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Inhibition of Hedgehog signaling pathway impedes cancer cell proliferation by promotion of autophagy

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ABSTRACT

Multiple lines of evidence implicate that aberrant activation of Hedgehog (Hh) signaling is involved in a variety of human cancers. However, the molecular mechanisms underlying how cancer cells respond to Hh inhibition remain to be elucidated. In this study, we found that blockade of Hh signaling suppresses cell proliferation in human cancer cells. Microarray analysis revealed that differentially expressed genes (DEGs) in human cancer cells are enriched in autophagy pathway in response to the inhibition of Hh signaling. Interestingly, inhibition of Hh signaling induced autophagy, whereas activation of Hh signaling by ligand treatments prevented the induction of autophagy. In addition, inhibition of autophagy by 3-methyladenine (3-MA) partially suppressed cytotoxicity induced by inhibition of Hh signaling. Finally, in autophagy deficient cells, cytotoxic effect triggered by inhibition of Hh signaling was partially reversed, indicating the modulation of autophagy by Hh signaling is autophagy-specific. These results suggest that inhibition of Hh signaling impedes cancer cell proliferation in part through induction of autophagy.

Introduction

The Hedgehog (Hh) signaling pathway is an evolutionarily conserved system that is involved in stem cell development and tissue regeneration (Barakat et al., 2010). As the downstream targets of Hh signaling are controlled by the dynamics of *GLI* transcription factors, Gli proteins are essential in Hh signal transduction processes (Hui and Angers, 2011). Increasing evidence shows that this Hh pathway may play an important role in carcinogenesis. Genetic dysfunction of Hh-related genes has been observed in different types of cancers, including basal cell carcinomas, medulloblastomas, and glioblastomas (Yang et al., 2010). In addition, the activation of Hh signaling has also been reported in tumor tissues of breast, pancreatic, prostate cancers as well as hematological cancers (Katoh and Katoh, 2009). These observations imply that deregulated Hh signaling is correlated with the rapid growth of cancer cells. Hence, inhibition of Hh signaling by various therapeutic agents has been explored in multiple human cancers (Lauth et al., 2007; Varnat et al., 2009; Von Hoff et al., 2009). Blockade of Hh signaling by cyclopamine (Von Hoff et al., 2009), Gli-ANTagonist 61 (GANT61) (Lauth et al., 2007) or Smo shRNA (Varnat et al., 2009) inhibits cell proliferation and suppresses tumor formation. GANT61 was identified from a cell-based screen for inhibitors of Gli-mediated transcription and had been demonstrated to be a potent Hh signaling inhibitor for treating various cancers (Lauth et al., 2007; Yan et al., 2013). Nevertheless, the mechanisms by which Hh signaling promotes tumor growth need to be further elucidated. One of the possible cellular mechanisms involves autophagy.

Autophagy is a highly conserved process that involves lysosomal degradation of cytoplasmic and cellular organelles (Mizushima, 2007). More than 30 ATG genes identified by yeast genetic analysis are involved in the autophagy and its related pathway (He and Klionsky, 2009; Yang and Klionsky, 2010). Many core ATG proteins such as the Beclin1-Vps34-Atg14L complex, the Atg5-Atg12-Atg16 complex and Atg8 homologs are required for autophagosome formation both in starvation-induced and basal autophagy (Lamark and Johansen, 2012). Many studies have demonstrated that autophagy occurs at low levels in normal conditions and can be

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up-regulated by starvation, or chemicals, such as rapamycin or trehalose (Ravikumar et al., 2010). Recent evidence has shown that autophagy is associated with cancer pathogenesis and pharmacologic manipulation of autophagic pathways may represent new therapeutic strategies for human cancers (Barakat et al., 2010; White and DiPaola, 2009). However, the role of autophagy in cancer is not fully defined. In addition, the functional relationship between Hh signaling and autophagy still remains to be addressed.

In this study, we performed a set of experiments to elucidate the molecular mechanisms by which the Hh signaling pathway regulates cancer cell proliferation and tumor growth. As a result, we found that inhibition of Hh signaling suppresses cancer cell proliferation in part through autophagy induction as detailed below.

Materials and methods

Reagents, antibodies and cells

GANT61, cyclopamine, trehalose, 3-methyladenine (3-MA), purmorphamine and bafilomycin A1 (bafA1) were purchased from Sigma-Aldrich (St. Louis, MO). The primary antibodies were purchased from Cell Signaling (Gli1, 2648S; SQSTM1/p62, 5114S; LC3-II, 3868; Atg3, 3415; Atg5, 4445S; Atg7, 8558), Abcam (Smo, ab38686; Ptch1, ab55629; Gli2, ab26056), and Millipore (GAPDH, mAb374). The BrdU cell proliferation assay kit was purchased from Millipore Chemicon (Billerica, MA). *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs were kindly gifted by Dr. Ye-Guang Chen (Ma et al., 2014; Mizushima et al., 2001). Glioma cell line H4, ovarian cancer cell line ES2, gastric cancer cell line MKN45 and colon cancer cell line HT29 were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as recommended by the supplier in a humidified incubator with 5% CO_2 at 37 °C. All the experiments were performed with cells in normal growth medium (nutrient-rich condition) unless specifically indicated.

