Gli3* siRNA sensitized colon cancer cells to treatment with anti-cancer agents (5-FU and bevacizumab). Taken together, our studies demonstrate that loss of Gli3 signaling leads to disruption of the MDM2–p53 interaction and strongly potentiate p53-dependent cell growth inhibition in colon cancer cells, indicating a basis for the rational use of Gli3 antagonists as a novel treatment option for colon cancer.

Introduction

The sonic hedgehog (Shh) signaling pathway plays a critical role in stem cell maintenance and specifying patterns of cell growth and differentiation during embryonic development [1–3]. Shh activates signaling by binding to the Shh receptors, Patched (Ptch) 1 and 2, to relieve Ptch repression of Smoothened (Smo), which is a membrane protein related to G protein-coupled receptors. Upon activation, Smo promotes nuclear translocation of a family of transcription factors (Ci in *Drosophila* and Glis [Gli1, Gli2, and Gli3] in vertebrates), and subsequently activates target genes through Glis [3,4].

Aberrant activation of the Shh signaling cascade has been associated with oncogenesis and maintenance of the malignant phenotype in multiple types of human cancers. Excessive activation mutations in the *Smo* gene, loss-of-function mutations in the *Ptch* gene, or amplification of *Glis* cause the majority of human and murine cancers [5,6], and inhibition of the pathway

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with a Smo inhibitor (cyclopamine) has been shown to retard the growth of tumors and to decrease tumor size [7,8]. In gastrointestinal cancers, transcriptional up-regulation of hedgehog ligands has been shown to be involved in tumorigenic potential and metastatic disease [9–12]. In addition, it was recently reported that Shh signaling participates in the development of colon cancer and in the metastatic process of advanced colon cancer [13–15]. However, very little is known regarding the specific role of Shh signaling in regulating cellular survival and proliferation in colon cancer and the molecular mechanisms underlying these effects.

The p53 tumor suppressor is a key regulator that controls multiple cellular processes, such as DNA repair, cell-cycle arrest, senescence, apoptosis, and angiogenesis [16]. p53 is negatively regulated by MDM2, which functions as an E3 ligase that mediates the ubiquitination and proteasome degradation of p53, and this p53–MDM2 regulatory feedback loop is essential in maintaining tight regulation of p53 levels [17–20]. Accordingly, interfering in the MDM2–p53 interaction could be an attractive strategy for activating wild-type p53, providing an effective approach toward enhancing cancer therapy. Recently, it has been shown that constitutively activated mutants of *Smo* down-regulate p53 by promoting p53 ubiquitination mediated by MDM2 and partially abrogate p53-mediated apoptosis and growth inhibition in oncogene-expressing mouse embryonic fibroblasts (MEFs) [21].

The purpose of this study was to investigate the critical regulation of Gli3–p53 in tumorigenesis of colon cancer cells and the molecular mechanisms underlying these effects. We demonstrate that inhibition of *Gli3* by treatment with *Gli3* siRNA leads to hyperactivation of the p53 pathway and a strong inhibition of proliferation in colon cancer cells. Furthermore, we showed that *Gli3* siRNA causes the stabilization and activation of p53 via the prevention of MDM2-mediated p53 ubiquitination and degradation. Taken together, our findings suggest a role and mechanism for Gli3–p53 signaling as it relates to the tumorigenic potential of colon cancer, which indicates that Gli3–p53 signaling has the potential to be a therapeutic molecular target to decrease tumorigenesis.

Material and methods

Cell lines and cultures

The human colon carcinoma cell lines, HCT-116, LOVO, and RKO were obtained from the Korea Cell Line Bank (KCLB; Seoul, Korea) and the cells were maintained according to manufacturer's instructions.

Clinical samples

The tissue samples were used from ten patients who had undergone surgical excision for colon cancer at Korea University Guro Hospital. After surgical resection, normal cancer and corresponding non-neoplastic mucosa were sampled from each patient and frozen immediately in liquid nitrogen. Total RNA was extracted from frozen tissues from normal cancer and corresponding non-neoplastic mucosa. This protocol was reviewed and permitted by Institutional Review Board of Guro Hospital.

Reagents

Cycloheximide, 5-FU, oxaliplatin, irinotecan, and bevacizumab were purchased from Sigma-Aldrich (St. Louis, MO, USA). MG-132 was purchased from Calbiochem (San Diego, CA, USA).

Cell proliferation assay

Cells transfected with Gli3 or control siRNA were seeded at a concentration of 4×10^3 cells per well in 96-well culture plates, then incubated for 24 h at 37 °C in an atmosphere of 5% CO_2. The medium was then changed to RPMI-1640 containing 0.5% FBS in the presence or absence of indicated drugs. After incubating for 24 or 72 h, the number of viable cells was determined in triplicate wells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche), according to the manufacturer's instructions.

Small interfering RNA transfection

Small interfering RNA (siRNA) duplex specific to *Gli3* and *p53* was synthesized at Invitrogen. The siRNA sequences used were as follows: *Gli3* siRNA, 5′-AAU GAG GAU GAA AGU CCU GGA TT-3′ and 5′-UCC AGG ACU UUC AUC CUC AUU TT-3′; and *p53* siRNA, 5′-UUC CGU CCC AGU AGA UUA CCA CUG G-3′ and 5′-CCA GUG GUA AUC UAC UGG GAC GGA A-3′. As a non-specific control siRNA, scrambled siRNA duplex was used. Transfection was done using LipofectAMINE 2000 reagent (Invitrogen), following the manufacturer's instruction.

RT-PCR analysis

Total RNA extraction was performed using the Trizol reagent (Life Technologies, Rockville, MD, USA), according to the manufacturer's instructions. Amplification of transcripts was performed using 1 μg/μl of total RNA and the reverse transcriptase polymerase chain reaction (RT-PCR) using Molony murine leukemia virus reverse transcriptase (MMLV; Gibco/BRL, Gaithersburg, MD, USA) and oligo-$d(T)_{15}$ primer (Roche). PCR amplification was performed using the following primers: *Shh*, forward : 5′-ATC AAG GAT CCC CTG CTG CTG GCG AGA TGT-3′, reverse : 5′-ATC AAA GCT TTC AGC TGG ACT TGA CCG C-3′; *Gli1*, forward : 5′-TGC CTT GTA CCC TCC TCC CGA A-3′, reverse : 5′-GCG ATC TGT GAT GGA TGA GAT TCC C-3′; *Gli3*, forward : 5′-AGG CTG CAC TAA GCG TTA CA-3′, reverse : 5′-CTT TCT AGT TTT ACG TGC TCC-3′; *Ptch*, forward : 5′-TTC TCA CAA CCC TCG GAA CCC A-3′, reverse : 5′-CTG CAG CTC AAT GAC TTC CAC CTT C-3′; *Smo*, forward : 5′-CTT CAG CTG CCA CTT CTA CG-3′, reverse : 5′-ACA GAA ATA TCC TGG GGC AG-3′; *p21*, forward : 5′-CCT CAA ATC GTC CAG CGA CCT T-3′, reverse : 5′-CAT TGT GGG AGG AGC TGT GAA A-3′; *MDM2*, forward : 5′-ATC TTG GCC AGT ATA TTA TG-3′, reverse : 5′-GTT CCT GTA GAT CAT GGT AT-3′; *β-actin*, forward : 5′-ACC CAG ATC ATG TTT GAG AC-3′, reverse : 5′-GGA GTT GAA GGT AGT TTC GT-3′. The cycling conditions were 95 °C for 10 min, followed by 30 cycles of amplification at 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 30 s. Experiments were independently repeated three times. The intensities of the DNA bands in the agarose gel were quantified using Image J software.

Cell cycle analysis

Cells transfected with *Gli3* or control siRNA were seeded in 100-mm dishes. Forty-eight hours after seeding, cells were fixed with