Western blot analysis

Following the designed program, cells treated with GANT61 or cyclopamine were harvested at indicated time points and subjected to Western blot analysis as described previously (Luo et al., 2008, 2009). All experiments were repeated at least three times with consistent results.

Cell viability and proliferation analysis

Cell viability was measured using a modified MTT assay (Yan et al., 2013). The relative survival rates were presented as the percentage of the control. For cell proliferation assay, cells were treated with GANT61 or cyclopamine for indicated time points. Cell proliferation was assessed using a BrdU cell proliferation assay kit according to the manufacturer's protocol (Yan et al., 2013).

RNAi expression plasmids and recombinant Shh ligand

Gli2-miRNAi expression vectors that suppress expression of Gli2 were generated using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (K4936-00, Invitrogen, Carlsbad, CA) (Luo et al., 2008, 2009). The oligonucleotide sequences for miRNAi constructs were as follows: for miR-Gli2-233 (targeting nucleotides 233 to 253 of Gli2; GenBank accession no. NM_005270), 5′-AAT GGT ACC TTC CTT CCT GGT-3′; for miR-Gli2-1127 (targeting nucleotides 1127 to 1147 of Gli2), 5′-TGT GAA TGG CGA CAG GGT TGA-3′; and for miR-Gli2-2058 (targeting nucleotides 2058 to 2078 of Gli2), 5′-ATC TCC ACG CCA CTG TCA TTG-3′. Vectors encoding short haipin RNAs that suppress expression of Atg5 were generated using the GV248 RNAi expression vector (GeneChem Co., Ltd, Shanghai, China). The oligonucleotide sequences for shRNA constructs were as follows: for shRNA-Atg5-420 (targeting nucleotides 420 to 438 of Atg5; GenBank accession no. NM_004849), 5′-AGA ACC ATA CTA TTT GCT T-3′; for shRNA-Atg5-915 (targeting nucleotides 915 to 934 of Atg5), 5′-TTT CAT TCA GAA GCT GTT T-3′; and for shRNA-Atg5-1170 (targeting nucleotides 1170 to 1188 of Atg5), 5′-TGA ACA GAA TCA TCC TTA A-3′. Transient transfection was performed with lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). Stable expression of Gli2 miRNAi was guaranteed by culturing cells in the presence of a selection antibiotic Blasticidin (8 μg/ml). The recombinant Shh ligand was prepared as described previously and used at 0.4 μg/ml (Chen et al., 2014; Yan et al., 2013).

RNA isolation and hybridization to Illumina gene expression arrays

Cancer cells (H4, ES2, MKN45 and HT29) were treated with or without GANT61 (5, 10 or 20 μM) for 24 h. Total RNA from each sample was isolated by the Trizol reagent, purified using an RNeasy Protect Mini Kit (QIAGEN), and amplified with the Total Prep Kit (Ambion Inc., Austin, TX). Biotinylated cRNA was hybridized to Sentrix Expression BeadChip (Illumina Inc., San Diego, CA). The arrays were scanned on the Illumina® BeadArray Reader. Image files were downloaded into BeadStudio for data visualization and analysis. All microarray data are available for download from the NCBI at GEO (Accession number: GSE 54936).

Bioinformatics analysis and real-time PCR

The combination of DiffScore and *P* was used to assess differences of gene expression between the two groups. Gene with $P < 0.01$ in any groups (DiffScore less than −20 or more than 20) was considered as the DEGs (differentially expressed genes). We performed DEGs enrichment analysis based on the Wikipathway database (Zhang et al., 2005). The gene-set with adjusted $P < 0.0005$ and involved at least four DEGs was considered as the enriched category. The expression levels of selected genes identified by cDNA microarray were validated by real-time PCR. Real-time PCR reaction

Table 1
Primers used for real-time PCR amplification.

Genes	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
GLI1	5′-TCCTACCAGAGTCCCAAGTT-3′	5′-CCCTATGTGAAGCCCTATTT-3′
GLI2	5′-CCTGGCATGACTACCACTATGAG-3′	5′-GGCTTGGCTGGCATGTTG-3′
PTCH1	5′-GGCTCCCTCATCTCGTAAC-3′	5′-ACTATGCTGTGGGTATTTCTGG-3′
MAP1LC3B/LC3-II	5′-AACGCATTTGCCATCACAGT-3′	5′-AGGAGTCAGGGACCTTCAGC-3′
GABARAPL1	5′-CGCAAGGAGACAGAAGGTGA-3′	5′-CCTCTATCAAAGCCAATCCAA-3′
SQSTM1/p62	5′-TGGCGGAGCAGATGAGGAAG-3′	5′-CTGGCGGGAGATGTGGGTAC-3′
CDKN1A	5′-TGTCCTTGGGCTGCCTGTT-3′	5′-TTGCTGCCGCATGGGTT-3′
IL8	5′-TGAAGATGCCAGTGAAA-3′	5′-AACCCTACAACAGACCC-3′
NFKBIA	5′-GAGGAGTACGAGCAGATGGTCAA-3′	5′-CAATTTCTGGCTGGTTGGTGAT-3′
GAPDH	5′-CAGGGCTGCTTTTAACTCTGGT-3′	5′-GATTTTGGAGGGATCTCGCT-3′